STUDIES ON THE PREPARATION OF ORGANOLEPTICALLY PALATABLE CASEIN AND WHEY PROTEIN HYDROLYSATES

THESIS SUBMITTED TO THE
ICAR- NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY), KARNAL
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRYING
(DAIRY TECHNOLOGY)

BY

SAURABH PRAKASH
M.Tech. (Dairy Technology)

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ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
KARNAL-132001 (HARYANA), INDIA

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Approved by

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This is to certify that the thesis entitled "Studies on the preparation of organoleptically palatable casein and whey protein hydrolysates" submitted by **Shri. SAURABH PRAKASH** towards the partial fulfilment for the award of the degree of **Doctor of Philosophy** in **Dairying (Dairy Technology)** of the **ICAR-National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 4th Dec., 2017

(S. K. Kanawjia)
Major Advisor and Chairman
(Guide)

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(Saurabh Prakash)

ABSTRACT

STUDIES ON THE PREPARATION OF ORGANOLEPTICALLY PALATABLE CASEIN AND WHEY PROTEIN HYDROLYSATES

Casein and whey proteins are two principal proteins in milk, which play nutritional as well as other functional roles. Milk proteins present amino acid profile superior to plant origin proteins, which make them suitable for the formulation of humanized milk products, infant formulas, etc. for infant nutrition. For several purposes, it is beneficial to hydrolyse proteins, for example, to reduce allergenicity, to achieve specific dietary requirements, or to improve functional properties. Nevertheless, casein- and whey-derived hydrolysates have not gained wide popularity due to its bitter & off-flavor generated during hydrolysis. Therefore, in the present study, enzymatic hydrolysis conditions were optimized using Response Surface Methodology (RSM) to develop casein and whey proteins hydrolysates using trypsin, alcalase and papain followed by debittering treatment using endo- and exo-proteases (Flavourzyme 500L, Flavourzyme 1000L and FlavorSEB). Further, developed debittered and optimized casein and whey protein hydrolysates were incorporated with strawberry flavour at the level of 2.5, 5.0 and 7.5% to develop flavoured milk beverage. Results revealed that there was a significant difference ($P<0.05$) among the degree of hydrolysis (%DH) of all casein as well as whey protein hydrolysed samples. Alcalase produced maximum %DH of casein hydrolysates under optimized conditions of pH 8.0, temperature 60°C, E/S ratio 0.004 and reaction time 4 h. Similarly, in case of whey protein hydrolysates, alcalase produced maximum %DH under the same conditions as in case of sodium caseinate. Alcalase showed highest degree of bitterness followed by trypsin and papain, respectively, irrespective of the protein type. Therefore, casein as well as whey protein hydrolysates catalysed by alcalase were selected for further treatment i.e. de-bittering using Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB. Results revealed that flavourzyme 1000L caused the maximum %DH and minimum biterrness of casein hydrolysates and whey protein hydrolysates. Therefore, Flavourzyme 1000L was finally selected as the best enzyme for the production of debittered casein and whey protein hydrolysates. The optimum conditions for developing debittered casein hydrolysates as well whey protein hydrolysates (alcalase catalysed) using Flavourzyme 1000L were as pH 7.0, temperature 50°C, E/S ratio 0.015 and reaction time 4 h, which was further flavoured with fruit essence in the formulation of milk beverage. No significant difference was observed among all sensory attributes of control and test samples, when milk beverage was fortified with optimized and debittered whey protein hydrolysates/casein hydrolysates, irrespective of the fortification level. It can be concluded that casein and whey protein hydrolysates developed using Alcalase and debittered using Flavourzyme 1000L under optimized conditions could be utilized successfully at the rate of 7.5% for developing strawberry flavoured milk beverage.

ईंद्रियेग्राही योग्य कैसीन और व्हे प्रोटीन अपघटित की तैयारी पर अध्ययन

सारांश

कैसीन और व्हे प्रोटीन दूध में प्रमुख प्रोटीन होते हैं, जो पौष्टिक होने के साथ साथ अन्य कार्यात्मक भूमिकाएं भी निभाते हैं। दूध प्रोटीन के अमीनो एसिड प्रोफ़ाइल अन्य पौधों के मूल प्रोटीनों से बेहतर है, जो उन्हें शिशु पोषण के लिए मानवकृत दूध उत्पादों, शिशु फार्मूले आदि के निर्माण के लिए उपयुक्त बनाती हैं। कई उद्देश्यों के लिए, प्रोटीन को अपघटित करने के अनेक लाभ है, उदाहरण के लिए, एलर्जि को कम करना, विशिष्ट आहार वाली आवश्यकताओं की पूर्ति करने में, और कार्यात्मक गुणों को बेहतर बनाने के लिए। फिर भी, अपघटित कैसीन - और व्हे प्रोटीन ने कड़वे स्वाद के कारण व्यापक लोकप्रियता प्राप्त नहीं की है। इसलिए, वर्तमान अध्ययन में, उत्प्रेरित अपघटन की परिस्थ्तियो को रिस्पांस सर्फेस मेथोडोलॉजी (आर एस एम) का उपयोग करके अपघटित कैसीन और व्हे प्रोटीन को ट्रिप्सिन, एल्केलेस और पपैन उत्प्रेरको के साथ विकसित करने के लिए अनुकूलित किया गया तथा इसके बाद अंततः और बाह्य-प्रोटीन अपघटक उत्प्रेरकों (फ्लेवरजायम 500 एल फ्लेवरजायम 1000 एल और फ्लेवर-एसईबी) का उपयोग किया गया था। इसके अलावा, विकसित और अपघटित कैसीन और व्हे प्रोटीन को 2.5, 5.0 और 7.5% के स्तर पर दूध में मिलाया गया ताकि स्ट्रॉबेरी फ्लेवर वाले दूध के पेय का विकास किया जा सके। परिणाम से पता चला है कि सभी अपघटित कैसीन और व्हे प्रोटीन नमूनों के बीच एक महत्वपूर्ण अंतर था। अल्कालेस ने pH 8.0, तापमान 60 डिग्री सेल्सियस, ई/एस अनुपात 0.004 और प्रतिक्रिया समय 4 घंटे की अनुकूल परिथितियों में कैसीन में अधिकतम अपघटन किया। इसी प्रकार, अपघटित व्हे प्रोटीन के संदर्भ में, अल्कालेस ने समान परिथितियों में अधिकतम अपघटन किया। दोनों प्रकार की प्रोटीन में, अल्कालेस ने क्रमशः ट्रिप्सिन और पपेन के बाद उच्चतम कड़वाहट उत्तपन की। इसलिए, अल्कालेस द्वारा उत्प्रेरित एवं अपघटित कैसीन और व्हे प्रोटीन को फ्लेवरजायम 500 एल, फ्लेवरजायम 1000 एल और फ्लेवर-एसईबी का उपयोग करके और कड़वाहट कम करने के लिए चुना गया। परिणामों से पता चला है कि फ्लेवरजायम 1000 एल ने कैसीन और व्हे प्रोटीन में अधिकतम अपघटन किया। अंततः फ्लेवरजायम 1000 एल को कम कड़वाहट वाले अपघटित कैसीन और व्हे प्रोटीन के उत्पादन के लिए सर्वश्रेष्ठ एंजाइम के रूप में चुना गया। अपघटित कैसीन और व्हे प्रोटीन (एल्कैलेज़ उत्प्रेरित) विकसित करने के लिए इष्टतम परिथितिया पी॰एच॰ 7.0, तापमान 50 डिग्री सेल्सियस, ई / एस अनुपात 0.015 और प्रतिक्रिया समय 4 घंटे पायी गयी, जिसको उचित दूध पेय पदार्थ बनाने में उपयोग किया गया। जब दुग्ध पेय में अनुकूलित एवं कड़वाहट-रहित एवं अपघटित कैसीन एवं व्हे प्रोटीन मिलाए गये, तब नियंत्रण और परीक्षण के सभी संवेदी गुणों के बीच कोई महत्वपूर्ण अंतर नहीं पाया गया। अंततः यह निष्कर्ष निकाला जा सकता है की अल्कालेस का उपयोग करके तथा अनुकूल परिथितियों में फ्लेवरजायम 1000 द्वारा कड़वाहट-रहित करके उत्पन्न अपघटक को स्ट्राबेरी स्वाद वाले दूध पेय के विकास के लिए 7.5% की दर से सफलतापूर्वक उपयोग किया जा सकता है।

Dedicated
To My
Loving Wife, Brothers
&
Beloved Parents

CONTENTS

List of abbreviation

ANOVA : Analysis of variance
AOAC : Association of Official Analytical Chemists
APV : Adequate precision value
AR : Analytical reagents
CCD : Central Composite Design
CCRD : Central Composite Rotatable Design
df : Degree of freedom
DH : Degree of hydrolysis
E/S : Enzyme/Substrate ratio
et al. : and co-workers
g : Gram
h : Hour
HTST : High-temperature short-time
i.e. : That is
Kg : Kilogram
kg : Kilogram
L : Liters
LMW : Low molecular weight
M : Molar
min : Minute
ml : milliliters
MS : Mean square
MSNF : Milk solids-not-fat
N : Normal
NDRI : National Dairy Research Institute
p : Probability
R^2 : coefficient of determination
RSM : Response Surface Methodology
sec : Second
SMP : Skimmed milk powder
SNF : Solids-not-fat

viz.	:	Namely
w/v	:	Weight/volume
w/w	:	Weight/weight
WPC	:	Whey protein concentrates
WPC-80	:	Whey protein concentrate of 80% protein
WPH	:	Whey Protein hydrolysates
α-la	:	Alpha-lacalbumin
β-lg	:	Beta-lactoglobulin
%	:	Percent
@	:	At the rate of
°C	:	Degree-Celsius
µg	:	microgram

CHAPTER –1

Introduction

INTRODUCTION

Proteins are nutritionally important as a source of nitrogen and essential amino acids. Milk proteins have high nutritional value compared to other proteins because of their relatively high content of essential amino acids and good digestibility. Casein and whey proteins are the two principal proteins in milk, which play nutritional as well as other functional roles. The nutritional roles of the casein fraction of milk including the supply of essential amino acids, adequate calcium, and phosphate, are well recognized. Similarly, whey proteins have high biological value (BV-106-159) and Protein digestibility-corrected amino acid score (PDCAAS-1) (Boutrif *et al.*, 1991); and helps in transfer of vitamin A, lactose synthesis, etc. They present amino acid profile superior to plant origin proteins, which make them suitable for the formulation of g, infant formulas, etc. geriatric food, body building persons, sports person, enteral feed for hospital patients, etc.

For several purposes, it is beneficial to hydrolyse proteins, for example, to reduce allergenicity, to achieve specific dietary requirements, or to improve their functional properties. Hydrolysis of proteins leads to numerous alterations in functional characteristics, like changes in solubility, viscosity, emulsion and foam forming and foam stability. Milk protein hydrolysates have many advantages over the intact proteins in terms of enhanced functional (emulsification, gelling, etc.) and biological properties. Enzymatic hydrolysis has been widely used to modify the solubility, foaming, emulsification and gelation properties of caseins and whey proteins. Endoproteases such as Alcalase, Papain and Trypsin are used to make protein hydrolysates and are mainly obtained from animal, plant and microbial sources. These endoproteases provide mild hydrolysis condition and are very specific to control the degree of hydrolysis (DH). Enzymatic hydrolysis of whey proteins has been shown various activities such as antihypertension, anticancer, immunomodulation, opiod, mineral binding, antioxidative, etc. (Chatterjee *et al.*, 2014; Lacroix *et al.*, 2016). Furthermore, the small peptides present in protein hydrolysates are absorbed more rapidly from the intestine than free amino acids or intact proteins.

Nevertheless, casein- and whey-derived hydrolysates have not gained wide popularity. The main reason for this lack of market penetration is the very bitter off-taste, which is generated during protein hydrolysis. A bitter off-taste is common for all protein hydrolysates, but large differences occur depending on the protein source used. According to Ney’s Q-hypothesis, the degree of bitterness that develops during hydrolysis is connected with the level of hydrophobic amino acids present in the protein substrate used (Ney, 1971). More recent views link bitterness to the formation of a few exceptionally bitter peptides rather than to an array of moderately bitter peptides. Several researchers observed that peptides with hydrophobic amino acids at C- or N-terminal positions appear less bitter than the peptides having hydrophobic amino acid in-between i.e. when both ends of hydrophobic amino acid were blocked by forming peptide linkages. Consequently solutions of peptides and peptide mixtures are bitterer than the equimolar mixture of amino acids. Casein is relatively rich in hydrophobic amino acids and especially in proline. Peptides 61-67, 84-89, and 193-208 of α-casein, which are isolated from cheese, have been identified as extremely bitter. The latter peptides are comprised of several proline residues and are very hydrophobic and bitter in taste. The extent of bitterness is correlated with the DH, source of protein, concentration of peptides & hydrophobic amino acid, etc. This bitter taste of hydrolysates represents a technical problem that limits its utilization in the formulation of energy drinks, supplements, nutritious beverages, etc.

Removal of bitter peptides from protein hydrolysates using different matrices or extraction methods has been proposed but these methods suffer from losses of some amino acids. Several workers have done a lot of experiments to reduce the bitterness of milk protein hydrolysates, but the bitterness could not be removed efficiently for use for human nutrition. Bitterness can be reduced by solvent extraction, adsorption by activated carbons/ matrices, masking the off- or bitter flavour using microencapsulation technique, or by further hydrolysis using exoproteases such as Flavourzyme 500L, FlavorSEB-P, Prolyve 1000, etc. (Sujith and Hymavathi, 2011; Bumberger and Belitz, 1993). Microencapsulation technology increases the cost of the production as well as it may destroy the bioactive properties of the peptides. FitzGerald & O’Cuinn (2006) and Saha & Hayashi, 2001 reported that bitterness can be masked in hydrolysates by the addition of polyphosphate or specific amino acids such as aspartic and glutamic acids. Several bitter taste inhibitors have been found effective in masking the bitter taste of WPH (low and high DH) and in beverages supplemented with WPH (Leksrisompong *et al.*, 2012).

Sweeteners (sucralose, fructose and sucrose) effectively inhibited bitterness of WPH and enhanced flavour of vanilla and chocolate beverages containing these hydrolysates.

Use of exoproteases provides the advantage of reduced cost with no loss of amino acids during the processing. Exoproteases such as Flavourzyme 500L, Flavourzyme 1000L and FlavorSEB-P provides hydrolysis of the terminal peptide bonds thus freeing the hydrophobic amino acid from the N-terminus or the C-terminus resulting in the debittering of protein hydrolysates. Spellman *et al.*, (2009) compared the bitterness of whey protein hydrolysates generated by three commercially available *Bacillus* proteinases; and revealed that Alcalase 2.4L hydrolysates were bitterer than those obtained from the action of prolyve 1000 and corolase 7089 on WPC. These differences were attributed to the presence of a glutamyl endopeptidase activity in the alcalase preparation but not found in the other two enzymes (Spellman *et al*., 2009). Debittering can be mediated using exopeptidases including amino- and carboxypeptidases with special emphasis on proline exopeptidases. Overall, debittering of casein and whey protein hydrolysates can be performed by using different exoproteases under optimized substrate: enzyme ratio, pH and temperature conditions. After debittering, protein hydrolysates could further be exploited to develop nutritional beverage, dry supplements, nutraceuticals, etc.

Although optimization can be done by conventional method or Response Surface Methodology (RSM); But the use of RSM has gained prominence in optimisation and different process design owing to the ease of operation, reliability and reproducibility of the model parameters as well as the availability of a range of user-friendly computer software packages that employ complex algorithms (Kalil *et al*., 2000). Factorial design of a limited set of variables is advantageous over conventional method since such approach frequently fails to locate the optimal conditions for the process due to its failure to consider the effect of possible interactions between factors.

By taking all these factors into consideration, the present study was proposed with the following objectives:

(i) Standardization of enzymatic hydrolysis of casein and whey proteins

(ii) Process optimization for debittering of casein and whey protein hydrolysates.

(iii) Formulation of functional dairy beverage using developed non-bitter casein and whey protein hydrolysates.

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

2.1 Milk Proteins

Milk provides all the nutrients necessary for the growth and development. Milk supply macronutrients as well as immunity compounds and micronutrients (Walzem *et al.,* 2002). Milk contains a combination of two major protein groups, whey and casein; each has specific functional and nutraceutical properties. Bovine milk contains on average 3.4% protein, which is primarily 80% casein and 20% whey.

2.1.1 Casein

Casein accounts for 80% of the protein in bovine milk and has four major subgroups (αs1, αs2, β, κ), each has multiple bioactive peptides (Shag, 2000) casein is a complete protein and also contains the minerals calcium and phosphorous. It has a PDCAAS rating of 1.23 (generally reported as a truncated value of 1.0) (Deutz *et al.*, 1998). Casein exists in milk in the form of a micelle, which is a large colloidal particle. An attractive property of the casein micelle is its ability to form a gel or clot in the stomach. The ability to form this clot makes it very efficient in nutrient supply. The clot is able to provide a sustained slow release of amino acids into the blood stream, sometimes lasting for several hours (Boirie *et al.*, 1997). This provides better nitrogen retention and utilization by the body as casein is absorbed more slowly producing a much less dramatic rise in plasma amino acid concentrations.

2.1.2 Whey Protein

In recent years, milk constituents have become recognized as functional foods, suggesting their use has a direct and measurable effect on health outcomes (Marshall, 2004) contain a relatively high proportion of branched-chain amino acids (BCAA-26%) (Bos *et al.*, 2000). The high sulfur-containing amino acids content of whey proteins appears important to their ability to enhance immune function and antioxidant status via modulation of the sulfur-containing tripeptide glutathione (Bounous and Gold, 1991). Also, because whey proteins have a relative surplus of some essential amino acids (lysine, threonine, methionine, isoleucine), The abundance of leucine in whey plays a distinct role in protein metabolism and has been identified as a key signal in the translation initiation pathway of muscle protein synthesis (Anthony *et al.,*2001). Amino acids supplied by whey relative to the amino acid composition of human skeletal muscle (USDA.2001; Bergstrom *et al.,* 1966; Rasmussen *et al.,* 2000).

2.2 Hydrolysis

Protein hydrolysates are one of the extensively studied functional foods. They usually contribute to several purposes: medical diets (Schmidt *et al.,* 1994; Clemente, 2000), protein supplementation (Frokjaer, 1994; Clemente, 2000) and food use (Lahl and Braun, 1994). Because of their broad application, there have been many studies to investigate the production of protein hydrolysates from different sources. Casein (Chobert *et al.,*1988a; Gallagher *et al.,* 1994a; Morato *et al.,* 2000 ; Pedroche *et al.,* 2004), whey (Jost and Monti 1977; Smyth and FitzGerald, 1998; Doucet *et al.,* 2003a) and soy (Deeslie and Cheryan 1988; Aaslyng *et al.,*1998; Lee *et al.,* 2001) have been excellent resources while other materials, such as chickpea (Clemente *et al.,* 1999), fish (Beddows *et al.,* 1976; Diniz and Martin, 1996; Imm and Lee, 1999; Ravallec-Ple *et al.,* 2000), fish waste (Benjakul and Morrissey, 1997; Liaset *et al.,*2000; Guerard *et al.,* 2002; Liaset *et al.,* 2003; Deng *et al.,* 2004; Aspmo *et al.,* 2005; Nilsang *et al.,* 2005; Dumay *et al.,* 2006), shrimp (Simpson *et al.,* 1998; Mukhin and Novikov, 2001) and shrimp waste (Jaswal, 1989; Balogun and Akegbejo-Samsons, 1992; Cancre *et al.,* 1999; Gildberg and Stenberg, 2001; Mizani *et al.,* 2005; deHolanda and Netto, 2006 ; Ruttanapornvareesakul *et al.,* 2006; Coward-Kelly *et al.,* 2006; Cavalheiro *et al.,* 2007) have also gained popularity recently.

2.2.1 Protein Hydrolysates

Protein hydrolysates, with their improved nutritional and functional qualities, can be used as protein supplements (Frokjaer, 1994 and Clemente, 2000), medical diets (Schmidt *et al.,* 1994, Clemente, 2000) as well as general food use (Lahl and Braun, 1994). They can not only benefit patients with disorders such as digestion difficulty, absorption problems and poor amino acid metabolism, but can also help to reduce allergic reactions in hypersensitive individuals (Clemente, 2000). Table 2.1 lists some examples of dietary uses of protein hydrolysates in human nutrition**.**

Table 2.1 Examples of dietary uses of protein hydrolysates in human nutrition as protein supplements or for specific clinical conditions by Clemente (2000)

Protein Supplementation	Clinical Use
Energy drinks Geriatric products Sport nutrition Weight-control diets	Phenylketonuria (PKU) Hypoallergenic infant formula Acute and chronic liver disease Short bowel syndrome Crohn's disease Pancreatitis Ulcerative colitis

2.2.2 Type of Hydrolysis

Scientists have developed different processes to produce hydrolysates such as acidic hydrolysis, alkaline hydrolysis and enzymatic hydrolysis (Khanna and Gupta, 1996).

2.2.2.1 Acid Hydrolysis of Proteins

The first reported acid hydrolysis goes back to 1820 by Braconnot (Hill, 1965). It took several decades for commercialization and is still in practice representing one of the older processes. The governing factors like temperature, pressure, acid concentration, type of acid employed and presence of non-protein components can affect the degree of acid hydrolysis (Hill, 1965). All of these independently and combined will have an impact on quality of the product. Hydrochloric and sulfuric acid are mainly used to hydrolyze proteins, the most common being hydrochloric acid. With the acid hydrolysis some of the essential amino acids such as tryptophan, methionine, cystine and cysteine are destroyed. Further, glutamine and aspargine are converted to glutamic acid and aspartic acid (Bucci and Unlu, 2000).

2.2.2.2 Alkaline Hydrolysis of Proteins

In the food industry, alkaline hydrolysate is not for any practical use. Some amino acids like serine and threonine are destroyed during alkaline hydrolysis but tryptophan is intact. First protein is solubilized by heating followed by the addition of alkaline agents like calcium, sodium or potassium hydroxide and maintaining the temperature to a desired set point (80–130°C). The hydrolysis will be continued for several hours and then the product is evaporated, pasteurized and spray dried. So under alkaline conditions, there is formation of amino acid residues, e.g. Lysinoalanine (Cheftel *et al.*, 1985) which is not desirable.

2.2.2.3 Enzymatic Hydrolysis of Proteins

Most of the enzymes used to make protein hydrolysates are obtained from animal, plant and microbial sources. The main advantages of enzyme hydrolysis of proteins is that the hydrolysis conditions are mild and enzymes are more specific to control the degree of hydrolysis and tailor made products for the end users.

A wide variety of proteolytic enzymes are commercially available from animal, plant and microbial source. The most commonly used enzymes for protein hydrolysates from animal sources are pancreatin, trypsin, pepsin & plant sources are papain and bromelain and from fermentation sources, bacterial and fungal proteases. The hydrolysis of proteins can be achieved by a single enzymatic step or a sequential enzyme hydrolysis using multiple enzymes. The choice of enzyme depends on the protein source and end user requirements. For example, if the protein has a higher content of hydrophobic amino acids then the enzyme of choice should be the one that preferentially cleaves the hydrophobic amino acids (Adler-Nissen, 1986).

For nutritional applications, enzymatic hydrolysis is preferred over the relatively harsh chemical techniques (Kinsella, 1976). Because of mild process conditions and relative specificity of the various enzymes used, enzymatic hydrolysis produces hydrolysates with well-defined peptide profile. Chemical hydrolysis (acid and alkali) can destroy L-form of amino acids, producing D-from as well as trigger the formation of nutritionally undesirable substances such as Iysinoalanine. This degrades the nutritional quality of the resulting hydrolysates (Prendergast, 1974; Lahl and Braun, 1994).

2.2.2.4 Significant Advantage of Enzymatic Hydrolysis over Acid and Alkaline Hydrolysis of Proteins

- Requirement of small amounts of enzymes that can be easily deactivated after the hydrolysis.
- Mild operating conditions like temperature and pH

- No destruction of amino acids
- No production of chloropropanols
- Hydrolysis of certain amino acids resulting in less complex mixtures of peptides that can be relatively easily purified
- Availability of several choices of enzymes.

2.3 Enzymatic Production for Casein and Whey protein Hydrolysates

2.3.1 Factor Affecting Enzymatic Hydrolysis

Many factors need to be considered during enzymatic hydrolysis. Enzymatic hydrolysis involves the use of proteases to hydrolyze materials to smaller peptides or single amino acids. Hence, factors that can affect the activity of the chosen protease will have an impact on the hydrolysis reaction. Enzyme performance depends on pH and temperature because each protease has its own optimal operating condition at which its activity can reach the highest. Other factors such as the nature of the substrate, water-to-substrate ratio, amount of enzyme used, incubation time as well as the proteases can affect the overall performance of the hydrolysis reaction. Many studies have been carried out to investigate the optimal conditions for hydrolysates production through enzymatic hydrolysis.

Four hydrolysis parameters must be controlled to accomplish the desired degree of hydrolysis. These are: substrate concentration, enzyme-substrate ratio, pH and temperature.

2.3.1.1 Water-to-Substrate Ratio

Even though water-to-substrate ratio is not as influential as other factors in the hydrolysis reaction, several studies have documented different values indicating its potential importance in affecting hydrolytic performance. Numerous studies have shown the use of lower water-to-substrate ratios.

Mahmoud and Cordle (2000) reported that a high water-to-substrate ratio concentration of the substrate usually produce relative viscous slurry, that may contain undissolved material, which limits proper diffusion of enzyme. Also ,dispersing the substrate at relatively high concentration tend to excessive foaming that interferes with subsequent processing operations such as heat treatment and filtration of digest. On the other hand too diluted water-to-substrate ratio not ensure substrate saturation and gives inefficiently low yield. In most protein hydrolysis, the water-to-substrate ratio concentration generally ranges from 10-14% on protein basis.

2.3.1.1.1 Casein

Casein 10-12% (Khanna and Gupta ,1991;Mohamed *et al.,* 1992; Damle *et al.,* 2010; Zhao *et al.*, 2006; Slattery & Fitzgerald ,1998) 8% (Wang *et al.,* 2013) 6% (Cogan , 1981) 5 % (Pralea *et al.,* 2011; Nishiwaki *et al.*, 2002) 4% (Minagawa *et al.,* 1989) 2.5% (Umetsu *et al.*, 1983) 8% (Wang, 2013)

2.3.1.1.2 Whey protein

Whey protein 10% (Sinha *et al.,* 2007; Silvestre *et al.,*2012 and Leksrisompong *et al.,* 2012;) 5% (Silva *et al.,* 2007 ; Nakamura *et al.,* 1993; Sindayikengera *et al.*, 2006; Guadix *et al.*, 2006;) 1% (Maehashia *et al.,* 2008).

The value of water-to-substrate ratio used can relate to the nature of the substrate. However, none of these work looked at the effect of substrate concentration on the quality of final hydrolysate products.

2.3.1.2 Heat Treatment of Protein

2.3.1.2.1 Heat Treatment of Sodium Caseinate

Gaucheron *et al.* (2001) studied the proteolysis of sodium caseinate solution (24.5 g/l) induced by heat treatment at 120°C at different pH Increase in proteolysis, deduced from the increase in soluble nitrogen content, was observed with heating time (10, 20 and 30 min) and pH (6.0, 7.0, 8.0 and 9.0). Result show that the release of peptides containing tryptophan was minimal at pH 7.0. And found, eighteen low molar mass peptides were characterized, of which four came from κ-casein, nine from β-casein and five from α_{s1}-casein. Peptides were preferentially released under alkaline conditions.

2.3.1.2.2 Heat Treatment of Whey Proteins

Whey proteins, which consist principally of β-lactoglobulin, α-lactalbumin and bovine serum albumin, have globular structures. Upon heat treatment above 70°C, these proteins unfold and aggregate (Sava *et al.,* 2005; Singh and Creamer, 1991). β-lactoglobulin (β-LG) is the main protein in whey, constituting about 50% of the total whey proteins in bovine milk. Many authors consider β-LG responsible for the allergenic reaction because of its absence in human milk (Guo *et al.,* 1994). Native protein is not hydrolyzed easily by pepsin, but is susceptible for chymotrypsin and trypsin hydrolysis, under non denaturing conditions (Phillips *et al.,* 1994). The relative resistance to proteolysis is generally explained by the compact tertiary structure of the protein that protects most of the enzyme susceptible peptide bonds. Physical and/or chemical denaturation of β-LG generally leads to a higher rate of hydrolysis by various enzymes (Reddy *et al.,* 1988; Iung *et al.,* 1991; Dufour *et al.,* 1996; Chobert *et al.*, 1995; Stapelfeldt *et al.,* 1996; Maynard *et al.*, 1998; Kananen *et al.,* 2000; Sitohy *et al.,* 2001, Stanciuc *et al.*, 2008). After denaturation, the β-LG conforms may represent the ideal substrates for the proteases, because ample regions of the hydrophobic protein core are unfolded and accessible for the enzyme, contrarily to what happens in the native form or in the aggregated products of extensive thermal denaturation (Iametti *et al.,* 2002).

Jost and Monti (1977) however reported that Pre denaturation of the WPC by heat did not improve digestibility by trypsin.

Lin *et al*. (2012) studied that pepsin, trypsin, alcalase and flavourzyme enzymatic hydrolysis reaction and preheat treatment of WPC at 95°C for 5 or 10min. increased DH in all experiment.

2.3.1.3 Temperature and pH

Sufficient evidence has suggested temperature and pH are essential parameters to consider for hydrolysis conditions. Temperature and pH adjustments should be maintain throughout process. Depending on the nature of the substrate, the pH may need to be adjusted by acid or base to reach the optimal pH of the protease.

2.3.1.3.1 Temperature and pH for Casein Hydrolysis

Temperature and pH are important factors affecting the activity of the protease in casein hydrolysis. Several studies have looked at the temperature and pH effect on casein hydrolysate production.

Jost and Monti, (1977) reported that digestion at pH 8.0 and 55°C for approximately 3 h, at an enzyme -substrate ratio of 1:100. Under these conditions, 500 μmoles of titratable protons were liberated per g of substrate in the course of the reaction. Digestion at 40°C generated only about 400 μmoles of acid.

Loosen *et al*. (1991) and Chataud *et al*. (1987, 1988) study support the employment of temperatures as high as 50°C for preparing peptide rich hydrolysates using subtilisin.

Morato *et al.* (2000) also studied the effect of temperature on the isolated action of subtilisin over casein. Changing the temperature from 40 to 50°C , increased the large (from 14 to 21%) and medium (from 46 to 61%) peptide contents, and also decreased the amount of di- and tri peptides (from 36 to 12%), while no important change was observed in the level of free amino acid .

Barbosa *et al.* (2004) prepared casein hydrolysates using temperature of 37°C and an E:S ratio of 2% and conclude it was that probably the most economical condition for use in large-scale manufacture.

This behavior can probably be explained by the charge of the amino acid residues on the enzyme and the casein molecules indifferent pH values affecting their binding and the availability of peptide bonds to the enzyme attack and consequently the issued products.

2.3.1.3.2 Temperature and pH for WPC Hydrolysis

Custódio *et al.* (2005) studied that optimum hydrolysis conditions for cheese whey proteins was at 60ºC and pH 9.0 for trypsin nor chymotrypsin alone hydrolyzed whey proteins in less than 3 h; rates of hydrolysis of α-La by trypsin, and of BSA by chymotrypsin were both very low.

Mota *et al.* (2006) reported that hydrolysis of whey protein concentrates (WPCs) using trypsin under different combinations of temperatures and pH values in three experiments, namely experiment A at 37°C and pH 8, experiment B at 37°C and pH 9, and experiment C at 50°C and pH 8 ,difference was observed between rate of hydrolysis of α-lactalbumin and β-lactoglobulin; in the former case hydrolysis was complete by 15min in experiments B and C and by 120 min in experiment A.

Britten *et al.* (1994) studied whey protein isolate modified by proteolysis using protease in combination with heat treatment of the hydrolysate. Half of β-lactoglobulin content was hydrolyzed when DH reached 5.1%. α-lactalbumin and BSA were not hydrolyzed by the enzyme. Heating of the hydrolysate resulted in the formation of small and large aggregates. Whey protein hydrolysis at a degree of hydrolysis of 1.7%, followed by heating at pH 8.0, resulted in the highest amount of accessible sulfhydryl groups, due to unfolded structure of the aggregates. Averaged hydrolysate solubility at pH 7 was 98% when pH during heating was 4.0 or 8.0. Solubility of the hydrolysate at pH 4.5 was higher when pH during heating was adjusted to 4.0. Solubility of the hydrolysate heated at pH 6.0 and 8.0 improved with degree of hydrolysis > 1.7%.

Jost and Monti, (1977) also studied hydrolysed whey protein with trypsin at pH 8.0 at 55°C to increase the solubility of whey protein as well as heat denatured whey protein. Also Wroblewska *et al.* (2008) use different proteinase Alcalase 2.4L FG and cross-linked transglutaminase and reported that WPC hydrolysate obtained with Alcalase contained proteins and peptides characterized mostly by low molecular weight peptides (MW < 14.4 kDa) in the pH range of 3-10.

2.3.1.4 Protease

In choosing proteases for use in food processing, pH optimum, the heat stability, the presence of activators or inhibitors, the cost and availability, and the specificity of the enzyme are all crucial factors to consider.

2.3.1.4.1 Proteases Used and their Specificities

Proteases refer to a group of enzymes whose catalytic function is to hydrolyze (breakdown) proteins. Proteases are separated into two types: Endo-protease and Exo-protease. Endopeptidases hydrolyze specific peptide bond within the polypeptide chain, exopeptidase catalyze the formation of free amino acids or small peptides from the N-terminal or C-terminal end of polypeptide substrate.

2.3.1.4.2 Endo-protease

The activity of a protease is determined by whether it hydrolyzes a particular protein, that cleave the peptide bonds within the interior of the protein molecule (endopeptidases), resulting in smaller peptide chains, are most widely used. The catalytic efficiency of the protease is determined by the rate at which it hydrolyzes under standard pH, temperature and enzyme concentration (Whitaker, 2003). The proteins in enzymatic hydrolysates are only partially hydrolyzed due to the inability of most proteases to cleave glycoproteins, phosphoproteins, or protein domains that contain numerous disulfide bridges (Gibbs *et al.,* 2004).

However, they are occasionally combined with exopeptidase (cleave single amino acids or dipeptides from either N or C terminus of the protein) to accomplish a more thorough and rapid hydrolysis. This becomes particularly important in the case of extensive hydrolysis for the production of hypoallergenic protein hydrolysates. Most commercial protease preparations contain a mixture of enzymes with endo- and exopeptidase activity.

There are many ways to categorize proteases as there are enzymes themselves. A simple classification is based on the protease source: animal, plant or microbial; or the pH range where the enzyme exhibits its optimum activity such as acid, neutral or alkaline proteases. More sophisticated methods of classification are based on the nature of the catalytic residue involved in the nucleophilic attack at the carbonyl carbon of the scissile bond. Accordingly, there are four groups of proteinases. These are: serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23) and metalloproteases (EC 3.4.24) (Wong, 1995; Howell, 1996).

2.3.1.4.2.1 Aspartic

Aspartic proteases derived from fungal sources *(Aspergillus, Rhizopus, Mucor, Penicillium spp.)* have carboxylic group from aspartic acid in the active site, tend to have maximum activity at acidic pH range (2-6) and exhibit broad specificity. Pepsin obtained from swine or bovine stomach, is also an aspartic protease with a typical pH activity range of 1-4, and has broad specificity but preference for splitting peptide bonds near phenylalanine and leucine. Because of the nature of their acidic pH requirements, fungal protease as well as pepsin is not commonly used for producing dietetic protein hydrolysates.

2.3.1.4.2.2 Cysteine

Plant proteases such as papain (from papaya), bromelain (from pineapple), and ficin (from fig) are cysteine proteases. They contain-SH in the active site, exhibit maximum activity near neutral pH (5-8) and hydrolyze a wide range of peptide bonds (Wong, 1995).

2.3.1.4.2.3 Serine

Bacterial proteases (from *Bacillus sp.)* such as Alcalase and Subtilisin are serine proteases. These enzymes have maximum activity at alkaline pH (6-10) with broad specificity but preference for peptide bonds adjacent to hydrophobic or aromatic amino acids. One of the most commonly used protease preparations for manufacturing dietetic protein hydrolysates, particularly for hypoallergenic infant formulas, is pancreatin. This enzyme preparation, obtained from swine or bovine pancreas glands, is a mixture of trypsin, chymotrypsin, elastase and carboxypeptidase A or B with both endo-and exopeptidase activities. Trypsin is highly specific for hydrolyzing the peptide bonds linking the carboxylic groups of lysine and arginine, while chymotrypsin is specific for cleaving the peptide bonds of the aromatic amino acids tryptophan, phenylalanine and tyrosine (Turgeon and Gauthier, 1990).

2.3.1.4.2.4 Metalloproteases

Metallopeptidases or metalloproteinase represent an extensive class of hydrolases .Most metalloproteases are characterized by a catalytic zinc ion. However, in some enzymes this function is undertaken by manganese, cobalt, nickel or even copper ions. In some metalloproteases two metal ions act co-catalytically. The metal ion is complexed by three conserved amino acid residues that can be His, Asp, Glu or Lys.

The selection of a given protease or a mixture of proteases for manufacturing protein as nutritional ingredient is primarily based on the relative degree of specificity of the protease(s) used, the desired extent of hydrolysis as well as the intended use of the resulting hydrolysate. Other considerations include regulatory approval for food use, enzyme cost and the need for the final product to meet special dietary rules.

2.3.1.4.3 Commercial Food Grade Endo-enzyme

Several food-grade enzyme preparations have evidence suggesting their efficiency in the enzymatic hydrolytic process are Alcalase® ,Trypsin , Flavourzyme® , Kojizyme™ , Neutrase® , papain , Protamex® have all demonstrated their abilities in producing protein hydrolysates.

2.3.1.4.3. 1 Alcalase 2.4L ®

Alcalase® 2.4 L FG (E.C. 3.4.21.62), also called subtilisin, is a serine protease derived from *Bacillus licheniformi* (Novozymes A/S 2001a). It has an activity of 2.4 Anson Units per gram (AU/g) and functions best at pH 8.0 - 8.5 and at temperature 55 – 60°C (Novozymes A/S 2001a). The preferential cleavage sites of this enzyme have been suggested to be at hydrophobic side chains near the carboxyl side and Doucet *et al.* (2003a) have shown that this enzyme has specificity for Phe, Trp, Tyr, Glu, Met, Leu, Ala, Ser and Lys residues. Alcalase has been used in different types of hydrolysis reactions mainly because of its ability to produce smaller peptides in comparison to other commercially available enzymes (Smyth and FitzGerald, 1998; Aspmo *et al.,* 2005).

2.3.1.4.3.2 Papain

Papain (3.4.22.2), is a Cysteine protease derived from papaya latex. It has an activity of 1.0 Anson Units per gram (AU/g) and functions best at pH 7.0-9.0 and at temperature 50 - 60°C (Sangeetha and Abraham, 2006; Wroblewska and Troszyñska, 2005). The preferential cleavage sites of this enzyme have been suggested to be at hydrophobic side chains near the carboxyl side and Doucet *et al.* (2003b) have shown that this enzyme has specificity for Arg, Lys and Phe-X residues. Papain has been used in different types of hydrolysis reactions mainly because of its ability to produce a wide range of peptide in comparison to other commercially available.

2.3.1.4.3.3 Trypsin

Trypsin (3.4.21.4) is a serine protease derived from Bovine pancreatic (Novozymes). It has an activity of 2.4 Anson Units per gram (AU/g) and functions best at pH 7.0 – 9.0 and at temperature 55-60°C (Novozymes A/S 2001a). The preferential cleavage sites of this enzyme have been suggested to be at hydrophobic side chains near the carboxyl side and Srinivasan, (2012) have shown that this enzyme has specificity for Arg and Lys residues. Trypsin has been used in different types of hydrolysis reactions mainly because of its ability to produce partly or extensively hydrolysates in comparison to other commercially available enzymes).

2.3.1.4.3.4 Endo-protease used in casein hydrolysis

Hydrolysates produced by pepsin were resolved into three main fractions of high retention times, while trypsin hydrolysates were resolved into nine major peaks and Alcalase® hydrolysates were resolved into 12 major peaks, presenting a wide range of polarities and sizes.

Trypsin enzyme to substrate [E/S] 2500 U/g was used for casein hydrolysates. Enzymatic hydrolysis was carried out at constant pH 8.0 (Wang *et al.*, 2013). Casein and whey protein were treated with trypsin shows increase emulsifying and solubility. Increase solubility of casein hydrolysates at pH 4.0-5.0 and whey hydrolysates at all pH. Emulsifying capacity of casein hydrolysates was increased at isoelectric point, while whey protein hydrolysates Emulsifying capacity was increase above pH-7 (Chobert *et al.* 1988ab).

According to Ribadeau-Dumas *et al.* (1972) and Mercier *et at.,* (1973), several subtilisin potential cleavage points may be found on the casein molecule and many structural factors are involved in the hydrolysis process.

2.3.1.4.3.4 Endo-protease Used in Whey hydrolysis

Lin *et al.* (2012) reported that, analyzed of WPH by polyacrylamide gel electrophoresis and high performance liquid chromatography indicated that the α-La was hydrolyzed completely by pepsin, trypsin and alcalase and was resistant to flavourzyme to some extent; β-lactoglobulin was only completely hydrolyzed by trypsin and alcalase.

Pintado (1999) studied that Hydrolysis with Trypsin led to release of high amounts of Lys. Quantitative depletion of b-lactoglobulin was observed by 2 h and hydrolysis of a-lactalbumin was slower when Trypsin was employed. Formation of peptides was more extensive under the action of Trypsin than of Protease 2A, and the major peptides released by the former had molecular weights mainly in the ranges 7500±8000 and 4000±4500 Da.

Shin *et al.(*2007) studied that when four enzyme combinations (1:1) trypsin + papain W-40 (TP), trypsin + neutrase 1.5 (TN), papain W-40 + protease S (PP) and papain W-40 + neutrase 1.5 (PN) were added at the rate of 1% of the cow whey protein (CWP) and it was incubated for 15, 30, 60, 90, 120 and 180 min at 50°C. The lowest antigenicity in CWP hydrolysates was observed with the use of trypsin-containing enzyme mixtures compared with other enzyme combinations.

2.3.1.5 Enzyme Substrate Ratio

The amount of proteases in a hydrolytic reaction can dramatically affect the final products. Enzymes act as cleaving agents in the hydrolysis reaction to break down protein molecules into smaller peptides or single amino acids. An increase in the amount of protease added will increase the efficiency of the hydrolysis.

2.3.1.5.1 Enzyme Substrate Ratio for Casein

Morais *et al.* (2004) claimed that use of papain enzyme for casein hydrolysis at temperature of 37°C and E:S ratio 2% is probably the most economical condition for production. A casein hydrolysate with 75% of di- and tripeptide and less than 5% of free amino acids was prepared by Loosen *et al.* (1991), by employing the subtilisin in a 2% E:S ratio.

2.3.1.5.2 Enzyme Substrate Ratio for Whey

Kim *et al.* (2007) reported that *β*-lactoglobulin in heated WPC was partially hydrolyzed by the 0.1 and 0.5% pepsin and trypsin treatments and was completely degraded by the 1% pepsin and trypsin treatment.

Morato *et al.* (2000) studied subtilisin and reported that two-fold increase of E: S ratio from 2 to 4% , changed significantly (P <0.05) the composition of all fractions, producing a decrease in the large peptide (from 30 to 14%) and free amino acid (from 16 to 4%) contents, and also an increase of medium peptide (from 31 to 46%) and di- and tripeptide (from 23 to 36%) contents, but no nutritional advantage was observed by the two-fold increase in the E :S ratio of subtilisin from 2 to 4% and of trypsin from 10 to 20% Thus, it can be inferred that this increase in enzyme concentration produced no further hydrolysis of the protein molecule.

De-Souza *et al.* (2008) studied on the effect of the enzyme- to-substrate ratio (E/S) of 0.01, 0.02 & 0.03 on the hydrolysis of whey protein. They found that 0.01(E/S) more advantageous for the nutritional point of view.

Even though increase in enzyme-to-substrate ratio often offers higher degree of hydrolysis and higher recovery of soluble matters, a high amount of proteases will imply a higher cost of production. Therefore, it is necessary to maintain a balance between the amount of protease used and the desired level of hydrolysis to be achieved to keep a low cost of production.

2.3.1.6 Time of Hydrolysis

Time of hydrolysis is another widely studied factor in controlling hydrolysis reaction. A long hydrolysis time will lead to the breakdown of protein molecules into smaller peptides and single amino acid.

2.3.1.6.1 Time of Hydrolysis for Casein

Wang *et al.* (2013) studied that degree of hydrolysis with Trypsin in the casein hydrolysates increased rapidly in the initial 20 minutes, reached a plateau after 45 minutes, and then kept relative constant for the rest of the hydrolysis.

Mahmoud *et al.* (1992) studied that casein was enzymatically hydrolyzed for potential use in a hypoallergenic infant formula. Casein digest at different hydrolysis time intervals were tested for antigenicity, hydrophobicity, net charge (zeta potential), and emulsifying activity. Casein antigenicity loss could be approximated by three-phased first-order kinetics. Extensive antigenicity loss occurred during the first 10% of hydrolysis time and relatively small changes occurred during the remaining 90% of hydrolysis time. Hydrophobicity and emulsifying activity decreased, while the magnitude of the net charge increased with increasing degree of hydrolysis.

2.3.1.6.2 Time of Hydrolysis for Whey Protein

Pintado *et al.* (1999) studied that hydrolysis of whole whey with Trypsin for 12 h, depletion of β-lactoglobulin was observed by 2 h under all processing conditions, and hydrolysis of α-lactalbumin was slow.

Kim *et al.* (2007) studied that the *α*-lactalbumin in native WPC was almost completely hydrolyzed within 60 min of incubation with 0.5% pepsin and then with 0.5% trypsin

Li-jun *et al.* (2008) also studied that enzymatic hydrolysis and molecular weight (MW) distribution of whey protein hydrolysates were investigated. Whey protein hydrolysates were first gained by the alkaline protease alcalase for 7 h at temperature (50°C), ranged from 300 to 1400 Da, and most of whey peptide was under 1000 Da.

However, similar to percent enzyme, incubation time involves energy expenditure so investigation on the hydrolysis time required to produce the products is crucial**.**

2.3.1.7 Degree of Hydrolysis

The degree of hydrolysis (DH) plays an important role in predicting bitter taste, with higher percentage of low molecular weight peptides contributing to higher bitter taste intensity (Ziajka and Dzwolak, 1999). Larger peptides can block hydrophobic sites by hydrophobic interactions forming U-shaped peptides or clusters of peptides and are thus not perceived as bitter (Adler-Nissen, 1979). Further degradation of these peptides into smaller peptides increases the bitter taste intensity. Extensive hydrolysis leads to free amino acids or terminal hydrophobic amino acids, which can result in decrease of bitterness (Adler-Nissen, 1979).

The degree of hydrolysis (DH) is used to represent the extent of hydrolysis during a catalytic reaction. DH represents the percentage of peptide bonds cleaved during the hydrolysis reaction and it can be described using the following formula:

$$DH = h/h_{tot}*100\%$$

Where h is called the hydrolysis equivalent expressed as mill equivalent per gram of protein which represents the number of peptide bonds cleaved during a hydrolytic process while h_{tot} can be determined by calculating the amount of free amino acids per gram of protein (Adler-Nissen, 1986).

2.3.1.7.1 Degree of Hydrolysis of Casein

Wang *et al.* (2013) 8% (w/v) studied that solution of casein was incubated at water bath at 40°C for 10 min. then trypsin with enzyme to substrate (E/S) 2500U/g was added. Enzymatic hydrolysis was carried out at constant pH 8.0. DH increased rapidly in the first 20 min, due to high protein content and enzyme concentration. The DH reached a plateau after 45 min (DH = 45.2%) and kept relative constant for the rest of the hydrolysis reaction.

2.3.1.7.2 Degree of Hydrolysis of Whey Protein

Kim *et al.* (2007) reported that when WPC (2% protein solution) was heated for 10 min at100°C and then incubated with 2% each of Alcalase, Flavourzyme, papain, and trypsin for 30, 60, 90, 120, 150, 180, and 240 min at 50°C The highest hydrolysis of WPC was observed after 240 min of incubation with Alcalase (12.4%), followed by Flavourzyme (12.0%), trypsin (10.4%), and papain (8.53%).

Kim *et al.* (2007) reported that two step hydrolysis of WPC was increased with an increasing level of enzymes and higher incubation times. The highest hydrolysis (25.23%) was observed in heated WPC incubated with 1% pepsin and then with 1% trypsin for 120 min.

Li-jun *et al.* (2008) reported that whey protein hydrolysis at pH (8.0) and E/S (3%) show dissociative amino acid content was 56.09 mmol/mL with the hydrolysis degree of 20.04%.

Pintado *et al.* (1999) studied that the DH (8%) was highest for the higher concentration of trypsin E/S 4% and lowest DH (7%) for the lower concentration of Trypsin E/S 2%. The degree of hydrolysis by Trypsin tended to level off by 2 h, and no further increase in DH with time.

Mutilangi *et al.* (1996) reported that heat-denatured whey protein isolate was hydrolyzed with trypsin, α-chymotrypsin, Alcalase or Neutrase to 2.8, 4.3, 6.0 or 8.0% degree of hydrolysis for Functional Properties.

2.4 Production of Bitter Peptides

Protein hydrolysates frequently exhibit bitter taste caused by some peptide products. This problem limits the utilization of proteases as processing aids in the food industry (Bumberger and Belitz, 1993). Most proteins, in intact forms, do not give a bitter sensation. The reason is that the hydrophobic side chains are usually buried in the interior part of the protein due to hydrophobic interaction. The stereochemistry, hydrophobicity as well as size of the peptides, are considered crucial to the sensation of bitterness (Ramos de Armas *et al.,* 2004). As a result of hydrolysis, protein molecules are degraded, exposing the hydrophobic side chains to interact with the taste buds and generate a bitter taste. Peptides have been shown to be responsible for the elicitation of bitter taste in protein hydrolysates (Aubes-Dufau *et al.,* 1995; Lee and Warthesen , 1996; Kim *et al.,* 1999; Maehashi *et al.,* 1999; Kim *et al.,* 2003; Cho *et al.,* 2004). During the hydrolytic process, the protein molecules are hydrolyzed into smaller peptide chains or even single amino acids while at the same time, bitterness is gradually elicited and enhanced during the process.

2.4.1 Bitterness in Casein Hydrolysates

The bitter taste of extensively hydrolyzed casein has been reported (Matoba *et al.,* 1970; Clegg *et al.,* 1974). The bitter flavor of the peptides was attributed to their hydrophobic amino acid content. Murray and Baker (1952) found that the hydrolysis of casein with various proteolytic enzymes brought about an increase in bitter taste. Furthermore, many bitter taste have been reported by different worker from enzymatic hydrolysates of casein (Carr *et al.,* 1956; Clegg *et al.,* 1974; Gordon and Speck, 1965; Belitz and Sparrer, 1971; Minamiura *et al.,* 1972; Pelissier *et al.,* 1974)

Hydrolyzed casein is also used as a protein source in infant formulas to produce hypoallergenic food for babies with cow's milk allergies, which tend to be induced by intact proteins and not derivative peptides reviewed in (Hays and wood, 2005; Host and Halken, 2004; Osborn and Sinn, 2003). A major problem with using hydrolyzed casein is its bitter flavour (Pedrosa *et al., 2006*; Sawatzki *et al., 1994*). Infants older than 4 months, like older children and adults, strongly reject hydrolysate formulas on their first presentation (Mennella *et al., 1996*; Mennella *et al., 2004)*,.Likely, this negative response is due to the bitter and sour tastes and the unappealing smell of the hydrolysate formulas

2.4.2 Bitterness in Whey Protein Hydrolysates

Whey protein hydrolysates (WPH) are used as an ingredient in many food applications for their biological properties and functional properties. However, usage limitations lie in the flavor and bitter taste of WPH (Pedrosa *et al.,* 2006; Leksrisompong *et al.,* 2010). Leksrisompong *et al.* (2010) characterized the flavor of WPH and reported that key sensory attributes in WPH were potato/brothy, malty, and animal flavors and bitter taste. Bitter taste negatively impact consumer acceptability of protein fortified foods such as protein bars and beverages (Childs *et al.,* 2008). Thus, it is crucial to minimize bitter taste contributed by functional ingredients. Bitter taste associated with WPH and other hydrolyzed proteins are caused by generation of smaller peptide chains during the enzymatic hydrolysis processes (Maehashi and Huang, 2009). Many parameters impact bitter taste intensity of a peptide, from the native protein sequence itself, peptide sequences, to the processing/enzymatic treatment steps. Hydrophobicity also plays a major role in bitter taste (Ishibashi *et al.,* 1987a, 1988a). Other parameters such as degree of hydrolysis, concentration and location of bitter taste residues, number of carbons on the R group of branched chain amino acids, and conformation of amino acids have been associated with bitterness of peptides (Shinoda *et al.,* 1985; Ishibashi *et al.,* 1987a, 1987b, 1988a, 1988b; Ohyama *et al.,* 1988). Many methods can be applied to hydrolysates to reduce the bitter taste of peptides. Removal of hydrophobic peptides, the key determinant of bitter taste, by enzymatic hydrolysis (Komai *et al.*,2007) or processing methods reduce bitter taste in protein hydrolysates (Ziajka *et al.*,1994; Cheison *et al.*,2007).

2.4.3 Debittering of casein and whey protein hydrolysates

Casein and whey hydrolysates manufactured by enzymatic process are clam to be more superior to their counterparts, viz., acid and alkaline hydrolysates. However, bitterness problem is the main obstacle for their practical use in food preparation (Carr *et al.,* 1956; Clegg *et al.,* 1974a; Gordon and Speck, 1965; Belitz and Sparrer, 1971; Minamiura *et al.,* 1972; Pelissier *et al.,* 1974; Pedrosa *et al.,* 2006; Leksrisompong *et al.,* 2010; Leksrisompong *et al.,* 2012)

2.4.3.1 Methods for Debittering

Deferent methods are used for debittering of casein and whey protein hydrolysates to preventing either the formation or bitter peptides or removing them if present.

2.4.3.1.1 Carbon Treatment

Peptides and amino acids removed when Carbon treatment applied to protein hydrolysates (Silvestre *et al*., 2009).Treatment of the enzymic hydrolysate with activated charcoal reduction of 63 % in tryptophan content and of *36* %in phenylalanine content resulted in a product of markedly inferior quality of casein hydrolysates (Cogan *et* *al.*,1981).The content of tryptophan and phenylalanine in casein can be regarded nutritionally adequate for the growing rat but there is no excess of these two amino acids (Rama-Rao *et al.*,1959; Orr and Watt., 1957).

Khanna and Gupta (1996) reported that the bitterness was removed using activated carbon treatment. Minimum 15% activated carbon treatment was necessary for debittering the casein hydrolysate, though this treatment resulted in 40.90% N loss through adsorption on activated carbon. The yield and recovery of liquid casein hydrolysate were 47.98% and 46.23%, respectively

2.4.3.1.2 Enzymatic Treatment (Exo-proteases)

Enzymatic treatment involves the use of Exo proteases enzymes to aid in producing non bitter hydrolysates. Some enzymes, like, aminopeptidase, alkaline and neutral protease, carboxypeptidase, have been suggested to reduce the bitterness of protein hydrolysates (Saha and Hayashi, 2001). Studies performed by Minagawa *et al.* (1989); Izawa *et al.* (1997) explored the use of aminopeptidase to lower the bitterness in protein hydrolysates. The multi stage enzymatic hydrolysis method involves the use of two or more enzymes to hydrolyze the protein and a combination of endopeptidases and exopeptidases are usually used. Endopeptidases cleave peptide bonds in the interior part of the chain; exopeptidases, on the other hand, prefer to hydrolyze terminal peptide bonds (Hanson *et al.*, 1966). This combination methodology will ensure a considerable degree of hydrolysis and a selective cleavage of hydrophobic amino acids by specific exopeptidases; therefore, the resulting hydrolysate products will contain minimal bitter tastes.

Treatment of the hydrolysates with Exo-proteases has found more economic, nutritive, safe promising and most effective in the elimination of bitter taste which develops during the course of hydrolysis (Thiansilakul *et al.*, 200; Damle *et al.*, 2010; Izawa *et al.*, 1997; Fernandez-Espia and Rul, 1999).

2.4.3.1.3 Classification of Exopeptidases

The exopeptidases require a free N-terminal amino group, C-terminal carboxyl group or both, and hydrolyse a bond not more than three residues from the terminus. The exopeptidases are further divided into aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, tripeptidylpeptidases and dipeptidases. There are no known exopeptidases that are aspartic or glumatic peptidases.

2.4.3.1.3.1. Aminopeptidases.

An aminopeptidase liberates a single amino acid residue from the unblocked N-terminus of its substrate.

2.4.3.1.3.1.1 Dipeptidases.

A dipeptidase hydrolyses a dipeptide, and requires that both termini be free: Xaa + Xaa. Examples are dipeptidase A and membrane dipeptidases.

2.4.3.1.3.1.2 Dipeptidyl-peptidases.

A dipeptidyl-peptidase hydrolyses a dipeptidyl bond, *i.e.* it releases an N-terminal dipeptide from its substrate: dipeptide + peptide (*i.e.* (Xaa)2 + (Xaa)n. These enzymes are sometimes erroneously called aminopeptidases or dipeptidases.

Examples are dipeptidyl-peptidase I and dipeptidylpeptidase III.

2.4.3.1.3.1.3 Tripeptidyl-peptidases.

A tripeptidyl-peptidase hydrolyses a tripeptidyl bond, releasing a tripeptide from the N-terminus of its substrate: tripeptide + peptide (*i.e.* $(Xaa)^3$ + (Xaa)n.

2.4.3.1.3.1.4 Peptidyl-dipeptidases.

A peptidyl-dipeptidases hydrolyses a dipeptide from the Cterminus of its substrate: peptide + dipeptide (*i.e.* (Xaa)n+ (Xaa)2.

2.4.3.1.3.1.5 Carboxypeptidases.

A carboxypeptidase hydrolyses a single residue from the unblocked C-terminus of its substrate: peptide + Xaa (or more precisely: (Xaa)n + Xaa). Examples are carboxypeptidase A1 (M14.001), cathepsin X (C01.013) and carboxypeptidase Y.

2.4.3.1.3.1.6 Other Terminologies

Several other terms have been introduced for peptidases. The commonest of these extra terms is tripeptidase. A tripeptidase is a peptidase that is known only to degrade a tripeptide; however, the known tripeptidases are specialized aminopeptidases that release an N-terminal amino acid and a dipeptide and are consequently also known as “aminotripeptidases”. An example is peptidaseT (M20.003)

2.4.3.1.3.2 Flavourzyme®

2.4.3.1.3.2.1 Flavourzyme® 1000 L

Flavourzyme® 1000 L is an exopeptidase/exopeptidase complex derived from the fungus *Aspergillus oryzae* and it has an activity of 1000 Leucine Amino Peptidase Unit per gram (LAPU/g) (Novozymes A/S 2001b). This enzyme preparation contains several exopeptidases and endopeptidases that are active at different pH. The endoproteases have both neutral and acid proteases, giving a broad operating pH (Pommer, 1995). Flavourzyme's optimal pH and temperature are at 5.5-7.5 and 50-55°C, respectively (Novozymes A/S 2001b). In contrast to other proteases, it functions better at a natural drifting pH because different exopeptidases in the complex will be activated at different pH making the hydrolysis more efficient (Pommer 1995; Imm and Lee 1999). This nature of the enzyme preparation not only eliminates the trouble of adjusting the pH during the hydrolytic reaction, but also produces non-bitter hydrolysates with very diverse peptide mixtures which are mostly small in size (Aaslyng *et al.,* 1998 and Novozymes A/S 2001b).

2.4.3.1.3.2.2 Flavourzyme® 500 L

Flavourzyme® 500 L is an exopeptidase/endopeptidases complex derived from the fungus *Aspergillus oryzae* and it has Leucine Amino Peptidase Unit per gram (LAPU/g). This enzyme preparation contains five exopeptidases and four endopeptidases that are active at different pH. The endoproteases have both neutral and acid proteases, giving a broad operating pH. Flavourzyme's optimal pH and temperature are at 4-10 and 50-55°C, respectively. In contrast to other proteases, it functions better at a natural drifting pH because different exopeptidases in the complex will be activated at different pH s making the hydrolysis more efficient. This nature of the enzyme preparation is to produces non-bitter hydrolysates with very diverse peptide mixtures which are mostly small in size (Aaslyng *et al.,* 1998; Novozymes A/S 2001b). Flavourzyme 500 L inactivated at 75°C for 10 minutes

2.4.3.1.3.2.3 FlavourSEB-P

FlavourSEB-P is an exopeptidase/exopeptidase complex derived from the fungus *Aspergillus oryzae* and it has an activity of 1000 Leucine Amino Peptidase Unit per gram (LAPU/g) . This enzyme preparation contains several exopeptidases and endopeptidases that are active at different pH. The endoproteases have both neutral and acid proteases, giving a broad operating pH. FlavourSEB-P optimal pH and temperature are at 7-9 and 55-65°C, respectively. In contrast to other proteases, it functions better at a natural drifting pH because different exopeptidases in the complex will be activated at different pH making the hydrolysis more efficient. This nature of the enzyme preparation to produces non-bitter hydrolysates with very diverse peptide mixtures which are mostly small in size. FlavouSEB inactivated at 75°C for 10 minutes

2.4.4 Debittering of Hydrolysates with Exopeptidases

Yamamoto, (1975) observed that individual endopeptidases like pepsin, papain, trypsin and pancreatin do not split all or even a majority of the peptide bonds in a protein system leading to the formation of bulky, hydrophobic side chains which give bitter taste. Exopeptidases like carboxypeptidases A and Leucine aminopeptidase are reported to hydrolyse carboxyl and amino terminal amino acids of such peptides, thus eliminating the bitter taste.

Minagawa *et al.* (1989) also observed that bitter peptide fraction present in casein hydrolysates obtained by using three proteases (subtilisin, papain and trypsin) was treated with aminopeptidase T from *Thermus aquaticus* YT-1. The bitterness of the bitter peptide fraction could be decreased, and it sometimes disappeared completely, with an increase in free amino acids. The percentages of total free amino acids released from each bitter peptide fraction (subtilisin, papain and trypsin) by aminopeptidase digestion for 20 hr were approximately 11%, 8.7%, and 6.5%, respectively.

Minamiura *el al.* (1972) reported that an aminopeptidase isolated from pronase to be more effective than a carboxypeptidases (Sigma Chemical Co.) in the breakdown of the bitter peptides of o proteolytic hydrolysates of casein. Aminopeptidase hydrolyzes dipeptides more rapidly than carboxypeptidases.

Minagawa *et al.* (1989) studied the debittering mechanism in bitter peptides of enzymatic hydrolysates from milk casein. The bitter peptide fraction present in casein hydrolysates obtained by using three protease (subtilisin, papain and trypsin) was treated with aminopeptidase T, an exopeptidase. The bitterness of bitter peptide fraction could be decreased, with an increase in free amino acids.

Thiansilakul *et al.* (2006) analyzed the composition and functional properties of a protein hydrolysates prepared from defatted round scads using flavourzyme, with a degree of hydrolysis of 60%. The protein hydrolysates had a high protein content (48%) and had arginine and lysine as dominant amino acids.

Damle *et al.* (201 0) carried out a study of debittering of protein hydrolysates using immobilized chicken intestinal mucosa. Enzymatic hydrolysates of casein and soybean were treated with alginate immobilized chicken intestinal mucosa, as an aminopeptidase source, to bring about debittering. The mucosa was hygienised by irradiation (20 kGy) which brought about a complete decontamination of the tissue accompanied by a 20% loss in aminopepidase activity. The effectiveness of the process was demonstrable by a higher acceptability and a marked reduction in bitterness scores for casein (from 4.4 to 2.5) and soybean (from 3.8 to 2.2) in organoleptic analysis. The action of aminopeptidases to bring about this change was corroborated by a concomitant increase in free amino acids and a decrease in average peptide length of the samples after treatment. The RP HPLC profiles of casein and soybean protein hydrolysates before and after treatment showed a higher content of peaks in the hydrophilic region suggesting a decrease in hydrophobic peptides, responsible for bitter taste.

Izawa *et al*. (1997) reported that aminopeptidase from *Aeromonas caviae* T-64 hydrolyzed bitter peptides containing hydrophobic amino acids in the N-terminal region of protease hydrolyzates of milk casein and soy protein. Bitterness of the peptides were reduced by removal of these amino acids.

Fernandez-Espia and Rul (1999) reported that an aminopeptidase (PepS) from *Streptococcus thermophilus* exhibits a high specificity towards peptides possessing Arg or aromatic amino acids at the N-terminus.

Donnell *et al*, (1997) reported that purified and characterized an aminopeptidase P from L. lactis subsp. cremoris. The purified enzyme removed the N-terminal amino acid from peptides only where Pro (and in one case Ala) was present in the penultimate position. As bitter casein-derived peptides are likely to contain single Pro or pairs of Pro, aminopeptidase P appears to be an important enzyme for debittering.

Tan *et al.* (1993) reported that trypsin digest of β-CN exhibits a strong bitter taste, which corresponds to the strong hydrophobicity of several peptides in the trypsin digest of β-CN. The degradation of the trypsin digest by aminopeptidase N from L. lactis subsp. cremoris WG2 resulted in the decrease of hydrophobic peptides and a drastic decrease of bitterness of the reaction mixture.

Watanabe *et al.* (1990) noticed that the bitter flavor of a trypsin hydrolysate of casein was decreased after it was incubated in the presence of *Erwinia ananas*, an ice nucleation-active bacterium. This is due to the action of aminopeptidase on these peptides at low temperature.

Park *et al.* (1995) investigated the effects of crude enzyme extract of *L. casei subsp. casei* LLG on the water-soluble peptides of enzyme-modified cheddar cheese (EMC). The crude enzyme extract contained a wide range of peptidolytic activities (aminopeptidase 1616 U/mL, x-prolyldipeptidyl peptidase 67 U/mL, proline-iminopeptidase 39 U/mL). They found that supplementation of cheddar cheese slurries with crude enzyme extract in addition to Neutrase produced cheese without bitter taste. They concluded that both aminopeptidase and Pro-specific peptidases present in the crude extract were responsible for degrading the hydrophobic peptides in bitter EMC.

Gallagher *et al.* (1994b) notice that used two protease preparations, papain and neutral Bacillus (*B. subtilis*) separately to produce bitter α-CN hydrolyzate. The action of a food grade fungal peptidase (*Aspergillus oryzae*) was then studied by observing changes in the peptide profiles of the bitter α-CN hydrolyzate. The production of novel savory (hydrophilic) peptides in the α-CN hydrolyzate was evident from their study.

The purified aminopeptidase II from *Penicillium caseicolum* had a wide specificity for various substrates, and it was capable of cleaving amino-terminal Leu and Phe residues of dipeptides and oligopeptides (Matsuoka *et al.*, 1991). The enzyme treatment for 3 h was effective in debittering the bitter peptide fraction from peptic casein.

Minagawa *et al.* (1989) treated the bitter peptide fraction present in casein hydrolysates obtained by using three proteases (subtilisin, papain, and trypsin) with aminopeptidase T from *Thermus aquaticus* YT-1. The bitterness of the bitter peptide fraction was decreased and sometimes disappeared completely, with an increase in free amino acids. The percentage of total free amino acids released from each bitter peptide fraction (subtilisin, papain, and trypsin) by aminopeptidase digestion for 20 h were approximately 11, 8.7, and 6.5, respectively. Further, a bitter peptide [αs_1-CN (f 91–100)] with a threshold value of bitterness of 2.9 ppm (w/v) was isolated from a trypsin hydrolysate of casein. The peptide [αs_1-CN (f 96–100)], obtained from the aminopeptidase digestion of this bitter peptide, was not bitter.

2.5 Masking of bitter Hydrolysates

2.5.1 Masking of bitter casein hydrolysates

Spellman *et al*. (2005) reported that whey protein hydrolysates were generated at different total solids (TS) levels (50-300 g/l) using the commercially available proteolytic preparation Debitrase HYW20, while enzyme to substrate ratio, pH and temperature were maintained constant. Hydrolysis proceeded at a faster rate at lower TS reaching a degree of hydrolysis (DH) of 16.6% at 300 g TS/l, compared with a DH of 22.7% at 50 g TS/l after 6 h hydrolysis

Ge *et al.* (1996) notice that enzymatic complete hydrolysis of casein was investigated by using immobilized endopeptidase and exopeptidase packed in the jacketed column reactors. The immobilized pancreatic and kidney exopeptidase could lead to a 20% increase of free amino acids. The free amino acid content of casein hydrolysates was 34.81% after processing and could reach to 64% if the column length was doubled, but 100% hydrolysis was impossible as the reverse reaction was also taking place. The casein hydrolysates was characterized by its high degree of hydrolysis and high content of free amino acids. It can be applied in infant formula, element diet, and as a protein ingredient for food industry.

Subtil *et al.* (2012) studied that spray drying hydrolysed casein using gum Arabic as the carrier agent, in order to decrease the bitter taste. Three formulations with differing proportions of hydrolysed casein: gum Arabic (10:90, 20:80 and 30:70) were prepared and characterized. They were evaluated for their moisture content, water activity, hygroscopicity, dispersibility in water and in oil, particle size and distribution, particle morphology, thermal behaviour (DSC) and bitter taste by a trained sensory panel using a paired-comparison test (free samples vs. spray dried samples). The proportion of hydrolysed casein did not affect the morphology of the microspheres. The spray drying process increased product stability and modified the dissolution time, but had no effect on the ability of the material to dissolve in either water or oil. The sensory tests showed that the spray drying process using gum Arabic as the carrier was efficient in attenuating or masking the bitter taste of the hydrolysed casein.

Morais *et al.* (2004) noticed that encapsulation in lipospheres and in liposomes was used with the aim of masking the bitterness of casein hydrolysates for dietetic of pharmaceutical purposes. Papain was used for preparing three casein hydrolysates, employing different temperatures and E:S ratios. The percentage of encapsulation of these preparations was measured by second derivative spectrophotometry (SDS). Lipid oxidation over a period of 60 days was followed by quantifying the 2-thiobarbituric acid reactive substances (TBARS). Other analysis was performed for characterizing these vesicles, including the sensory evaluation and the measure of hydrophobicity. The results showed that these two encapsulation systems were equally efficient in reducing the hydrophobicity and the bitterness of casein hydrolysates. SDS was a useful tool in the characterization of these preparations. It is a quick and relatively low-cost method, and indicated that the encapsulation rate in lipospheres (65%) was higher than in liposomes (59%). The TBARS method revealed the chemical stability of these preparations over the period of study (60 days).

Barbosa *et al.* (2004) study on some reaction parameters were tested in the hydrolysis of casein by papain, in order to prepare hydrolysates with high oligopeptide contents, for either dietetic or pharmaceutical purposes. Casein hydrolysates were prepared. Masking of the bitterness, based on the encapsulation in lipospheres, was used. Also, second derivative spectrophotometry was used for the first time to measure the extent of encapsulation of protein hydrolysates, which changed from 50% to 83%. Which showed a reduction of hydrophobicity and bitterness of all samples, as well as good chemical stability during 60 days of storage under refrigeration. The electron microscopical analysis of lipospheres showed an average size around 5.0 ± 1.0 µm.

Morais *et al.* (2005) tested for hydrolytic parameters to prepare casein hydrolysates with high oligopeptide content for dietetic purpose, using papain. Employing a temperature of 37 °C and an E:S ratio of 2% was used. The encapsulation in liposomes was used for masking the bitterness, and was also able to reduce the hydrophobicity as well as to keep the chemical stability during 60 days of storage. The UV spectrometry with second derivative transformation was used to measure the encapsulation rate, which changed from 56% to 62%. The size distribution of vesicles was in the range of 500 to 1000 nm.

2.5.2 Masking of bitter whey protein hydrolysates

Leksrisompong (2012) study of Effectiveness of bitter taste inhibitors ($P < 0.05$) of both WPH were sucralose, fructose, sucrose, adenosine 5' monophosphate (5'AMP), adenosine 5'monophosphate disodium (5'AMP Na(2)), sodium acetate, monosodium glutamate, and sodium gluconate. Sodium chloride inhibited bitter taste of WPH with high

DH but not WPH with low DH. Amino acids (l-Lysine, l-arginine) inhibited bitter taste of quinine but not WPH. All effective inhibitors in rehydrated WPH were also effective in the beverage applications.

2.6 Utilization of Developed Hydrolysates Enriched in the Formulation of Functional

Dairy Beverage

A study by Moriarty *et al*. (1985) is frequently cited, concluding that the molecular form of protein elicits no difference in nitrogen balance in healthy humans. This was hardly surprising as the basis of question relates to the rapidity of absorption of amino acids. In the Moriarty study this was not an issue because feeding patterns were essentially unconstrained. Under conditions of rapid intestinal infusion, amino acids from protein hydrolysates appear in the portal circulation faster than even free amino acids Monchi & Rerat. (1993).The most sophisticated study to date demonstrated that a 35 g dose of rapidly absorbed casein hydrolysate is ~30% more effective in stimulating skeletal muscle protein synthesis than intact casein when measured over the 6 h period Kim and Egan (2008).

2.6.1 Utilization of Developed Casein Hydrolysates Enriched in the Formulation of Functional Dairy Beverage

Zhao *et al.* (2006) study the effects of casein hydrolysates by papain on yogurt fermentation have shown that strongly decreased the fermentation and coagulation time of the yogurts. The post-fermentation acidification was retarded by the hydrolysates and show the hydrolysates increased the probiotic counts during initial fermentation stage.

Helbig *et al.* (1980) study the carbon adsorption methods for debittering skim milk hydrolysates were utilized in an attempt to develop acid-soluble milk solids for fortification of beverages which appear and taste like the original beverages after fortification of the milk solids up to the level of skim milk

Rizvi and Action (1982) noticed that addition of hydrolysed casein at 4, 8 and 12% to navy beans, rice and black eye peas for increasing amino-acid content.

Iwasawa *et al.* (1982) studied on use of prepared cheese where incorporation of hydrolysate was made with calcium caseinate, butterfat and emulsifier. The hydrolysate at 5% level gave the best results for flavour and texture.

2.6.2 Utilization of Developed Whey Protein Hydrolysates Enriched in the Formulation of Functional Dairy Beverage

Sinha *et al.* (2007) studies on use of WPC hydrolysates in a beverage was formulated with hydrolyzed WPC, skim milk powder, cocoa, liquid glucose, sugar and vegetable fat .Formulated beverage had 52% protein, 10% fat and 6.6% ash. Chandan *et al.* (1982) reported that either hydrolyzed or unhydrolyzed WP could be usedin the formation of a brine replacer in canned beans, with beans canned in WP solutions retaining firmness better than those canned in control solutions. They also found that hydrolyzed WP could be successfully used as a syrup replacer for canned plums without significant differences from control samples (Chandan *et al.,* 1982). Athira (2012) reported that three types of whey drink were prepared i.e. without WPH (control), with0.5% WPH and 1% WPH. Lemon extract and flavor was used for masking the salty taste of whey protein hydrolysate. It was clear that 1% WPH fortified whey drink was prepared with the addition of more flavour and extract to mask the salty taste than the 0.5 % WPH fortified product.

John (2007) used10% protein solution of whey protein is hydrolyzed with E:S ratio of 1:50produce DH 5% was spray drying and was finally incorporated into milk @ 20% V/V to develop an acceptable product of enriched milk functional peptide. Kishor & Mann (2009) noticed that incorporation of WPHs in the skim milk (1-2%) raised its antioxidant activity from 21% to 88% and ACE inhibitory activity was 35 times higher than the skim milk. Khorshid & Rao (1971) reported the use of whey protein hydrolysate in accelerating the ripening of Domaiti cheese. The addition of protein hydrolysate (at 0.5% in buffalo milk) accelerated the ripening and improved cheese quality without affecting the pH, acidity or moisture content of cheese.

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

This chapter deals with the materials and methodologies employed during the present investigation relating to the technological, analytical, sensory, statistical aspects and formulation of dairy based beverage incorporated with milk protein hydrolysates. Details of equipments / instruments used in this study are also given. All the trials were conducted at laboratory scale.

3.1 Location of the study

The present investigation was carried out in the Dairy Technology Division of National Dairy Research Institute, Karnal, Haryana, India.

3.2 Materials

3.2.1 Sodium Caseinate

Sodium Caseinate was purchased from Ace International LLP, New Delhi, India. Sodium Caseinate was claimed containing 91.0% protein (on dry basis), 1.5% fat, 3.8% moisture, 0.2% lactose and 3.5% ash content with particle size of 80 mesh.

3.2.2 Whey protein concentrate-80 (WPC-80)

WPC-80 (Davisco, USA) was purchased from Ace International LLP, New Delhi, India. WPC-80 was claimed containing 82.5% protein (on dry basis), 6.4% fat, 1.2% moisture, 7.5% lactose and 2.4% ash content.

3.2.3 Enzymes

Alcalase 2.4L (2.4 U/ml), Trypsin, Flavourzyme 500L (500 U/ml) and Flavourzyme 1000L (1000 U/ml) were provided donated by Novozymes, Denmark. FlavourSEB L was provided by Advanced Enzyme Technologies Ltd, USA. FlavourSEB L is a protease and peptidase preparation for production of de-bittered protein hydrolysates. Papain was procured from Sisco Research Lab (SRL), India.

3.2.4 Milk

Milk was procured from the Experimental Dairy of National Dairy Research Institute (NDRI), Karnal. Sample was prepared by separating whole cow milk at 55-60°C in a hermetically sealed cream separator (Alfa Laval- S2181M, capacity, 5000 L/h) followed by standardization (fat 3.2% and SNF 8.7%) & pasteurization at 72°C/15s in

HTST pasteurizer (Westfalia Separators India Pvt. Ltd. - A Division of GEA Specialty Products).

3.2.5 Sugar

Commercial grade cane sugar was procured from central store of Experimental Dairy of the institute.

3.2.6 Strawberry Colour and flavor

Strawberry red powder (IH 7804) was procured from Bush India Pvt. Ltd. and Strawberry no. 1 flavour was procured from International Flavour & Fragrance India Pvt. Ltd., Chennai.

3.2.7 Apparatus and glassware

All volumetric flasks, pipettes and burettes were washed, cleaned and dried before use. Burette (50 ml), funnels (small and large), measuring cylinder (10, 50, 100, 250, 500 and 1000 ml), separating funnels (100, 250 and 500 ml), beakers (100, 150 and 250 ml), conical flasks (10, 25, 50, 100, 150 and 250 ml) and volumetric flasks (25, 50, 100, 250 and 500 ml) were purchased from The Laboratory Glass Co. (LABCO), Ambala, India. Glass pipettes (1, 2, 5, 10 and 25 ml), glass test tubes, flat bottom flasks (150 and 250 ml) and round bottom flask (500 ml) were procured from Borosil India Ltd., Mumbai, India; filter papers (Whatman filter paper no. 1, 4 and 41) from Whatman International Ltd., Kent, England. Packaging material- Aluminum foil laminates having thickness 90-100 µm were purchased from local market, Karnal, Haryana, India.

3.3 Equipments

a) Auto pipettes: Fisher, India

b) Hand blender: Hovels, India

c) Homogenizer: Goma Engineering Pvt. Ltd., India

d) Muffle Furnace: Scientruric Instruments, New Delhi

e) Halogen moisture analyzer: WENSAR, HMB 100, India

f) pH meter: EUTECH Instruments, Cyberscan, Singapore

g) High precision water bath: LABCO (Laboratory Glassware Co.) Ambala, India

h) Magnetic stirrer: Remi Stirrer, Mumbai, India

i) Rotary flash evaporator: Metrex Scientific Instruments, India

j) Hot air oven: Metrex Scientific Instruments, India

k) Hunter Lab Colorflex Colorimeter: Colorflex- colorimeter, supplied by Hunterlab (Hunter Associates Laboratory), Reston, VA, USA

l) Weighing balance: Precisa XB 220A,Kolkata, India

m) Vortex shaker: Remi Laboratory Instruments, Mumbai, India

n) Refrigerated water bath: LKB, BROMMA, Multitemp II, Sweden

o) Halogen Moisture Analyzer : Wensar

3.4 Chemicals

Other chemicals were of analytical grade purchased from Hi-media, India and Merck, Germany, used for chemical analyses.

3.5 Experimental design for optimization of different variable for developing organoleptically palatable Hydrolysate of Casein and Whey Protein

Response Surface Methodology (RSM) was used to reduce the number of experiments without affecting the accuracy of results and to determine interactive effect of independent variable on dependent variable. RSM with Central Composite Rotatable Design (CCRD) for four independent variables; pH, Temperature, Enzyme: substrate (E/S) ratio and time of hydrolysis was adopted to optimize the hydrolysis condition. Degree of Hydrolysis (DH) was taken as dependent variable to optimize the development of non-bitter palatable casein and whey protein hydrolysates using Endoproteases and Exoproteases enzymes, independently.

3.6 Preparation of Casein and Whey Protein hydrolysates

Protein hydrolysates were prepared by the method of Cheison *et al.,* (2007) with slight modifications. Fresh 10% protein solutions were prepared dissolving Na-caseinate and WPC-80 separately in distilled water and heated at 72^{o}C for 10 min. As per the RSM combinations, hydrolysis was carried out for the specified time period, pH, enzyme to substrate ratio and temperature. pH was maintained at 7.0 ± 0.04 using 0.5 M or 1 M NaOH (Cheison *et al.,* 2007). Enzyme/Substrate ratio was different for each enzyme viz., Trypsin (0.01-0.3), Alcalase (0.004-0.012) & Papain (3-5). After achieving the hydrolysis time (as per the set of runs of RSM), enzyme activity was terminated by heating the solution at 90^{o}C for 10 min. Samples were cooled (36^{o}C) and analyzed for sensory attributes such as taste, flavor and bitterness. Sensory panel observed an intense bitter taste for both type of hydrolysates. Therefore, the prepared hydrolysates were further treated with exoproteases for debittering.

10% aqueous solution of Sodium Caseinate/ Whey protein concentrate-80

↓

Heating in a temperature controlled water bath at 72°C/10 min

↓

Continuous stirring with constant pH 7.0 by adding 0.5 or 1.0 N NaOH

↓

Trypsin (0.01-0.03)/Alcalase (0.004-0.012)/Papain (3-5) with varying time (1-4 h) and temperatures (50-60°C)

↓

0.1 N NaOH was used to maintain constant pH 7.0

↓

Reaction was terminated by immersing in water bath at 90°C for 10 min

↓

Reaction mixture was cooled and stored at -20°C till further analysis/processing

Fig. 3.1. The process flow diagram for the preparation of protein hydrolysates

Similar experiment (as discussed above) was performed for debittering of casein and whey protein hydrolysates, prepared in above step. Hydrolysate solutions (as obtained in former step) were heated again to 72^{o}C for 10 min. Enzyme/Substrate ratio was in kept separate for each enzyme viz., Flavourzyme1000L (0.1), FlavouSEB L (0.1) & Flavourzyme 500L (0.03). The required hydrolysis time (as per the set of runs of RSM), enzyme activity was terminated by heating the solution at 90^{o}C for 10 min. Samples were cooled and stored at -20^{0}C till further analysis.

Hydrolysate solution of Na- Caseinate/ Whey protein concentrate-80 (obtained in step 1)

↓

Heating in a temperature controlled water bath at 72°C/10 min

↓

Continuous stirring with constant pH 7.0 by adding 0.5 or 1.0 N NaOH

↓

Flavourzyme1000L (0.015-.025)/ FlavouSEB L (0.1-.30)/ Flavourzyme 500L (0.03-.05)
with varying time (1-4 h) and temperatures (50-60°C)

↓

0.1 N NaOH was added to keep constant pH 7.0

↓

Reaction was terminated by immersing in water bath at 90°C for 10 min

↓

Mixture was cooled and stored at -20°C till further analysis

Fig. 3.2. The process flow diagram for the preparation of debittered protein hydrolysates

3.7 Physico-Chemical analyses of raw material (sodium caseinate and WPC-80)

3.7.1 Determination of moisture content

The moisture content of sodium caseinate and WPC-80 was determined by a Halogen moisture analyzer (Wensar) standardized at 108°C for 5 min. Each analysis was repeated three times.

3.7.2 Determination of protein by Macro Kjeldahl method

Protein content of Na-Caseinate and WPC-80 was determined by Micro Kjeldahl method (IDF 20B: 1993). Around 1 g of the accurately weighed sample was transferred to a digestion tube and digested after adding 1 g of digestion mixture (1:4::$CuSO_4$:K_2SO_4) and 10 ml of concentrated sulphuric acid till the content becomes clear. After digestion, the flask was cooled in air followed by addition of 200 ml of water gradually. A piece of granulated Zinc or anti-bump granules were added into the flask. A small amount of sodium hydroxide solution (50% W/W) was added to make the contents strongly alkaline (about 110 ml). Connect the flask to a distillation apparatus incorporating an efficient flash head and condenser. Contents of the digestion flask was mixed and distalled until a volume of 150 ml distilled into the receiver. Five drops of methyl red indicator were added followed by titration with 0.1 N HCL. Blank determination was conducted using distilled water in place of sample. The total nitrogen and percent protein were calculated as follows:

$$\text{Total Nitrogen } (\%) = \frac{14.007 \times (\text{X} - \text{Y}) \times \text{N} \times 10}{\text{W}} \times 100$$

Where,

X = ml of HCl used for sample;

Y = ml of HCl used for blank;

N = normality of HCl used;

W = weight of sample in g, expressed to nearest 0.1mg.

Percentage total protein = Percentage total nitrogen × 6.38

3.7.3 Determination of fat content by Rose - Gottlieb Method

Principle

Fat (%) was determined by Rose - Gottlieb Method (AOAC, 2000b). The sample was treated with ammonia and ethyl alcohol; the former to dissolve the protein and the latter to help precipitate the proteins. Fat was extracted with diethyl ether and petroleum ether. Mixed ethers were evaporated and the residues weighed.

Apparatus

i) Mojonnier fat extraction flask or any other suitable extraction tube (as per IS specification).

ii) Cork or stopper of synthetic rubber unaffected by usual fat solvents.

iii) 100 ml flat bottom flask with G/G joint or stainless steel or aluminium dishes of 5.5 cm height and 9 cm diameter or glass bowl.

Reagents

a) Ammonia Sp. gr. 0.8974 at 16 ^{0}C

b) Ethyl alcohol (95%)

c) Diethyl ether, peroxide-free

d) Petroleum ether, boiling range 40-60 ^{0}C

Procedure

1 g of the sample was taken in a Mojonnier flask or suitable extraction flask. Nine ml of warm distilled water was added and mixed properly to dissolve the protein. An adequate of 1.0 ml of ammonia (sp. gr. 0.8974) was added into the flask and mixed well in the lower bulb thoroughly. 10 ml of ethyl alcohol was added and mixed by allowing the liquid to flow backwards and forward between the two bulbs. The flask was allowed to cool in cold, running water or by immersing in chilled water. An adequate of 25 ml of diethyl ether (peroxide free) was added, and the flask was closed with glass stopper and mixed vigorously for 1 minute. After that, 25 ml of light petroleum ether was added, and mixed again vigorously for a minute. Flask was kept undisturbed until the upper ethereal layer was clear and separated completely from the aqueous layer.

Upper layer was separated into a suitable flask by gradually bringing the cylindrical bulb of the tube into a horizontal position. Extraction was repeated by using 15 ml of each of ether and 15 ml of petroleum ether. After separation, solvents were evaporated from the flask by keeping it on a hot plate (50-60ºC), and flask was dried containing the residual fat, which was further dried in an air oven at 102 ± 2°C for two hours. After cooling, the flask was weighted and the % fat was calculated according to the following equation:

$$\text{Fat \% (w/w)} = - \quad \frac{\text{Weight of fat}}{\text{Weight of Sample}} \times 100$$

3.7.4 Estimation of lactose content

Lactose content of Na-Caseinate, WPC-80, casein & whey protein hydrolysates, debittered casein and debittered whey protein hydrolysates and flavoured milk beverage was determined by subtracting fat, protein and ash content from the total solids content as per the following equation:

$$\textbf{\% Lactose} = \textbf{\% Total solids} - (\textbf{\% Fat} + \textbf{\%Protein} + \textbf{\% Ash})$$

3.7.5 Determination of total Ash content

10 g of the sample was accurately measured in a previously heated and cooled silica dish. It was charred on a heater or flame, and afterwards, kept in a muffle furnace maintained at 525-550°C for approximately 5-6 h or until white ash was obtained (AOAC, 2000).

Calculation

$$\text{Total Ash \%} = \frac{W1}{W} \times 100$$

W = Weight of the sample

W1 = Weight of the residue after ashing

3.8 Physico-chemical characteristics of protein hydrolysates

3.8.1 Determination of Degree of hydrolysis (DH) by pH stat method

In protein hydrolysis, the key parameter for monitoring the reaction is the degree of hydrolysis (DH). DH is defined as the percentage of cleaved peptide bonds:

$$\text{DH (\%)} = h/h_{tot} \times 100\%$$

Where, h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. h_{tot} is dependent on the amino-acid composition of the raw material. Depending on the pH during hydrolysis, hydrogen ions are either released or taken up due to enzyme action on the protein. The hydrolysate solution is titrated with alkali or acid to maintain optimal pH for the enzyme. The rate at which alkali or acid is added corresponds to the rate of hydrolysis. The total volume of alkali or acid added to maintain pH during hydrolysis can be used in calculating the degree of hydrolysis (Adler-Nissen, 1986).

The base used for maintaining constant pH was 1.0 N NaOH. The percentage DH was calculated using the following formula (Adler-Nissen, 1986):

$$\mathbf{DH\ (\%) = 100BNb\ (1/\alpha)\ (l/MP)\ (l/h_{tot})}$$

where B is the base consumption in mL, Nb the normality of the base, α the average degree of dissociation of the α-NH_2 groups, MP the mass of protein being hydrolyzed (g), and h_{tot} the total number of peptide bonds in the protein substrate (meqv g^{-1} protein).

The degree of dissociation (α) for the α-NH_2 groups was calculated as follows:

$$\boldsymbol{\alpha = 10\ (pH\text{-}pK)\ /1+10(pH\text{-}\ pK)}$$

Where, pK is the average dissociation value for the α-amino groups liberated during hydrolysis and is dependent on temperature, peptide chain length and the nature of the terminal amino acid. At 50^0C (the hydrolysis temperature used in the present study), the average pK value for the α-amino groups of peptides and proteins is 7.1 (Adler-Nissen, 1986). The parameter h_{tot} is expressed as milliequivalents (meqv) peptide bonds per gram of protein. This was calculated from amino acid analysis by summing the mmoles of each individual amino acid per gram of protein. The h_{tot} for whey protein concentrate and casein is 8.8 and8.2 meqv/g protein, respectively (Adler-Nissen, 1986).

3.9 Sensory evaluation of developed casein and whey protein hydrolysates

Sensory evaluation was done by a panel of ten judges of Dairy Technology and Dairy Chemistry Division of NDRI, Karnal, India. Developed hydrolysates were graded for bitterness on 4-point intensity scale, where 1 indicates most bitter and 4 indicates least bitter. Whey protein concentrate-80 and sodium caseinate on 10% protein basis solution were used as control for whey protein hydrolysates and casein hydrolysates, respectively.

3.10 Addition of developed debittered hydrolysates for the formulation of strawberry flavoured dairy beverage

Cow whole milk was standardized by mixing skimmed milk using Pearson square method in such a way that it contained 3.2% fat and 8.7% SNF after the addition of casein or whey protein hydrolysates at 2.5, 5.0 and 7.5% levels. For a particular addition level, fresh standardized milk was warmed to 45-50°C and then homogenized at 2500 psi pressure. The whole homogenized milk was divided into 4 batches. First batch was used as control and rest three batches were supplemented with three different debittered hydrolysates preparations each (casein & Whey protein). All the batches were pasteurized at 74°C for 15 s and then cooled to 4°C and storage at refrigerated temperature (4-7°C). A flow diagram for the preparation of flavored milk beverage is added with debittered hydrolysates given in Fig. 3.3

Mixed milk

↓

Standardization

(3.2% fat, 8.7% SNF)

↓

Preheating to 55°C

↓

Homogenization (2500 psi)

↓

Addition of debittered casein/ whey protein hydrolysates

(@2.5/5.0/ 7.5%)

↓

Addition of strawberry colour @ 1% (w/w) and strawberry flavor @ 1% (w/v)

↓

Addition of sugar (8%)

↓

Pasteurization

(74°C/15 s)

↓

Cooling and storage at 4-7°C

Fig. 3.3 Flow diagram for the preparation of containing debittered hydrolysates added flavored milk beverage

3.11 Sensory evaluation of developed debittered hydrolysates added flavored milk beverage

Sensory evaluation of developed containing debittered hydrolysates added flavored milk beverage (final optimized product) was evaluated on composite sensory score card. All the samples were evaluated for colour and appearance, flavour, consistency, taste, bitterness and overall acceptability by ten semi-trained judges from the Faculty of Dairy Technology and Dairy Chemistry Division of NDRI, Karnal, India.

CHAPTER –4

Results and Discussion

RESULTS AND DISCUSSION

There are a wide variety of enzymes that can be used to hydrolyze casein and whey proteins, which includes trypsin, pepsin and chymotrypsin, as well as plant derived enzymes such as papain and bromelain, and bacterial and fungal enzymes, such as subtilisin, bacillopeptidases, and aspergillopepsin (Lahl and Braun, 1994; Nnanna and Wu, 2007; Morais *et al.*, 2014). The hydrolysis process can include a single enzymatic step, or a combination of multiple enzymes to achieve a specific desired end product. Apart from the type and specificity of the enzyme used, the degree of hydrolysis (DH), the percentage of peptides bonds cleaved by an enzyme, depends on multiple factors, such as pH, Temperature, time of expose and Enzyme/Substrate (E/S) ratio (Morais *et al.*, 2014). In present study, Trypsin, Alcalase & Papain were used for the development and optimization of casein and whey protein hydrolysates under different specified conditions of pH, Temperature, time and E/S ratio. Later on, Flavourzyme1000L, FlavourSEB L and Flavourzyme 500L were used for debittering the developed hydrolysates.

As mentioned above, in order to obtain the optimum hydrolysis conditions, optimization was done by using Response Surface Methodology (RSM). One of the reasons for using RSM in the optimization of hydrolysis conditions is that it generates a mathematical model that accurately describes the overall processes with significant estimation ability.

The proposed study was investigated into following steps as mentioned below:

1. Three enzymes (Trypsin, Alcalase & Papain) were selected to induce controlled hydrolysis of sodium caseinate and whey protein concentrate-80.
2. Optimization was performed using four independent variables (pH, Time, Temperature and E/S ratio) in terms of dependent variable (response) i.e. Degree of Hydrolysis.
3. Further, three enzymes (Flavourzyme1000L, FlavourSEB-P & Flavourzyme 500L) were used for debittering of hydrolysates developed and optimized in step 1 and 2.
4. Debittered casein and whey protein hydrolysates were developed and optimized under the same controlled and independent variables (pH, Time, Temperature and E/S ratio), in terms of degree of hydrolysis (dependent response).

5. Optimized and debittered casein and whey protein hydrolysates were utilized for the formulation of flavoured dairy beverage.

The proximate composition of sodium caseinate and whey protein concentrate-80 is given in Table 4.1.1 and 4.1.2 respectively.

Table 4.1.1 Composition of Sodium Caseinate

Constituents	Value
Moisture (%, w/w)	3.8%
Fat (%, w/w)	1.1%
Protein (%, w/w)	91.4%
Ash (%, w/w)	3.6%
Lactose (%, w/w)	0.1%

Table 4.1.2 Composition of Whey Protein Concentrate (WPC)-80

Constituents	Value
Moisture(%, w/w)	4.5%
Fat (%, w/w)	8.0%
Protein (%, w/w)	78.0%
Ash (%, w/w)	4.0%
Lactose (%, w/w)	5.5%

4.1 Optimization of hydrolysis conditions by using RSM

The response surface methodology (RSM) was used to optimize the enzymatic hydrolysis conditions of sodium caseinate and WPC-80 solutions. On the basis of four independent variables, total of 30 experiments were run using central composite design (CCD) with independent factors, namely, pH (A), Temperature (°C, B), E/S ratio (C) and time (h, D) and one response degree of hydrolysis (%DH) was obtained. A wide range of literature suggested that these are the most significant parameters for optimizing the hydrolysis reaction of proteins. As above mentioned factors can affect response individually and/or in combinations, RSM can be considered the best approach for optimization the conditions and process variables. Now-a-days, RSM has become a statistical and mathematical tool that has been successfully used to determine the effects of several independent and dependents variables and to optimize the formulations and process conditions. Several researchers have used RSM for the preparation of milk proteins' and other food protein hydrolysates (Tavares *et al.*, 2011; Chatterjee, 2015, Silvestre *et al.*, 2012; Jamil *et al.*, 2016; Mullally, 1996) and concluded that it is a potential tool for the optimization of process conditions.

4.1.1 Optimization of hydrolysis conditions of Casein Hydrolysate using Trypsin

Table 4.1.3 Boundaries of the experimental domain and spacing of the compositional variables level

Independent Variables	Coded Value	Coded Levels				
		-2α	-1	0	+1	+2α
pH	A	6.5	7.0	7.5	8.0	8.5
Temp (^oC)	B	45	50	55	60	65
E/S	C	0	0.01	0.02	0.03	0.04
Time (h)	D	-0.5	1	2.5	4	5.5

Various independent variables and their coded value and levels are shown in Table 4.1.3. The central value (coded levels) of four independent variables i.e. pH, Temp, E/S and time coded as A, B, C and D respectively were taken as 7.5, 55°C, 0.02 and 2.5h. As per the general rule of RSM, 4 independent variables were taken; therefore, a total of 30 experiments were run in randomised order that included 16 factorial, 8 axial and 6 centre points (Table 4.1.4). It is clear that response (%DH) is a function of experimental factors (pH, Temp, E/S and time) or it can be expressed as y = f (A, B, C, D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, $\beta_{F1}, \beta_{F2}, \beta_{F3}, \beta_{F4}$ are the first order coefficients, $\beta_{F12}, \beta_{F13}, \beta_{F23}, \beta_{F14}, \beta_{F24}, \beta_{F34}$ are the cross product coefficients and $\beta_{F11}, \beta_{F22}, \beta_{F33}$ and β_{F44} are the second order coefficients.

Table 4.1.4 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Standard Run	Run Order	pH	Temp (°C)	E/S ratio	Time (h)	Coefficients assess
1	16	7	50	0.01	1	Factorial
2	2	8	50	0.01	1	Factorial
3	18	7	60	0.01	1	Factorial
4	24	8	60	0.01	1	Factorial
5	9	7	50	0.03	1	Factorial
6	19	8	50	0.03	1	Factorial
7	4	7	60	0.03	1	Factorial
8	22	8	60	0.03	1	Factorial
9	10	7	50	0.01	4	Factorial
10	12	8	50	0.01	4	Factorial
11	23	7	60	0.01	4	Factorial
12	17	8	60	0.01	4	Factorial
13	21	7	50	0.03	4	Factorial
14	28	8	50	0.03	4	Factorial
15	5	7	60	0.03	4	Factorial
16	14	8	60	0.03	4	Factorial
17	29	6.5	55	0.02	2.5	Axial
18	27	8.5	55	0.02	2.5	Axial
19	1	7.5	45	0.02	2.5	Axial
20	15	7.5	65	0.02	2.5	Axial
21	11	7.5	55	0.00	2.5	Axial
22	20	7.5	55	0.04	2.5	Axial
23	8	7.5	55	0.02	-0.5	Axial
24	30	7.5	55	0.02	5.5	Axial
25	6	7.5	55	0.02	2.5	Center
26	3	7.5	55	0.02	2.5	Center
27	25	7.5	55	0.02	2.5	Center
28	7	7.5	55	0.02	2.5	Center
29	13	7.5	55	0.02	2.5	Center
30	26	7.5	55	0.02	2.5	Center

Table 4.1.5 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Casein Hydrolysate, hydrolysed using Trypsin

Standard Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	Degree of Hydrolysis (DH)
1	16	7	50	0.01	1.0	3.13
2	2	8	50	0.01	1.0	7.67
3	18	7	60	0.01	1.0	8.66
4	24	8	60	0.01	1.0	5.75
5	9	7	50	0.03	1.0	3.65
6	19	8	50	0.03	1.0	8.88
7	4	7	60	0.03	1.0	9.63
8	22	8	60	0.03	1.0	7.64
9	10	7	50	0.01	4.0	7.72
10	12	8	50	0.01	4.0	13.59
11	23	7	60	0.01	4.0	16.94
12	17	8	60	0.01	4.0	11.23
13	21	7	50	0.03	4.0	8.21
14	28	8	50	0.03	4.0	16.01
15	5	7	60	0.03	4.0	17.89
16	14	8	60	0.03	4.0	12.97
17	29	6.5	55	0.02	2.5	9.31
18	27	8.5	55	0.02	2.5	14.15
19	1	7.5	45	0.02	2.5	10.12
20	15	7.5	65	0.02	2.5	13.12
21	11	7.5	55	0.00	2.5	0
22	20	7.5	55	0.04	2.5	9.11
23	8	7.5	55	0.02	-0.5	0
24	30	7.5	55	0.02	5.5	14.17
25	6	7.5	55	0.02	2.5	10.43
26	3	7.5	55	0.02	2.5	8.03
27	25	7.5	55	0.02	2.5	10.73
28	7	7.5	55	0.02	2.5	11.91
29	13	7.5	55	0.02	2.5	10.45
30	26	7.5	55	0.02	2.5	10.19

4.1.1.1 Response surface analysis for DH of Casein Hydrolysate debittered using Trypsin

Data of dependent variable, i.e. DH of Casein Hydrolysate, prepared using Trypsin is presented in Table 4.1.5. In total, 30 experiments were conducted with different combination of the independent variables as determined by RSM. It is clear from the said table that values of % DH of caseinate hydrolysates were observed in the range of 0 to 17.89. The minimum value (0) was obtained for the two combinations of independent variables; one of which had pH 7.5, Temp 55°C, E/S 0.00 and Time 2.5 h and second combination had pH 7.5, Temp 55°C, E/S 0.02 and Time -0.5 h. Data shows that the maximum value was obtained only for one combination of independent variables comprising of pH 7.0, Temp 60 °C, E/S 0.03 and Time 4 h.

The regression analysis of data as presented in Table 4.1.6 revealed that the coefficient of determination (R^2) was 0.923, which suggest that this model fits well to the used model. The Adequate Precision value (APV) was found to be 13.24, which was appreciably higher than the minimum desirable value (4.00) for the higher prediction ability.

The statistical significance of the proposed model could be obtained using the Fisher's test (F-test) (Maache-Rezzoug *et al.*, 2011). Table 4.1.6 shows the F-value (12.83), which indicates a significant model. The lack of fit test was used to predict the fitness of the model. In terms of the lack of fit value, Karki *et al.*, (2011) suggested a non-significant value, which means the model experienced a non-significant lack of fit. In the model of % DH, it was found that the p-value for the lack of fit was not significant ($p>0.05$) (0.205). Thus, the model was fit to determine the optimum hydrolysis condition of sodium caseinate using trypsin. Further, the statistical analysis indicated that the model fitted the data well (model F value=12.83. From the data, it is also revealed that the lack of fit (0.216) was non-significant ($P>0.05$), again supporting our hypothesis that this RSM model can be used to navigate the design.

To determine the significant or non-significant effect of various independent variables singly or in combinations on response (DH), the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) of all the four independent parameters were studied. It can be observed from Table 5.1.6, that the all the independent variables (pH, Temp, E/S and time) significantly ($P<0.05$) affected the degree of hydrolysis profile in linear terms

(P<0.01). However, the interactive effect of AB, AC, AD, BC and BD of all the four independent variables on the DH% was found to be non-significant (P>0.05). Similarly, the interactive effect in quadrant form was observed to be non-significant (P>0.05) on %DH response.

Table 4.1.6 Coefficient of the full second order polynomial model for coded responses for Casein Hydrolysate, prepared using Trypsin

ANOVA for Response Surface Quadratic model of Trypsin Casein Hydrolysate					
Source	**Sum of Squares**	**df**	**Mean of Sum of Squares**	**F-Value**	**p-value Prob > F**
Model	507.38	14	36.24	12.83	<0.0001
pH (A)	12.92	1	12.92	4.57	0.0493
Temp (B)	32.33	1	32.33	11.44	0.0041
E/S (C)	33.64	1	33.64	11.91	0.0036
Time (D)	252.87	1	252.87	89.52	<0.0001
AB	94.91	1	94.91	33.60	<0.0001
AC	1.17	1	1.17	0.41	0.5293^{ns}
AD	.21	1	0.21	0.074	0.7897^{ns}
BC	0.055	1	0.055	0.019	0.8912^{ns}
BD	1.66	1	1.66	0.59	0.4549^{ns}
CD	0.065	1	1.065	0.023	0.8811^{ns}
A^2	8.65	1	8.76	3.10	0.0985^{ns}
B^2	7.91	1	7.91	2.80	0.1150^{ns}
C^2	41.47	1	41.47	14.68	0.0016^{ns}
D^2	9.76	1	9.76	3.46	0.0828^{ns}
Residual	42.37	15	2.82		
Lack of Fit	34.40	10	3.44	2.16	0.205^{ns}
Pure Error	7.97	5	1.59		
Cor Total	549.75	29			
Other statistics					
R-squared					0.923
APV					13.24
Mean					9.71±1.68

If p value is <0.05, it indicates the value is significantly different. Significant (P<0.05); highly significant (P<0.01); non-significant (P>0.05).

4.1.1.2 Response surface plots and effects of factors for casein hydrolysate, obtained by trypsin hydroslysis on %DH

The model equation for yield and the response variable (Y) of %DH obtained was derived using the regression coefficient of linear and quadratic terms to fit a full response surface model. According to the model's regression analysis, the best explanatory model equation of %DH of casein hydrolysates as affected by different factors in terms of coded factors is as following:

$$DH = +10.29 + 0.73 * A +1.16 * B + 1.18 * C + 3.25 * D - 2.44 * A * B + 0.27 * A * C - 0.11 * A * D + 0.058 * B * C + 0.32 * B * D + 0.064 * C * D + 0.57 * A^2 + 0.54 * B^2 - 1.23 * C^2 - 0.60 * D^2$$

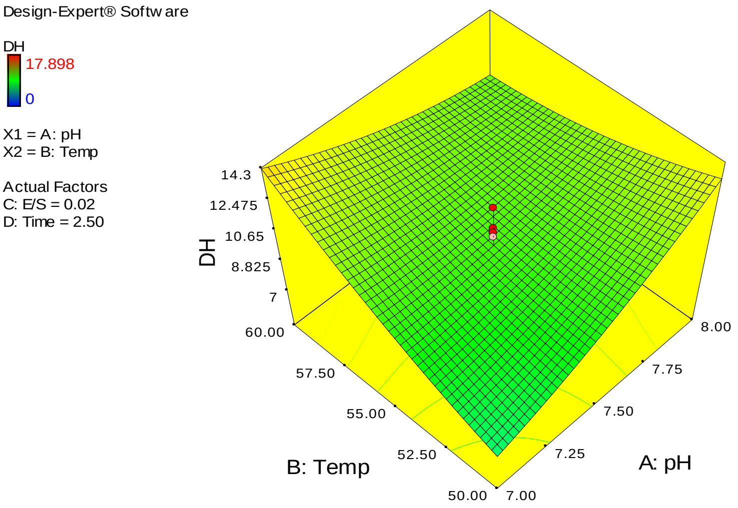

Fig 4.1.1 Response surface graph for %DH value of Casein Hydrolysate using Trypsin as a function of Temp and pH

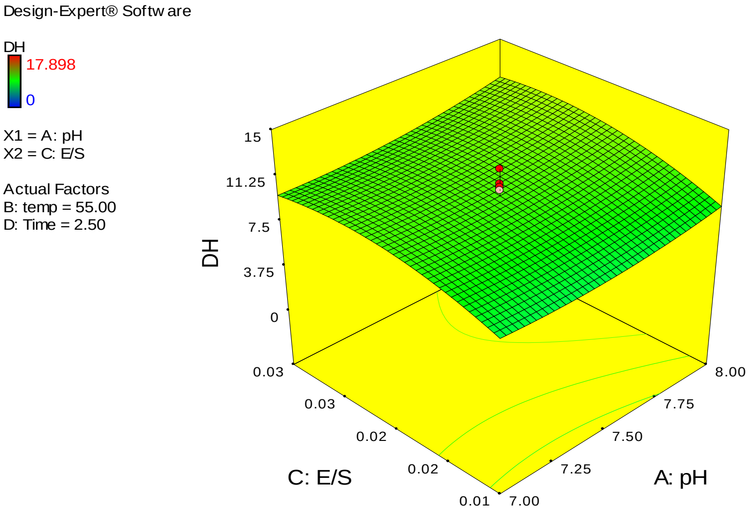

Fig 4.1.2 Response surface related to % DH of Casein Hydrolysate using Trypsin as influenced by E/S and pH

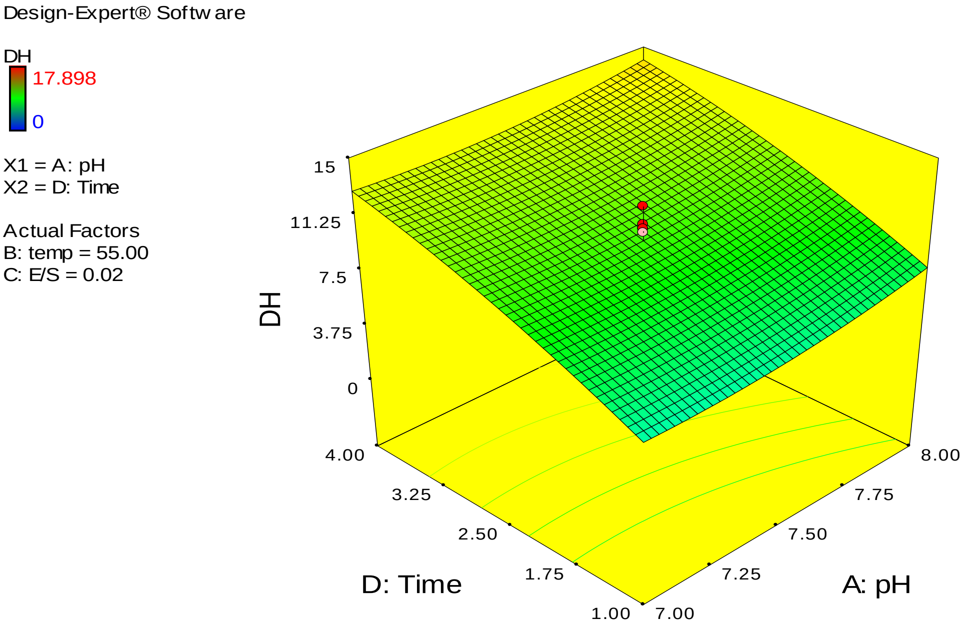

Fig 4.1.3 Response surface related to % DH value of Casein Hydrolysate using Trypsin as influenced by Time and pH

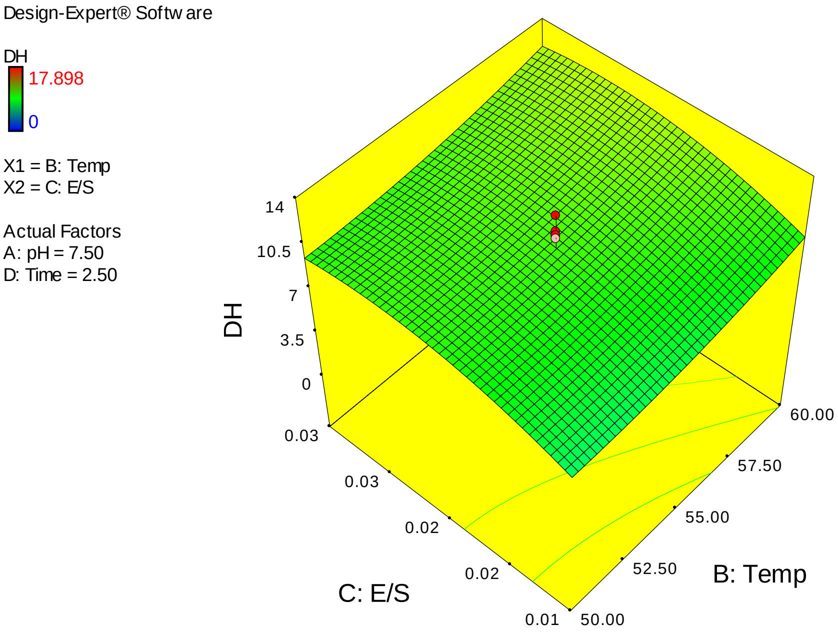

Fig 4.1.4 Response surface relate to % DH value of Casein Hydrolysate using Trypsin as influenced by Temp and E/S

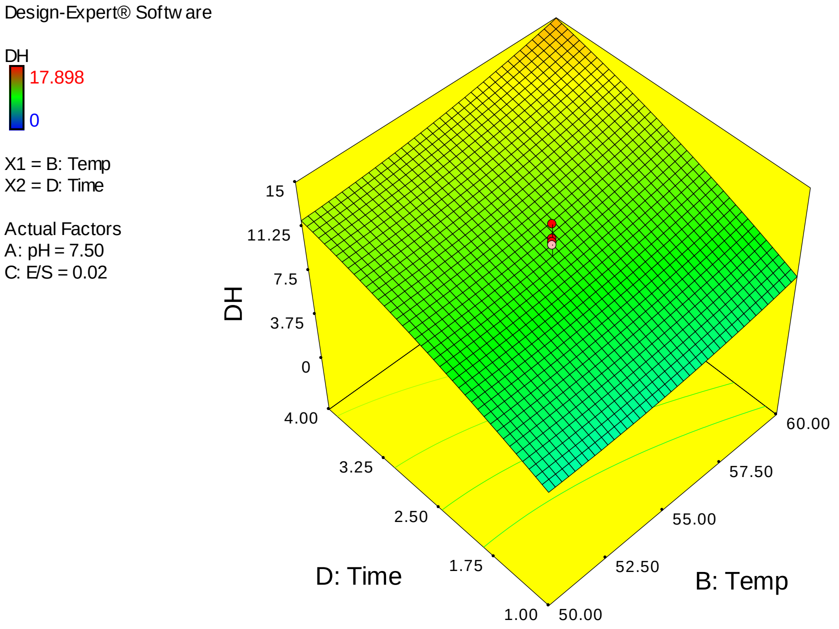

Fig 4.1.5 Response surface related to % DH value of Casein Hydrolysate using Trypsin as influenced by Temp and Time

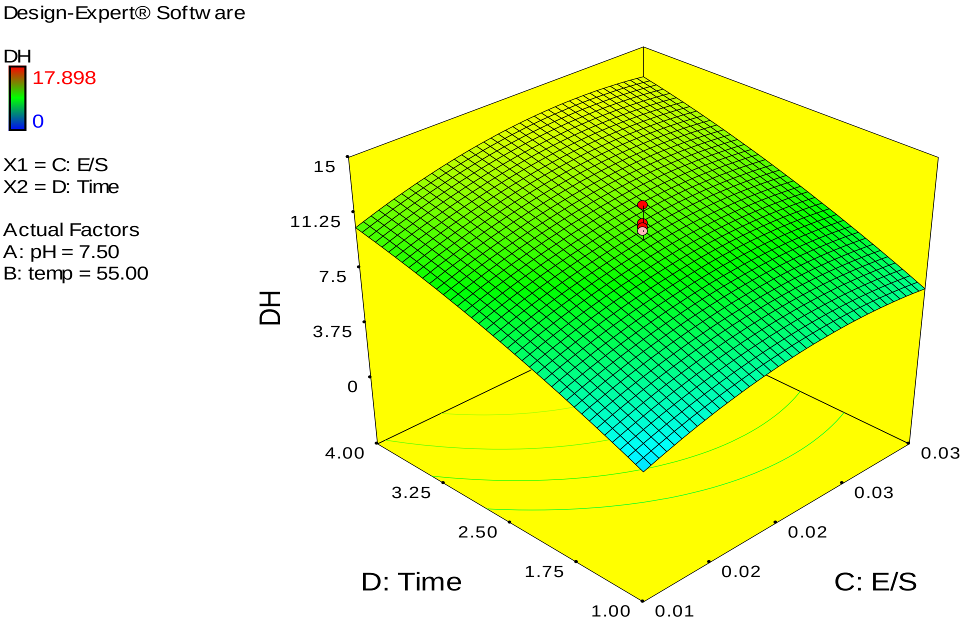

Fig 4.1.6 Response surface related to % DH value of Casein Hydrolysate using Trypsin as influenced by E/S and Time.

A 3-dimensional (3D) response was developed to study the effect between the two independent factors (Temp and pH; E/S and pH; Time and pH and so on) on %DH of sodium caseinate. Figures 4.1.1, 4.1.2, 4.1.3, 4.1.4, 4.1.5 & 4.1.6. show the 3D response surface graphs, which represent the effect of four independent factors (two for each graph) on %DH.

When E/S and Time were kept at centre point 0.02 and 2.5 h, respectively, increasing pH value from 7.0 to 8.0, significantly increased the %DH value. Similarly, increase in Temperature caused a significant increase in %DH. In another possible combination, when the Temperature and time were kept at centre point 55^{o}C and 2.5 h, respectively, increasing both the E/S and pH values led to noticeable increase in the DH value. Similarly, on keeping Temperature and E/S at the centre point 55^{o}C and 0.02, respectively, %DH increased significantly on increasing both time and pH values. In this way, observations can be made from the 3D graphs for the interaction of combined factors such as of E/S and Temperature, time and Temperature; and time and E/S on %DH. When pH and time were kept at centre point 7.5 and 2.5 h, respectively, increase in Temperature increased %DH slightly. However, it can be noted that on increasing E/S ratio first increased the %DH, but after reaching on optimum level, %DH showed a significant decrease ($P<0.05$). Our results are in accordance with the results of Shahidi *et al.*, (1995), who reported increased product formation (hydrolysates) during initial phase and no further increase in soluble hydrolysates on addition of enzyme further. Pralea *et al.*, (2011), tested the functional properties of sodium caseinate hydrolysates as affected by the extent of chymotrypsinolysis. In their study, 5% (w/v) aqueous solution of casein sodium salt was adjusted to pH 8.0 and digested with 0.25% (w/w of substrate) α-chymotrypsin for 10 to 80 min at 37°C. A rapid hydrolysis was observed within the first 30 min of reaction. Thereafter, a slower rate of hydrolysis was recorded. The maximum extent of hydrolysis (DH 8.7±0.7%) was observed after 60 min of hydrolysis Pralea et al., (2011).

When pH and E/S were kept at centre point 7.5 and 0.02, respectively, increase in Temperature did not significantly affect the %DH. However, time showed a significant effect ($P<0.05$) and positive correlation with %DH. Overall, it can be concluded from the results discussed above based on Table 5.1.5., that %DH of casein hydrolysates, hydrolysed with trypsin, was obtained in the range 0 to 17.89 with a mean of 9.71.

4.1.1.3 Optimization for formulation of Casein Hydrolysate, Hydrolysed by trypsin, using most desirable combination of independent variables

From the results obtained through an array of hydrolysates prepared using various combinations of independent variables, as given by the software package Design Expert® (version 7.0.0), the most desirable combination of independent variables was selected for further investigation by verification of its sought functional attributes. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.1.7 Criteria chosen for optimizing values of independent variables in Casein Hydrolysate using Trypsin

Name		Goal	Lower limit	Upper limit	Importance
Factors	**pH**	In range	7	8	3
	Temp (^oC)	In range	50	60	3
	E/S	Minimize	0.01	0.03	3
	Time (h)	In range	1.00	4.00	3
Response	**DH**	Maximize	0	17.89	3

For optimization, goal for factors as well as for response was selected. Three of the four factors viz. pH, Temp and Time were kept ‘In range’ and E/S was kept ‘Minimize’. The goal (Table 4.1.7) for DH was set ‘Maximize’. Twenty nine different solutions were obtained out of which six solutions with different values for dependent and independent variables are presented in Table 4.1.8. The desirability of the solution ranged from a minimum of 0.907 to a maximum of 0.917. With intention to get maximum value of DH percentage with a high desirability value, solution 1 was shortlisted for comparing and finalising the formulation.

Table 4.1.8 Suggested solutions and predicted dependent variable values of Casein Hydrolysate, prepared using Trypsin as given by Design Expert software package

Number	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7	60	0.01	4	15.05	0.917
Solution 2	7.01	60	0.01	4	15.00	0.915
Solution 3	7.01	59.87	0.01	4	14.91	0.912
Solution 4	7	60	0.01	3.9	14.90	0.912
Solution 5	7	60	0.01	3.94	15.11	0.909
Solution 6	7.01	59.74	0.01	3.99	14.74	0.907

Verification of the optimized formulation was done by preparing the selected sample (solution 1) in triplicate and thereafter evaluating the product on similar lines as done while optimizing the formulation. The result revealed (Table 4.1.9) that the difference (t-value) between the predicted and the actual values of independent variables was statistically non-significant (p>0.05).

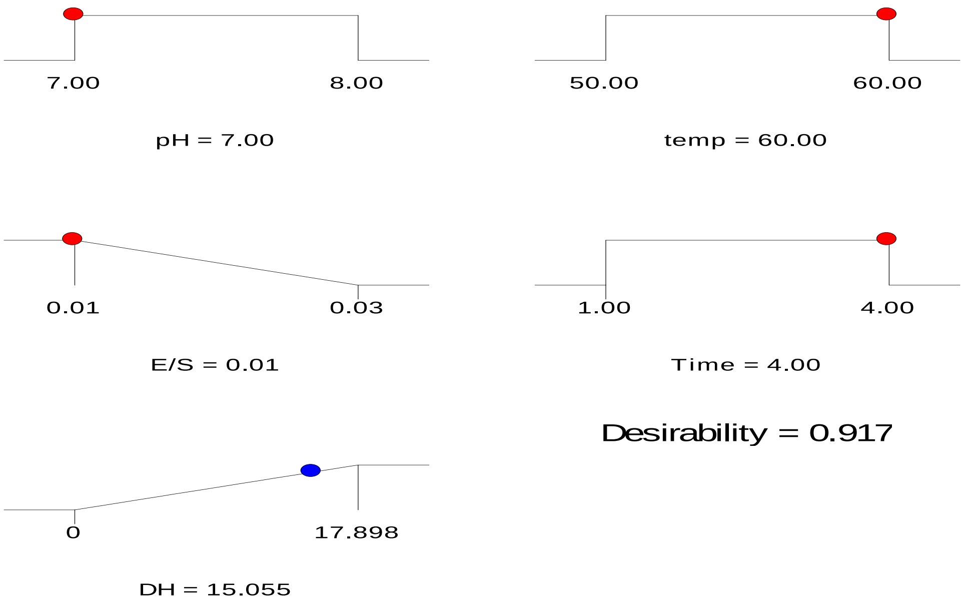

Fig. 4.1.7 Ramp graph for Casein Hydrolysate, prepared using Trypsin

Thus, it can be concluded that maximum degree of hydrolysis was observed when adjusting pH 7.0, Temp. 60°C, E/S 0.03 and time 4 h. Therefore, these can be considered optimum conditions for the production of casein hydrolysates using trypsin. Mahmoud *et al.,* (1992) studied the casein hydrolysis by porcine pancreatic enzyme and observed a very high DH (48%) in first 10% of hydrolysis time at pH 7.0. They also observed a further decrease in degree of hydrolysis with increase in time, as observed in present study. Similarly, Kim *et al.,* (1990), investigated hydrolysis of soy protein isolate by trypsin, and observed a rapid rate of hydrolysis (about 65% of the total) during the initial 17% of hydrolysis time, which decreased thereafter.

Table 4.1.9 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	15.05	16.79±1.273	2.396^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized Casein Hydrolysate, using Trypsin; t-value found non-significant at 1% level; ^{ns} non-significant

4.1.2 Optimization of hydrolysis conditions of WPC-80 using Trypsin

Whey proteins have superior nutritional quality, as a result of which, it have wide applications in formulations of nutritious and healthy beverages, infant formula, food supplements, etc. However, these proteins (α-lactalbumin and β-lactoglobulin) are likely to provoke allergic responses (Wroblewska and Troszynska, 2005). To reduce hypersensitivity caused by whey proteins, enzymatic hydrolysis is generally carried out using various proteolytic enzymes. Selection of the proteolytic enzyme(s) for the preparation of hydrolysates, hydrolysis condition and DH depends on the target uses of the hydrolysates. Hydrolysates are usually classified according to DH such as partial hydrolysates and extensive hydrolysates. Partial (limited) hydrolysis of proteins aims to improve the functional properties of proteins, while extensively hydrolysed proteins are mainly used for nutritional purposes. As, in present study, the aim is to develop non-bitter protein hydrolysates and its application in the formulation of dairy beverage using developed hydrolysates; Therefore, WPC-80 was hydrolysed by trypsin under different conditions of pH, Temp, E/S ratio and time and process conditions were optimized using response surface methodology (RSM).

Table 4.1.10 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2α	**-1**	**0**	**+1**	**+2α**
pH	A	6.5	7.0	7.5	8.0	8.5
Temp (°C)	B	45	50	55	60	65
E/S	C	0	0.01	0.02	0.03	0.04
Time (hr)	D	-0.5	1	2.5	4	5.5

As discussed in Section 4.1, total 30 experiments were run using four independent variables to get the value of dependant response. Various independent variables and their coded value and levels are shown in Table 4.1.10. The central value of the coded levels of four independent variables i.e. pH, Temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.02 and 2.5 h, respectively. Results revealed that out of 30 experiments, which were run in randomised order, included 16 factorial, 8 axial and 6 centre points (Table 5.1.11). In present study, only one response (y, degree of hydrolysis, DH) was measured, which can be considered a function of experimental and independent factors (pH, Temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and β_{F11}, β_{F22}, β_{F33} and β_{F44} are the 2nd order coefficients.

Table 4.1.11 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Standard Run	Run Order	pH	Temp (^{o}C)	E/S	Time(h)	Coefficients assess
1	16	7	50	0.01	1	Factorial
2	2	8	50	0.01	1	Factorial
3	18	7	60	0.01	1	Factorial
4	24	8	60	0.01	1	Factorial
5	9	7	50	0.03	1	Factorial
6	19	8	50	0.03	1	Factorial
7	4	7	60	0.03	1	Factorial
8	22	8	60	0.03	1	Factorial
9	10	7	50	0.01	4	Factorial
10	12	8	50	0.01	4	Factorial
11	23	7	60	0.01	4	Factorial
12	17	8	60	0.01	4	Factorial
13	21	7	50	0.03	4	Factorial
14	28	8	50	0.03	4	Factorial
15	5	7	60	0.03	4	Factorial
16	14	8	60	0.03	4	Factorial
17	29	6.5	55	0.02	2.5	Axial
18	27	8.5	55	0.02	2.5	Axial
19	1	7.5	45	0.02	2.5	Axial
20	15	7.5	65	0.02	2.5	Axial
21	11	7.5	55	0.00	2.5	Axial
22	20	7.5	55	0.04	2.5	Axial
23	8	7.5	55	0.02	-0.5	Axial
24	30	7.5	55	0.02	5.5	Axial
25	6	7.5	55	0.02	2.5	Center
26	3	7.5	55	0.02	2.5	Center
27	25	7.5	55	0.02	2.5	Center
28	7	7.5	55	0.02	2.5	Center
29	13	7.5	55	0.02	2.5	Center
30	26	7.5	55	0.02	2.5	Center

Table 4.1.12 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of WPC-80 hydrolysed using Trypsin

Standard Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	**Degree of Hydrolysis (**DH)
1	16	7	50	0.01	1.0	4.65
2	2	8	50	0.01	1.0	9.13
3	18	7	60	0.01	1.0	10.01
4	24	8	60	0.01	1.0	7.25
5	9	7	50	0.03	1.0	4.98
6	19	8	50	0.03	1.0	10.59
7	4	7	60	0.03	1.0	11.12
8	22	8	60	0.03	1.0	9.05
9	10	7	50	0.01	4.0	9.32
10	12	8	50	0.01	4.0	16.07
11	23	7	60	0.01	4.0	17.43
12	17	8	60	0.01	4.0	12.79
13	21	7	50	0.03	4.0	9.07
14	28	8	50	0.03	4.0	18.99
15	5	7	60	0.03	4.0	20.29
16	14	8	60	0.03	4.0	14.47
17	29	6.5	55	0.02	2.5	10.89
18	27	8.5	55	0.02	2.5	15.65
19	1	7.5	45	0.02	2.5	11.62
20	15	7.5	65	0.02	2.5	14.62
21	11	7.5	55	0.00	2.5	0
22	20	7.5	55	0.04	2.5	10.61
23	8	7.5	55	0.02	-0.5	0
24	30	7.5	55	0.02	5.5	15.67
25	6	7.5	55	0.02	2.5	11.93
26	3	7.5	55	0.02	2.5	9.89
27	25	7.5	55	0.02	2.5	12.23
28	7	7.5	55	0.02	2.5	13.31
29	13	7.5	55	0.02	2.5	10.01
30	26	7.5	55	0.02	2.5	11.69

4.1.2.1 Response surface analysis for DH of WPC-80 prepared using Trypsin.

Table 4.1.12 shows the data of response (Degree of hydrolysis) for WPC-80, hydrolysed using Trypsin. As discussed above, total 30 experiments were conducted using CCDM of RSM with different combination of the independent variables. Results revealed that %DH of whey proteins were observed in the range of 0 to 20.29. Two combinations (run 21 and 23) of independent variables had minimum value of %DH, i.e. 0; one of which (run 21) had pH 7.5, Temp 55°C, E/S 0.00 and Time 2.5 h while second combination comprised of pH 7.5, Temp 55°C, E/S 0.02 and Time -0.5 h. It can also be observed that the maximum value was obtained for one combination of independent variables which had pH 7.0, Temp 60°C, E/S 0.03 and Time 4 h.

The regression coefficients and the response surfaces were used to study the effects of various parameters on %DH. The significance of each coefficient was determined by p-values which are listed in Table 4.1.13. To know the statistical significance of the used model, F-value is one of the best parameter (Maache-Rezzoug *et al.,* 2011). The goodness of fit for the model was also evaluated by the coefficient of determination (R^2) and the lack of fit test for the model from the analysis of variance (ANOVA). The regression analysis of data revealed that the coefficient of determination (R^2) was 0.907, which indicates the suitability of this model for optimizing the conditions of WPC hydrolysates. The Adequate Precision value (APV) was found to be 11.91, which was significantly ($P<0.05$) higher than the minimum desirable value (4.00) for the higher prediction ability.

ANOVA data showed that the F-value was 10.44, suggesting that model is appropriate for optimizing process conditions. In a similar study, Athira *et al.,(*2015) studied the production and characterisation of whey protein hydrolysate from cheese whey using RSM and reported similar findings. In the model of %DH, it was found that the p-value for the lack of fit was not significant ($p>0.05$) (0.1252). On the basis of overall findings of the analysis, it can be suggested that the model is adequate for the optimizing of process conditions for hydrolysing whey proteins concentrate using trypsin.

To determine the effect of independent variables (pH, Temp., E/S and time) on the dependant response (%DH) singly and/or in combination, the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) were studied. It can be observed from the Table 4.1.12, that all the independent variables (pH, Temp, E/S and time) had a significant effect ($P<0.05$) on the degree of hydrolysis in linear terms. On the contrary, the interactive effect of independent variables (AC, AD, BC, BD and CD) was found to be non-significant on %DH ($P>0.05$). It can be noted that the only combination, which had significant effect ($P<0.0001$) on %DH was pH and Temperature (AB). Similar results have been observed (as in case of sodium caseinate hydrolysed by trypsin) when the interactive effect in quadrant form was studied. All the quadrants (A^2, B^2, C^2, D^2) did not affect %DH significantly ($P>0.05$).

4.1.2.2 Response surface plots and effects of independent variables on %DH of WPC-80 using trypsin

The model equation and the response variable (Y) of %DH obtained, was derived using the regression coefficient of linear and quadratic terms to fit a full response surface model. According to the model's regression analysis, the best explanatory model equation of %DH of WPC-80 as affected by different independent variables in terms of coded factors is as following

$$DH = + 11.51 + 0.87*A + 1.07*B + 1.38*C + 3.46*D - 2.63*A*B + 0.24*A*C + 0.060*A*D + 0.19*B*C + 0.22*B*D + 0.16*C*D + 0.72*A^2 + 0.68*B^2 - 1.27*C^2 - 0.64*D^2$$

Table 4.1.13 Coefficient of the full second order polynomial model for coded responses for WPH using Trypsin

ANOVA for Response Surface Quadratic model of Trypsin WPH					
Source	Sum of Squares	df	Mean of Sum of Squares	F-Value	p-value Prob > F
Model	587.20	14	41.94	10.44	<0.0001
pH (A)	18.37	1	18.37	4.58	0.0493
Temp (B)	27.29	1	27.29	6.80	0.0198
E/S (C)	45.77	1	45.77	11.40	0.0042
Time (D)	287.23	1	287.23	71.52	<0.0001
AB	110.42	1	110.42	27.50	<0.0001.
AC	0.90	1	0.90	0.23	0.6418^{ns}
AD	0.058	1	0.058	0.014	0.9058^{ns}
BC	0.56	1	0.56	0.14	0.7144^{ns}
BD	0.74	1	0.74	0.18	0.6738^{ns}
CD	0.39	1	0.39	0.098	0.7584^{ns}
A^2	14.35	1	14.35	3.57	0.0782^{ns}
B^2	12.87	1	12.87	3.20	0.0937^{ns}
C^2	44.19	1	44.19	11.00	0.0047^{ns}
D^2	11.10	1	11.10	2.76	0.1172^{ns}
Residual	60.24	15	4.02		
Lack of Fit	51.40	10	5.14	2.91	0.1252
Pure Error	8.84	5	1.77		
Cor Total	647.44	29			
Other statistics					
R-squared					0.9070
APV					11.916
Mean					11.12±2.00

If p value is <0.05, it indicates the value is significantly different. Significant (P<0.05); Highly significant (P<0.01); non-significant (P>0.1).

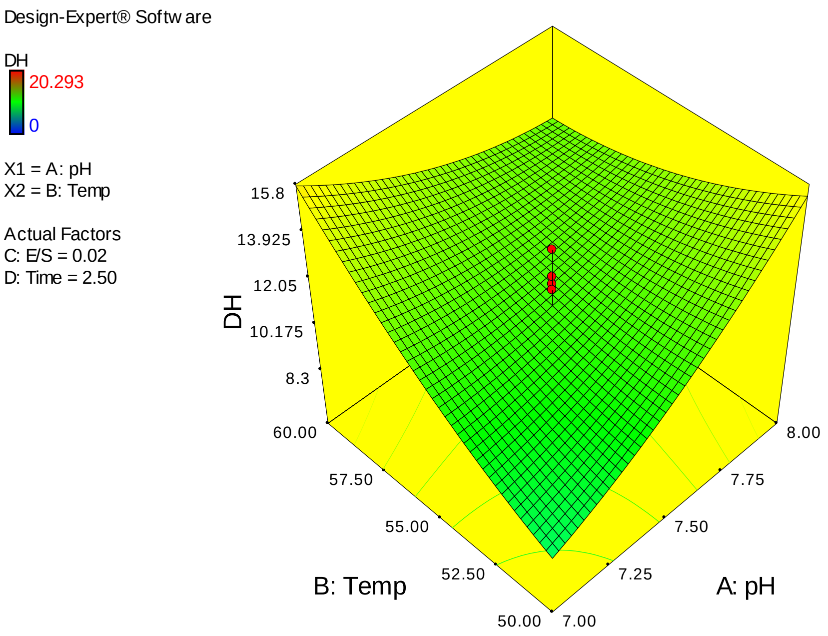

Fig 4.1.8 **Response surface related to % DH value of WPH, using Trypsin as influenced by Temp and pH**

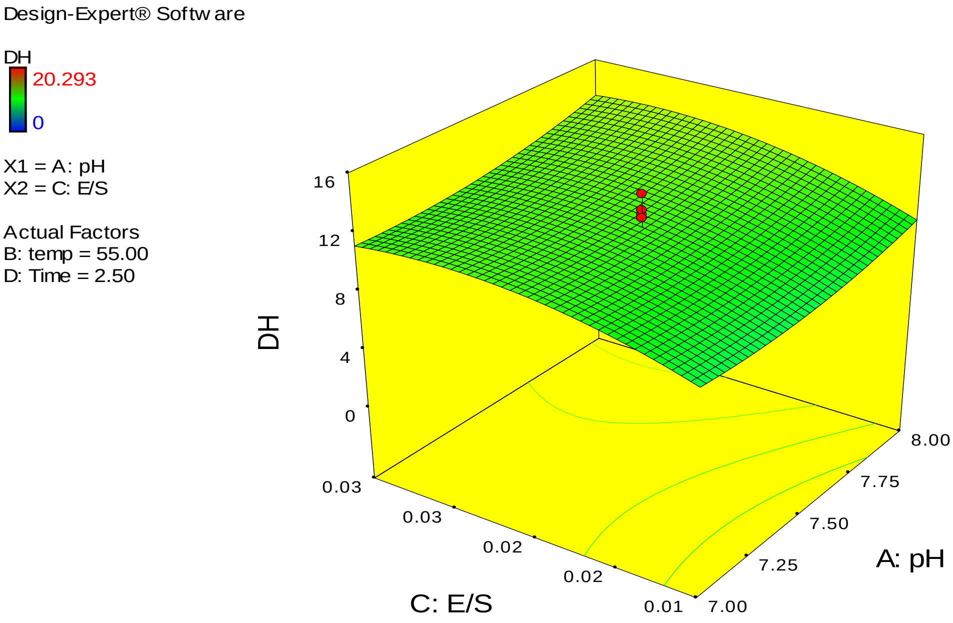

Fig 4.1.9 **Response surface related to % DH value of WPH using Trypsin as influenced by E/S and pH**

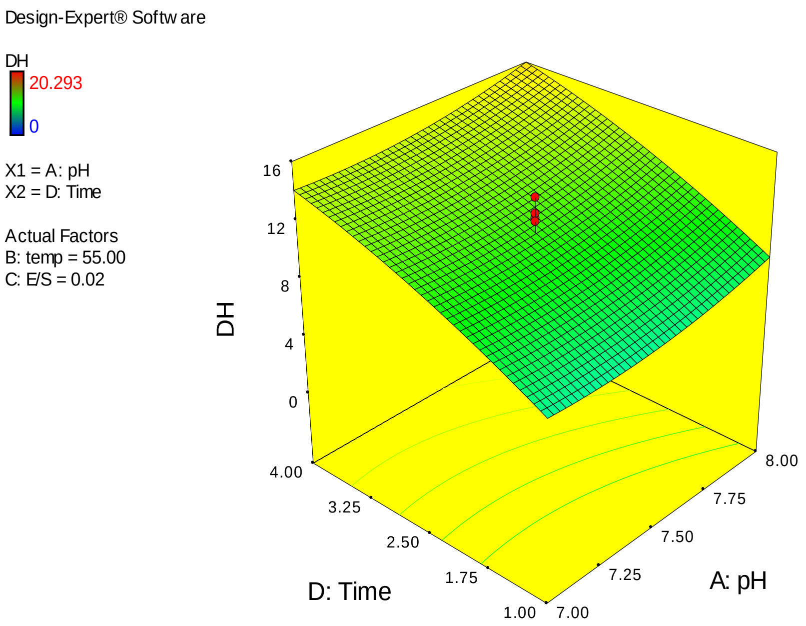

Fig 4.1.10 **Response surface related to % DH value of WPH using Trypsin as influenced by Time and pH**

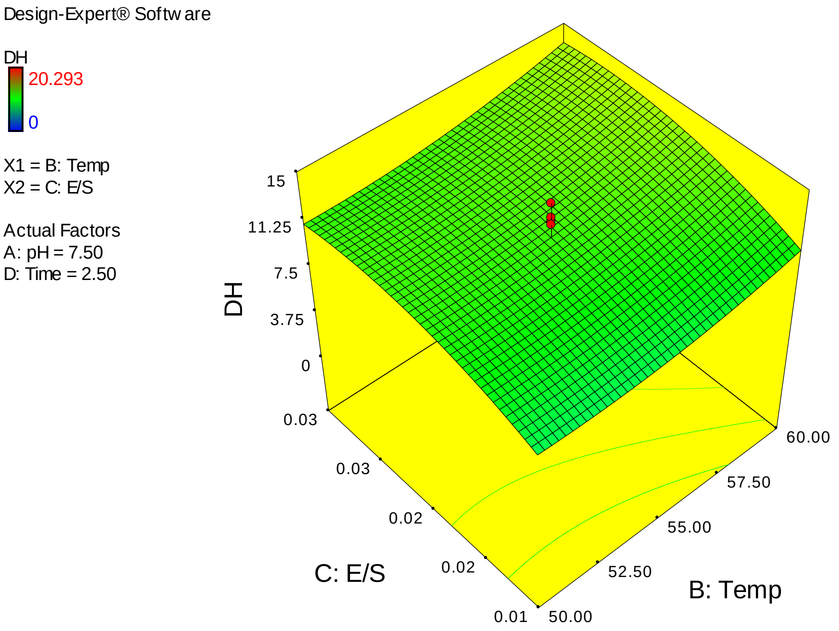

Fig 4.1.11 **Response surface related to % DH value of WPH using Trypsin as influenced by Temp and E/S**

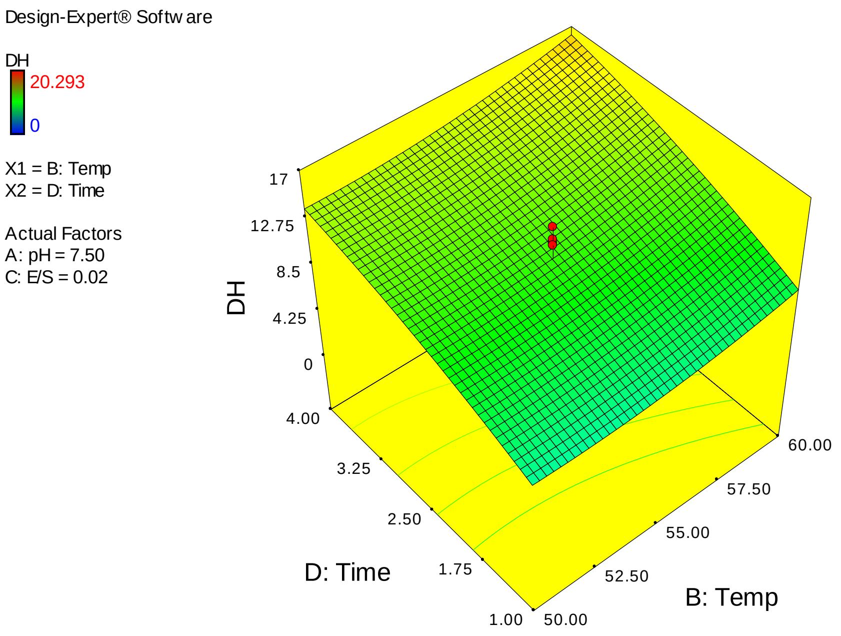

Fig 4.1.12 Response surface related to % DH value of WPH using Trypsin as influenced by Temp and Time

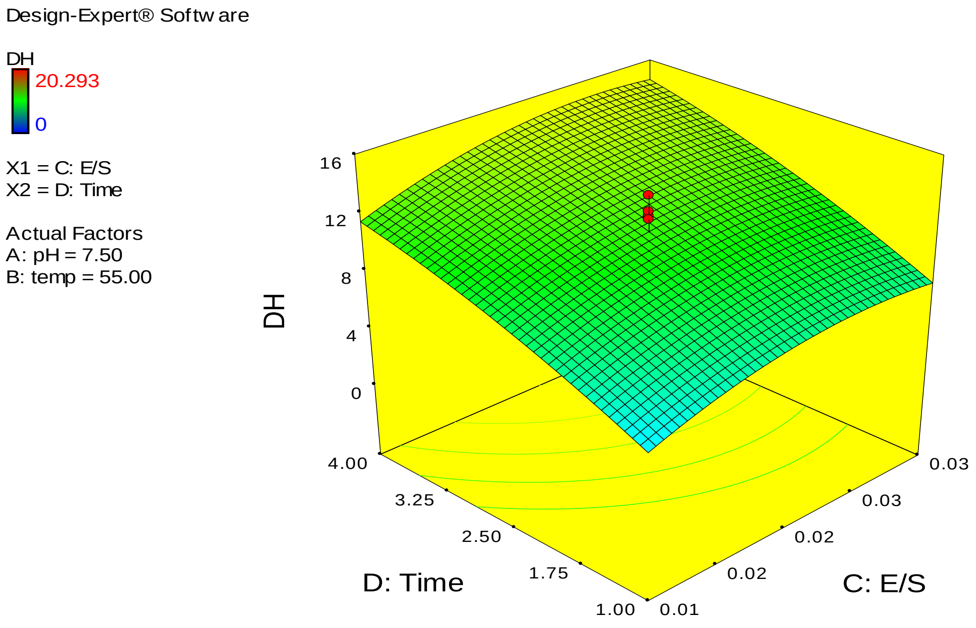

Fig 4.1.13 Response surface related to % DH value of WPH using Trypsin as influenced by E/S and Time

The influence of independent variables and their interaction effects can be analysed by using 3-D response graph. Figures 4.1.9, 4.1.10, 4.1.11, 4.1.12, 4.1.13 and 4.1.14 show the 3-D response graph for DH. The response surface graphs and perspective plots are drawn by varying two parameters and keeping the other two parameters at constant middle level (centre point).

Figure 4.1.9 shows the response graph for two varying parameters pH and Temperature by keeping other two parameters time and E/S ratio at constant middle level, which indicates that the increase in pH as well as Temperature significantly affected the %DH. A positive correlation was observed between the Temperature and %DH; and between pH and %DH.

However, when Temperature and time were kept at constant middle level, pH value did not significantly affect the %DH. On the other hand, on increasing E/S ratio from 0.01 to 0.03, %DH increased slightly, but not significantly. In continuation of the interpretation of response surface perspective graphs, effect of pH and time was studied, keeping constant rest of the variables (Temp. and E/S). It can be observed from the graph (Fig. 4.1.11) that pH and DH were not correlated under these specified variables. However, time showed a positive correlation with %DH under the same conditions. Response surface plot for %DH as affected by Temp and E/S did not show any correlation between %DH and the pertinent variables (Temp and E/S), when other two factors (pH and time) were kept at the constant middle point (Fig. 4.1.12). In another possible combination, when the pH and E/S were kept at centre point, increasing Temperature caused a non-significant ($P>0.05$) increase in the %DH value. Conversely, a positive association was observed between time and %DH. Fig. 4.1.13 shows the change in response (%DH) as affected by E/S and time, when pH and Temperature were kept at the centre point. It is interesting to note that both the studied variables showed a direct correlation with %DH.

Therefore, it can be concluded from the results discussed above based on data in Table 4.1.12., that %DH of WPC, hydrolysed with trypsin, was obtained in the range of 0 to 20.29. The value of DH varied with condition of hydrolysis. Our results are in accordance with the results reported by Kamau and Lu, (2011), who investigated the effect of enzymes and hydrolysis conditions conditions (WPC concentration 30 mg mL^{-1}, pH 8, temperature 50°C and hydrolysis time of 1 h, enzyme to substarte ratio 2% w/w) on degree of hydrolysis and 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of whey protein hydrolysates. They reported that Temperature, pH, substrate concentration and enzyme to substrate (E/S) ratio showed a significant influence on % DH and DPPH scavenging activity of the trypsin-derived WPH. Similar results have been reported by Kanu *et al.,* (2009), when they hydrolyzed sesame flour using different proteases. On the contrary, Silvestre *et al.,* (2013) studied the degree of hydrolysis and peptide profile of whey proteins using pancreatin and reported that DH of WPC proteins was weakly affected by period of hydrolysis. Further, they observed that DH increased sharply as the reaction time increased from 1 h (17.20%) to 2 h (24.86%) when using formol titration method. However, when using soluble protein content method, a slight decrease in the DH was observed when the reaction time increased from 4 h (35.68%) to 5 h (34.28%).

4.1.2.3 Optimization of hydrolytic conditions for formulation for whey protein hydrolysates developed by using Trypsin.

As discussed above, that %DH varied under different conditions of four independent variables, the most desirable combination of independent variables was selected for further investigation by verification of its sought after functional attributes. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.1.14 Criteria chosen for optimizing values of independent variables in WPH prepared using Trypsin

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	In range	7	8	3
	Temp (^{o}C)	In range	50	60	3
	E/S	Minimize	0.01	0.03	3
	Time (h)	In range	1.00	4.00	3
Response	DH	Maximize	0	20.293	3

To develop WPH under optimized conditions, three of the four factors viz. pH, Time and Temp were kept ‘In range’, while E/S was kept ‘minimize’. The goal (Table 4.1.14) for response (%DH) was set ‘Maximize’, as the goal of experiment is to get maximum DH under optimised conditions. Twenty nine different solutions were obtained, out of which six solutions with different values for dependent and independent variables are presented in Table 4.1.15. The desirability of the solution ranged from 0.872 to 0.886. With intention to get maximum value for %DH with a high desirability value, solution 1 was shortlisted for comparing and finalising the formulation.

Table 4.1.15 Suggested solutions and predicted dependent variable values of WPH using trypsin given by Design Expert software package

Number	pH	Temp (^{o}C)	E/S	Time(h)	DH	Desirability
Solution 1	7.00	60.00	0.01	4.00	15.97	0.88
Solution 2	7.00	60.00	0.01	4.00	16.02	0.88
Solution 3	7.00	60.00	0.01	3.85	15.71	0.88
Solution 4	7.05	60.00	0.01	3.99	15.62	0.87
Solution 5	7.00	60.00	0.01	4.00	16.31	0.87
Solution 6	7.05	60.00	0.01	4.00	15.67	0.87
Solution 7	7.00	59.96	0.01	3.71	15.47	0.87
Solution 8	7.03	60.00	0.01	3.78	15.45	0.87

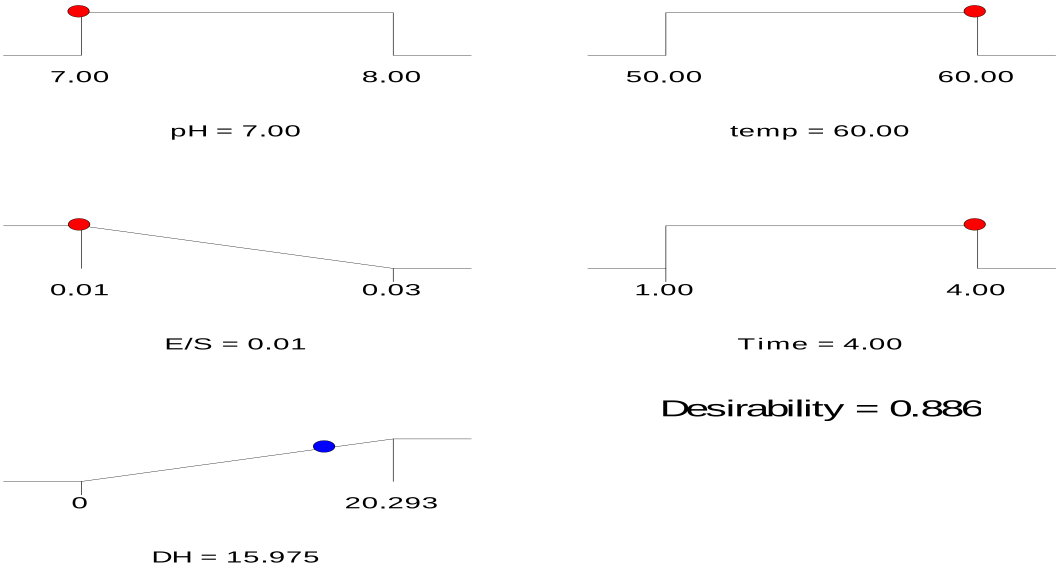

Fig. 4.1.14 Ramp graph for WPC-80, hydrolysed using Trypsin

Verification of the optimized conditions was done by preparing the selected sample (solution 1) in triplicate and then evaluating the product on similar lines as done while optimizing the formulation. The result revealed (Table 4.1.16) that the difference between the predicted and the actual values of independent variables were statistically non-significant. The optimized conditions of independent variables comprised pH 7, Temp 60°C, E/S 0.01 and Time 4 h which was selected for the preparation of WPH, using trypsin with maximum DH.

Table 4.1.16 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	15.97	17.42 ±1.385	1.814^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Whey protein hydrolysate, using Trypsin; t-value found to be non-significant at 1% level of significance; ^{ns} non-significant

4.1.3 Optimization of hydrolysis conditions of Casein Hydrolysate using Alcalase

Alcalase is a non-specific, endo-protease of the serine type, obtained from *Bacillus subtilis*. It is also known as subtilisin. It has very broad substrate specificity. In other words, it can hydrolyse most peptide bonds within a protein molecule. Alcalase is active between pH 6.5 and 8.5. It functions between 45 and 65 °C with maximum activity at about 60 °C, above which the activity falls rapidly. In the present study, sodium caseinate was hydrolysed using Alcalase and optimum conditions of the hydrolysis in terms of pH, Temperature, time and E/S ratio were investigated. Several researchers have worked on the development of casein hydrolysates using Alcalase and optimized the process conditions. A wide range of literature has suggested that most of the studies on casein hydrolysates and alcalase are related to ‘Plastein reaction’, which is not the scope of the present study. In present study, our goal was to develop non-bitter casein hydrolysates under optimized conditions using alcalase. Therefore, sodium caseinate was hydrolysed using Alcalase enzyme under different independent variables (pH, Temp, E/S and time) to determine maximum %DH (response) by RSM and the data was statistically analysed.

Table 4.1.17 Boundaries of the experimental domain and spacing of the compositional variables level.

Independent variables	Coded Value	Coded Levels				
		-2α	-1	0	+1	+2α
pH	A	6.5	7.0	7.5	8.0	8.5
Temp (°C)	B	45	50	55	60	65.
E/S	C	0	0.004	0.008	0.012	0.016
Time (h)	D	-0.5	1	2.5	4	5.5

An experimental design consisting of 30 runs and four independent variables at five different levels was applied in the study. The detailed design is listed in Tables 4.1.17 and 4.1.18, and all the experiments were carried out in triplicate. Various independent variables and their coded value are shown in Table 4.1.17. The central value (coded levels) of four independent variables i.e. pH, Temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.008 and 2.5 h, respectively. Results showed that out of 30 experiments, 16, 8 and 6 were factorial, axial and centre points, respectively (Table 4.1.18). It is clear that response (y, degree of hydrolysis, DH) is a function of experimental factors (pH, Temp, E/S and time; A, B, C and D resp.). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, $\beta_{F1,}$ $\beta_{F2,}$ $\beta_{F3,}$ β_{F4} are the first order coefficients, $\beta_{F12,}$ $\beta_{F13,}$ $\beta_{F23,}$ $\beta_{F14,}$ $\beta_{F24,}$ β_{F34} are the cross product coefficients and $\beta_{F11,}$ $\beta_{F22,}$ β_{F33} and β_{F44} are the second order coefficients.

Table 4.1.18 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Standard Run	Run Order	pH	Temp (^{o}C)	E/S	Time (h)	Coefficients assess
1	16	7	50	0.004	1	Factorial
2	2	8	50	0.004	1	Factorial
3	18	7	60	0.004	1	Factorial
4	24	8	60	0.004	1	Factorial
5	9	7	50	0.012	1	Factorial
6	19	8	50	0.012	1	Factorial
7	4	7	60	0.012	1	Factorial
8	22	8	60	0.012	1	Factorial
9	10	7	50	0.004	4	Factorial
10	12	8	50	0.004	4	Factorial
11	23	7	60	0.004	4	Factorial
12	17	8	60	0.004	4	Factorial
13	21	7	50	0.012	4	Factorial
14	28	8	50	0.012	4	Factorial
15	5	7	60	0.012	4	Factorial
16	14	8	60	0.012	4	Factorial
17	29	6.5	55	0.008	2.5	Axial
18	27	8.5	55	0.008	2.5	Axial
19	1	7.5	45	0.008	2.5	Axial
20	15	7.5	65	0.008	2.5	Axial
21	11	7.5	55	0.000	2.5	Axial
22	20	7.5	55	0.016	2.5	Axial
23	8	7.5	55	0.008	-0.5	Axial
24	30	7.5	55	0.008	5.5	Axial
25	6	7.5	55	0.008	2.5	Center
26	3	7.5	55	0.008	2.5	Center
27	25	7.5	55	0.008	2.5	Center
28	7	7.5	55	0.008	2.5	Center
29	13	7.5	55	0.008	2.5	Center
30	26	7.5	55	0.008	2.5	Center

Table 4.1.19 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Casein Hydrolysate, prepared using Alcalase

Standard Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	%DH
1	16	7.0	50	0.004	1.0	6.34
2	2	8.0	50	0.004	1.0	7.65
3	18	7.0	60	0.004	1.0	6.01
4	24	8.0	60	0.004	1.0	18.53
5	9	7.0	50	0.012	1.0	9.11
6	19	8.0	50	0.012	1.0	10.01
7	4	7.0	60	0.012	1.0	11.53
8	22	8.0	60	0.012	1.0	17.94
9	10	7.0	50	0.004	4.0	18.53
10	12	8.0	50	0.004	4.0	20.31
11	23	7.0	60	0.004	4.0	18.51
12	17	8.0	60	0.004	4.0	27.78
13	21	7.0	50	0.012	4.0	18.75
14	28	8.0	50	0.012	4.0	20.54
15	5	7.0	60	0.012	4.0	22.11
16	14	8.0	60	0.012	4.0	28.54
17	29	6.5	55	0.008	2.5	17.71
18	27	8.5	55	0.008	2.5	20.01
19	1	7.5	45	0.008	2.5	18.12
20	15	7.5	65	0.008	2.5	19.22
21	11	7.5	55	0.000	2.5	0
22	20	7.5	55	0.016	2.5	21.12
23	8	7.5	55	0.008	-0.5	0
24	30	7.5	55	0.008	5.5	24.95
25	6	7.5	55	0.008	2.5	15.23
26	3	7.5	55	0.008	2.5	18.78
27	25	7.5	55	0.008	2.5	18.19
28	7	7.5	55	0.008	2.5	24.51
29	13	7.5	55	0.008	2.5	20.78
30	26	7.5	55	0.008	2.5	24.51

4.1.3.1 Response surface analysis for %DH of Casein Hydrolysate, using Alcalase

Degree of hydrolysis of Casein Hydrolysate, prepared using Alcalase under various conditions of independent variables is presented in Table 4.1.19. Results indicated that %DH ranged from 0 to 27.78. The minimum value (0) was obtained for the two combinations of independent variables; one of which had pH 7.5, Temp 55°C, E/S 0.008 and Time -0.5 h and the second combination had pH 7.5, Temp 55°C, E/S 0.00 and Time 2.5 h. Data shows that the maximum value was obtained only for one combination of independent variables which had pH 8.0, Temp 60°C, E/S 0.004 and Time 4 h. It has already been discussed that Alcalase is active between pH 6.5 and 8.5 and functions between 45 and 65°C with optimum activity at about 60 °C. Thus, it supports the findings of the present study.

The experimental design and results of CCD are presented in Table 4.1.18. Response results were analyzed using Design Expert 7.0 and are shown in Tables 4.1.19 and 4.1.20. For the casein hydrolysates prepared by Alcalase, the coefficient of determination (R^2) was 0.851 as presented in Table 4.1.20, which indicated a high correlation between the independent variables and response (%DH). The probability p value was very low ($p<0.001$), indicating the significance of the model. The ANOVA also showed that there was a non-significant ($P>0.05$) lack of fit, which further validated the model. The APV was found to be 9.59, which was appreciably higher than the minimum desirable value (4.00) for the higher prediction ability.

To determine the effect of various independent variables singly or in combination on response (DH), the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) of all the four independent parameters were studied. It can be observed from Table 4.1.20, that the all the independent variables (pH, Temp, E/S and time) significantly ($P<0.05$) affected the degree of hydrolysis. However, the interactive effect of combinations (AB, AC, AD, BC, BD and CD) of all four independent variables on the %DH was found to be non-significant ($P>0.05$). Similar, non-significant effect was observed on %DH response, when quadrant forms were studied. Zhao and Li (2009) developed casein hydrolysates with plastein reaction catalyzed by Alcalase. In their experiment, the impact of four reaction conditions on plastein reaction of casein hydrolysates were studied, and then optimal conditions were determined using RSM with the decrease of free amino groups in the reaction mixture as response. The optimum conditions for the above decrease were found to be an E/S ratio of 7.7 kU g^{-1} proteins, reaction Temperature of 42.7°C and reaction time of 6 h; and reported that used model was fit with non-significant lack of fit and significant probability values. They also suggested that the studied independent variables (pH, Temp, concentration of substrate and E/S ratio) had a significant effect and positive correlation with DH, which was measured in terms of concentration of free amino acids.

4.1.3.2 Response surface plots and effects of factors on %DH of casein hydrolysates prepared by Alcalase

According to the model's regression analysis, the best explanatory model equation of %DH of casein hydrolysates as affected by different factors in terms of coded factors is as follows

$$DH = +20.34 + 1.88*A + 1.75*B + 2.38*C + 5.74*D + 1.80*A*B - 0.58*A*C - 0.12*A*D + 0.23*B*C - 0.13*B*D - 0.33*C*D - 0.16*A - 0.21*B2 - 2.24*C2 - 1.76*D2$$

Table 4.1.20 Coefficient of the full second order polynomial model for coded responses for Casein Hydrolysate prepared using Alcalase

ANOVA for Response Surface Quadratic model of Casein Hydrolysate developed using Alcalase					
Source	Sum of Squares	df	Mean of Sum of Square	F- Value	p-value Prob > F
Model	1346.18	14	96.16	6.14	0.0006
A-pH	84.43	1	84.43	5.39	0.0347
B-Temp	17.19	1	17.19	4.67	0.0472
C-E/S	135.86	1	135.86	8.68	0.0100
D-Time	791.95	1	791.95	50.57	<0.0001
AB	52.04	1	52.04	3.32	0.0883 ^{ns}
AC	5.47	1	5.47	0.35	0.5623 ^{ns}
AD	0.22	1	0.22	0.014	0.9080 ^{ns}
BC	0.86	1	0.86	0.055	0.8181 ^{ns}
BD	0.27	1	0.27	0.0017	0.8971 ^{ns}
CD	1.73	1	1.73	0.11	0.7445 ^{ns}
A^2	0.71	1	0.71	0.045	0.8340 ^{ns}
B^2	1.19	1	1.19	0.076	0.7866 ^{ns}
C^2	137.17	1	137.17	8.76	0.0097 ^{ns}
D^2	84.71	1	84.71	5.41	0.0345 ^{ns}
Residual	234.91	15	215.66		
Lack of Fit	166.84	10	16.68	1.23	0.4352 ^{ns}
Pure Error	68.07	5	13.61		
Cor Total	1581.08	29			
Other statistics					
R-squared					0.8514
APV					9.569
Mean					16.85±3.96

If p value is <0.05, it indicates the value is significantly different. Significant (P<0.05); Highly significant (P<0.01); non-significant (P>0.05).

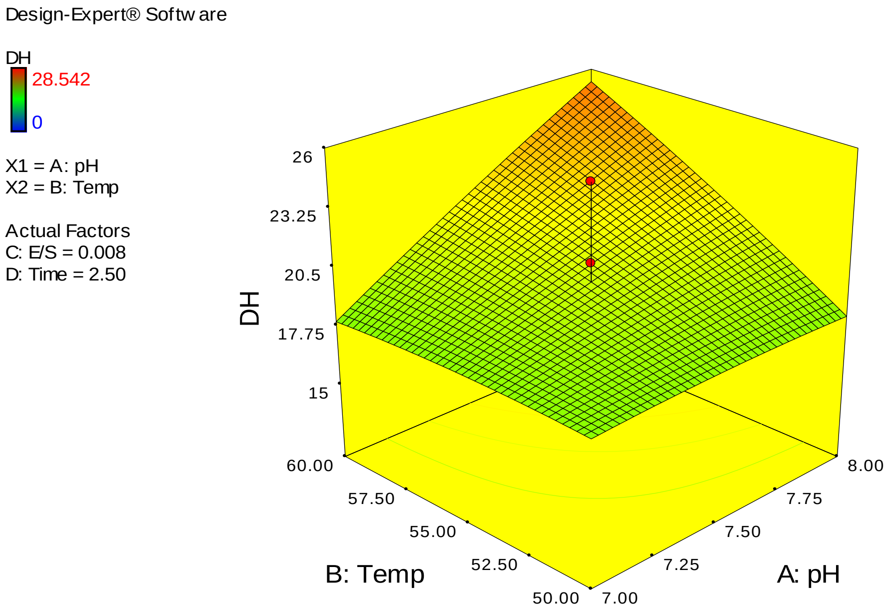

Fig 4.1.15 Response surface related to %DH value of Casein Hydrolysate using Alcalase as influenced by Temp and Ph

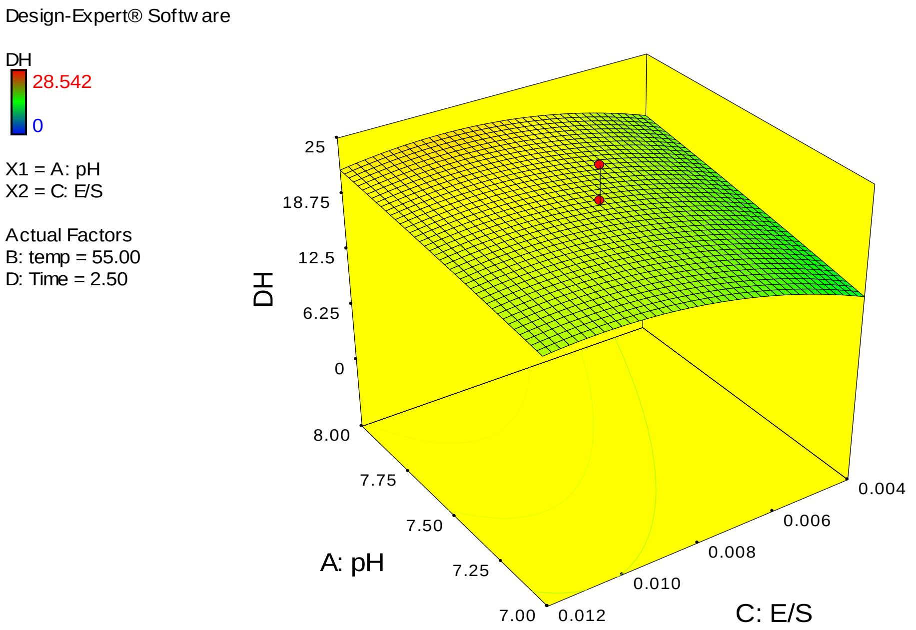

Fig 4.1.16 Response surface related to %DH value of Casein Hydrolysate using Alcalase as influenced by E/S and pH

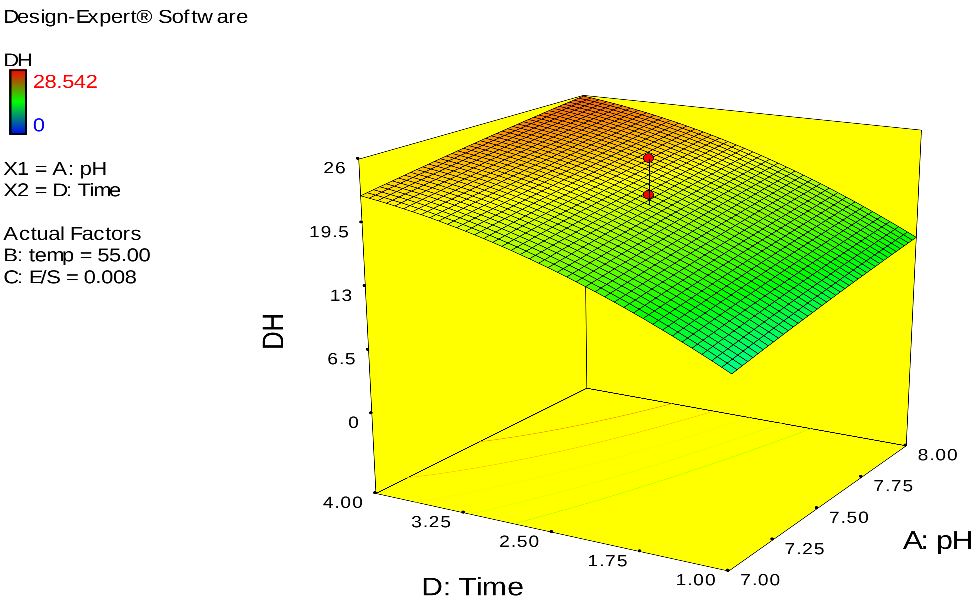

Fig 4.1.17 Response surface related to %DH value of Casein Hydrolysate using Alcalase as influenced by Time and pH

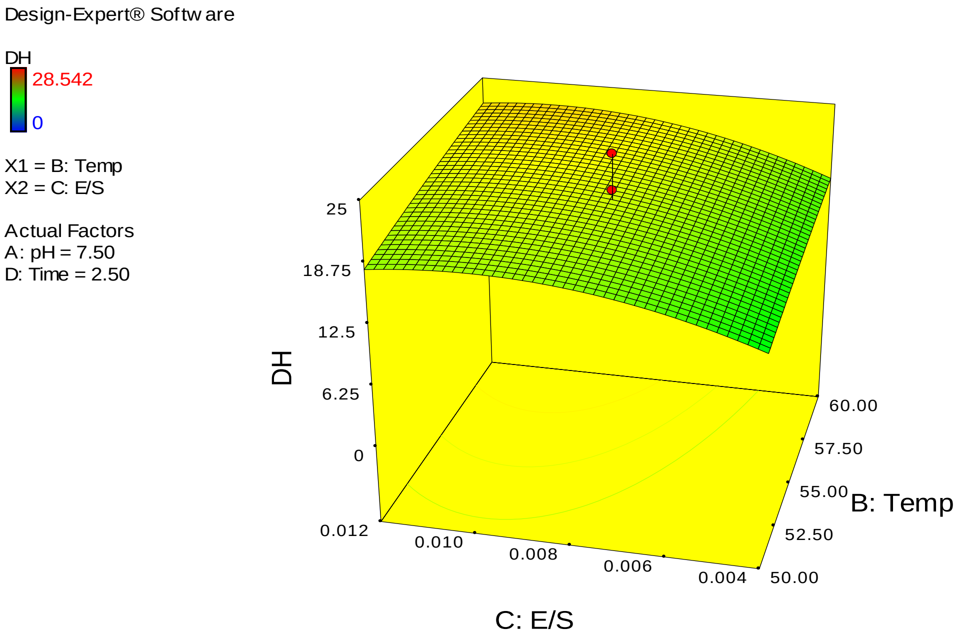

Fig 4.1.18 Response surface related to %DH value of Casein Hydrolysate using Alcalase as influenced by Temp and E/S

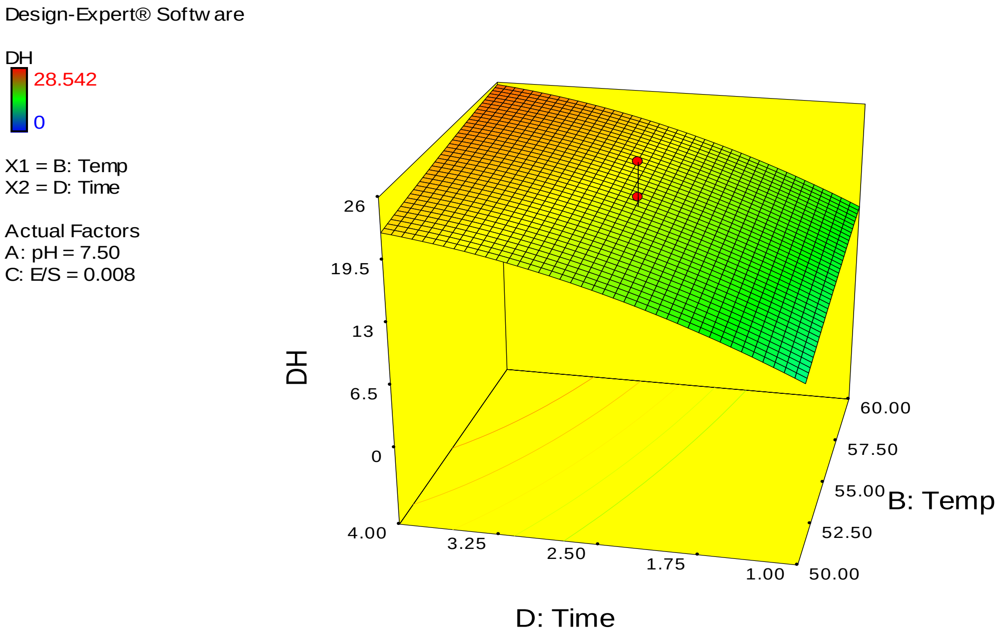

Fig 4.1.19 Response surface related to %DH value of Casein Hydrolysate using Alcalase as influenced by Temp and Time

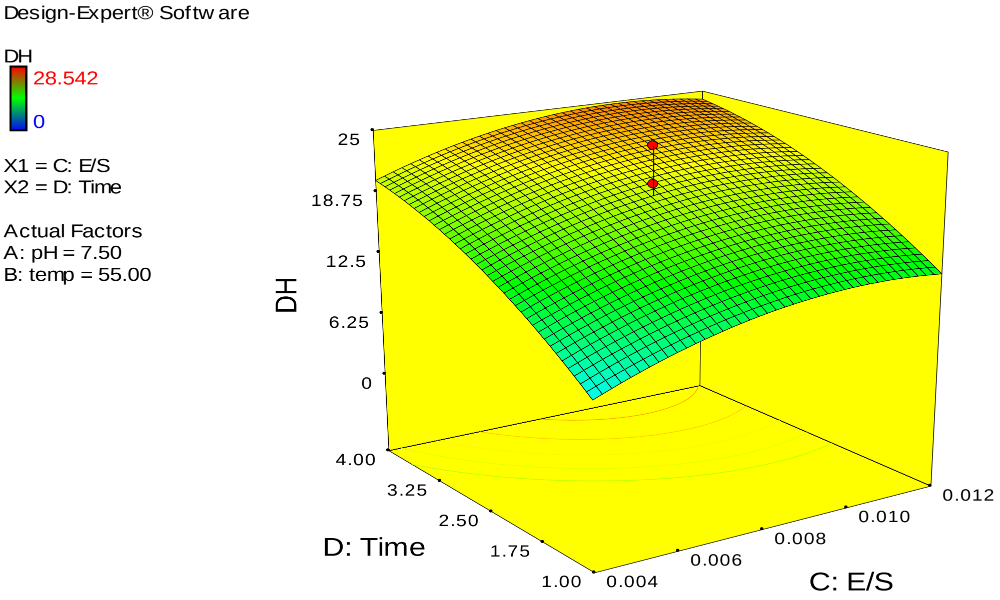

Fig 4.1.20 Response surface related to %DH value of Casein Hydrolysate using Alcalase as influenced by E/S and Time

Response surface graphs with perspective plots were developed to study the effect of independent variables, in combination, on %DH of sodium caseinate, hydrolysed using Alcalase. Figures 4.1.15 to 4.1.20 show the 3D response surface graphs, for which interpretation is described below:

It is clear from Fig. 4.1.15, that when E/S and time were kept at centre point 0.008 and 2.5 h, respectively, increasing pH and Temperature from 7.0 to 8.0 and 50-60°C, respectively did not significantly ($P>0.05$) affect the %DH. In another possible combination, when the Temperature and time were kept at centre point viz. 55^{o}C and 2.5 h, respectively, no correlation was observed between the pH and %DH (Fig. 5.1.18). However, a slight increase was observed in %DH on increasing enzyme concentration, followed by a gradual decrease in hydrolysis. It could be attributed to the optimum activity of alcalase at a particular enzyme concentration.

Response surface graph of %DH as affected by time and pH is shown in Fig. 4.1.17, which implied that on keeping Temperature and E/S at the centre point 55^{o}C and 0.008, respectively, %DH increased significantly ($P<0.05$) on increasing hydrolysis time. However, no relationship was noticed between the pH and %DH of sodium caseinate.

On keeping pH and time constant (at centre point) 7.5 and 2.5 h, respectively, increase in Temperature did not significantly ($P>0.05$) affect %DH. However, similar trend was observed between E/S and %DH, as discussed for Fig. 4.1.18. It indicates that on increasing E/S ratio first there is increase in the %DH, but after reaching on optimum level, %DH showed significant decrease ($P<0.05$).

When pH and E/S were kept at centre point viz. 7.5 and 0.02, respectively, increase in Temperature did not significantly affect the %DH. However, time showed a significant effect ($P<0.05$) and positive correlation with %DH (Fig. 4.1.19). Here, it is interesting to note that on keeping pH and Temperature constant at 7.5 and 55°C, respectively, E/S and time showed a positive and significant association with DH. From the results discussed above, it can be concluded that %DH ranged from 0 to 27.78 under varied conditions of independent variables. Li *et al.* (2010) prepared alcalase-catalyzed casein hydrolysates and reported that DH of casein hydrolysates increased from 0 to 11.4% during first 6 h and then remained constant till 12 h period. Similar results have been reported by Zhao and Li (2009).

4.1.3.3 Optimization for formulation of Casein Hydrolysate developed using Alcalase

From the results obtained through an array of hydrolysates prepared using various combinations of independent variables, as given by the software package Design Expert® (version 7.0.0), the most desirable combination of independent variables was selected for further investigation by verification of its sought after functional attributes. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.1.21 Criteria chosen for optimizing values of independent variables in Casein Hydrolysate, hydrolysed using Alcalase

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	In range	7	8	3
	Temp (^{o}C)	In range	50	60	3
	E/S	Minimize	0.004	0.012	3
	Time (h)	In range	1.00	4.00	3
Response	DH	Maximize	0	28.542	3

For optimization, goal for factors as well as for response was selected. Three of the four factors viz. pH, Temp and Time were kept 'In range' and E/S was kept at 'Minimize'. The goal (Table 4.1.21) for DH was set at 'Maximize'. Twenty nine different solutions were obtained out of which six solutions with different values for dependent and independent variables are presented in Table 4.1.22. The desirability of the solution ranged from a minimum of 0.931 to a maximum of 0.940. With intention to get maximum value of DH percentage with a high desirability value, solution 1 was shortlisted for comparing and finalising the formulation.

Table 4.1.22 Suggested solutions and predicted dependent variable values of Casein Hydrolysate using Alcalase as per Design Expert software package

Number	pH	Temp	E/S	Time	%DH	Desirability
Solution 1	8.00	60	0.004	4.00	25.197	0.940
Solution 2	7.98	60	0.004	4.00	25.059	0.937
Solution 3	7.98	60	0.004	4.00	25.030	0.936
Solution 4	7.97	60	0.004	4.00	24.961	0.935
Solution 5	7.96	60	0.004	4.00	24.926	0.935
Solution 6	8.00	60	0.004	3.83	24.913	0.934
Solution 7	7.95	60	0.004	4.00	24.840	0.933
Solution 8	8.00	60	0.004	3.73	24.718	0.931

To confirm the applicability of the model designed, confirmation runs using the calculated levels of the variables were carried out in triplicates. Although this actual value was higher than the expected value, there was no significant difference ($P<0.05$) between them (Table 4.1.23). This result also indicated that the second-order polynomial model could be used to predict the %DH of sodium caseinate hydrolysed using alcalase under different conditions.

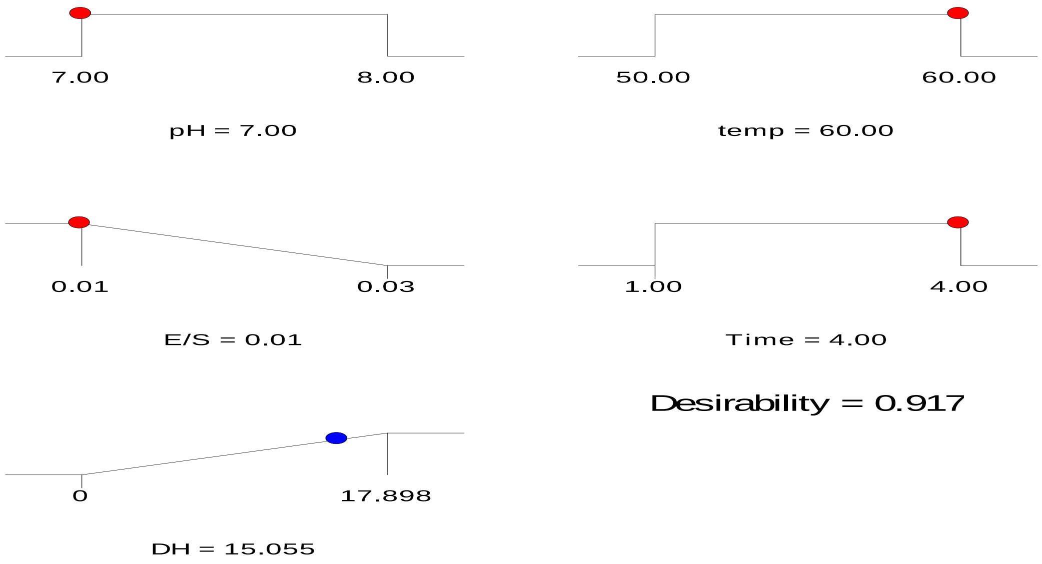

Fig. 4.1.21 Ramp graph for Casein Hydrolysate prepared using Alcalase

From the results, it can be concluded that maximum degree of hydrolysis (25.19) of sodium caseinate using alcalase, was observed under the optimum conditions of pH 8.0, Temp. 60°C, E/S 0.004 and time 4 h. In other words, these values of independent variables can be considered to be optimum condition for the production of casein hydrolysates catalysed by alcalase. Mahmoud *et al.* (1992) studied the casein hydrolysis by porcine pancreatin enzyme and observed a very high degree of hydrolysis (48%) in first 10% of hydrolysis time at pH 7.0.

Table 4.1.23 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	25.19	27.08±1.833	1.778^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Casein Hydrolysate, hydrolysed using Alcalase; t-value found to be non-significant at 1% level of significance; ^{ns} non-significant

4.1.4. Optimization of hydrolysis conditions of WPC-80 using Alcalase

Whey protein, a by-product recognized as valuable food ingredient with important nutritional and functional properties, is gaining acceptance as a functional food ingredient. Hydrolysis of whey protein is known to release bioactive peptides that can exhibit a number of physiological properties and enable them to exhibit antioxidative, antihypertensive, antimicrobial activity, opioid activity and antithrombotic activity (Clare and Swaisgood 2000; Pihlanto Leppala 2001; Le-Maux *et al.*, 2016; Mukhopadhya and Sweeney, 2016; Dalziel *et al.*, 2016; Bansal and Bhandari, 2016). WPHs had shown a broad range of antioxidant activity in an iron-catalysed liposome oxidation system depending on the type of protease used. Dryakova *et al.*, (2010) reported that on hydrolysis with microbial proteases (alcalase, flavourzyme, protamex and neutrase) the antioxidant activity of whey protein increased from 7.0–19.8 to 40.0–54.2 %. There are concerns about the potential health effects of synthetic antioxidants (such as BHA, gallates etc.) and therefore, the demand for natural antioxidants has recently increased. In present study, alcalase was used for the hydrolysis of whey proteins and process conditions were optimized by RSM.

Table 4.1.24 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2α	**-1**	**0**	**+1**	**+2α**
pH	A	6.5	7.0	7.5	8.0	8.5
Temp (°C)	B	45	50	55	60	65.
E/S	C	0	0.004	0.008	0.012	0.04
Time (h)	D	-0.5	1	2.5	4	5.5

In total, 30 runs and four independent variables at five different levels were applied in the RSM model. Various independent variables and their coded value are shown in Table 4.1.24. The detailed experimental design is shown in Tables 4.1.25 and 4.1.26. All experiments were carried out in triplicate. The central value (coded levels) of four independent variables i.e. pH, Temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.008 and 2.5 h, respectively. Results showed that out of 30 experiments, 16, 8 and 6 were factorial, axial and centre points, respectively (Table 4.1.25). It is clear that response (y, degree of hydrolysis, DH) is a function of experimental factors (pH, Temp, E/S and time). Second order polynomials was developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and β_{F11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.1.25 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Standard Run	Run Order	pH	Temp (°C)	E/S	Time (h)	Coefficients assess
1	16	7.0	50	0.004	1.0	Factorial
2	2	8.0	50	0.004	1.0	Factorial
3	18	7.0	60	0.004	1.0	Factorial
4	24	8.0	60	0.004	1.0	Factorial
5	9	7.0	50	0.012	1.0	Factorial
6	19	8.0	50	0.012	1.0	Factorial
7	4	7.0	60	0.012	1.0	Factorial
8	22	8.0	60	0.012	1.0	Factorial
9	10	7.0	50	0.004	4.0	Factorial
10	12	8.0	50	0.004	4.0	Factorial
11	23	7.0	60	0.004	4.0	Factorial
12	17	8.0	60	0.004	4.0	Factorial
13	21	7.0	50	0.012	4.0	Factorial
14	28	8.0	50	0.012	4.0	Factorial
15	5	7.0	60	0.012	4.0	Factorial
16	14	8.0	60	0.012	4.0	Factorial
17	29	6.5	55	0.008	2.5	Axial
18	27	8.5	55	0.008	2.5	Axial
19	1	7.5	45	0.008	2.5	Axial
20	15	7.5	65	0.008	2.5	Axial
21	11	7.5	55	0.000	2.5	Axial
22	20	7.5	55	0.016	2.5	Axial
23	8	7.5	55	0.008	-0.5	Axial
24	30	7.5	55	0.008	5.5	Axial
25	6	7.5	55	0.008	2.5	Center
26	3	7.5	55	0.008	2.5	Center
27	25	7.5	55	0.008	2.5	Center
28	7	7.5	55	0.008	2.5	Center
29	13	7.5	55	0.008	2.5	Center
30	26	7.5	55	0.008	2.5	Center

Table 4.1.26 Central Composite Design Matrix(CCDM) with the experimental data on dependent variables of whey protein hydrolysates developed using Alcalase

Std. Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	DH
1	16	7.0	50	0.004	1.0	7.61
2	2	8.0	50	0.004	1.0	8.04
3	18	7.0	60	0.004	1.0	6.33
4	24	8.0	60	0.004	1.0	19.83
5	9	7.0	50	0.012	1.0	9.26
6	19	8.0	50	0.012	1.0	10.02
7	4	7.0	60	0.012	1.0	12.36
8	22	8.0	60	0.012	1.0	19.00
9	10	7.0	50	0.004	4.0	19.23
10	12	8.0	50	0.004	4.0	22.42
11	23	7.0	60	0.004	4.0	20.25
12	17	8.0	60	0.004	4.0	29.59
13	21	7.0	50	0.012	4.0	18.52
14	28	8.0	50	0.012	4.0	21.34
15	5	7.0	60	0.012	4.0	22.02
16	14	8.0	60	0.012	4.0	30
17	29	6.5	55	0.008	2.5	19.47
18	27	8.5	55	0.008	2.5	20.12
19	1	7.5	45	0.008	2.5	17.12
20	15	7.5	65	0.008	2.5	19.34
21	11	7.5	55	0.000	2.5	0
22	20	7.5	55	0.016	2.5	21.31
23	8	7.5	55	0.008	-0.5	0
24	30	7.5	55	0.008	5.5	26.49
25	6	7.5	55	0.008	2.5	16.25
26	3	7.5	55	0.008	2.5	20.59
27	25	7.5	55	0.008	2.5	20.78
28	7	7.5	55	0.008	2.5	24.71
29	13	7.5	55	0.008	2.5	20.15
30	26	7.5	55	0.008	2.5	25.77

4.1.4.1 Response surface analysis for DH of whey protein hydrolysates developed using Alcalase

Response surface analysis data for %DH of whey proteins hydrolysates prepared using alcalase is discussed here in. The design of the model and experiment and results for the same are presented in Table 4.1.25. Design Expert 7.0 was used to interpret the response results and are shown in Tables 4.1.26 and 4.1.27. Degree of hydrolysis under various conditions of independent variables is presented in Tables 4.1.25 and 4.1.26. Results indicated that %DH ranged from 0 to 29.59. The minimum value (0) was obtained for the two combinations of independent variables; one of which had pH 7.5, Temp 55°C, E/S 0.00 and Time 2.5 h and the second combination had pH 7.5, Temp 55°C, E/S 0.008 and Time -0.5 h. Data shows that the maximum value was obtained only for one combination of independent variables which had pH 8.0, Temp 60°C, E/S 0.004 and Time 4 h.

Both these values of %DH were obtained at different levels of pH, Temperature, E/S and time. Such behaviour suggests possible interaction between the independent variables in the hydrolysis of whey proteins by Alcalase. Analysis of variance (F-test) showed that the second order model is well adjusted to the experimental data (Table 4.1.27). The coefficient of determination (R^2: 0.8404) implies that 84% of the behaviour variation could be explained by the fitted model. Moreover, the lack-of-fit test, which measures the fitness of the model obtained, did not result in a significant F-value, indicating that the model is sufficiently accurate for predicting the DH for independent variable values within the ranges studied. To determine the effect of various independent variables singly or in combinations on response (DH), the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) of all the four independent parameters were studied. It can be observed from Table 4.1.20, that the all the independent variables (pH, Temp, E/S and time) significantly ($P<0.05$) affected the degree of hydrolysis. However, the interactive effect of combinations (AB, AC, AD, BC, BD and CD) of all four independent variables on the %DH was found to be non-significant ($P>0.05$). It should be noted here that effect of quadratic terms of independent variables was non-significant ($p>0.05$) except for C^2 and D^2, for which p values were found to be <0.05. Overall, statistical analysis indicated that within each term all four hydrolysis factors had a strong influence on DH. In a study, Adler-Nissen (1986), investigating the hydrolysis of soy protein by bacterial proteases, pointed out that pH, Temperature and E/S ratio markedly influenced the peptide bond cleavage in the protein substrate. Diniz and Martin (1996) used RSM to describe the combined effects of pH, Temperature and E/S ratio on the hydrolysis of dogfish (*Squalus acanthias*) muscle, and reported similar results.

4.1.4.2 Response surface plots and effects of factors on %DH of casein hydrolysates prepared using Alcalase

The best explanatory equation for DH is given below. This was generated using the regression coefficients with statistical significance up to 5% probability level. The polynomial model shows that all linear terms and two quadrant forms (C^2 and D^2) contributed to the response. However, the interactive effects among independent variables did not appear to be significant in comparison with the linear and quadratic effects.

$$DH = 21.38 + 1.92 * A + 1.97 * B + 2.16 * C + 6.00 * D + 1.89 * A * B - 0.52*A*C + 0.13*A*D + 0.35*B*C - 0.14*B*D - 0.53*C*D - 0.10*A^2 - 0.49*B^2 - 2.39*C^2 - 1.74*D^2$$

Table 4.1.27 Coefficient of the full second order polynomial model for coded responses for WPH hydrolysed using Alcalase

ANOVA for Response Surface Quadratic model of Alcalase WPH					
Source	**Sum of Squares**	**df**	**Mean of Sum of Squares**	**F- Value**	**p-value Prob > F**
Model	1441.27	14	102.95	5.64	0.0010
A-pH	88.02	1	88.02	4.82	0.0442
B-Temp	93.60	1	93.60	5.13	0.0388
C-E/S	112.02	1	112.02	6.14	0.0256
D-Time	863.03	1	863.03	47.285	<0.0001
AB	57.16	1	57.16	3.13	0.0971 ^{ns}
AC	4.28	1	4.28	0.23	0.6351 ^{ns}
AD	0.25	1	0.25	0.014	0.9081 ^{ns}
BC	1.92	1	1.92	0.11	0.7503 ^{ns}
BD	0.31	1	0.31	0.017	0.8973 ^{ns}
CD	4.45	1	4.45	0.24	0.6287 ^{ns}
A^2	0.29	1	0.29	0.016	0.9013 ^{ns}
B^2	6.69	1	6.69	0.37	0.5540 ^{ns}
C^2	156.51	1	156.51	8.57	0.0104
D^2	83.11	1	83.11	4.55	0.0498 ^{ns}
Residual	273.80	15	18.25		
Lack of Fit	214.64	10	21.46	1.81	0.2651 ^{ns}
Pure Error	59.16	5	11.83		
Cor Total	1715.07	29			
Other statistics					
R-squared					0.8404
APV					9.088
Mean					17.60±4.27

Significant (P<0.05); Highly significant (P<0.01); non-significant (P>0.05)

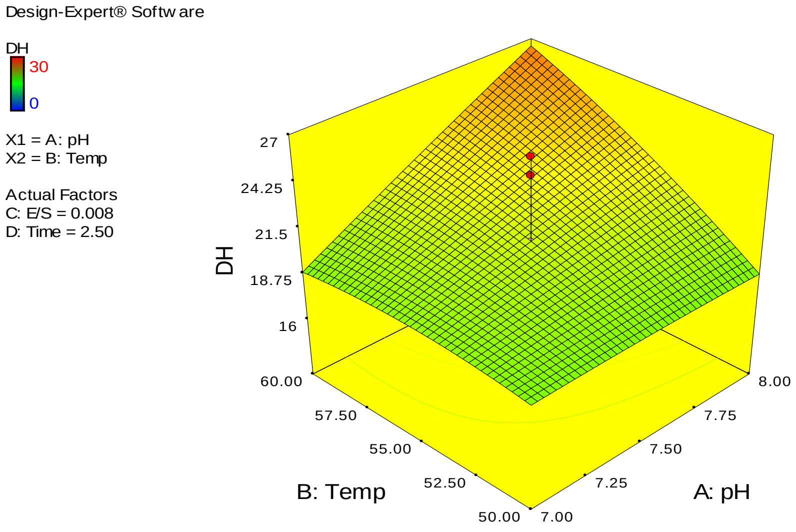

Fig 4.1.22 Response surface related to %DH value of WPH hydrolysed using Alcalase as influenced by Temp and pH

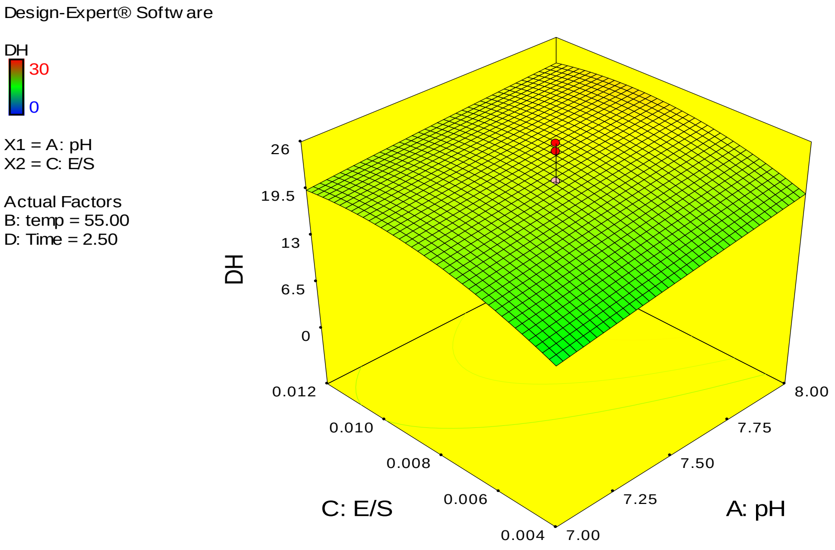

Fig 4.1.23 Response surface related to %DH value of WPH hydrolysed using Alcalase as influenced by E/S and pH

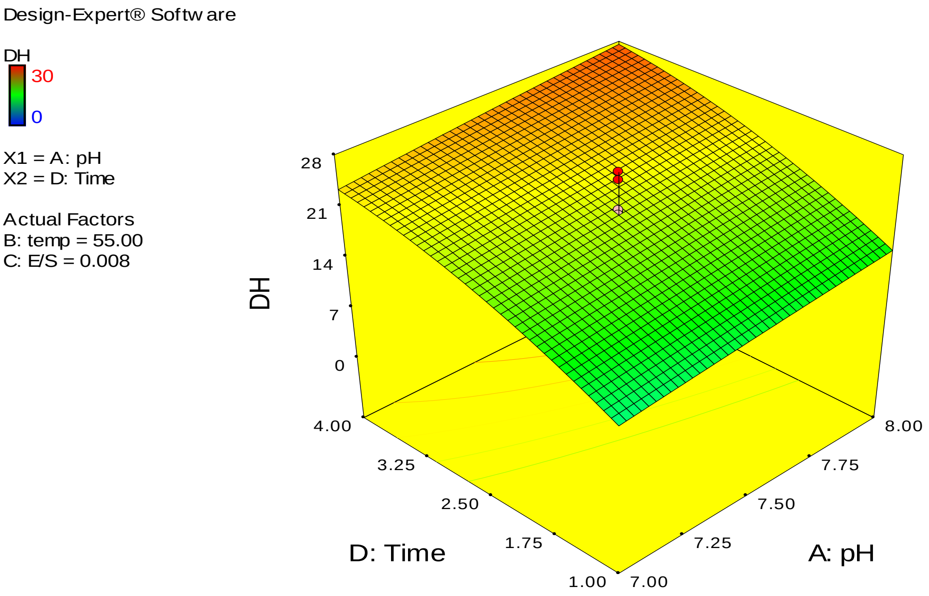

Fig 4.1.24 Response surface related to %DH value of WPH hydrolysed using Alcalase as influenced by Time and pH

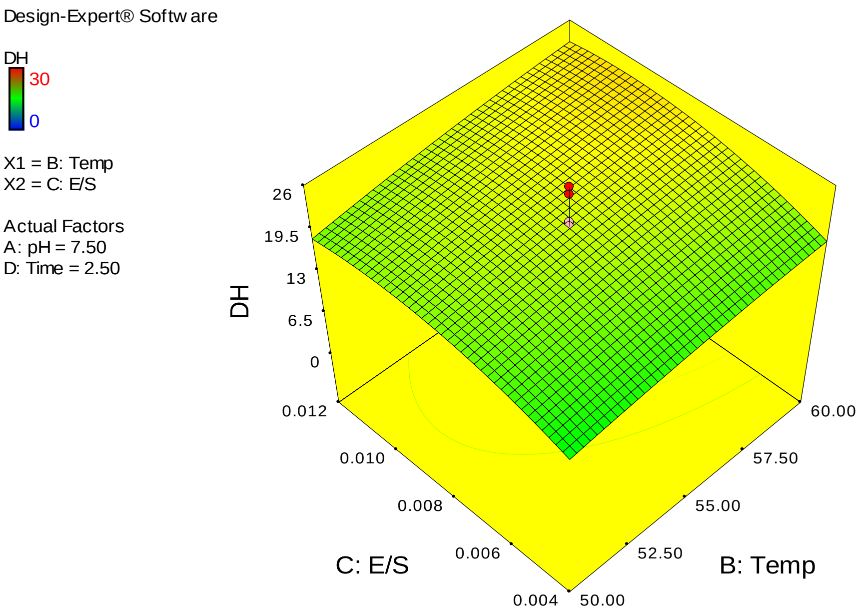

Fig 4.1.25 Response surface related to %DH value of WPH hydrolysed using Alcalase as influenced by Temp and E/S

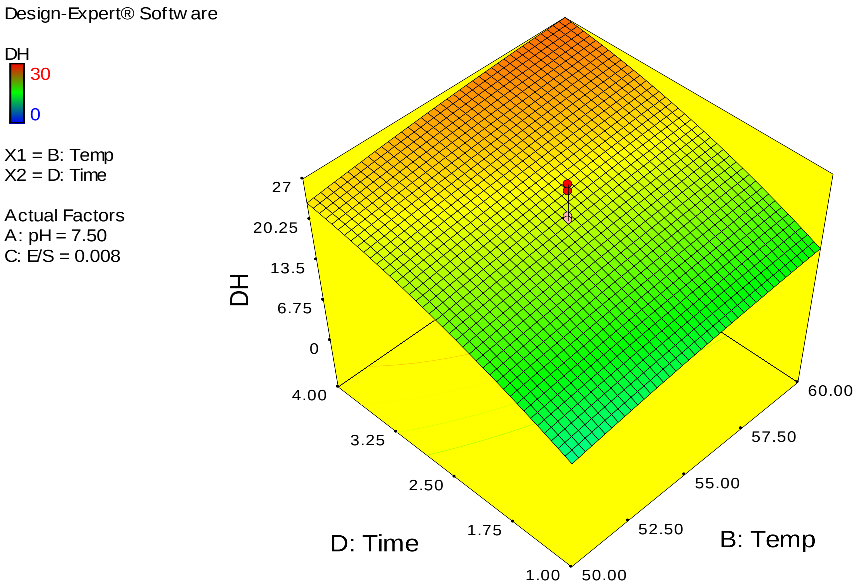

Fig 4.1.26 Response surface related to %DH value of WPH hydrolysed using Alcalase as influenced by Temp and Time

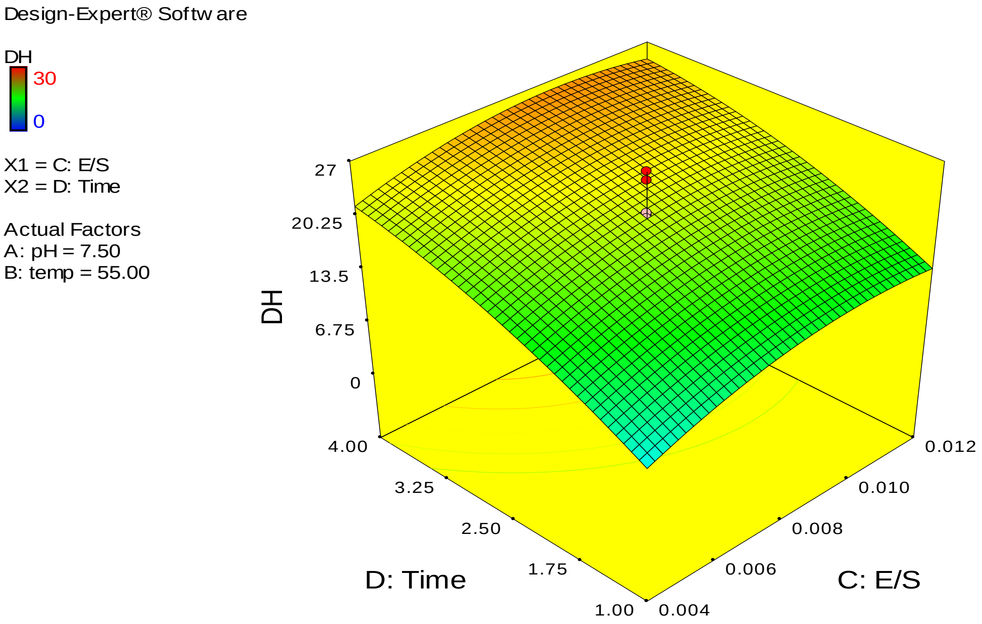

Fig 4.1.27 Response surface related to percentage DH value of WPH hydrolysed using Alcalase as influenced by E/S and Time

The effect of pH and Temperature is illustrated in the response surface plot (Fig. 4.1.25). It is clear from the said plot that when E/S and Time were kept at centre point 0.008 and 2.5 h, respectively, increasing pH from 7.0 to 8.0 and Temperature from 50-60°C did not significant ($P<0.05$) affect the %DH. However, when the Temperature (55^{o}C) and time (2.5 h) were kept at centre point, a significant effect of E/S was observed on %DH, but no correlation was observed between pH and %DH (Fig. 4.1.26).

Perspective plot of response i.e. %DH as affected by time and pH is shown in Fig. 5.1.27, which suggested that on keeping Temperature and E/S of 55^{o}C and 0.008, respectively, at centre point, %DH increased significantly ($P<0.05$) on increasing the hydrolysis time. However, no association was noticed between pH and %DH of whey proteins. Fig. 5.1.28 shows that on keeping pH and time constant (at centre point) 7.5 and 2.5 h, respectively, increase in Temperature did not significantly affect ($P>0.05$) the %DH. However, %DH first increased on increasing E/S ratio followed by a gradual but significant decrease ($P<0.05$) in %DH.

The effect of time and Temperature was observed by keeping pH and E/S constant at centre point i.e. (7.5 and 0.008, respectively). It was observed that an increase in the temperature did not significantly affect the %DH. However, time showed a significant effect ($P<0.05$) and positive correlation on %DH (Fig. 5.1.29). On the contrary, keeping pH and Temperature constant at 7.5 and 55°C, respectively, E/S and time showed a positive and significant association with DH. From the results discussed above, it can be concluded that %DH ranged from 0 to 29.59 under varied conditions of independent variables. In present study, a very high degree of hydrolysis was observed, when whey proteins were hydrolysed using alcalase under optimum conditions. Several researchers have reported that alcalase-assisted reactions exhibited higher DH compared to neutral or acid enzymes from plant, animal or microbial origin, such as papain, pepsin and Neutrase, respectively (Quaglia and Orban, 1987; Rebeca *et al.*, 1991; Sugiyama *et al.,* 1991). In another study conducted by Diniz and Martin (1996), hydrolysis variables were optimized at a pH of 8.3, a hydrolysis Temperature of 53.6°C and an E/S of 3.6 (w/w) and predicted DH value of 18.8% using alcalase.

4.1.4.3 Optimization for formulation for WPC-80 hydrolysed using Alcalase

To determine the optimized conditions for preparation of WPH using alcalase, the most desirable combination of independent variables was selected for further investigation. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.1.28 Criteria chosen for optimizing values of independent variables in WPH hydrolysed using Alcalase

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	In range	7	8	3
	Temp (°C)	In range	50	60	3
	E/S	Minimize	0.004	0.012	3
	Time (h)	In range	1.00	4.00	3
Response	DH	Maximize	0	30.0	3

To optimize the values of independent variables, three of the four factors viz. pH, Temp and Time were kept at 'In range' and E/S was kept at 'Minimize'. The goal (Table 5.1.21) for DH was set 'Maximize'. Twenty nine different solutions were obtained out of which six solutions with different values for dependent and independent variables are presented in Table 5.1.29. The desirability of the solution ranged from a minimum of 0.939 to a maximum of 0.946. Out of 6 solutions, solution 2 was shortlisted due to its highest value of %DH for comparing and finalising the formulation

Table 4.1.29 Suggested solutions and predicted dependent variable values of WPH, hydrolysed using Alcalase as per Design Expert software package

Number	pH	Temp	E/S	Time	DH	Desirability
Solution 1	8.00	59.83	0.004	4.00	26.87	0.946
Solution 2	8.00	60.00	0.004	4.00	27.05	0.945
Solution 3	8.00	59.68	0.004	4.00	26.80	0.945
Solution 4	7.99	60.00	0.004	3.94	26.75	0.944
Solution 5	8.00	59.47	0.004	4.00	26.68	0.943
Solution 6	8.00	60.00	0.004	3.81	26.53	0.940
Solution 7	8.00	60.00	0.004	3.79	26.45	0.939
Solution 8	8.00	59.98	0.004	4.00	27.35	0.939

The relevance and suitability of the model was verified by preparing the selected sample (solution 2) in triplicates. Although this actual value (29.033) was higher than the predicted value (27.059), there was no significant difference ($P<0.05$) between them (Table 5.1.30). This result also indicated that the that the model was capable of predicting the studied response, i.e. %DH of whey proteins hydrolysed by alcalase under different variables.

Finally, the optimum values of the four independent variables in order to get maximum degree of hydrolysis of whey proteins using alcalase are as follows: pH 8.0, Temperature 60°C, E/S 0.004 and time 4 h.

Table 4.1.30 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	27.059	29.03±1.983	1.724^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional WPH, hydrolysed using Alcalase; T-value was found to be non-significant at 1% level of significance; ^{ns} non-significant

4.1.5. Optimization of hydrolysis conditions of Casein Hydrolysate using Papain

Papain is a cysteine protease, which consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for activity of the enzyme. It shows the optimal pH for activity in the range of 6.0-7.0 and optimum Temperature at around 65°C. Although papain solutions have good Temperature stability, the solution stability is pH dependent. Papain solutions are unstable under acidic conditions, i.e., at pH values below 2.8, there is a significant loss in activity. Several researchers have reported the use of papain in developing casein hydrolysates (Barbosa *et al.,* 2004; Morais *et al.,* 2005; Zhao *et al.,* 2010; Zhang *et al.,* 2010; Shu *et al.,* 2015; Kumar *et al.,* 2016a; Kumar *et al.,* 2016b; Abdel-Hamid *et al.,* 2017; Lin *et al.,* 2017), and in the production of debittered amino-acids mixtures and peptides. Recently, a comprehensive review is published on the preparation, properties, and uses of enzymatic milk protein hydrolysates using various proteases such as papain, trypsin, alcalase, etc. (Abd El-Salam and El-Shibiny, 2017). Abd El-Salam and El-Shibiny, (2017) reported that when papain was immobilized with trypsin and rhozyme 41 , it caused hydrolysis of casein to a great extent within 30 min, demonstrating increased efficiency of immobilized enzymes. Hydrolysis of whey proteins with alcalase followed by treatment with papain was highly effective in reducing the immune reactivity and improving the sensory properties of the hydrolysate (Wröblewska et al., 2004).

Table 4.1.31 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2α	-1	0	+1	+2α
pH	A	6.5	7.0	7.5	8.0	8.5
Temp(°C)	B	45.0	50	55.0	60.0	65.0
E/S	C	2.0	3.0	4.0	5.0	6.0
Time (h)	D	-0.5	1.0	2.5	4.0	5.5

As discussed in previous section, boundaries of the experimental domain and spacing of the compositional variables level are shown in Table 5.1.31. Various independent variables and their coded value and levels are indicated in the said table. The central value of the coded levels of four independent variables i.e. pH, Temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 4.0 and 2.5 h, respectively. Results revealed that out of 30 experiments, which were run in randomised order, included 16 factorial, 8 axial and 6 centre points (Table 5.1.32). In the present study, one response (y, degree of hydrolysis, DH) was analysed, which can be considered as a function of experimental and independent factors (pH, Temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and βF_{11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.1.32 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Std. Run	Run Order	pH	Temp	E/S	Time	Coefficients
1	16	7.0	50	3.0	1.0	Fact
2	2	8.0	50	3.0	1.0	Fact
3	18	7.0	60	3.0	1.0	Fact
4	24	8.0	60	3.0	1.0	Fact
5	9	7.0	50	5.0	1.0	Fact
6	19	8.0	50	5.0	1.0	Fact
7	4	7.0	60	5.0	1.0	Fact
8	22	8.0	60	5.0	1.0	Fact
9	10	7.0	50	3.0	4.0	Fact
10	12	8.0	50	3.0	4.0	Fact
11	23	7.0	60	3.0	4.0	Fact
12	17	8.0	60	3.0	4.0	Fact
13	21	7.0	50	5.0	4.0	Fact
14	28	8.0	50	5.0	4.0	Fact
15	5	7.0	60	5.0	4.0	Fact
16	14	8.0	60	5.0	4.0	Fact
17	29	6.5	55	4.0	2.5	Axial
18	27	8.5	55	4.0	2.5	Axial
19	1	7.5	45	4.0	2.5	Axial
20	15	7.5	65	4.0	2.5	Axial
21	11	7.5	55	2.0	2.5	Axial
22	20	7.5	55	6.0	2.5	Axial
23	8	7.5	55	4.0	-0.5	Axial
24	30	7.5	55	4.0	5.5	Axial
25	6	7.5	55	4.0	2.5	Center
26	3	7.5	55	4.0	2.5	Center
27	25	7.5	55	4.0	2.5	Center
28	7	7.5	55	4.0	2.5	Center
29	13	7.5	55	4.0	2.5	Center
30	26	7.5	55	4.0	2.5	Center

Table 4.1.33 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Casein hydrolysate, hydrolysed using Papain

Std. Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	DH
1	16	7.0	50	3.0	1.0	7.05
2	2	8.0	50	3.0	1.0	3.98
3	18	7.0	60	3.0	1.0	3.01
4	24	8.0	60	3.0	1.0	4.97
5	9	70.0	50	5.0	1.0	8.23
6	19	8.0	50	5.0	1.0	6.97
7	4	7.0	60	5.0	1.0	5.79
8	22	8.0	60	5.0	1.0	8.75
9	10	7.0	50	3.0	4.0	11.59
10	12	8.0	50	3.0	4.0	8.54
11	23	7.0	60	3.0	4.0	10.78
12	17	8.0	60	3.0	4.0	10.57
13	21	7.0	50	5.0	4.0	14.43
14	28	8.0	50	5.0	4.0	11.45
15	5	7.0	60	5.0	4.0	12.64
16	14	8.0	60	5.0	4.0	10.85
17	29	6.5	55	4.0	2.5	11.73
18	27	8.5	55	4.0	2.5	9.47
19	1	7.5	45	4.0	2.5	8.75
20	15	7.5	65	4.0	2.5	5.75
21	11	7.5	55	2.0	2.5	7.23
22	20	7.5	55	6.0	2.5	11.03
23	8	7.5	55	4.0	-0.5	0
24	30	7.5	55	4.0	5.5	9.82
25	6	7.5	55	4.0	2.5	4.89
26	3	7.5	55	4.0	2.5	6.79
27	25	7.5	55	4.0	2.5	7.72
28	7	7.5	55	4.0	2.5	6.09
29	13	7.5	55	4.0	2.5	5.12
30	26	7.5	55	4.0	2.5	6.75

4.1.5.1 Response surface analysis for DH of Casein Hydrolysate prepared using Papain

RSM was used to develop a predicted model for optimizing the hydrolytic condition to develop casein hydrolysates using papain under various independent variables. The experimental conditions and the corresponding values from the experimental design are presented in Table 5.1.32 and 5.1.33. All tests were performed in triplicate. Results revealed that %DH of casein proteins were observed in the range of 0 to 14.43. It should be noted here that only one experiment (run 23) of independent variables had minimum value of %DH, i.e. 0 under the values of pH 7.5, Temp 55°C, E/S 4.0 and Time 0.5 h. Conversely, the maximum value was obtained for pH 7.0, Temp 50°C, E/S 5.0 and Time 4 h.

Table 5.1.34 showed the ANOVA for the hydrolytic conditions and %DH of sodium caseinate prepared using papain. It was found that the used statistical model was significant (R^2=0.94) at 99% confidence level ($p < 0.001$), which meant there was only a 0.01% chance that a “model F-value” could occur due to noise. As the test of lack of fit hypothesis was not significant ($P>0.05$) in model equations, the model was fitted well for the said experiment. An adequate precision is a measure of the range of predicted response relative to its associated error or, in other words, a single-to-noise ratio, whose value above 4 is considered to be desirable (Canettieri *et al.,* 2007). The Adequate Precision value (APV) was found to be 18.99, which was significantly higher, indicating the higher prediction ability. ANOVA data showed that the F-value was 18.52, again supporting our assumption that the model is appropriate for optimizing the process conditions. Yue *et al.,* (2013) developed casein hydrolysates using papain and studied the impact of extrinsic amino acids and solvent fractionation on the in vitro antioxidant activity of plastein reaction-stressed hydrolysates. They reported %DH in the range of 8.9±0.1 to 10.3±0.1 under conditions such as hydrolysis time of 5 h, E/S of 2 and temperature of 30°C. In our study, higher DH (14.43) could be attributed to higher hydrolytic temperature (50°C) and higher enzyme concentration than that studied by Yue *et al.,* (2013).

Statistical analysis for linear, quadratic and interaction of the 4 independent variables (A, B, C and D) on the response values (Y) is presented in Table 5.1.34. P value of the model was less than 0.0001, implying that the model was significant and could be used to optimize hydrolysis conditions. For the P values of four independent variables, E/S and time showed the lowest values as compared to pH and temperature; suggesting most significant effect of enzyme concentration and time on %DH. On the contrary, the interactive effect of independent variables (AB, AC, AD, BC, BD and CD) was found to be non-significant on %DH ($P>0.05$). Unlike previous experiments, this showed a quadrant effect of A^2 and C^2 on response (%DH). However, other B^2 and D^2 were not shown to effect %DH significantly.

4.1.5.2 Response surface plots and effects of factors on %DH of casein hydrolysates prepared using Papain

The best explanatory equation for DH is given below. This was generated using the regression coefficient with statistical significance up to 5% probability level. The polynomial model shows that all linear terms and two quadrant forms (A^2 and C^2) contributed to the response. However, the interactive effects among independent variables did not appear to be significant in comparison with the linear and quadratic effects.

$$DH = +6.23 - 0.50*A - 0.45*B + 1.09*C + 2.57*D + 0.83*A*B + 0.081*A*C - 0.54*A*D - 0.077*B*C + 0.16*B*D - 0.18*C*D + 1.22*A^2 + 0.38*B^2 + 0.85*C^2 - 0.20*D^2$$

Table 4.1.34 Coefficient of the full second order polynomial model for coded responses for Casein hydrolysate, hydrolysed using Papain

ANOVA for Response Surface Quadratic model					
Source	**Sum of Squares**	**df**	**Mean of Sum of Square**	**F-Value**	**p-value Prob > F**
Model	275.71	14	19.69	18.52	<0.0001
A-pH	5.95	1	5.95	5.59	0.0319
B-Temp	4.93	1	4.93	4.64	0.0480
C-E/S	28.64	1	28.64	26.93	0.0001
D-Time	158.88	1	158.88	149.40	<0.0001
AB	11.02	1	11.02	10.37	0.0057
AC	0.1	1	0.1	0.099	0.7579 ^{ns}
AD	4.64	1	4.64	4.37	0.0541 ^{ns}
BC	0.094	1	0.094	0.089	0.7698 ^{ns}
BD	0.41	1	0.41	0.39	0.5440 ^{ns}
CD	0.51	1	0.51	0.48	0.5003 ^{ns}
A^2	40.74	1	40.74	38.30	<0.0001
B^2	3.97	1	3.97	3.74	0.0723 ^{ns}
C^2	19.85	1	19.85	18.67	0.0006
D^2	1.15	1	1.15	1.08	0.3156 ^{ns}
Residual	15.95	15	1.06		
Lack of Fit	10.07	10	1.01	0.86	0.6109 ^{ns}
Pure Error	5.88	5	1.18		
Cor Total	291.66	29			
Other statistics					
R-squared					0.9453
APV					18.997
Mean					8.03±1.03

Significant (P<0.05); Highly significant (P<0.01); non-significant (P>0.1)

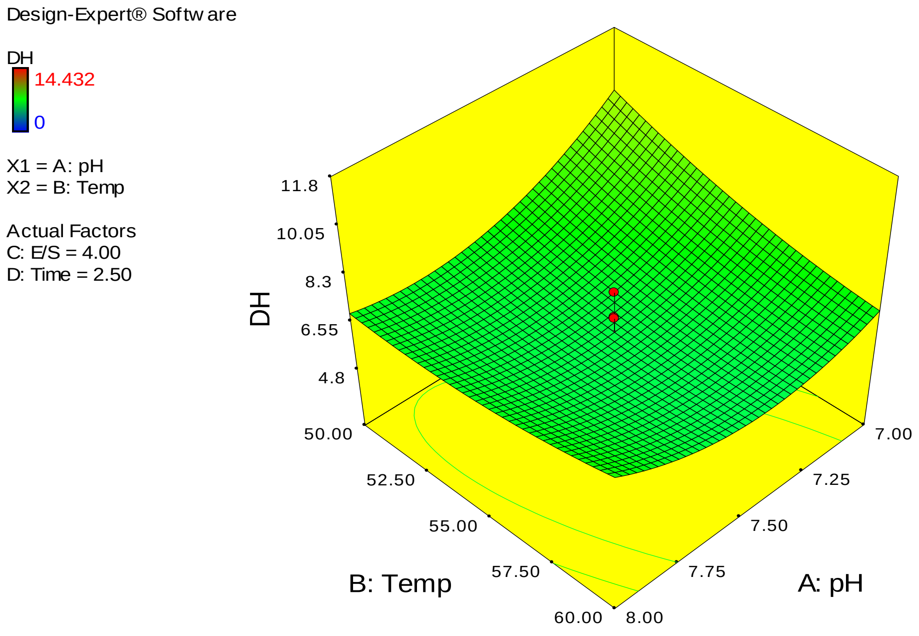

Fig 4.1.28 Response surface related to %DH value of Casein Hydrolysate using Papain as influenced by Temp and pH

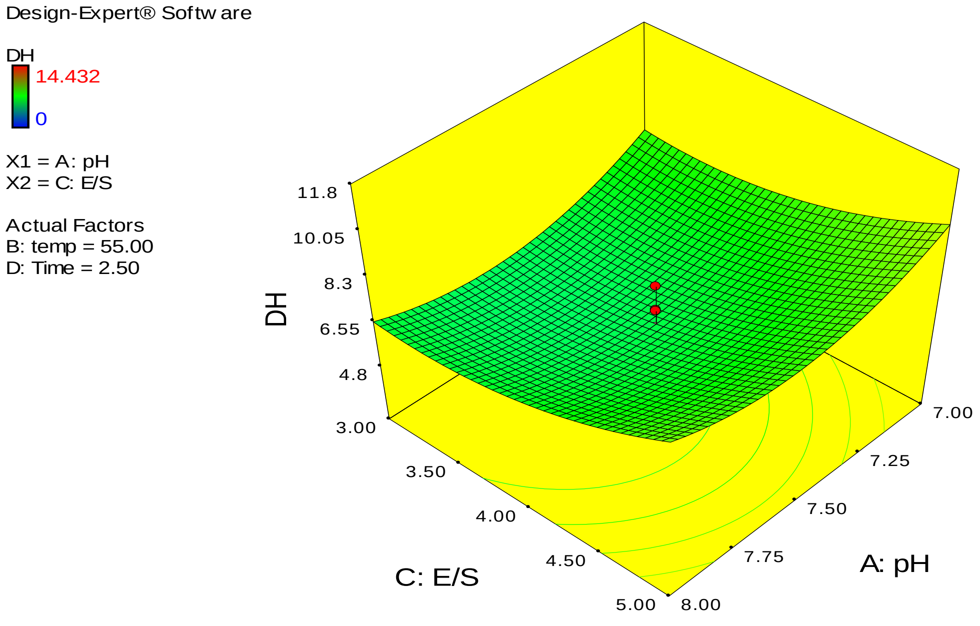

Fig 4.1.29 Response surface related to %DH value of Casein Hydrolysate using Papain as influenced by E/S and pH

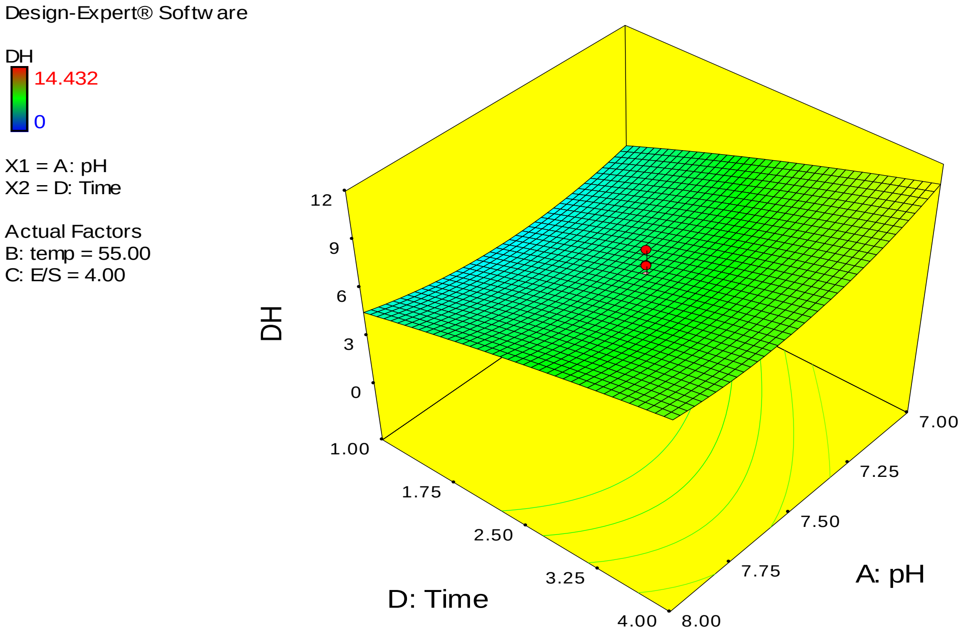

Fig 4.1.30 Response surface related to %DH value of Casein Hydrolysate using Papain as influenced by Time and pH

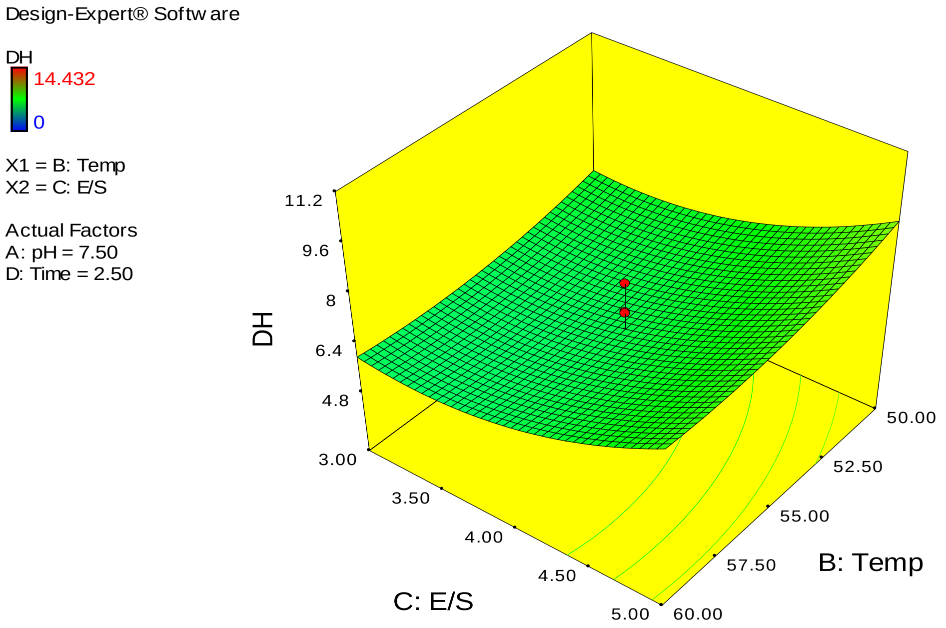

Fig 4.1.31 Response surface related to %DH value of Casein hydrolysate using Papain as influenced by Temp and E/S

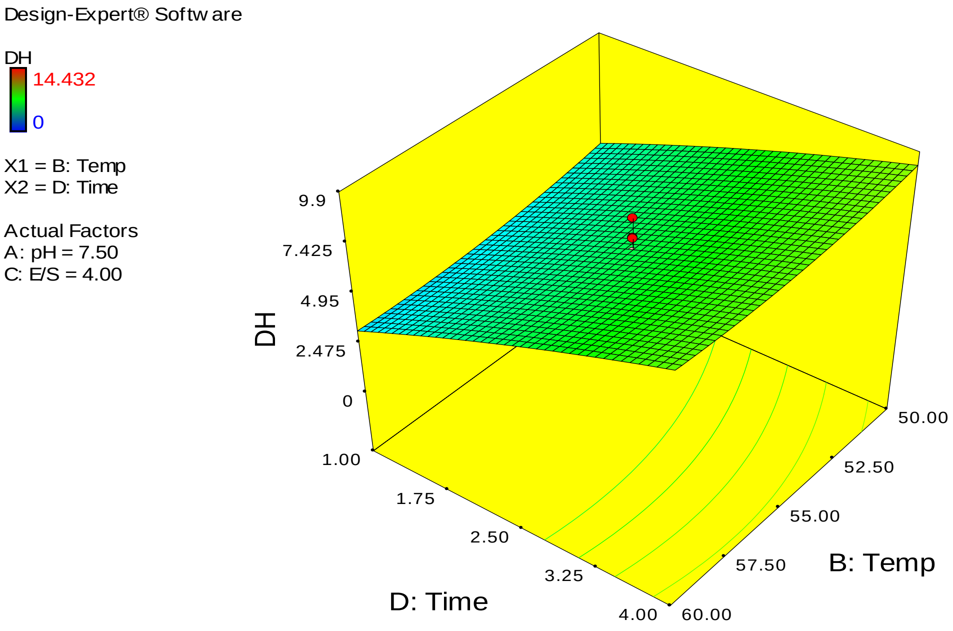

Fig 4.1.32 Response surface related to %DH value of Casein Hydrolysate using Papain as influenced by Temp and Time

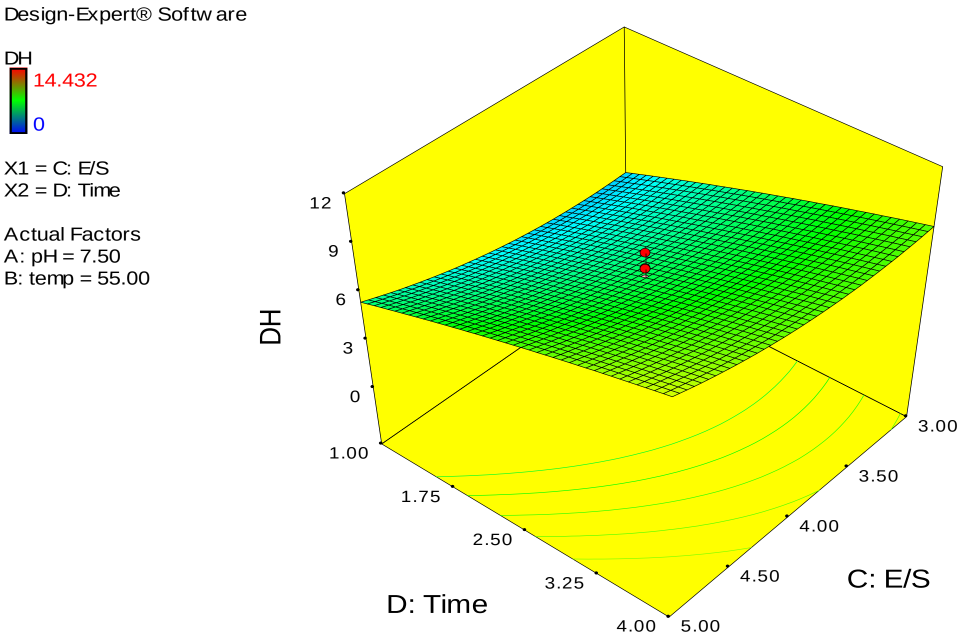

Fig 4.1.33 Response surface related to %DH value of Casein Hydrolysate using Papain

4.1.5.3 Optimization for formulation for Casein Hydrolysate hydrolysed using Papain

3D response are the graphical representations of regression equation. RSM perspective 3D plots have been used to investigate the influence of different hydrolysis variables (in combination), including E/S ratio, temperature, pH and time on %DH of casein hydrolysates prepared using papain. All of the response surface plots shown in Fig. 4.1.28 to 4.1.33, suggested that there were well-defined optimum conditions to obtain maximum %DH (Shao *et al.,* 2007).

Figure 4.1.28 presents the effects of pH (A) and temperature (B), where the E/S ratio and reaction time are set on constant values of 4 and 2.5 h, respectively. It is clear from the said figure that the %DH decreases with an increase in the pH, followed by a gradual increase in %DH once again. It indicates that when the pH is 7.25-7.50, the minimal degree of hydrolysis occurs. Liu *et al.*, (2013) investigated the effects of pH, temperature, E/S ratio and reaction time on the antigenicity of casein hydrolysates prepared by papain. They found similar 3D response curve suggesting similar results. This suggests that papain may have different effects on different substrates; and for different purposes, optimum conditions are different even using the same enzyme.

The effect of pH and E/S ratio is shown in the response surface plot (Figure 4.1.29) where the temperature and reaction time are set at constant values of 55°C and 2.5 h, respectively. It is clear from the figure that the %DH decreases initially with an increase in the E/S ratio and then the DH increases once again.

Figure 4.1.30 illustrated the effect of pH and reaction time, where the temperature and E/S ratio are set on constant values of 55°C and 4, respectively. It shows that when reaction time is 4 h and pH is 7.0, the DH was maximum.

Figures 4.1.30, 4.1.31 and 4.1.32 show, respectively, the effect of temperature and E/S, temperature and reaction time, E/S and reaction time on the response (degree of hydrolysis). It can be comprehensively concluded from the three figures that %DH obtained was maximum under the conditions when the temperature, E/S ratio and reaction time were 50°C, 5 and 4 h, respectively.

The effects of the four independent variables (pH, temperature, E/S ratio and reaction time) are different on DH of sodium caseinate when hydrolysed by papain. As predicted by the model, optimum values of the four independent variables to reach maximum levels of DH for sodium caseinate were pH 7.0, temperature 50°C, E/S 5.0 and reaction time 4.0 h. Under the above hydrolysis conditions, the DH for sodium caseinate was 14.43%.

4.1.5.4 Optimization for formulation of Casein Hydrolysate developed using Papain

To verify the optimized conditions for preparation of casein hydrolysates using papain, the most desirable combination of independent variables was selected for further investigation. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.1.35 Criteria chosen for optimizing values of independent variables in Casein Hydrolysate prepared using Papain

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	In range	7	8	3
	Temp (^oC)	In range	50	60	3
	E/S	Minimize	3	5	3
	Time (h)	In range	1.00	4.00	3
Responses	DH	Maximize	0	28.54	3

To optimize the values of independent variables, three of the four factors viz. pH, Temp and Time were kept ‘In range’ and E/S was kept ‘Minimize’. The goal (Table 4.1.35) for DH was set at ‘Maximize’. Twenty nine different solutions were obtained out of which six solutions with different values for dependent and independent variables are presented in Table 4.1.36. The desirability of the solution ranged from a minimum of 0.870 to a maximum of 0.923. Out of 6 solutions, solution 1 was shortlisted due to its highest value of %DH for comparing and finalizing the formulation.

Table 4.1.36 Suggested solutions and predicted dependent variable values of Casein hydrolysate prepared using Papain as given by Design Expert software package

Number	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7.00	50.00	3	4	12.29	0.923
Solution 2	7.00	50.24	3	4	12.21	0.920
Solution 3	7.00	51.37	3	4	11.83	0.905
Solution 4	7.00	52.01	3	4	11.63	0.898
Solution 5	7.08	50.00	3	4	11.59	0.896
Solution 6	7.00	53.15	3	4	11.31	0.885
Solution 7	7.16	50.00	3	4	10.99	0.872
Solution 8	7.00	54.77	3	4	10.91	0.870

To validate the predicted model, the tests were carried out at optimal conditions for pH, temperature, E/S ratio and hydrolysis time in triplicate. The predicted response was experimentally verified. The adequacy of the response surface equations was indicated by a comparison between the experimental value and the predicted data. The result revealed (Table 4.1.37) that the difference between the predicted and the actual values of independent variables was statistically non-significant. The hydrolytic conditions comprising of pH 7, temp 50°C, E/S 3.0 and time 4 h were finally selected for the preparation of casein hydrolysate, using Papain with maximum DH (12.43±0.60). Fang *et al.,* (2012) optimized antioxidant hydrolysate production from flying squid muscle protein using RSM. In their study, optimum conditions obtained were E/S ratio of 1.74, temperature of 51◦C and time of 46 min, under which, maximum DPPH radical scavenging activity of 74.25% was obtained, which was further correlated with DH.

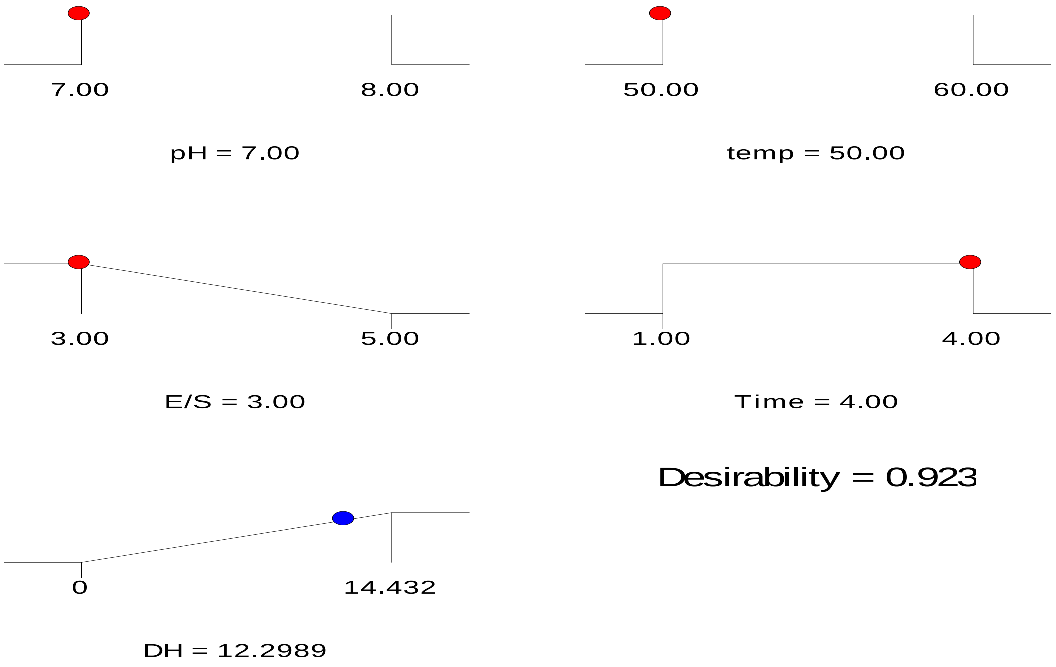

Fig. 4.1.34 Ramp graph for Casein Hydrolysate using Papain

Table 4.1.37 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	12.29	12.43±0.6007	0.3867^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Casein hydrolysate, hydrolysed using Papain; t-value was found to be non-significant at 1% level of significance; ^{ns} non-significant

4.1.6. Optimization of hydrolysis conditions of WPC-80 using Papain

Hydrolysis of whey proteins generates various bioactive peptides, which are associated with health benefits such as antidiabetic, antioxidant and antihypertensive properties (Hernandez-Ledesma *et al.*, 2014; Korhonen and Pihlanto, 2006; Li-Chan, 2015; Nongonierma and FitzGerald, 2015; Power *et al.*, 2013). The main approach used to generate bioactive peptides is enzymatic hydrolysis, however, the outcome in terms of the peptides released may be highly dependent on the processing conditions employed

(Wei and Zhimin, 2006; Whitehurst and Law, 2002). Parameters such as type of enzyme, enzyme concentration, pH, duration of the process and incubation temperature have been shown to influence the peptide composition of hydrolysates. Therefore, the processing condition used for the generation of protein hydrolysates can impact the release of peptides and thus may alter the bioactive profile of the resultant hydrolysate (Van der Ven *et al.,* 2002).

Table 4.1.38 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	**Coded Value**	**Coded Levels**				
		-2α	**-1**	**0**	**+1**	**+2α**
pH	A	6.5	7.0	7.5	8.0	8.5
Temp (°C)	B	45	50	55	60	65
E/S	C	2	3	4	5	6
Time (h)	D	-0.5	1	2.5	4	5.5

Table 5.1.38 shows various independent variables and their coded value. The central value of the coded levels of four independent variables i.e. pH, temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 4.0 and 2.5 h, respectively. Results revealed that out of 30 experiments, which were run in randomised order, included 16 factorial, 8 axial and 6 centre points (Table 4.1.39). Degree of hydrolysis (%DH) was analysed as a response, which can be considered a dependant function of experimental and independent parameters (pH, temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and βF_{11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.1.39 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental which factors expressed in coded units

Standard Run	Run Order	pH	Temp	E/S	Time	Coefficients
1	16	7.0	50	3.0	1.0	Fact
2	2	8.0	50	3.0	1.0	Fact
3	18	7.0	60	3.0	1.0	Fact
4	24	8.0	60	3.0	1.0	Fact
5	9	7.0	50	5.0	1.0	Fact
6	19	8.0	50	5.0	1.0	Fact
7	4	7.0	60	5.0	1.0	Fact
8	22	8.0	60	5.0	1.0	Fact
9	10	7.0	50	3.0	4.0	Fact
10	12	8.0	50	3.0	4.0	Fact
11	23	7.0	60	3.0	4.0	Fact
12	17	8.0	60	3.0	4.0	Fact
13	21	7.0	50	5.0	4.0	Fact
14	28	8.0	50	5.0	4.0	Fact
15	5	7.0	60	5.0	4.0	Fact
16	14	8.0	60	5.0	4.0	Fact
17	29	6.5	55	4.0	2.5	Axial
18	27	8.5	55	4.0	2.5	Axial
19	1	7.5	45	4.0	2.5	Axial
20	15	7.5	65	4.0	2.5	Axial
21	11	7.5	55	2.0	2.5	Axial
22	20	7.5	55	6.0	2.5	Axial
23	8	7.5	55	4.0	-0.5	Axial
24	30	7.5	55	4.0	5.5	Axial
25	6	7.5	55	4.0	2.5	Center
26	3	7.5	55	4.0	2.5	Center
27	25	7.5	55	4.0	2.5	Center
28	7	7.5	55	4.0	2.5	Center
29	13	7.5	55	4.0	2.5	Center
30	26	7.5	55	4.0	2.5	Center

Table 4.1.40 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of WPH, hydrolysed using Papain

Std Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	DH
1	16	7.0	50	3.0	1.0	7.02
2	2	8.0	50	3.0	1.0	4.16
3	18	7.0	60	3.0	1.0	3.13
4	24	8.0	60	3.0	1.0	5.37
5	9	7.0	50	5.0	1.0	9.11
6	19	8.0	50	5.0	1.0	7.21
7	4	7.0	60	5.0	1.0	6.28
8	22	8.0	60	5.0	1.0	9.23
9	10	7.0	50	3.0	4.0	13.55
10	12	8.0	50	3.0	4.0	8.69
11	23	7.0	60	3.0	4.0	12.12
12	17	8.0	60	3.0	4.0	11.35
13	21	7.0	50	5.0	4.0	15.78
14	28	8.0	50	5.0	4.0	12.54
15	5	7.0	60	5.0	4.0	13.23
16	14	8.0	60	5.0	4.0	11.97
17	29	6.5	55	4.0	2.5	12.33
18	27	8.5	55	4.0	2.5	10.34
19	1	7.5	45	4.0	2.5	9.33
20	15	7.5	65	4.0	2.5	4.76
21	11	7.5	55	2.0	2.5	5.71
22	20	7.5	55	6.0	2.5	11.86
23	8	7.5	55	4.0	-0.5	0
24	30	7.5	55	4.0	5.5	9.82
25	6	7.5	55	4.0	2.5	5.71
26	3	7.5	55	4.0	2.5	7.96
27	25	7.5	55	4.0	2.5	5.54
28	7	7.5	55	4.0	2.5	7.02
29	13	7.5	55	4.0	2.5	6.26
30	26	7.5	55	4.0	2.5	5.68

4.1.6.1 Response surface analysis for DH of WPH using Papain

Experimental data using CCDM showed that among the 30 hydrolysates evaluated (Table 4.1.40), 10 experiments showed DH more than 10.00% and twenty showed DH in the range of 0-10%. It can be noted that out of 26 experiments, 30 runs showed between 5 to 10%. It is also noteworthy that the highest results for the DH were obtained when papain was used at pH of 7.0, temperature of 50°C, E/S ratio of 5.0 and 4 h reaction time. However, lowest DH was observed under the conditions of 7.5 pH, 55°C, E/S ratio of 4 and 0.5 h reaction time.

The analysis of variance was performed to evaluate the significance of the model and whether the second-order polynomial equation was adequate for the experimental data given in Table 4.1.41. The analysis of variance demonstrated that the regression coefficient was significant (R^2=0.934), which meant there was only 0.065% chance that a "model F-value" could occur due to noise. An adequate precision is a measure of the range of predicted response relative to its associated error or, in other words, a single-to-noise ratio, whose value above 4 is considered to be desirable (Canettieri *et al.*, 2007). The APV used abbrev earlier was found to be 16.97, which was significantly higher, indicating the higher prediction ability. ANOVA data showed that the F-value was 15.39, again supporting our assumption that the model is appropriate for optimizing the process conditions.

ANOVA results for linear, quadratic and interaction of the 4 independent variables (A, B, C and D) on the response values (Y) is presented in Table 4.1.41. It can be noted that all the four independent variables had a significant ($P<0.05$) effect on DH. For the P values of four independent variables, E/S and time showed the lowest values as compared to pH and temperature; suggesting most significant effect of enzyme concentration and time on %DH. On the contrary, except AB (pH and temperature), the interactive effect of independent variables (AC, AD, BC, BD and CD) was found to be non-significant on %DH ($P>0.05$). Analysis data revealed that the quadratic model in terms of A^2 and C^2 was highly significant ($P<0.0001$), which was evidenced by the Fisher's F-test with a very low probability value. The lack-of-fit F value of 0.2011 was non-significant ($P>0.05$), which implied that the model was fitted to all data. The non-significant value of lack of fit ($P>0.05$) revealed that the quadratic model (for pH and E/S) was statistically significant for the response and it could be used for further study. P value of the model was less than 0.0001, implying again that the model was significant and could be used to optimize hydrolysis conditions.

Table 4.1.41 Coefficient of the full second order polynomial model for coded responses for WPH using Papain

ANOVA for Response Surface Quadratic model Papain WPH					
Source	**Sum of Squares**	**Df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	350.99	14	102.95	15.39	<0.0001
A-pH	7.82	1	7.82	4.80	0.0446
B-Temp	8.78	1	8.78	5.39	0.0347
C-E/S	43.39	1	43.39	26.64	0.0001
D-Time	189.08	1	189.08	116.08	<0.0001
AB	16.02	1	16.02	9.84	0.0068
AC	0.49	1	0.49	0.30	0.5920 ^{ns}
AD	6.97	1	6.97	4.28	0.0562 ^{ns}
BC	0.38	1	0.38	0.24	0.6341 ^{ns}
BD	0.16	1	0.16	0.098	0.7580 ^{ns}
CD	1.18	1	1.18	0.73	0.4073 ^{ns}
A^2	59.87	1	59.87	36.76	<0.0001
B^2	4.48	1	4.48	2.75	0.1178 ^{ns}
C^2	19.35	1	19.35	11.88	0.0036
D^2	0.46	1	0.46	0.28	0.6018 ^{ns}
Residual	24.43	15	18.25		
Lack of Fit	19.88	10	21.46	2.18	0.2011 ^{ns}
Pure Error	4.55	5	11.83		
Cor Total	3.75.42	29			
Other statistics					
R-squared					0.9349
APV					16.977
Mean					8.44±1.28

Significant (P<0.05); Highly significant (P<0.01); non-significant (P>0.1)

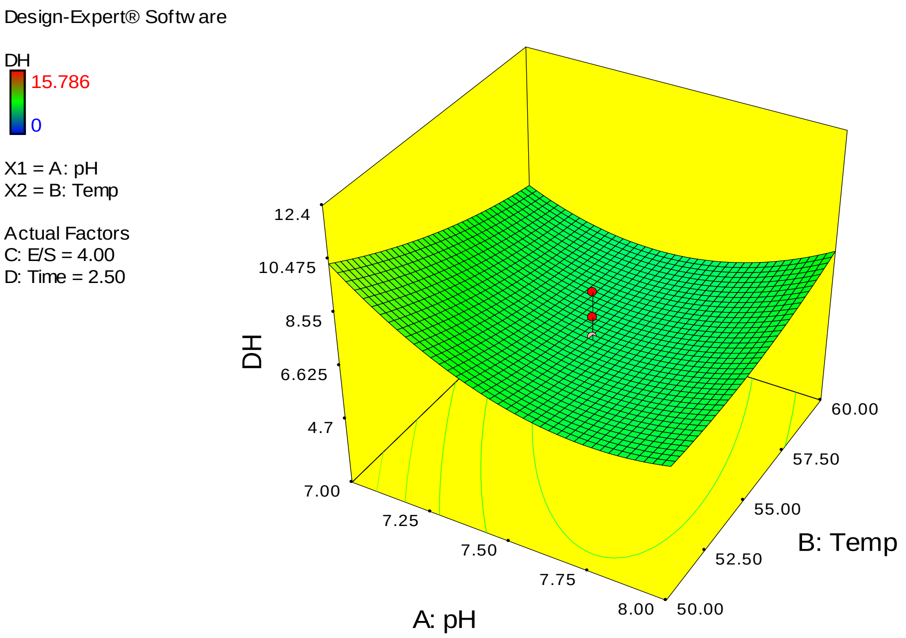

Fig 4.1.35 Response surface related to %DH value of whey proteins hydrolysates developed using Papain as influenced by Temp and pH

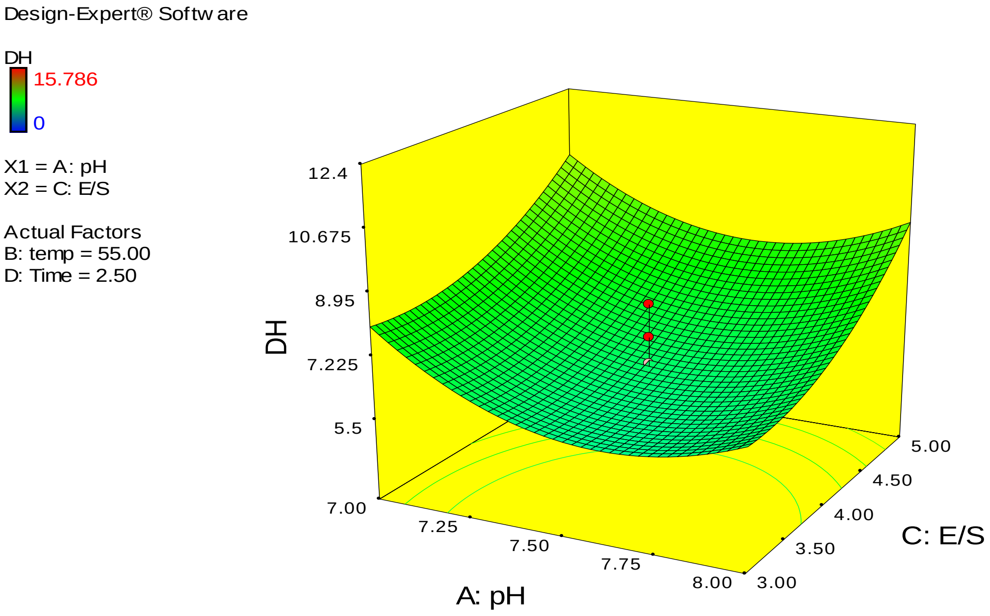

Fig 4.1.36 Response surface related to %DH value of whey proteins hydrolysates developed using Papain as influenced by E/S and pH

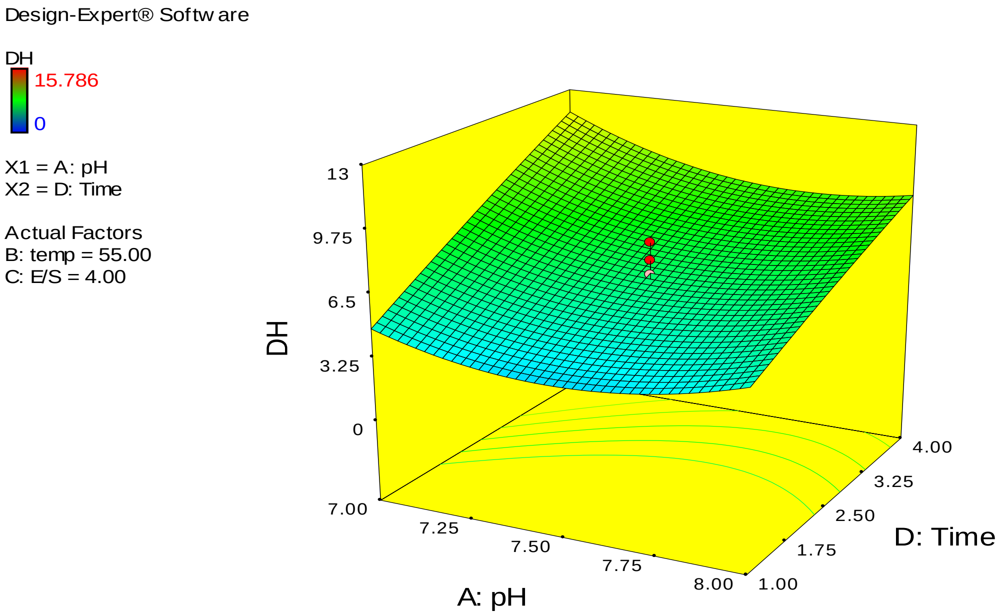

Fig 4.1.37 Response surface related to %DH value of whey proteins hydrolysates developed using Papain as influenced by Time and pH

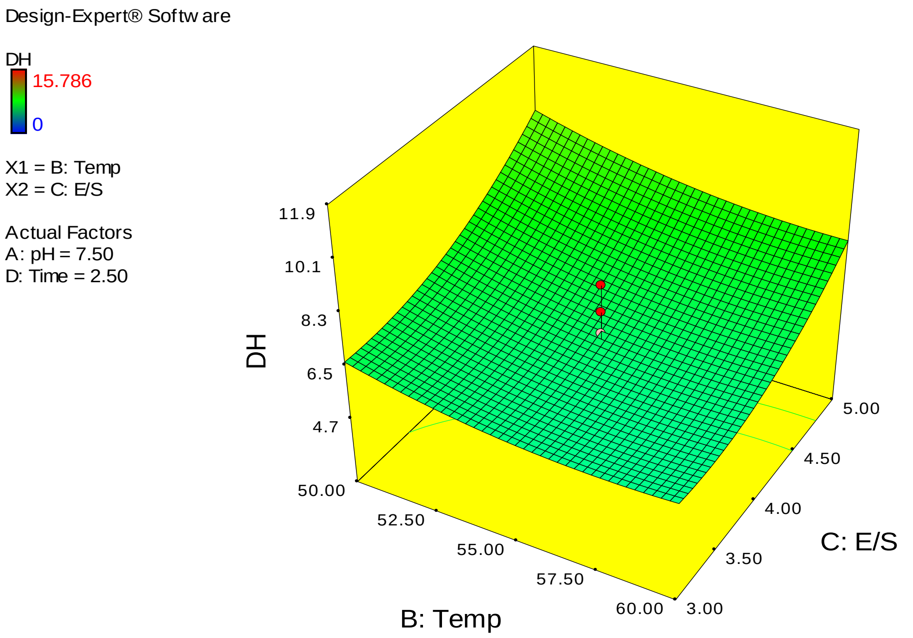

Fig 4.1.38 Response surface related to %DH value of whey proteins hydrolysates developed using Papain as influenced by Temp and E/S

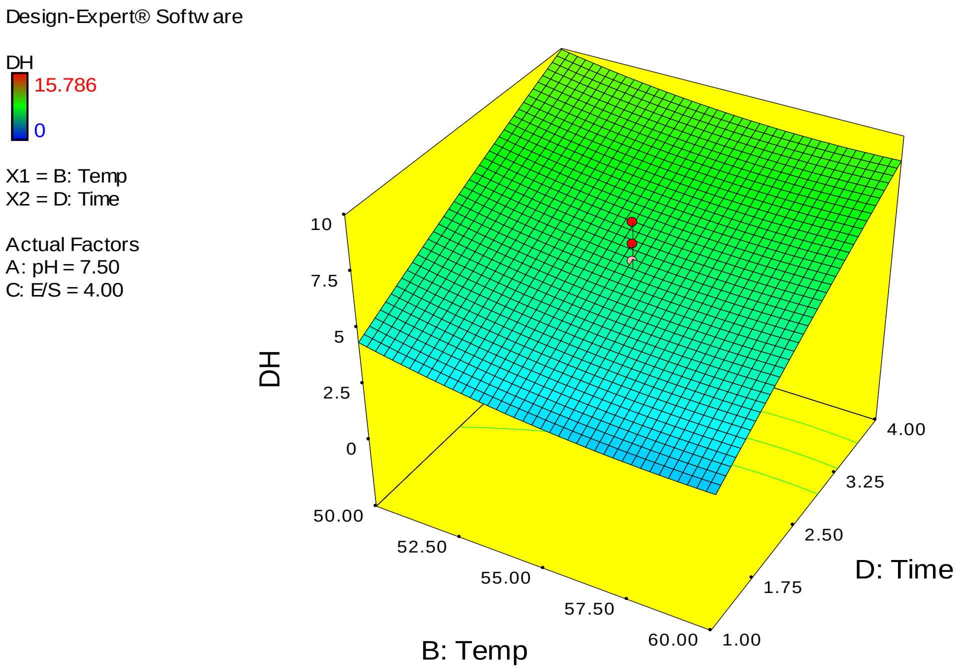

Fig 4.1.39 Response surface related to %DH value of whey proteins hydrolysates developed using Papain as influenced by Temp and Time

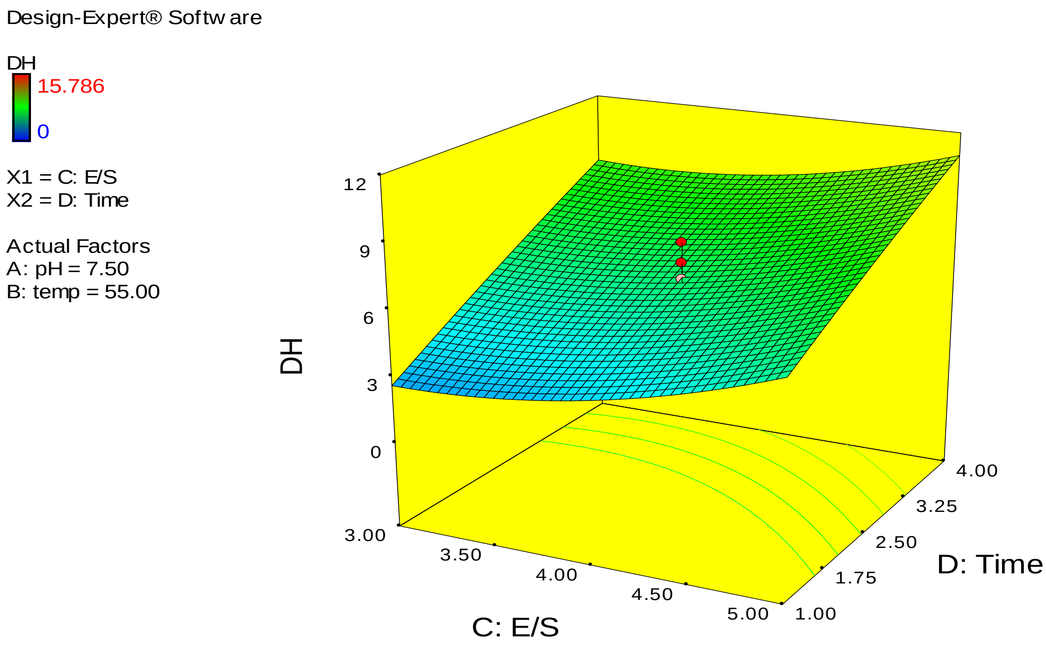

Fig 4.1.40 Response surface related to %DH value of whey proteins hydrolysates developed using Papain as influenced by E/S and Time

4.1.6.2 Response surface plots and effects of factors on %DH of whey protein hydrolysates prepared using Papain

The polynomial model of the DH was regressed and shown as below (in terms of coded factors):

$$DH = + 6.37 - 0.57*A -0.61*B + 1.34*C + 2.81*D +1.00*A*B + 0.17*A*C - 0.66*A*D - 0.15*B*C + 0.10*B*D -0.27*C*D + 1.48*A^2 +0.40*B^2 + 0.84*C^2 - 0.13*D^2$$

The optimal value of the selected test variables was obtained by solving above equation and analyzing the three dimensional (3D) surface plots.

3D response surface plots as a function of two factors, maintaining all other factors at fixed levels, were more helpful in understanding both the main and the interaction effects of these two factors. In order to gain better understanding of the effects on the independent variables and their interactions, 3D response surface plots of the measured responses were formed based on the model equation. All of the response surface plots are shown in Figures 4.1.35 to 4.1.40. Figure 4.1.35 presents the effects of pH (A) and temperature (B), where the E/S ratio and reaction time are set on constant values of 4 and 2.5 h, respectively. It is clear from the said figure that the %DH increased with the decrease in pH, indicated an inverse relationship between pH and DH. It indicates that when the pH was 7.0, the DH was maximum (10.47%). However, there was a slight but no significant effect of temperature on DH. The effect of pH and E/S is shown in the response surface plot (Figure 4.1.36) where the temperature and reaction time are set on constant values of 55°C and 2.5 h, respectively. It is clear from the figure that the %DH decreased initially with an increase in pH and then %DH increased. On the contrary, enzyme concentration (E/S ratio) was found to effect DH positively. Maximum DH was observed at E/S of 5.0 ratio. Figure 4.1.37 demonstrated the effect of pH and reaction time, where the temperature and E/S ratio are set on constant values of 55°C and 4 h respectively. It shows that when reaction time is 4 h and pH is 7.0 or 8.0, the DH was maximum. Figures 4.1.38, 4.1.39 and 4.1.40 show, respectively, the effect of temperature and E/S, temperature and reaction time, E/S and reaction time on the response (degree of hydrolysis). It can be comprehensively concluded from the three figures that %DH was maximum under the conditions when the temperature, E/S ratio and reaction time were 50°C, 5 and 4 h, respectively. Our results are in accordance with the results reported by Guo *et al.* (2014), who optimized the enzymatic hydrolysis for the preparation of shrimp flavour precursor using RSM using Dispase (protease which cleaves collagen). The parameters, namely, enzyme concentration, reaction temperature and initial pH, on the DH were optimized using CCRD. The obtained optimum conditions were initial pH value of 6.5, Dispase concentration 2.0%, reaction temperature 57°C and hydrolysis time 3 h with material : water ratio 1:10. Under the optimized conditions, the DH reached 57.65%.

To sum up all of these 3D response surface plots demonstrated that there were considerable interactions between each of the independent variables and DH. From these graphs, It is understood that enzyme hydrolysis of substrate was affected by various factors. Enzyme showed good activity at specific temperature and pH. As predicted by the model, optimum values of the four independent variables to reach maximum levels of DH for whey proetins were pH 7.0, temperature 50°C, E/S 5.0 and reaction time 4.0 h. Under the above hydrolysis conditions, the DH for whey proteins was 15.78%.

4.1.6.3 Optimization for formulation for WPH, hydrolysed using Papain

For the validation of the optimized conditions for preparation of whey protein hydrolysates using papain, the most desirable combination of independent variables was selected for further investigation. The selection was made using optimization command of the software package Design Expert$^{®}$ (version 7.0.0).

Table 4.1.42 Criteria chosen for optimizing values of independent variables in WPH, proposed using Papain

Attributes		Goal	Lower limit	Upper limit	Importance
Factors	pH	In range	7	8	3
	Temp (^{o}C)	In range	50	60	3
	E/S	Minimize	3	5	3
	Time (h)	In range	1.00	4.00	3
Responses	DH	Maximize	0	15.786	3

For the optimization of independent variables, three of the four factors viz. pH, Temp and Time were kept ‘In range’ and E/S was kept ‘Minimize’. The goal (Table 4.1.42) for DH was set at ‘Maximize’. Twenty nine different solutions were obtained out of which six solutions with different values for dependent and independent variables are presented in Table 4.1.43. The desirability of the solution ranged from a minimum of 0.880 to a maximum of 0.922. On the basis of results, solution 1 was selected due to its highest value of %DH for comparing and finalizing the formulation.

Table 4.1.43 Suggested solutions and predicted dependent variable values of WPH using Papain as per Design Expert software package

Number	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7.00	50.00	3.00	4.00	13.417	0.922
Solution 2	7.00	50.27	3.00	3.99	13.307	0.918
Solution 3	7.00	50.69	3.00	4.00	13.156	0.913
Solution 4	7.00	50.01	3.00	3.83	13.060	0.910
Solution 5	7.05	50.00	3.00	4.00	12.913	0.904
Solution 6	7.00	50.00	3.00	3.72	12.818	0.901
Solution 7	7.09	50.02	3.00	4.00	12.497	0.890
Solution 8	7.00	50.00	3.17	4.00	13.366	0.880

To know the adequacy of the predicted model, the tests were carried out at optimal conditions for pH, temperature, E/S ratio and hydrolysis time in triplicate. The predicted response was experimentally verified. The suitability of the response surface equations was indicated by a comparison between the experimental value and the predicted data. The result revealed (Table 4.1.44) that the difference between the predicted and the actual values of independent variables was statistically non-significant. The hydrolytic conditions consisting pH 7, temp 50°C, E/S 3.0 and time 4 h were finally selected for the preparation of whey protein hydrolysates, prepared using Papain with maximum degree of hydrolysis (13.199±0.328).

Table 4.1.44 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	13.417	13.19±0.328	1.153^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Whey protein hydrolysate, hydrolysed using Papain; t-value was found to be non-significant at 1% level of significance; ^{ns} non-significant

4.1.7 Optimized conditions for developing casein hydrolysates and whey protein hydrolysates

In this way, conditions of independent varaibles were optimized for developing casein and whey proteins hydrolysates using trypsin, alcalase and papain, which are shown in Table 4.1.45 and 4.1.46. As the main aim of the present study was to develop non-bitter protein hydrolysates, so that it can be utilized for the formulation of dairy beverage; therefore, developed casein and whey protein hydrolysates were analysed on the basis of sensory evaluation.

Table 4.1.45 Optimized conditions for developing casein hydrolysates

Enzyme used		Optimized conditions			
	pH	Temperature (°C)	E/S ratio	Time (h)	DH (%)
Trypsin	7	60	0.010	4	16.79±1.27^{a}
Alcalase	8	60	0.004	4	27.08±1.83^{b}
Papain	7	50	3.000	4	12.43±0.60^{c}

*Values with different superscripts along with the column differ significantly ($P<0.05$)

4.1.46. Optimized conditions for developing whey protein hydrolysates

Enzyme used		Optimized conditions			
	pH	Temperature (°C)	E/S ratio	Time (h)	DH (%)
Trypsin	7	60	0.010	4	17.42±1.38^{a}
Alcalase	8	60	0.004	4	29.03±1.93^{b}
Papain	7	50	3.000	4	13.19±0.38^{c}

*Values with different superscripts along with the column differ significantly ($P<0.05$)

4.1.8 Sensory evaluation of casein and whey proteins hydrolysate developed under optimized conditions using Trypsin, Alcalase and Papain

Samples of casein hydrolysate, optimized using Trypsin, Alcalase and Papain were analyzed for bitterness on the basis of 4-point intensity scale, where 1 indicates most bitter and 4 indicates least bitter. The results of the sensory evaluation are shown in Table 4.1.47. It is clear from the said table that Alcalase catalyzed casein hydrolysates were found to be highest in terms of bitterness. There was significant difference ($P<0.05$) among the bitterness of alcalase, trypsin & papain catalyzed casein hydrolysates. Alcalase showed highest degree of bitterness followed by Trypsin and Papain, respectively.

Table 4.1.47 Bitterness intensity scores of casein hydrolysates on based 4 point intensity scale

Parameters	Trypsin	Alcalase	Papain
Bitterness scores **(DH %)**	1.6 ± 0.05^{a} (16.79 ± 1.27^{a})	1.0 ± 0.03^{b} (27.08 ± 1.83^{b})	1.8 ± 0.04^{c} (12.43 ± 0.60^{c})

Values with different small superscripts (a, b, c) differ significantly ($P<0.05$) within the rows. Score of 1 indicates most bitter and 4 indicate least bitter.

Table 4.1.48 Bitterness intensity scores of whey protein hydrolysates based on 4 point intensity scale

Parameters	Trypsin	Alcalase	Papain
Bitterness score **(DH %)**	1.5 ± 0.2^{a} (17.42 ± 1.385^{a})	1.0 ± 0.0^{b} (29.03 ± 1.983^{b})	1.7 ± 0.2^{c} (13.19 ± 0.328^{c})

Values with different small superscripts (a,b,c) differ significantly ($P<0.05$) within the rows. score 1 of indicates most bitter and 4 indicate least bitter.

Table 4.1.47 illustrated that the enzyme which showed the maximum degree of hydrolysis (Alcalase: 27.08±1.83% DH) was noted/ perceived to be most bitter among all the samples. Similar results were observed in case of whey protein hydrolysates (Table 4.1.48). Alcalase catalysed whey protein hydrolysates had the maximum bitterness among all the samples followed by hydrolysates prepared using trypsin and papain (Table 4.1.48). For better understanding, results of bitterness score and DH (%) are depicted in Fig. 4.1.41. Casein hydrolysates, catalyzed by Alcalase (CHA) showed lowest bitterness score, among all the samples (Fig. 4.1.41). Alcalase has a broad specificity for peptide bonds formed by hydrophobic amino acids, which may have been responsible for the formation of a more hydrophobic hydrolysate having the most intense bitterness.

The findings of the present study are in accordance with the results of Humiski and Aluko (2007), who investigated the bitterness properties of enzymatic pea protein hydrolysates using alcalase, flavourzyme, papain, trypsin and α-chymotrypsin. Alcalase and Flavourzyme produced protein hydrolysates with significantly higher DH as compared to other proteases. Alcalase produced hydrolysates that had the highest level of aromatic amino acids, and were found to be most bitter among all the samples.

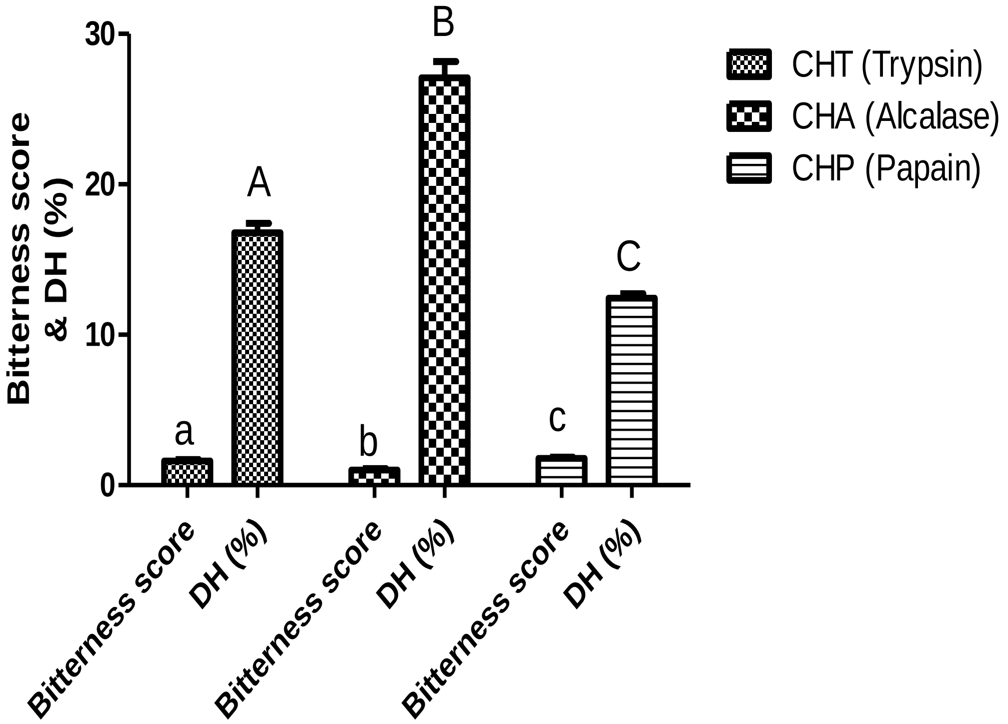

Fig. 4.1.41. **Bitterness score and %DH of optimized casein hydrolysates prepared with different enzymes. CHT: CHA, CHP Casein hydrolysates using trypsin, alcalase and papain respectively. Values are means±SEM (n=3). Means that do not share a common alphabet indicate statistical difference at P <0.05.**

Liu *et al.*, (2016) studied the effect of sequential hydrolysis of wheat gluten with endo-and exo-peptidase on bitterness of hydrolysates prapared using a Alcalase, Flavourzyme, Neutrase and Protamex. They concluded that the short-chain bitter peptides prepared by Neutrase and Alcalase had low bitter taste thresholds. Neutrase and Alcalase had higher (TD) factors than Papain and Trypsin. Their results indicated that the stereoscopic structure of long-chain peptides hindered the exposure of hydrophobic amino acids. Thus, the long-chain bitter peptides did not play a major role in the bitterness intensity of the wheat gluten hydrolysates. In contrast, short-chain bitter peptides, limed at greater degree of hydrolysis, were the main contributor to the bitterness of the substances.

In this way, it is clear why alcalase catalysed hydrolysates, with maximum degree of hydrolysis, produced the maximum bitterness in present study. The present work was planned to develop non-bitter protein hydrolysates with maximum DH. Therefore, out of the studied samples, casein as well as whey protein hydrolysates catalysed by alcalase were selected for further treatment i.e. de-bittering using enzymes.

4.2 Process optimization for debittering of Casein Hydrolysate and Whey Protein Hydrolysate prepared using Alcalase

As discussed in above section, Alcalase was selected for obtaining the casein and whey protein hydrolysates, which were further treated with exopeptidases (Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB) to reduce the bitterness. First, casein hydrolysates were prepared using endo-peptidase (Alcalase) under optimized conditions (as described above) followed by enzymatic hydrolysis using Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB. Similar treatments were performed for whey proteins.

4.2.1 Process optimization of Casein Hydrolysate (Alcalase catalysed) using Flavourzyme 500L

Flavourzyme® 500L is an exopeptidase/endopeptidase complex derived from the fungus *Aspergillus oryzae*. This enzyme preparation contains five exopeptidases and four endopeptidases that are active at different pH (Ref.). The endoproteases have both neutral and acid proteases, giving a broad operating pH. Flavourzyme’s optimal pH and temperature ranged from 4-10 and 50-55^{o}C, respectively (Ref.). In contrast to other proteases, it functions better at a natural drifting pH because different exopeptidases in the complex will be activated at different pH making the hydrolysis more efficient. This nature of the enzyme preparation is to produce non-bitter hydrolysates with very diverse peptide mixtures which are mostly small in size. In this phase, optimization was done to debitter casein hydrolysates (alcalase catalysed) using Flavourzyme 500L under different pH, temperature, E/S ratio and reaction time using RSM. Boundaries of the experimental domain and spacing of the compositional variables level are shown in Table 4.2.1.

Table 4.2.1 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2a	**-1**	**0**	**+1**	**+2a**
pH	A	6.5	7.0	7.5	8.0	8.5
Temp(°C)	B	45	50	55	60	65
E/S	C	0.02	0.03	0.04	0.05	0.06
Time	D	-0.5	1	2.5	4	5.5

The central value of the coded levels of four independent variables i.e. pH, temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.04 and 2.5 h, respectively. Results revealed that out of 30 experiments, which were run in randomised order, included 16 factorial, 8 axial and 6 centre points (Table 4.2.2). Response (y, degree of hydrolysis, DH) was analysed, which can be considered as a function of experimental and independent factors (pH, temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^{2.}$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and β_{F11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.2.2 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Std Run	Run Order	pH	Temp(^oC)	E/S	Time(h)	Coefficients
1	8	7.00	50.00	0.03	1.00	Factorial
2	18	8.00	50.00	0.03	1.00	Factorial
3	24	7.00	60.00	0.03	1.00	Factorial
4	7	8.00	60.00	0.03	1.00	Factorial
5	13	7.00	50.00	0.05	1.00	Factorial
6	15	8.00	50.00	0.05	1.00	Factorial
7	29	7.00	60.00	0.05	1.00	Factorial
8	20	8.00	60.00	0.05	1.00	Factorial
9	16	7.00	50.00	0.03	4.00	Factorial
10	26	8.00	50.00	0.03	4.00	Factorial
11	2	7.00	60.00	0.03	4.00	Factorial
12	5	8.00	60.00	0.03	4.00	Factorial
13	23	7.00	50.00	0.05	4.00	Factorial
14	6	8.00	50.00	0.05	4.00	Factorial
15	12	7.00	60.00	0.05	4.00	Factorial
16	30	8.00	60.00	0.05	4.00	Factorial
17	3	6.50	55.00	0.04	2.50	Axial
18	19	8.50	55.00	0.04	2.50	Axial
19	1	7.50	45.00	0.04	2.50	Axial
20	10	7.50	65.00	0.04	2.50	Axial
21	21	7.50	55.00	0.02	2.50	Axial
22	17	7.50	55.00	0.06	2.50	Axial
23	28	7.50	55.00	0.04	-0.50	Axial
24	11	7.50	55.00	0.04	5.50	Axial
25	27	7.50	55.00	0.04	2.50	Center
26	9	7.50	55.00	0.04	2.50	Center
27	14	7.50	55.00	0.04	2.50	Center
28	4	7.50	55.00	0.04	2.50	Center
29	25	7.50	55.00	0.04	2.50	Center
30	22	7.50	55.00	0.04	2.50	Center

Table 4.2.3 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Casein hydrolysate hydrolysed using Flavourzyme 500L

Std Order	Run Order	Coefficients assessed by	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	DH
1	8	Factorial	7.00	50.00	0.03	1.00	29.00
2	18	Factorial	8.00	50.00	0.03	1.00	27.95
3	24	Factorial	7.00	60.00	0.03	1.00	28.65
4	7	Factorial	8.00	60.00	0.03	1.00	27.34
5	13	Factorial	7.00	50.00	0.05	1.00	32.50
6	15	Factorial	8.00	50.00	0.05	1.00	27.50
7	29	Factorial	7.00	60.00	0.05	1.00	28.95
8	20	Factorial	8.00	60.00	0.05	1.00	27.12
9	16	Factorial	7.00	50.00	0.03	4.00	33.75
10	26	Factorial	8.00	50.00	0.03	4.00	29.98
11	2	Factorial	7.00	60.00	0.03	4.00	30.75
12	5	Factorial	8.00	60.00	0.03	4.00	30.12
13	23	Factorial	7.00	50.00	0.05	4.00	33.95
14	6	Factorial	8.00	50.00	0.05	4.00	30.11
15	12	Factorial	7.00	60.00	0.05	4.00	29.43
16	30	Factorial	8.00	60.00	0.05	4.00	27.55
17	3	Axial	6.50	55.00	0.04	2.50	30.32
18	19	Axial	8.50	55.00	0.04	2.50	27.30
19	1	Axial	7.50	45.00	0.04	2.50	28.23
20	10	Axial	7.50	65.00	0.04	2.50	27.29
21	21	Axial	7.50	55.00	0.02	2.50	27.89
22	17	Axial	7.50	55.00	0.06	2.50	30.23
23	28	Axial	7.50	55.00	0.04	-0.50	27.00
24	11	Axial	7.50	55.00	0.04	5.50	30.95
25	27	Central	7.50	55.00	0.04	2.50	30.00
26	9	Central	7.50	55.00	0.04	2.50	30.25
27	14	Central	7.50	55.00	0.04	2.50	30.00
28	4	Central	7.50	55.00	0.04	2.50	28.50
29	25	Central	7.50	55.00	0.04	2.50	29.23
30	22	Central	7.50	55.00	0.04	2.50	31.00

4.2.1.1 Response surface analysis for DH of Casein Hydrolysate prepared using Flavourzyme 500L

The experimental conditions and the corresponding values from the experimental design are presented in Table 4.2.2 and 4.2.3. All tests were performed in triplicate. Results revealed that %DH of casein proteins were observed to be in the range 27 to 33.75. It should be noted here that only one experiment (run 23) of independent variables had minimum value of %DH, i.e. 27 at pH 7.5, Temp 55°C, E/S 0.04 and Time 0.5 h. Conversely, the maximum value was obtained at pH 7.0, Temp 50°C, E/S 0.03 and Time 4 h.

Table 5.2.4 showed the ANOVA for the hydrolytic conditions and %DH of sodium caseinate hydrolysed by Flavourzyme 500L. It was found that the statistical model used was significant (R^2=0.822) at 99% confidence level ($p < 0.001$). As the test of lack of fit hypothesis was not significant ($P>0.05$) in model equations, the model was fitted well for the said experiment. An adequate precision is a measure of the range of predicted response relative to its associated error or, in other words, a single-to-noise ratio, whose value above 4 is considered to be desirable (Canettieri *et al.,* 2007). The Adequate Precision value (APV) was found to be 9.21, which was significantly higher, indicating the higher prediction ability. ANOVA data showed that the F-value was 4.97, again supporting our assumption that the model is appropriate for optimizing process conditions.

Statistical analysis for linear, quadratic and interaction of the 4 independent variables (A, B, C and D) on the response values (Y) is presented in Table 5.2.4. P-value of the model was less than 0.05, implying that the model was significant and could be used to optimize hydrolysis conditions. For the P values of four independent variables, E/S showed no significant effect on DH. However, all other independent variables significantly affected %DH. On the contrary, the interactive effect as well as quadrant effect of independent variables (AB, AC, AD, BC, BD and CD) was found to be non-significant on %DH ($P>0.05$).

4.2.1.1.1 Response surface plots and effects of factors on %DH of casein hydrolysates prepared using Flavourzyme 500L

The best explanatory equation for DH is given below. This was generated using the regression coefficients with statistical significance up to 5% probability level. The polynomial model shows that all linear terms contributed to the response. However, the interactive as well as quadrant effects among independent variables did not appear to be significant.

Multiple regression equation generated to predict the DH as affected by different factors in terms of coded factors is as follows:

$$DH = + 29.83 - 1.06 * A - 0.70 * B + 0.18 * C + 1.02 * D + 0.50 * A * B - 0.36 * A * C - 0.058 * A * D - 0.45 * B * C - 0.32 * B * D - 0.42 * C * D - 0.086 * A^2 - 0.35 * B^2 - 0.023 * C^2 - 0.045 * D^2$$

Table 4.2.4 Coefficient of the full second order polynomial model for coded responses for Casein hydrolysate using Flavourzyme 500L

Analysis of variance table [Partial sum of squares - Type III]					
Source	**Sum of Squares**	**Df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	81.41	14	5.82	4.97	0.0019
A-pH	26.78	1	26.78	22.90	0.0002
B-Temp	11.63	1	11.63	9.95	0.0065
C-E/S	0.75	1	0.75	0.64	0.4349^{ns}
D-Time	25.07	1	25.07	21.45	0.0003
AB	4.01	1	4.01	3.43	0.0838^{ns}
AC	2.10	1	2.10	1.79	0.2006^{ns}
AD	0.054	1	0.054	0.046	0.8326^{ns}
BC	3.23	1	3.23	2.76	0.1172^{ns}
BD	1.59	1	1.59	1.36	0.2612^{ns}
CD	2.80	1	2.80	2.39	0.1427^{ns}
A^2	0.20	1	0.20	0.17	0.6831^{ns}
B^2	3.33	1	3.33	2.85	0.1121^{ns}
C^2	0.015	1	0.015	0.013	0.9111^{ns}
D^2	0.055	1	0.055	0.047	0.8315^{ns}
Residual	17.54	15	1.17		
Lack of Fit	13.80	10	1.38	1.85	0.2580^{ns}
Pure Error	3.73	5	0.75		
Cor Total	98.95	29			
Other statistics					
R-squared					0.8228
APV					9.211
Mean					29.43±1.08

Significant (P<0.05); Highly significant (P<0.01); non-significant(P>0.1)

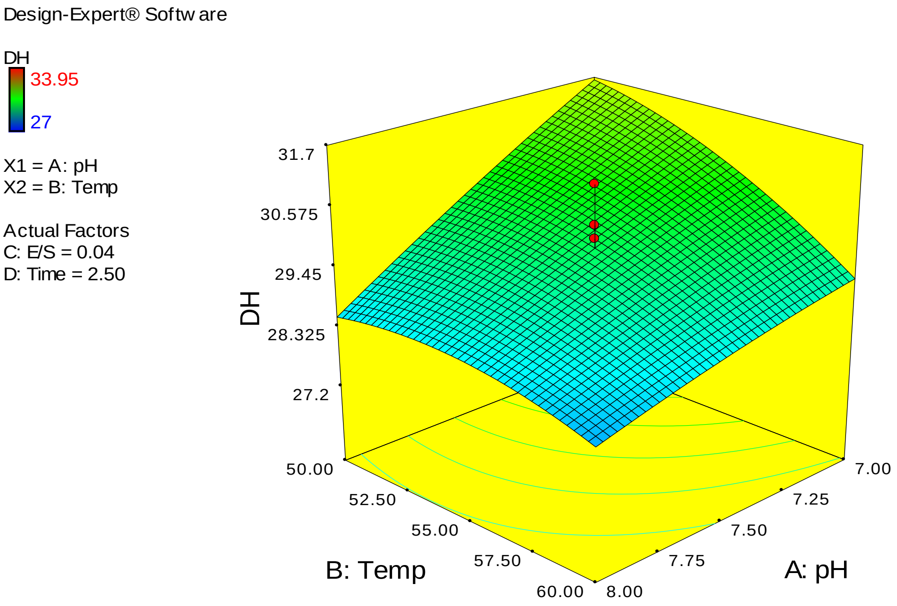

Fig 4.2.1 Response surface related to %DH value of Casein hydrolysate hydrolysed using Flavourzyme 500L as influenced by Temp and pH

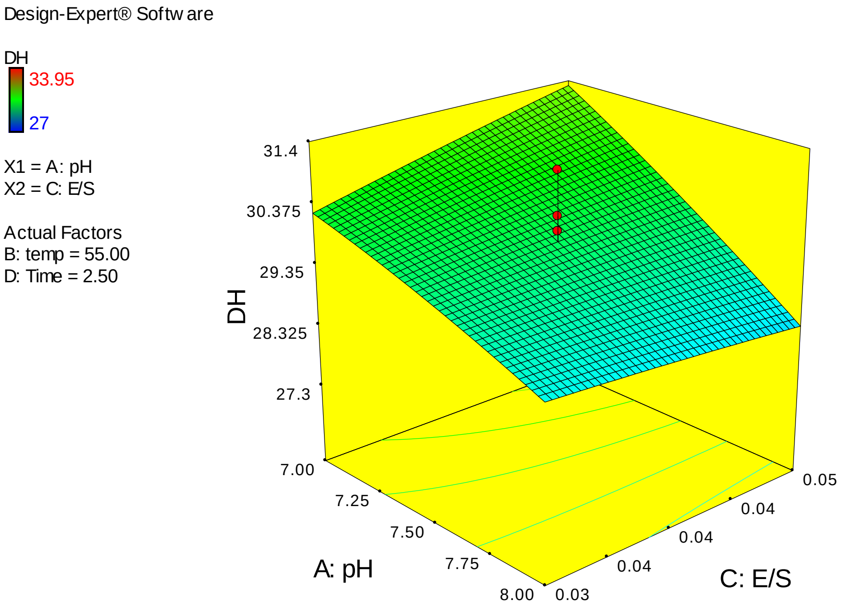

Fig 4.2.2 Response surface related to %DH value of Casein hydrolysate hydrolysed using Flavourzyme 500L as influenced by E/S and pH

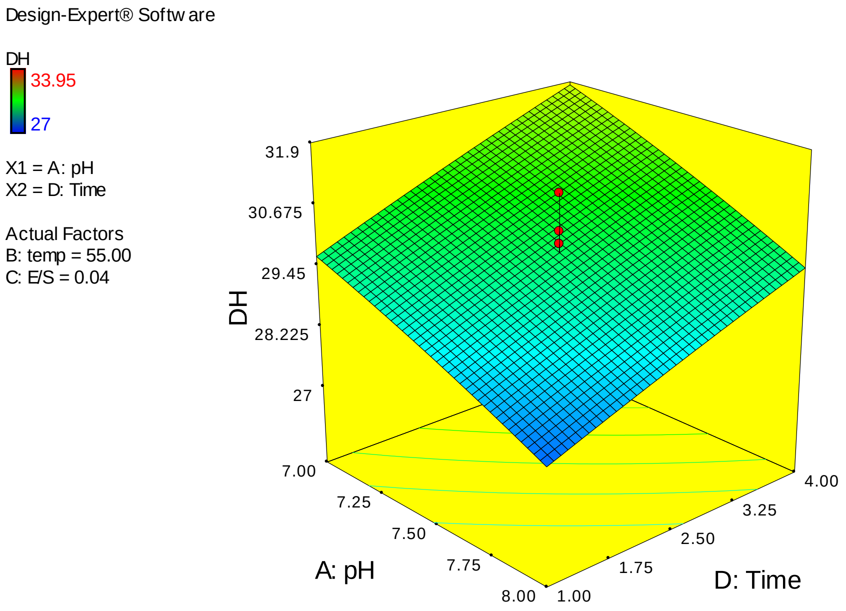

Fig 4.2.3 Response surface related to %DH value of Casein hydrolysate hydrolysed using Flavourzyme 500L as influenced by Time and pH

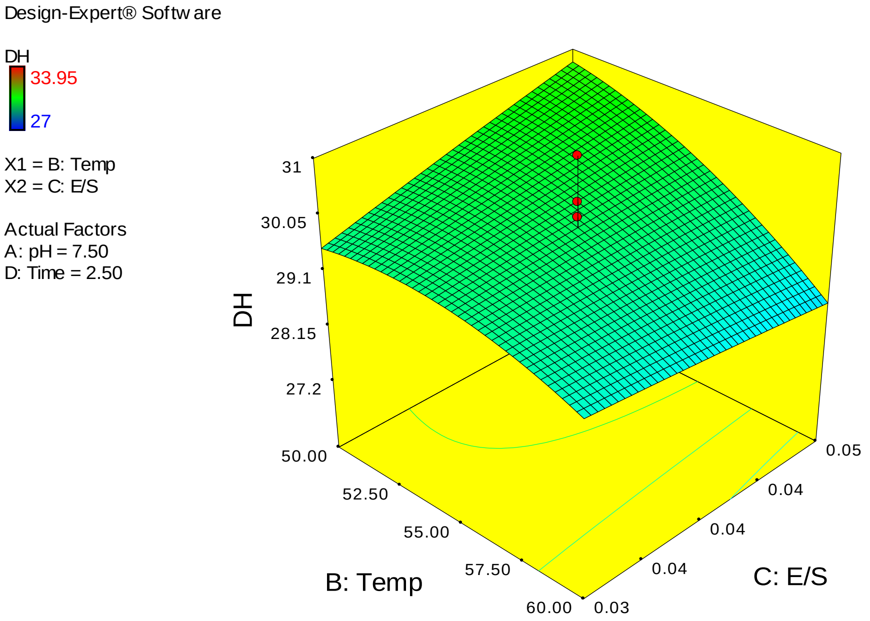

Fig 4.2.4 Response surface related to %DH value of Casein hydrolysate hydrolysed using Flavourzyme 500L as influenced by Temp and E/S

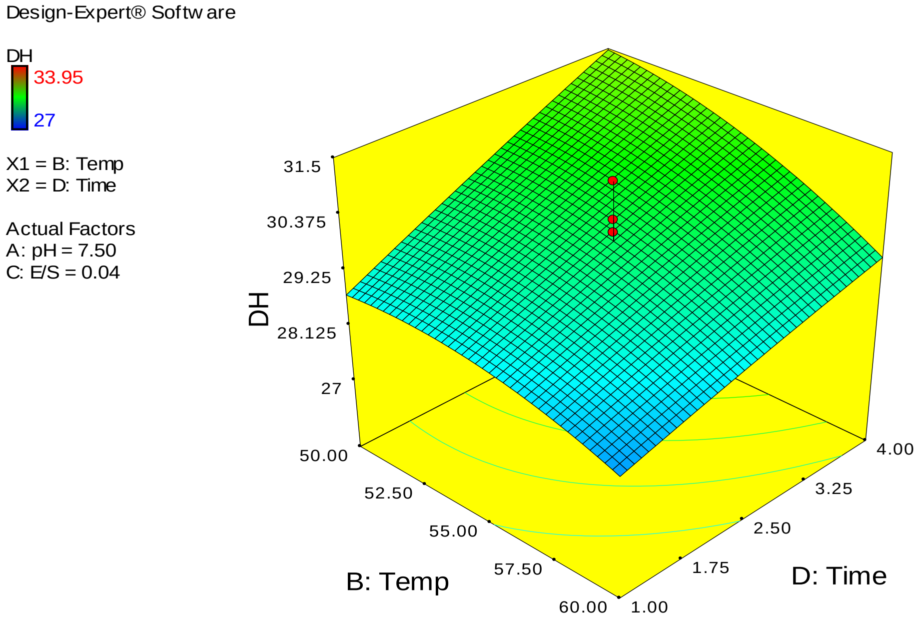

Fig 4.2.5 **Response surface related to %DH value of Casein hydrolysate hydrolysed using Flavourzyme 500L as influenced by Temp and Time**

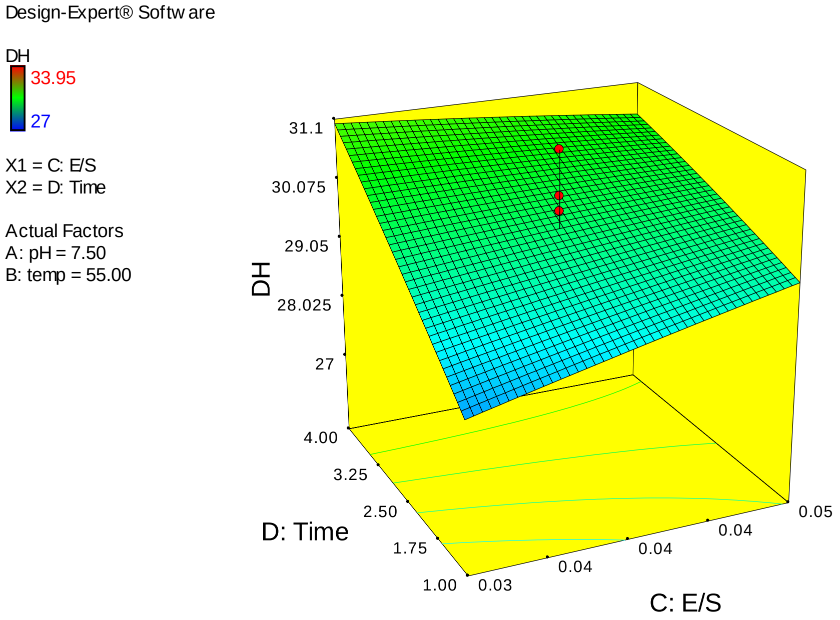

Fig 4.2.6 **Response surface related to %DH value of Casein hydrolysate hydrolysed using Flavourzyme 500L as influenced by E/S and Time**

Response surface plots shown in Fig. 4.2.1 to 4.2.6, suggested that there were well-defined optimum conditions to obtain maximum %DH. Figure 5.2.1 represents the effects of pH (A) and temperature (B), where as the E/S ratio and reaction time are set on constant values of 0.04 and 2.5 h, respectively. It is clear from the said figure that the %DH increased with decrease in the pH of what? However, optimum temperature was observed to be 55°C under these conditions. Upon increase or decrease beyond this specific temperature, %DH was found to be affected showing a gradual decrease.

The effect of pH and E/S ratio is shown in the response surface plot (Figure 4.2.2) where the temperature and reaction time are set at constant values of 55°C and 2.5 h, respectively. It is clear from the figure that the %DH was almost constant and not significantly affected by the change in E/S ratio. However, %DH showed a significant sharp increase with decrease in pH of medium. Figure 4.2.3 illustrated the effect of pH and reaction time, where the temperature and E/S ratio were set at constant values of 55°C and 0.04, respectively. It shows that when reaction time is 4 h and pH is 7.0, the DH was maximum.

Figures 4.2.4, 4.2.5 and 4.2.6 show the effect of temperature and E/S, temperature and reaction time, and E/S and reaction time on the response (DH), respectively. It can be concluded from the three figures that %DH was maximum under the conditions when the temperature, E/S ratio and reaction time were 50°C, 0.05 and 4 h, respectively.

The effects of the four independent variables (pH, temperature, E/S ratio and reaction time) were different on the DH of sodium caseinate hydrolysed using Flavourzyme 500L. As predicted by the model, optimum values of the four independent variables to reach maximum levels of DH for sodium caseinate were pH: 7.0, temperature 50°C, E/S 0.30 and reaction time 4.0 h. Under such hydrolysis conditions, the DH for sodium caseinate was 33.75%.

4.2.1.1.2 Optimization for formulation of Casein Hydrolysate prepared using Flavourzyme 500L

To verify the optimized conditions for preparation of casein hydrolysates using Flavourzyme 500L, the most desirable combination of independent variables was selected for further investigation. The selection was made using optimization command of the software package Design Expert$^{®}$ (version 7.0.0).

Table 4.2.5 Criteria chosen for optimizing values of independent variables in Casein hydrolysed using Flavourzyme 500L

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	Minimize	50	60.00	3
	Temp	Minimize	7	8.00	3
	E/S	Minimize	0.03	0.05	3
	Time	In range	1.00	4.00	3
Responses	DH	Maximize	27	33.95	3

To optimize the values of independent variables, three of the four factors viz. pH, Temp and E/S were kept 'Minimize' and time was kept 'in range'. The goal (Table 4.2.5) for DH was set at 'Maximize'. Twenty nine different solutions were obtained out of which 8 solutions with different values for dependent and independent variables are presented in Table 4.2.6. The desirability of the solution ranged from a minimum of 0.920 to a maximum of 0.939. Out of 8 solutions, solution 1 was shortlisted due to its highest value of %DH for comparing and finalizing the formulation.

Table 4.2.6 Suggested solutions and predicted dependent variable values for Casein Hydrolysate prepared using Flavourzyme 500L

Name	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7.00	50.00	0.03	4.00	32.40	0.939
Solution 2	7.00	50.03	0.03	3.98	32.38	0.938
Solution 3	7.00	50.00	0.03	3.98	32.42	0.933
Solution 4	7.00	50.00	0.03	4.00	32.47	0.928
Solution 5	7.00	50.48	0.03	4.00	32.36	0.926
Solution 6	7.00	50.00	0.03	4.00	32.51	0.923
Solution 7	7.05	50.09	0.03	3.99	32.29	0.921
Solution 8	7.00	50.00	0.03	3.90	32.38	0.920

To validate the predicted model, the tests were carried out at optimal conditions for pH, temperature, E/S ratio and hydrolysis time in triplicate. The predicted response was experimentally verified. The adequacy of the response surface equations was indicated by a comparison between the experimental value and the predicted data. The result revealed (Table 4.2.7) that the difference between the predicted and the actual values of independent variables was statistically non-significant. The hydrolytic conditions consisting of pH 7, temp 50°C, E/S 0.03 and time 4 h were finally selected for the preparation of debittered casein hydrolysate, hydrolysed using Flavourzyme 500L with maximum DH (31.85±0.67).

Table 4.2.7 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	32.403	31.852±0.675	1.413 ^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Casein hydrolysate, hydrolysed using Alcalase; t-value found non-significant at 1% level of significance; ^{ns} non-significant

4.2.2 Process optimization for debittering of Whey Protein Hydrolysate (Alcalase catalysed) using Flavourzyme 500L

Alcalase catalysed whey protein hydrolysates were treated with Flavourzyme 500L to develop debittered protein hydrolysates under different conditions, results of which are discussed below.

Table 4.2.8 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2α	-1	0	+1	+2α
pH	A	6.5	7.0	7.5	8.0	8.5
Temp (°C)	B	45	50	55	60	65
E/S	C	0.02	0.03	0.04	0.05	0.06
Time (h)	D	-0.5	1	2.5	4	5.5

Total, 30 runs and four independent variables at five different levels were applied in the RSM model. Various independent variables and their coded value are shown in Table 4.2.8. The detailed experimental design is shown in Tables 4.2.9 and 4.2.10. All experiments were carried out in triplicate. The central value (coded levels) of four independent variables i.e. pH, temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.04 and 2.5 h, respectively. Results showed that out of 30 experiments, 16, 8 and 6 were factorial, axial and centre points, respectively. It is clear that response (y, DH) is a function of experimental factors (pH, temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, $\beta_{F1,}$ $\beta_{F2,}$ $\beta_{F3,}$ β_{F4} are the first order coefficients, $\beta_{F12,}$ $\beta_{F13,}$ $\beta_{F23,}$ $\beta_{F14,}$ $\beta_{F24,}$ β_{F34} are the cross product coefficients and $\beta_{F11,}$ $\beta_{F22,}$ β_{F33} and β_{F44} are the second order coefficients.

Table 4.2.9 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Std Run	Run Order	pH	Temp(^{o}C)	E/S	Time(h)	Coefficients
1	27	7.0	50	0.03	1.0	Fact
2	22	8.0	50	0.03	1.0	Fact
3	30	7.0	60	0.03	1.0	Fact
4	7	8.0	60	0.03	1.0	Fact
5	6	7.0	50	0.05	1.0	Fact
6	9	8.0	50	0.05	1.0	Fact
7	13	7.0	60	0.05	1.0	Fact
8	14	8.0	60	0.05	1.0	Fact
9	17	7.0	50	0.03	4.0	Fact
10	21	8.0	50	0.03	4.0	Fact
11	18	7.0	60	0.03	4.0	Fact
12	16	8.0	60	0.03	4.0	Fact
13	26	7.0	50	0.05	4.0	Fact
14	2	8.0	50	0.05	4.0	Fact
15	5	7.0	60	0.05	4.0	Fact
16	19	8.0	60	0.05	4.0	Fact
17	3	6.5	55	0.04	2.5	Axial
18	10	8.5	55	0.04	2.5	Axial
19	23	7.5	45	0.04	2.5	Axial
20	12	7.5	65	0.04	2.5	Axial
21	25	7.5	55	0.02	2.5	Axial
22	29	7.5	55	0.06	2.5	Axial
23	4	7.5	55	0.04	-0.5	Axial
24	11	7.5	55	0.04	5.5	Axial
25	15	7.5	55	0.04	2.5	Center
26	8	7.5	55	0.04	2.5	Center
27	1	7.5	55	0.04	2.5	Center
28	28	7.5	55	0.04	2.5	Center
29	20	7.5	55	0.04	2.5	Center
30	24	7.5	55	0.04	2.5	Center

Table 4.2.10 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Whey Protein Hydrolysate prepared using Flavourzyme 500L

Std Order	Run Order	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	DH
1	27	7.0	50	0.03	1.0	31.113
2	22	8.0	50	0.03	1.0	29.956
3	30	7.0	60	0.03	1.0	30.358
4	7	8.0	60	0.03	1.0	29.347
5	6	7.0	50	0.05	1.0	33.523
6	9	8.0	50	0.05	1.0	29.546
7	13	7.0	60	0.05	1.0	30.958
8	14	8.0	60	0.05	1.0	29.129
9	17	7.0	50	0.03	4.0	35.606
10	21	8.0	50	0.03	4.0	31.983
11	18	7.0	60	0.03	4.0	32.754
12	16	8.0	60	0.03	4.0	32.125
13	26	7.0	50	0.05	4.0	35.739
14	2	8.0	50	0.05	4.0	32.116
15	5	7.0	60	0.05	4.0	31.438
16	19	8.0	60	0.05	4.0	29.552
17	3	6.5	55	0.04	2.5	30.325
18	10	8.5	55	0.04	2.5	29.308
19	23	7.5	45	0.04	2.5	30.236
20	12	7.5	65	0.04	2.5	29.291
21	25	7.5	55	0.02	2.5	31.451
22	29	7.5	55	0.06	2.5	32.232
23	4	7.5	55	0.04	-0.5	29.0
24	11	7.5	55	0.04	5.5	32.953
25	15	7.5	55	0.05	2.5	32.111
26	8	7.5	55	0.05	2.5	32.106
27	1	7.5	55	0.05	2.5	32.013
28	28	7.5	55	0.05	2.5	30.504
29	20	7.5	55	0.05	2.5	31.235
30	24	7.5	55	0.05	2.5	33.128

4.2.2.1 Response surface analysis for DH of Whey Protein Hydrolysate prepared using Flavourzyme 500L

Response surface analysis data for %DH of whey proteins hydrolysates debittered using Flavourzyme 500L is discussed here. The design of the model and experiment and results for the same are presented in Table 4.2.11 Degree of hydrolysis under various conditions of independent variables is presented in Table 4.2.11. Results indicated that %DH ranged from 29.00 to 35.73. The minimum value of %DH was obtained at conditions pH 7.5, Temp 55°C, E/S 0.04 and Time 0.5 h. Data shows that the maximum value was obtained only for one combination of independent variables which had pH 7.0, Temp 50°C, E/S 0.05 and Time 4 h.

Both these values of %DH were obtained at different levels of pH, temperature, E/S and time. Such behaviour suggests possible interaction between the independent variables in the hydrolysis of whey proteins by Flavourzyme 500L. Analysis of variance (F-test) showed that the second order model is well adjusted to the experimental data (Table 4.2.11). The coefficient of determination (R^2: 0.812) implies that 81% of the behaviour variation could be explained by the fitted model. Moreover, the lack-of-fit test, which measures the fitness of the model obtained, did not result in a significant F-value (0.365), indicating that the model is sufficiently accurate for predicting the DH for independent variable values within the ranges studied. To determine the effect of various independent variables singly or in combination on response (DH), the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) of all the four independent parameters were studied. It can be observed from the said Table that except for E/S ratio, all the independent variables (pH, temp and time) significantly ($P<0.05$) affected the DH. However, the interactive effect of combinations (AB, AC, AD, BC, BD and CD) of all four independent variables on the %DH was found to be non-significant ($P>0.05$). Similarly, the effect of quadratic terms of independent variables was non-significant ($p>0.05$). Overall, statistical analysis indicated that within each term all four hydrolysis factors had a strong influence on DH.

4.2.2.1.1 Response surface plots and effects of factors on %DH of whey protein hydrolysates prepared using Flavourzyme 500L

The best explanatory equation for DH is given below. This was generated using the regression coefficients with statistical significance up to 5% probability level. The polynomial model shows that all linear terms contributed to the response. However, the interactive effects among independent variables as well as quadratic terms did not appear to be significant.

$$DH = + 31.85 - 0.91*A - 0.74*B + 0.097*C + 1.14*D + 0.56*A*B - 0.43*A*C - 0.24*A*D - 0.49*B*C - 0.45*B*D - 0.25*C*D - 0.32*A^2 - 0.34*B^2 + 0.18*C^2 - 0.034*D^2$$

Table 4.2.11 Coefficient of the full second order polynomial model for coded responses for Whey Protein Hydrolysate Alcalase, prepared using Flavourzyme 500L

ANOVA for Response Surface Quadratic model of Whey protein Hydrolysate prepared using Flavourzyme 500L					
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	66.46	14	4.75	4.65	0.0027
A-pH	14.68	1	14.68	14.37	0.0018
B-Temp	9.14	1	9.14	8.95	0.0091
C-E/S	0.073	1	0.073	0.071	0.7933 ^{ns}
D-Time	24.58	1	24.58	24.07	0.0002
AB	2.27	1	2.27	2.22	0.1568 ^{ns}
AC	2.17	1	2.17	2.13	0.1654 ^{ns}
AD	0.039	1	0.039	0.038	0.8483 ^{ns}
BC	2.87	1	2.87	2.81	0.1146 ^{ns}
BD	1.12	1	1.12	1.09	0.3121 ^{ns}
CD	1.57	1	1.57	1.53	0.2347 ^{ns}
A^2	3.47	1	3.47	3.40	0.0852 ^{ns}
B^2	3.73	1	3.73	3.65	0.0752 ^{ns}
C^2	0.62	1	0.62	0.61	0.4472 ^{ns}
D^2	0.12	1	0.12	0.12	0.7385 ^{ns}
Residual	15.32	15	1.02		
Lack of Fit	11.34	10	1.13	1.42	0.3657 ^{ns}
Pure Error	3.98	5	0.80		
Cor Total	81.78	29			
R-squared					0.8127
APV					8.371
Mean					31.34±1.01

Significant (P<0.05); Highly significant (P<0.01); non-significant (P>0.5)

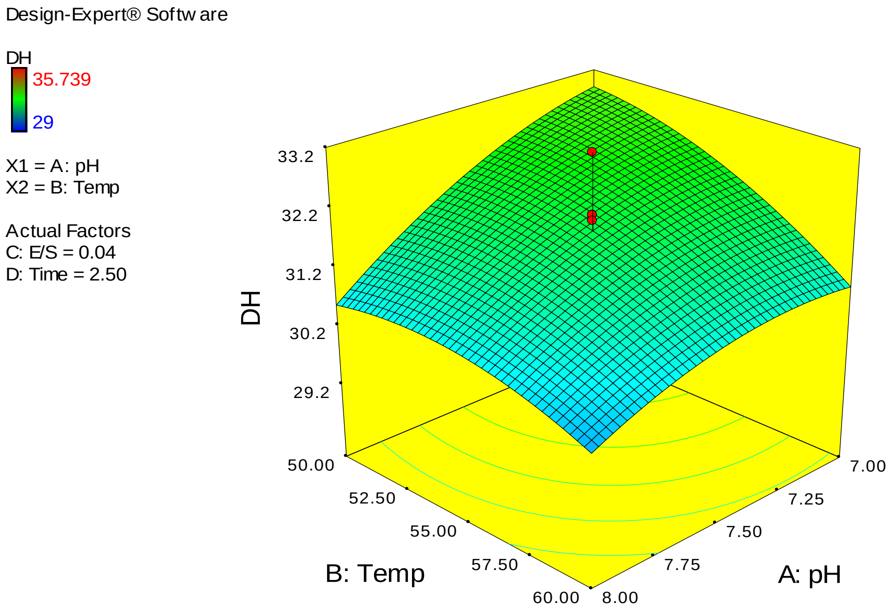

Fig 4.2.7 Response surface related to %DH value of Whey protein hydrolysate prepared using Flavourzyme 500L as influenced by Temp and pH

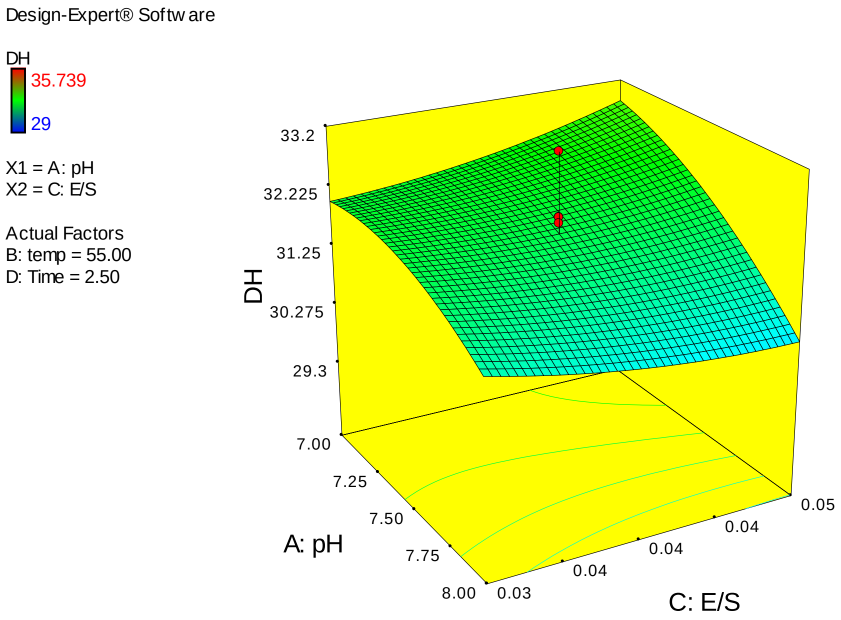

Fig 4.2.8 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 500L as influenced by E/S and pH

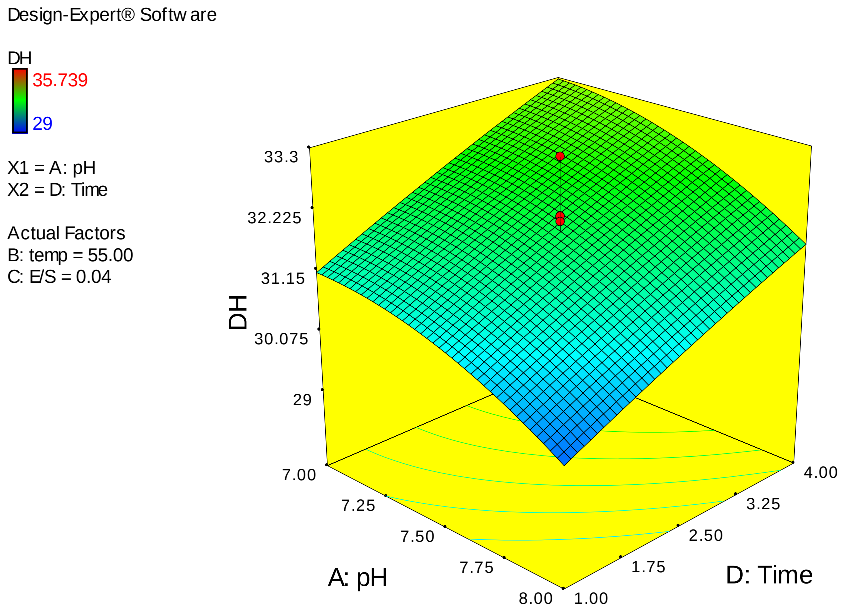

Fig 4.2.9 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 500L as influenced by Time and pH

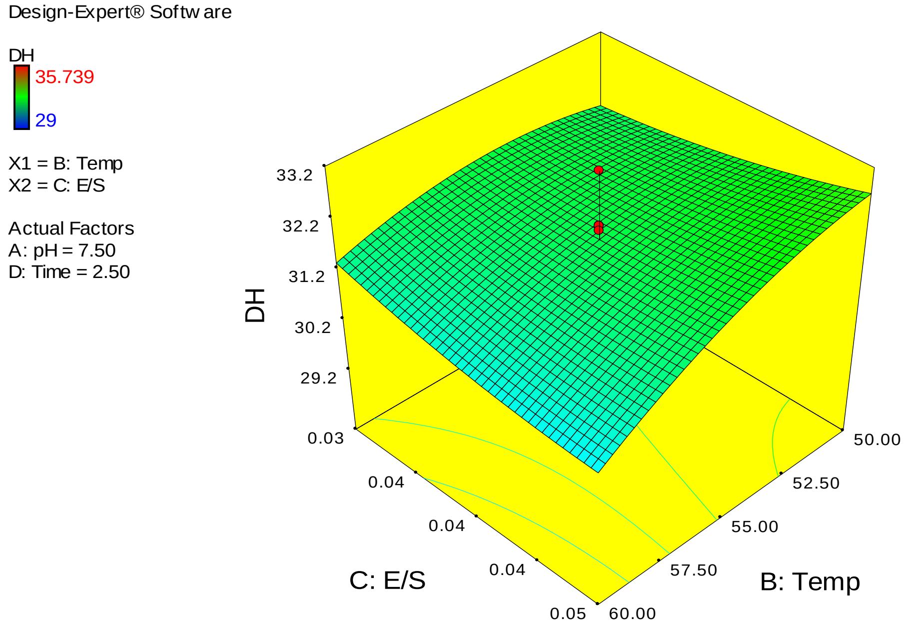

Fig 4.2.10 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 500L as influenced by Temp and E/S

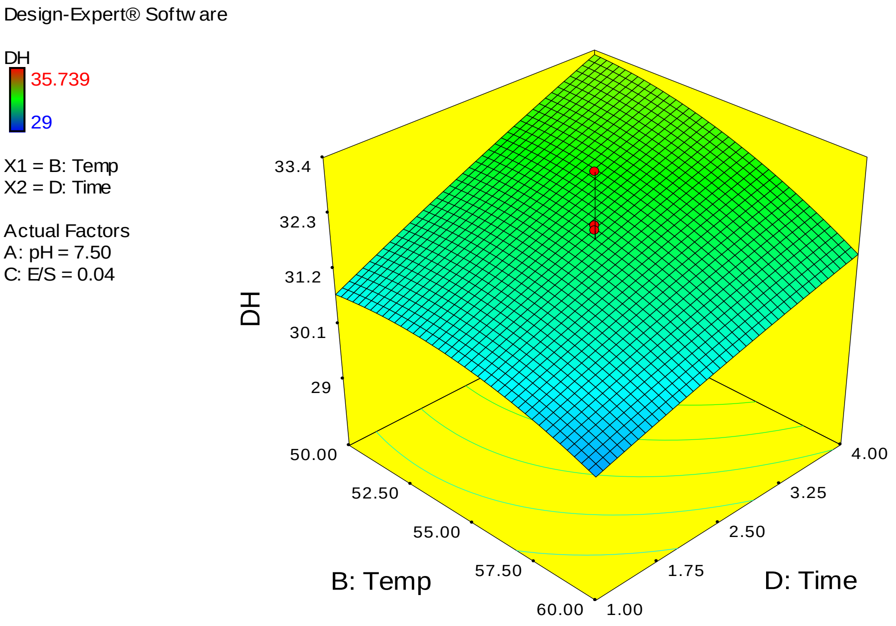

Fig 4.2.11 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 500L as influenced by Temp and Time

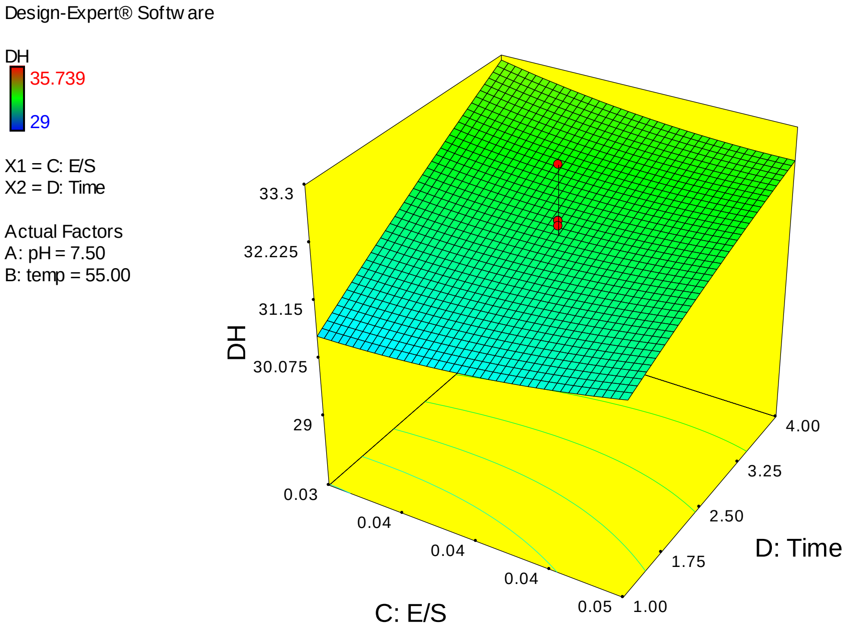

Fig 4.2.12 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 500L as influenced by E/S and Time

The effect of pH and temperature is illustrated in the response surface plot (Fig. 4.2.7). It is clear from the said plot that when E/S and time were kept at centre point 0.04 and 2.5 h, respectively, increasing pH from 7.0 to 8.0 and temperature from 50-60°C had a significant ($P<0.05$) effect on %DH. It can be observed from the said figure that %DH first increased gradually followed by a decrease, with increase in pH and temperature under specified conditions. However, when the temperature (55^{o}C) and time (2.5 h) were kept at centre point, a significant effect of pH was observed on %DH, but no correlation was observed between E/S and %DH (Fig. 4.2.8).

Perspective plot of response i.e. %DH as affected by time and pH is shown in Fig. 4.2.9, which suggested that on keeping temperature and E/S at 55^{o}C and 0.04, respectively, at centre point, %DH increased significantly ($P<0.05$) with increase in time and decrease in pH. Fig. 4.2.9 shows that on keeping pH and time constant (at centre point) at 7.5 and 2.5 h, respectively, increase in temperature significantly affected ($P>0.05$) the %DH. However, no significant correlation was observed between %DH and E/S ratio.

The effect of time and temperature was observed by keeping pH and E/S constant at centre point 7.5 and 0.04, respectively. It was observed that an increase in the temperature significantly ($P<0.05$) increased %DH. Similarly, reaction time showed a significant effect ($P<0.05$) and positive correlation on %DH (Fig. 4.2.11). On the contrary, keeping pH and temperature constant at 7.5 and 55°C, respectively, the reaction time had a positive effect while E/S ratio had no significant effect on %DH (Fig. 4.2.12).

4.2.2.1.2 Optimization for debittering WPC-80 hydrolysates prepared using Flavourzyme 500L

To determine the optimized conditions for debittering WPC-80 hydrolysates, prepared using Flavourzyme 500L, the most desirable combination of independent variables was selected for further investigation. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.2.12 Criteria chosen for optimizing values of independent variables in Whey protein Hydrolysate prepared using Flavourzyme 500L

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	Minimize	50	60	3
	Temp	Minimize	7	8	3
	E/S	Minimize	0.03	0.05	3
	Time	In range	1.00	4.00	3
Responses	DH	Maximize	29	35.739	3

To optimize the values of independent variables, three of the four factors viz. pH, Temp and E/S were kept at 'Minimize' and time was kept at 'In range'. The goal (Table 4.2.12) for DH was set at 'Maximize'. Twenty nine different solutions were obtained out of which six solutions with different values for dependent and independent variables are presented in Table 4.2.13. The desirability of the solution ranged from a minimum of 0.899 to a maximum of 0.918. Out of 6 solutions, solution 1 was shortlisted due to its highest value of %DH for comparing and finalising the formulation

Table 4.2.13 Suggested solutions and predicted dependent variable values of Whey Protein Hydrolysate prepared using Flavourzyme 500L

Name	pH	Temp(^{o}C)	E/S	Time(h)	DH	Desirability
Solution 1	7	50.0	0.03	4.0	33.776	0.918
Solution 2	7	50.3	0.03	4.0	33.771	0.910
Solution 3	7	50.0	0.03	4.0	33.800	0.908
Solution 4	7	50.0	0.03	4.0	33.814	0.902
Solution 5	7	50.0	0.03	4.0	33.817	0.901
Solution 6	7	50.0	0.03	3.6	33.401	0.899

The relevance and suitability of the model was verified by preparing the selected sample (solution 1) in triplicates. There was no significant difference ($P<0.05$) between the predicted value and actual value (Table 4.2.14). This result also indicated that the model was capable of predicting the studied response, i.e. %DH. Finally, the optimum values of the four independent variables in order to get maximum DH of whey proteins using alcalase were pH 7.0, Temperature 50°C, E/S 0.03 and time 4 h.

Table 4.2.14 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	33.77	33.58±0.85	0.3956 ^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Whey protein hydrolysate, hydrolysed using Trypsin; t-value was found to be non-significant at 1% level of significance; ^{ns} non-significant

4.2.3 Process optimization for debittering of Casein Hydrolysate prepared using Flavourzyme 1000L

Flavourzyme® 1000L contains both endoprotease and exopeptidase activitiesis, which is derived from the fungus *Aspergillus oryzae (Ref.)*. Experimental design for the process optimization for debittering casein hydrolysates using Flavourzyme 1000L is discussed below:

Table 4.2.15 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2a	-1	0	+1	+2a
pH	A	6.5	7.0	7.5	8.0	8.5
Temp(°C)	B	45	50	55	60	65
E/S	C	0.01	0.015	0.02	0.025	0.03
Time	D	-0.05	1	2.5	4	5.5

Various independent variables and their coded value and levels are shown in Table 4.2.15. The central value (coded levels) of four independent variables i.e. pH, temp, E/S and hydrolysis time coded as A, B, C and D were taken as 7.5, 55°C, 0.02 and 2.5 h, respectively. As per the general rule of RSM, 4 independent variables were taken; therefore, a total of 30 experiments were run in randomised order that included 16 factorial, 8 axial and 6 centre points (Table 4.2.16). It is clear that response (y, DH) is a function of experimental factors (pH, temp, E/S and time; A, B, C and D) or it can be expressed as y = f (A, B, C, D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and β_{F11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.2.16 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Std Run	Run Order	pH	Temp (^oC)	E/S	Time(h)	Coefficients assessed by
1	8	7.00	50.00	0.015	1.00	Factorial
2	18	8.00	50.00	0.015	1.00	Factorial
3	24	7.00	60.00	0.015	1.00	Factorial
4	7	8.00	60.00	0.015	1.00	Factorial
5	13	7.00	50.00	0.025	1.00	Factorial
6	15	8.00	50.00	0.025	1.00	Factorial
7	29	7.00	60.00	0.025	1.00	Factorial
8	20	8.00	60.00	0.025	1.00	Factorial
9	16	7.00	50.00	0.015	4.00	Factorial
10	26	8.00	50.00	0.015	4.00	Factorial
11	2	7.00	60.00	0.015	4.00	Factorial
12	5	8.00	60.00	0.015	4.00	Factorial
13	23	7.00	50.00	0.025	4.00	Factorial
14	6	8.00	50.00	0.025	4.00	Factorial
15	12	7.00	60.00	0.025	4.00	Factorial
16	30	8.00	60.00	0.025	4.00	Factorial
17	3	6.50	55.00	0.02	2.50	Axial
18	19	8.50	55.00	0.02	2.50	Axial
19	1	7.50	45.00	0.02	2.50	Axial
20	10	7.50	65.00	0.02	2.50	Axial
21	21	7.50	55.00	0.01	2.50	Axial
22	17	7.50	55.00	0.03	2.50	Axial
23	28	7.50	55.00	0.02	-0.50	Axial
24	11	7.50	55.00	0.02	5.50	Axial
25	27	7.50	55.00	0.02	2.50	Center
26	9	7.50	55.00	0.02	2.50	Center
27	14	7.50	55.00	0.02	2.50	Center
28	4	7.50	55.00	0.02	2.50	Center
29	25	7.50	55.00	0.02	2.50	Center
30	22	7.50	55.00	0.02	2.50	Center

Table 4.2.17 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Casein Hydrolysate prepared using Flavourzyme 1000L

Std Order	Run Order	pH (A)	Temp (B)(^{o}C)	E/S ratio (C)	Time (D)(h)	DH
1	28	7.0	50	0.015	1.0	30.00
2	21	8.0	50	0.015	1.0	28.95
3	29	7.0	60	0.015	1.0	29.70
4	30	8.0	60	0.015	1.0	28.35
5	11	7.0	50	0.025	1.0	32.49
6	14	8.0	50	0.025	1.0	28.51
7	23	7.0	60	0.025	1.0	29.97
8	15	8.0	60	0.025	1.0	28.12
9	7	7.0	50	0.015	4.0	35.98
10	25	8.0	50	0.015	4.0	30.98
11	16	7.0	60	0.015	4.0	31.80
12	4	8.0	60	0.015	4.0	31.08
13	19	7.0	50	0.025	4.0	36.65
14	6	8.0	50	0.025	4.0	31.20
15	9	7.0	60	0.025	4.0	30.44
16	1	8.0	60	0.025	4.0	28.50
17	24	6.5	55	0.02	2.5	31.33
18	5	8.5	55	0.02	2.5	28.32
19	27	7.5	45	0.02	2.5	29.22
20	8	7.5	65	0.02	2.5	28.30
21	22	7.5	55	0.01	2.5	30.44
22	17	7.5	55	0.03	2.5	31.19
23	10	7.5	55	0.02	-0.5	27.00
24	12	7.5	55	0.02	5.5	31.13
25	26	7.5	55	0.02	2.5	31.01
26	3	7.5	55	0.02	2.5	31.12
27	20	7.5	55	0.02	2.5	31.00
28	18	7.5	55	0.02	2.5	29.49
29	2	7.5	55	0.02	2.5	30.24
30	13	7.5	55	0.02	2.5	31.95

4.2.3.1 Response surface analysis for DH of Casein Hydrolysate prepared using Flavourzyme 1000L

Data of dependent variable, i.e. Degree of hydrolysis of Casein Hydrolysate, hydrolysed using Flavourzyme 1000L is presented in Table 4.2.17. In total, 30 experiments were conducted with different combination of the independent variables as determined by RSM. It is clear from the said table that values of %DH of caseinate hydrolysates were observed in the range of 27.00 to 36.65. The minimum value was obtained for the combination of independent variables which had pH 7.5, Temp 55°C, E/S 0.02 and Time 0.5 h Data shows that the maximum value was obtained only for one combination of independent variables which had pH 7.0, Temp 50°C, E/S 0.025 and Time 4 h.

The regression analysis of data as presented in Table 4.2.18 revealed that the coefficient of determination (R^2) was 0.834, which suggest that this model fits well to the used model. The APV was found to be 9.59, which was appreciably higher than the minimum desirable value (4.00) for the higher prediction ability.

The statistical significance of the proposed model could be obtained using the Fisher's test (F-test) (Maache-Rezzoug *et al.*, 2011). Table 4.2.18 shows the F-value (5.40), which indicates a significant model. The lack of fit test was used to predict the fitness of the model. In the model of %DH, it was found that the p-value of 0.0012 for the lack of fit was not significant ($p>0.05$). Thus, the model was fit to determine the optimum hydrolysis condition of sodium caseinate using Flavourzyme 1000L.

To determine the significant or non-significant effect of various independent variables singly or in combinations on response (DH), the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) of all the four independent parameters were studied. It can be observed from Table 4.2.18, that except for E/S ratio, all the independent variables (pH, temp and time) significantly ($P<0.05$) affected the degree of hydrolysis in linear terms ($P<0.01$). However, the interaction effect of AB, AC, AD, BC and BD of all four independent variables on the DH% was found to be non-significant ($P>0.05$). Similarly, the interaction effect in quadrant form was observed to be non-significant ($P>0.05$) on %DH response.

4.2.3.1.1 Response surface plots and effects of factors for casein hydrolysate prepared using Flavourzyme 1000L on %DH

The model equation for yield and the response variable (Y) of %DH obtained was derived using the regression coefficient of linear and quadratic terms to fit a full response surface model. According to the model's regression analysis, the best explanatory model equation of %DH of casein hydrolysates as affected by different factors in terms of coded factors is as follows:

Multiple regression equation generated to predict the DH as affected by different factors in terms of coded factors is as following:

$$DH = + 29.83 - 1.14 * A - 0.78 * B + 0.24 * C + 1.11 * D + 0.63 * A * B - 0.45 * A * C - 0.19 * A * D - 0.54 * B * C - 0.45 * B * D - 0.33 * C * D - 0.064 * A^2 - 0.33 * B^2 - 1.771E - 003 * C^2 - 0.023 * D^2$$

Table 4.2.18 Coefficient of the full second order polynomial model for coded responses for Casein Hydrolysate prepared using Flavourzyme 1000L

ANOVA for Response Surface Quadratic model					
Source	**Sum of Squares**	**Df**	**Mean Square**	**F-Value**	**p-value Prob > F**
Model	104.98	14	7.50	5.40	0.0012
A-pH	31.26	1	31.26	22.53	0.0003
B-Temp	14.59	1	14.59	10.51	0.0055
C-E/S	0.014	1	0.014	0.10	0.9205 ^{ns}
D-Time	34.73	1	34.73	25.03	0.0002
AB	5.74	1	5.74	4.14	0.0600
AC	1.60	1	1.60	1.16	0.2993 ^{ns}
AD	1.45	1	1.45	1.05	0.3222 ^{ns}
BC	2.88	1	2.88	2.08	0.1701 ^{ns}
BD	5.20	1	5.20	3.75	0.0719 ^{ns}
CD	1.69	1	1.69	1.22	0.2877 ^{ns}
A^2	0.066	1	0.066	0.047	0.8309 ^{ns}
B^2	2.70	1	2.70	1.94	0.1837 ^{ns}
C^2	1.10	1	1.10	0.79	0.3871 ^{ns}
D^2	1.57	1	1.57	1.13	0.3048 ^{ns}
Residual	20.82	15	1.39		
Lack of Fit	17.28	10	1.73	2.44	0.1684 ^{ns}
Pure Error	3.54	5	0.71		
Cor Total	125.80	29			
Other statistics					
R-squared					0.8345
APV					9.599
Mean					30.48±1.18

Significant(P<0.05); Highly significant (P<0.01); non-significant(P>0.1)

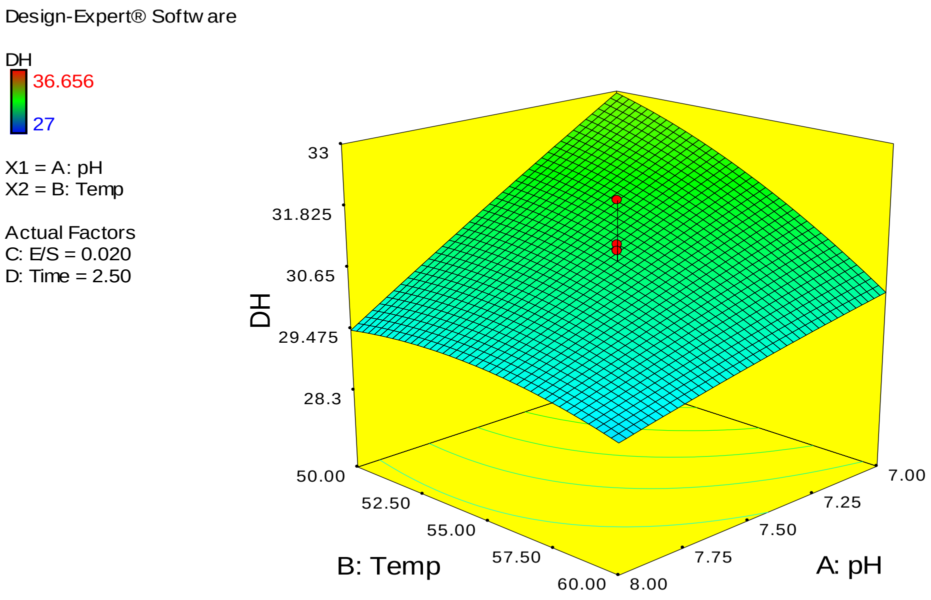

Fig 4.2.13 Response surface related to %DH of Casein hydrolysate developed using Flavourzyme 1000L as influenced by Temp and pH

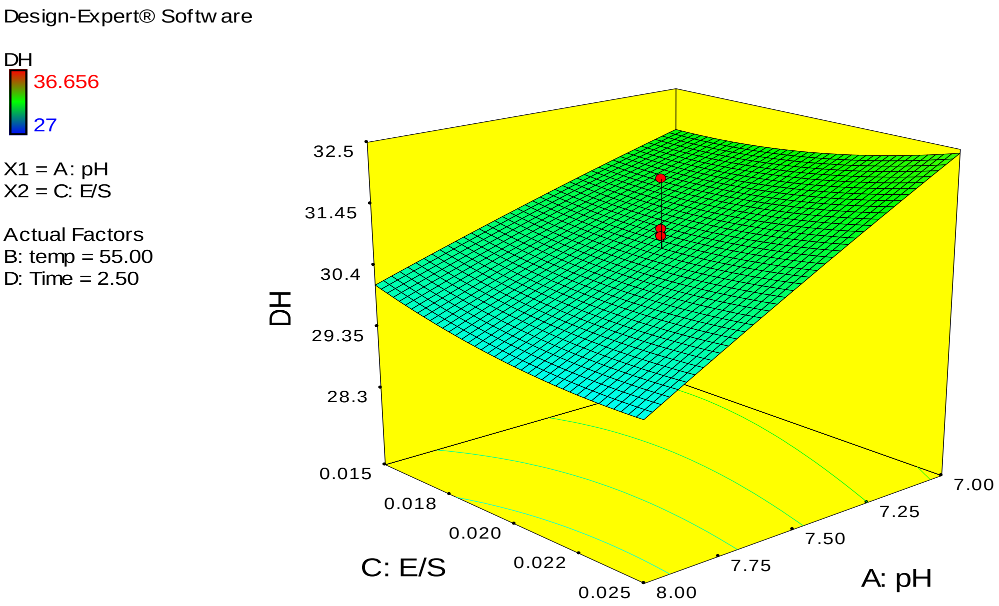

Fig 4.2.14 Response surface related to %DH Casein hydrolysate developed using Flavourzyme 1000L as influenced by E/S and pH

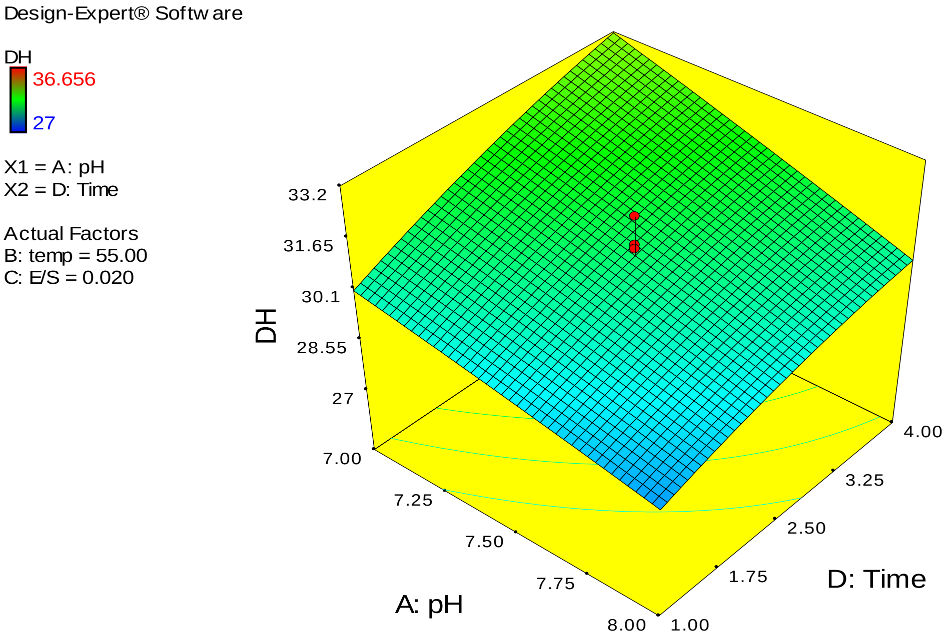

Fig 4.2.15 Response surface related to %DH of Casein hydrolysate prepared using Flavourzyme 1000L as influenced by Time and pH

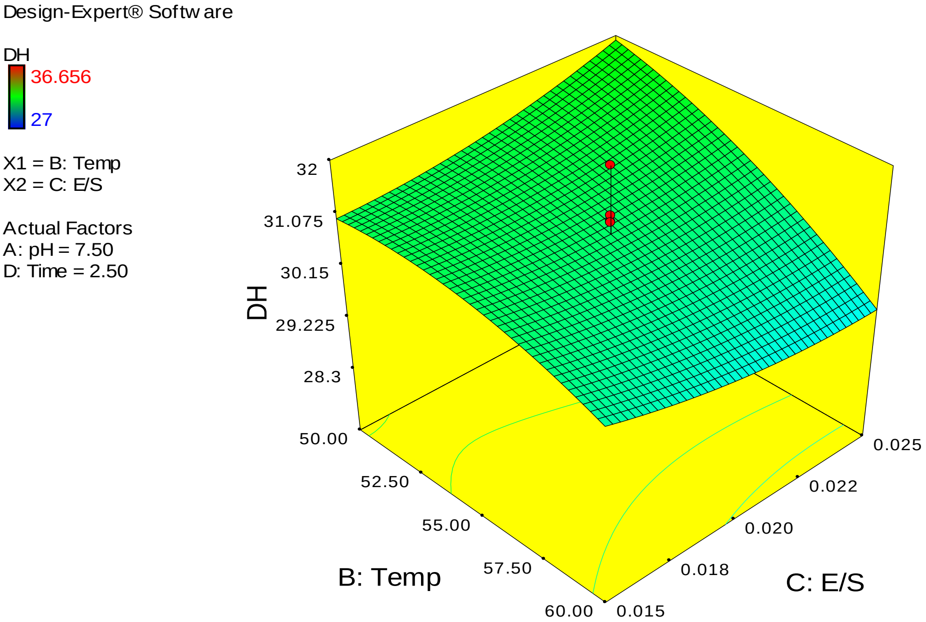

Fig 4.2.16 Response surface related to %DH of Casein hydrolysate preparedusing Flavourzyme 1000L as influenced by Temp and E/S

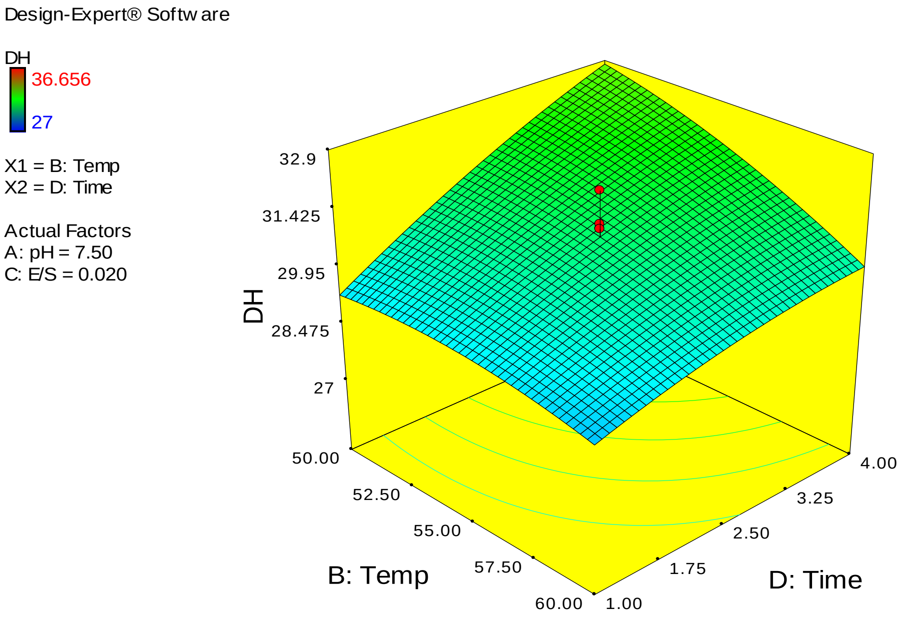

Fig 4.2.17 Response surface related to %DH of Casein hydrolysate developed using Flavourzyme 1000L as influenced by Temp and Time

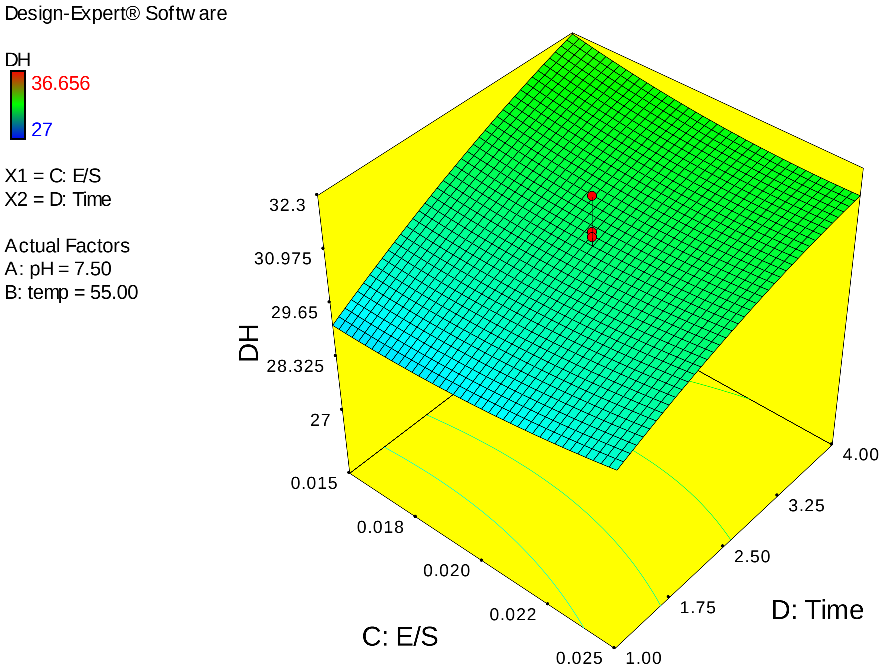

Fig 4.2.18 Response surface related to %DH of Casein hydrolysate developed using Flavourzyme 1000L as influenced by E/S and Time

A 3-dimensional (3D) response was developed to study the effect between the two independent factors (Temp and pH; E/S and pH; Time and pH and so on) on %DH of sodium caseinate. Figures 4.2.13 to 4.2.18 show the 3D response surface graphs, which represent the effect of four independent factors (two for each graph) on %DH.

When E/S and Time were kept at centre point 0.02 and 2.5 h, respectively, increasing pH value from 7.0 to 8.0, significantly decreased the %DH value. However, no significant effect was observed of temperature on %DH. In another possible combination, when the temperature and time were kept at centre point 55°C and 2.5 h, respectively, increasing pH values marked noticeable decrease in the DH value. Again, however, no significant effect was observed of E/S ratio on %DH. On keeping temperature and E/S at the centre point 55°C and 0.02, respectively, %DH increased significantly on increasing reaction time from 1 to 4 h, but decreased with increase in pH values. In this way, observations can be made from the 3D graphs for the interaction of combined factors of E/S and temperature, time and temperature; and time and E/S on %DH. When pH and time were kept at centre point 7.5 and 2.5 h, respectively, increase in temperature significantly decreased %DH. However, it can be noted that on increasing E/S ratio, %DH did not affected significantly and remained almost constant. When pH and E/S were kept at centre point 7.5 and 0.020, respectively, temperature did not significantly affect the %DH. However, time showed a significant effect ($P<0.05$) and positive correlation with %DH. Overall, it can be concluded from the results discussed above, that %DH of casein hydrolysates, hydrolysed with Flavourzyme 1000L, was obtained in the range of 27.00 to 36.65 under conditions discussed above.

4.2.3.1.2 Optimization for formulation of Casein Hydrolysate prepared by Flavourzyme 1000L using most desirable combination of independent variables

From the results obtained through an array of hydrolysates prepared using various combinations of independent variables, as given by the software package Design Expert® (version 7.0.0), the most desirable combination of independent variables was selected for further investigation by verification of its sought functional attributes. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

Table 4.2.19 Criteria chosen for optimizing values of independent variables in functional Casein Hydrolysate prepared using Flavourzyme 1000L

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	Minimize	7	8	3
	Temp	Minimize	50	60	3
	E/S	Minimize	0.015	0.025	3
	Time	In range	1.00	4.00	3
Responses	DH	Maximize	27	36.656	3

For optimization, goal for factors as well as for response was selected. Three of the four factors viz. pH, Temp and E/S were kept ‘Minimize’ and time was kept ‘In range’. The goal (Table 4.2.19) for DH was set at ‘Maximize’. Twenty nine different solutions were obtained out of which 8 solutions with different values for dependent and independent variables are presented in Table 4.2.20. The desirability of the solution ranged from a minimum of 0.912 to a maximum of 0.940. With intention to get maximum value of DH percentage with a high desirability value, solution 1 was shortlisted for comparing and finalising the formulation.

Table 4.2.20 Suggested solutions and predicted dependent variable values of Casein Hydrolysate prepared using Flavourzyme 1000L

Name	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7.00	50	0.015	4	34.55	0.940
Solution 2	7.00	50.05	0.015	4	34.54	0.939
Solution 3	7.00	50	0.015	3.98	34.54	0.934
Solution 4	7.03	50	0.015	3.99	34.46	0.932
Solution 5	7.03	50	0.015	3.96	34.42	0.929
Solution 6	7.00	50	0.015	3.63	34.07	0.924
Solution 7	7.00	50.01	0.016	4	34.56	0.923
Solution 8	7.00	50.92	0.015	3.97	34.34	0.912

Verification of the optimized formulation was done by preparing the selected sample (solution 1) in triplicate and thereafter evaluating the product on similar lines as done while optimizing the formulation. The result revealed (Table 4.2.21) that the difference (t-value) between the predicted and the actual values of independent variables was statistically non-significant (p>0.05).

Thus, it can be concluded that maximum DH was observed at conditions of pH 7.0, temp. 50°C, E/S 0.015 and time 4 h. Therefore, these can be considered optimum condition for the production of casein hydrolysates using Flavourzyme 1000L.

Table 4.2.21 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	34.5577	35.304±0.9305	1.389 ^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Casein hydrolysate, hydrolysed using Alcalase; t-value was found to be non-significant at 1% level of significance;^{ns} non-significant

4.2.4 Process optimization for debittering of Whey Protein Hydrolysate prepared using Flavourzyme 1000L

Various independent variables and their coded value and levels are shown in Table 5.2.22. The central value of the coded levels of four independent variables i.e. pH, temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.02 and 2.5h, respectively. Results revealed that out of 30 experiments, which were run in randomised order, included 16 factorial, 8 axial and 6 centre points (Table 4.2.23).

Table 4.2.22 Boundaries of the experimental domain and spacing of the compositional variables level

Independent variables	Coded Value	Coded Levels				
		-2a	-1	0	+1	+2a
Ph	A	6.5	7.0	7.5	8.0	8.5
Temp(°C)	B	45	50	55	60	65
E/S	C	0.01	0.015	0.020	0.025	0.03
Time	D	-0.05	1	2.5	4	5.5

In present study, only one response (y, degree of hydrolysis, DH) was measured, which can be considered a function of experimental and independent factors (pH, temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and β_{F11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.2.23 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Std Run	Run Order	pH	Temp (^oC)	E/S	Time(h)	Coefficients assessed by
1	16	7.0	50	0.015	1.0	Fact
2	2	8.0	50	0.015	1.0	Fact
3	18	7.0	60	0.015	1.0	Fact
4	24	8.0	60	0.015	1.0	Fact
5	9	7.0	50	0.025	1.0	Fact
6	19	8.0	50	0.025	1.0	Fact
7	4	7.0	60	0.025	1.0	Fact
8	22	8.0	60	0.025	1.0	Fact
9	10	7.0	50	0.015	4.0	Fact
10	12	8.0	50	0.015	4.0	Fact
11	23	7.0	60	0.015	4.0	Fact
12	17	8.0	60	0.015	4.0	Fact
13	21	7.0	50	0.025	4.0	Fact
14	28	8.0	50	0.025	4.0	Fact
15	5	7.0	60	0.025	4.0	Fact
16	14	8.0	60	0.025	4.0	Fact
17	29	6.5	55	0.02	2.5	Axial
18	27	8.5	55	0.02	2.5	Axial
19	1	7.5	45	0.02	2.5	Axial
20	15	7.5	65	0.02	2.5	Axial
21	11	7.5	55	0.01	2.5	Axial
22	20	7.5	55	0.03	2.5	Axial
23	8	7.5	55	0.02	-0.5	Axial
24	30	7.5	55	0.02	5.5	Axial
25	6	7.5	55	0.02	2.5	Center
26	3	7.5	55	0.02	2.5	Center
27	25	7.5	55	0.02	2.5	Center
28	7	7.5	55	0.02	2.5	Center
29	13	7.5	55	0.02	2.5	Center
30	26	7.5	55	0.02	2.5	Center

Table 4.2.24 Central Composite Design matrix (CCDM) with the experimental data on dependent variables of Whey Protein Alcalase Hydrolysate prepared using Flavourzyme 1000L

Std Run	Run Order	pH (A)	Temp (B) (^oC)	E/S ratio (C)	Time (D)(h)	DH
1	16	7.0	50	0.015	1.0	31.00
2	2	8.0	50	0.015	1.0	30.00
3	18	7.0	60	0.015	1.0	30.34
4	24	8.0	60	0.015	1.0	29.34
5	9	7.0	50	0.025	1.0	33.50
6	19	8.0	50	0.025	1.0	29.51
7	4	7.0	60	0.025	1.0	30.90
8	22	8.0	60	0.025	1.0	29.01
9	10	7.0	50	0.015	4.0	36.71
10	12	8.0	50	0.015	4.0	31.96
11	23	7.0	60	0.015	4.0	32.74
12	17	8.0	60	0.015	4.0	32.09
13	21	7.0	50	0.025	4.0	37.69
14	28	8.0	50	0.025	4.0	32.00
15	5	7.0	60	0.025	4.0	31.41
16	14	8.0	60	0.025	4.0	29.49
17	29	6.5	55	0.02	2.5	30.30
18	27	8.5	55	0.02	2.5	29.29
19	1	7.5	45	0.02	2.5	30.22
20	15	7.5	65	0.02	2.5	29.28
21	11	7.5	55	0.01	2.5	31.44
22	20	7.5	55	0.03	2.5	32.21
23	8	7.5	55	0.02	-0.5	29.01
24	30	7.5	55	0.02	5.5	32.96
25	6	7.5	55	0.02	2.5	32.11
26	3	7.5	55	0.02	2.5	32.00
27	25	7.5	55	0.02	2.5	32.11
28	7	7.5	55	0.02	2.5	32.00
29	13	7.5	55	0.02	2.5	30.61
30	26	7.5	55	0.02	2.5	33.09

4.2.4.1 Response surface analysis for DH of Whey Protein Hydrolysate prepared using Flavourzyme 1000L

Table 4.2.24 shows the DH for WPC-80 using Flavourzyme 1000L. As discussed above, total 30 experiments were conducted using CCDM of RSM with different combination of the independent variables. Results revealed that %DH of whey proteins were observed to be in the range of 0 to 20.293. Two combinations (run 21 and 23) of independent variables had minimum value of %DH, i.e. 0; one of which (run 21) had pH 7.5, Temp 55°C, E/S 0.00 and Time 2.5 h and second combination had pH 7.5, Temp 55°C, E/S 0.02 and Time 0.5 h. It can also be observed that the maximum value was obtained for one combination of independent variables which had pH 7.0, Temp 60°C, E/S 0.03 and Time 4 h.

The regression analysis of data revealed that the coefficient of determination (R^2) was 0.8029, which indicates the suitability of this model for optimizing conditions of WPC hydrolysates. APV was found to be 8.52, which was significantly higher than the minimum desirable value (4.00) for the higher prediction ability. ANOVA data showed that the F-value was found to be 4.36, suggesting that model is appropriate for optimizing process conditions. In the applied model, it was found that the p-value of 0.1001 for the lack of fit was not significant ($p>0.05$). On the basis of overall findings of the analysis, it can be suggested that the model is adequate for the optimizing of process conditions for whey proteins concentrate using Flavourzyme 1000L.

To determine the effect of independent variables (pH, temp., E/S and time) on the dependant response (%DH) in singly and/or in combinations, the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) were studied. It can be observed from the Table 5.2.25, that all the independent variables (except E/S ratio) had a significant effect ($P<0.05$) on DH in linear terms. On the contrary, the interactive effect of independent variables as well as quadrant terms was found to be non-significant on %DH ($P>0.05$).

4.2.4.1.1 Response surface plots and effects of independent variables on %DH of WPC-80 hydrolysed by Flavourzyme 1000L

According to the model's regression analysis, the best explanatory model equation of %DH of WPC-80 as affected by different independent variables in terms of coded factors is as following:

$$DH = + 31.99 - 0.96 * A - 0.79 * B + 0.036 * C + 1.18 * D + 0.62 * A * B - 0.38 * A * C - 0.32 * A * D - 0.42 * B * C - 0.51 * B * D - 0.32 * C * D - 0.36 * A^2 - 0.37 * B^2 + 0.15 * C^2 - 0.061 * D^2$$

Table 4.2.25 Coefficient of the full second order polynomial model for coded responses for Whey Protein Hydrolysate prepared using Flavourzyme 1000L

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	97.28	14	6.95	4.36	0.0038
A-pH	21.90	1	21.90	13.75	0.0021
B-Temp	14.91	1	14.91	9.36	0.0079
C-E/S	0.032	1	0.032	0.020	0.8900 ^{ns}
D-Time	33.62	1	33.62	21.11	0.0004
AB	6.22	1	6.22	3.90	0.0669 ^{ns}
AC	2.31	1	2.31	1.45	0.2466 ^{ns}
AD	1.63	1	1.63	1.02	0.3278 ^{ns}
BC	2.82	1	2.82	1.77	0.2032 ^{ns}
BD	4.22	1	4.22	.2.65	0.1242 ^{ns}
CD	1.65	1	1.65	1.03	0.3254 ^{ns}
A^2	3.50	1	3.50	2.20	0.1587 ^{ns}
B^2	3.72	1	3.72	2.34	0.1471 ^{ns}
C^2	0.62	1	0.62	0.39	0.5427 ^{ns}
D^2	0.10	1	0.10	0.064	0.8035 ^{ns}
Residual	23.89	15	1.59		
Lack of Fit	20.74	10	2.07	3.29	0.1001 ^{ns}
Pure Error	3.15	5	0.63		
Cor Total	121.17	29			
Other statistics					
R-squared					0.8029
APV					8.520
Mean					31.48±1.26

Significant($P<0.05$); Highly significant ($P<0.01$); non-significant($P>0.1$)

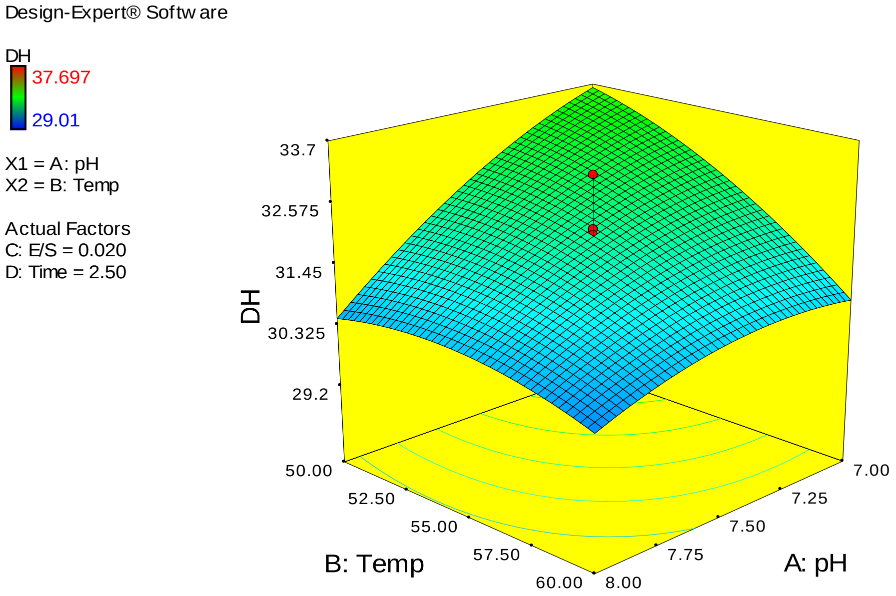

Fig 4.2.19 Response surface related to %DH value of Whey protein Hydrolysate prepared using Flavourzyme 1000L as influenced by Temp and pH

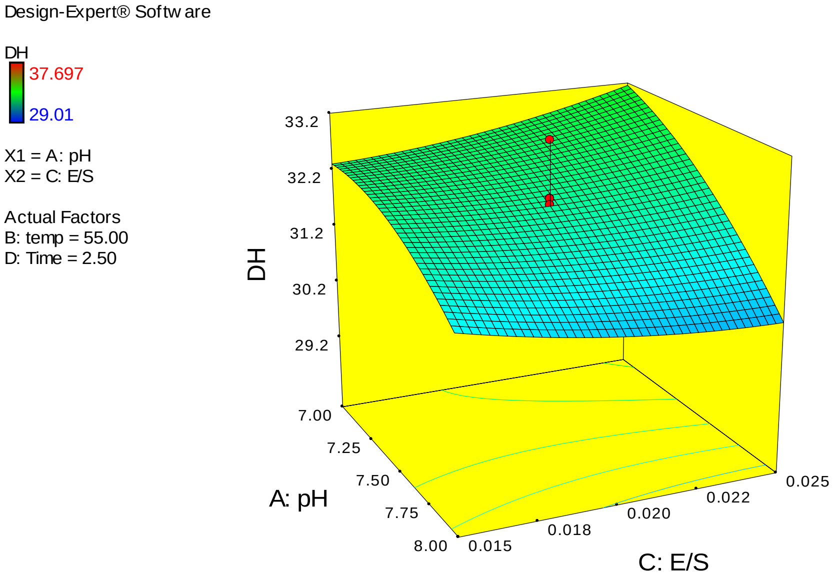

Fig 4.2.20 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 1000L as influenced by E/S and pH

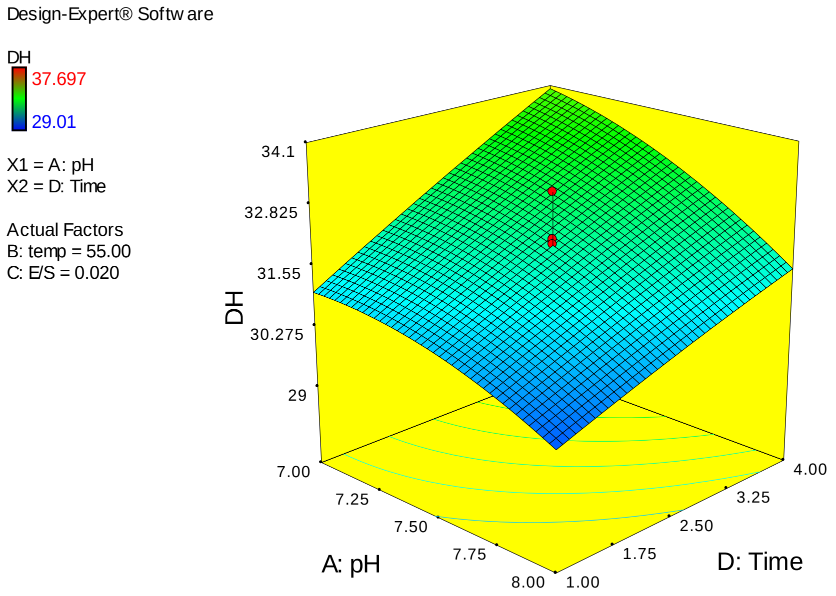

Fig 4.2.21 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 1000L as influenced by Time and pH

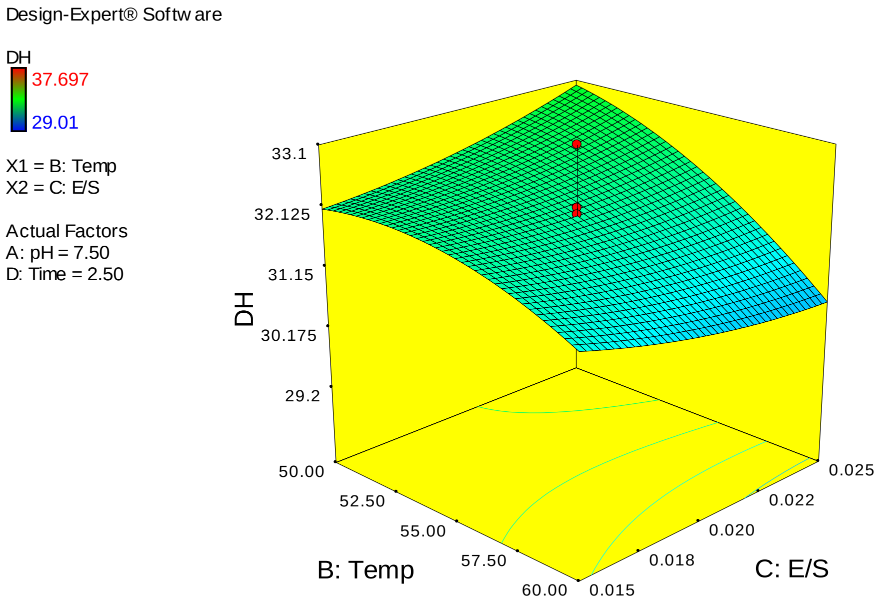

Fig 4.2.22 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 1000L as influenced by Temp and E/S

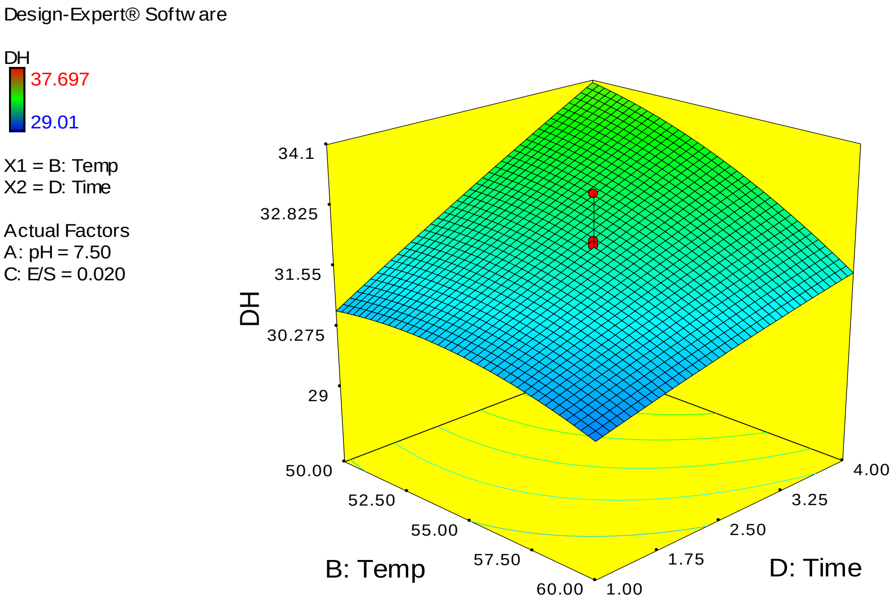

Fig 4.2.23 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 1000L as influenced by Temp and Time

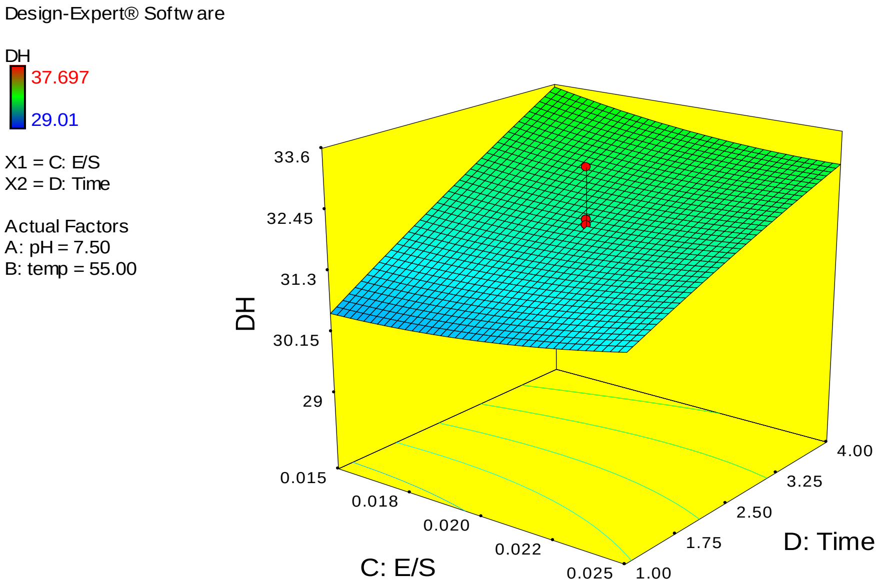

Fig 4.2.24 Response surface related to %DH value of Whey Protein Hydrolysate prepared using Flavourzyme 1000L as influenced by E/S and Time

The influence of independent variables and their interaction effects was analysed using 3-D response graph. Figures 4.2.19 to 4.2.24 show the 3-D response graph for DH. The response surface graphs are drawn by varying two parameters and keeping other two parameters at constant middle level (centre point).

Figure 4.2.19 shows the response graph for two varying parameters pH and temperature by keeping other two parameters time and E/S ratio at constant middle level, which indicates that the increase in pH as well as temperature significantly affected the %DH. A positive correlation was observed between the temperature and %DH; as well as between pH and %DH.

However, when temperature and time were kept at constant middle level (Fig. 4.2.26), %DH significantly ($P<0.05$) decreased with an increase in pH value. On the other hand, on increasing E/S ratio, no significant change was observed in %DH. In continuation of the interpretation of response surface graphs, effect of pH and time was studied keeping rest of the variables constant (temp. and E/S). It can be observed from the graph (Fig. 4.2.19) that DH was positively correlated with pH and reaction time under these specified variables. Response surface plot for %DH as affected by temp and E/S did not showed any significant correlation between %DH and the variables studied (temp and E/S), when other two factors (pH and time) were kept at the constant middle point (Fig. 4.2.21). In another possible combination, when the pH and E/S were kept at centre point, increasing temperature caused a non-significant ($P>0.05$) increase in %DH followed by a gradual (non-significant) decrease in DH. Conversely, a positive association was observed between time and %DH. Fig. 4.2.24 shows the change in response (%DH) as affected by E/S and time, when pH and temperature were kept at the centre point. It is interesting to note that reaction time showed a direct correlation with %DH. However, no significant effect was observed for E/S on %DH.

4.2.4.1.2 Optimization of hydrolytic conditions for preparing whey protein hydrolysates using Trypsin

As discussed above that %DH varied under different conditions of four independent variables, the most desirable combination of independent variables was selected for further investigation by verification of its sought functional attributes. The selection was made using optimization command of the software package Design Expert$^{®}$ (version 7.0.0).

Table 4.2.26 Criteria chosen for optimizing values of independent variables in Whey Protein using Hydrolysate, Alcalase prepared using Flavourzyme 1000L

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	Minimize	7	8	3
	Temp	Minimize	50	60	3
	E/S	Minimize	0.015	0.025	3
	Time	In range	1.00	4.00	3
Responses	DH	Maximize	29.01	37.697	3

To develop WPH under optimized conditions, three of the four factors viz. pH, temp and E/S were kept at ‘Minimize’, while time was kept at ‘In range’. The goal (Table 4.2.26) for response (%DH) was set at ‘Maximize’. Twenty nine different solutions were obtained out of which 8 solutions with different values for dependent and independent variables are presented in Table 4.2.27. The desirability of the solution ranged from 0.903 to 0.919. In orders to get maximum value for %DH with a high desirability value, solution 1 and/or 2 was shortlisted for comparing and finalising the formulation.

Table 4.2.27 Suggested solutions and predicted dependent variable values of Whey Protein Hydrolysate prepared using Flavourzyme 1000L

Name	pH	Temp	E/S	Time (h)	DH	Desirability
Solution 1	7.00	50.00	0.015	4.00	35.22	0.919
Solution 2	7.00	50.00	0.015	4.20	35.19	0.919
Solution 3	7.01	50.01	0.015	4.00	35.21	0.916
Solution 4	7.00	50.00	0.015	4.00	35.23	0.915
Solution 5	7.00	50.18	0.015	4.00	35.19	0.914
Solution 6	7.00	50.00	0.016	4.00	35.25	0.905
Solution 7	7.05	50.00	0.015	3.99	35.12	0.905
Solution 8	7.00	50.00	0.015	3.71	34.79	0.903

Verification of the optimized conditions was done by preparing the selected sample (solution 1) in triplicate and thereafter evaluating the product on similar lines as done while optimizing the formulation. The result revealed (Table 4.2.28) that the difference between the predicted and the actual values of independent variables was statistically non-significant. The optimized conditions of independent variables comprised of pH 7, Temp 50°C, E/S 0.015 and Time 4 h which were finally selected for the preparation of WPH, prepared using Flavourzyme 1000L yielding maximum DH.

Table 4.2.28 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	35.221	36.46±1.31	1.62 ^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Whey protein hydrolysate, hydrolysed using Trypsin; t-value was found to be non-significant at 1% level of significance; ^{ns} non-significant

4.2.5 Process optimization for debittering of Casein Hydrolysate using FlavourSEB

Flavour SEB is a protease and peptidase preparation, which is generally used for the production of yeast extracts and de-bittering of protein hydrolysates (Ref.). Results relating to the combinations of independent variables and their effects on response (DH), analysed by RSM are discussed below:

Table 4.2.29 Boundaries of the experimental domain and spacing of the compositional variables level.

Independent variables	Coded Value	Coded Levels				
		-2α	-1	0	+1	+2α
Ph	A	6.5	7.0	7.5	8.0	8.5
Temp(°C)	B	45	50	55	60	65
E/S	C	0.00	0.10	0.20	0.30	0.40
Time	D	-0.5	1	2.5	4	5.5

The detailed design is listed in Tables 5.2.29 and 5.2.30 and all the experiments were carried out in triplicate. The central value (coded levels) of four independent variables i.e. pH, temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.20 and 2.5 h, respectively. Results showed that out of 30 experiments, 16, 8 and 6 were factorial, axial and centre points, respectively. It is clear that response (y, DH) is a function of experimental factors (pH, temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta F_{23}*B*C + \beta F_{14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta F_{11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$

Where β_{F0} is the intercept, $\beta_{F1,}$ $\beta_{F2,}$ $\beta_{F3,}$ β_{F4} are the first order coefficients, $\beta_{F12,}$ $\beta_{F13,}$ $\beta F_{23,}$ $\beta F_{14,}$ $\beta_{F24,}$ β_{F34} are the cross product coefficients and $\beta F_{11,}$ $\beta_{F22,}$ β_{F33} and β_{F44} are the second order coefficients.

Table 4.2.30 Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units

Std Run	Run Order	pH	Temp (^oC)	E/S	Time (h)	Coefficients
1	11	7	50	0.1	1	Fact
2	9	8	50	0.1	1	Fact
3	17	7	60	0.1	1	Fact
4	27	8	60	0.1	1	Fact
5	3	7	50	0.3	1	Fact
6	5	8	50	0.3	1	Fact
7	29	7	60	0.3	1	Fact
8	12	8	60	0.3	1	Fact
9	25	7	50	0.1	4	Fact
10	24	8	50	0.1	4	Fact
11	13	7	60	0.1	4	Fact
12	23	8	60	0.1	4	Fact
13	7	7	50	0.3	4	Fact
14	15	8	50	0.3	4	Fact
15	2	7	60	0.3	4	Fact
16	26	8	60	0.3	4	Fact
17	22	6.5	55	0.2	2.5	Axial
18	14	8.5	55	0.2	2.5	Axial
19	20	7.5	45	0.2	2.5	Axial
20	18	7.5	65	0.2	2.5	Axial
21	19	7.5	55	0.4	2.5	Axial
22	21	7.5	55	0.4	2.5	Axial
23	30	7.5	55	0.2	-0.5	Axial
24	16	7.5	55	0.2	5.5	Axial
25	1	7.5	55	0.2	2.5	Centre
26	6	7.5	55	0.2	2.5	Centre
27	10	7.5	55	0.2	2.5	Centre
28	28	7.5	55	0.2	2.5	Centre
29	8	7.5	55	0.2	2.5	Centre
30	4	7.5	55	0.2	2.5	Centre

Table 4.2.31 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of debittered Casein Hydrolysate prepared using FlavourSEB

Std Run	Run Order	pH	Temp (^oC)	E/S	Time (h)	DH
1	11	7.0	50	0.1	1.0	29.50
2	9	8.0	50	0.1	1.0	28.45
3	17	7.0	60	0.1	1.0	29.2
4	27	8.0	60	0.1	1.0	27.80
5	3	7.0	50	0.3	1.0	32.00
6	5	8.0	50	0.3	1.0	28.01
7	29	7.0	60	0.3	1.0	29.47
8	12	8.0	60	0.3	1.0	27.62
9	25	7.0	50	0.1	4.0	34.14
10	24	8.0	50	0.1	4.0	30.48
11	13	7.0	60	0.1	4.0	31.30
12	23	8.0	60	0.1	4.0	30.58
13	7	7.0	50	0.3	4.0	35.05
14	15	8.0	50	0.3	4.0	30.70
15	2	7.0	60	0.3	4.0	29.94
16	26	8.0	60	0.3	4.0	28.00
17	22	6.5	55	0.2	2.5	30.83
18	14	8.5	55	0.2	2.5	27.82
19	20	7.5	45	0.2	2.5	28.72
20	18	7.5	65	0.2	2.5	27.80
21	19	7.5	55	0.4	2.5	27.0
22	21	7.5	55	0.4	2.5	30.69
23	30	7.5	55	0.2	-0.5	27.0
24	16	7.5	55	0.2	5.5	30.63
25	1	7.5	55	0.2	2.5	30.51
26	6	7.5	55	0.2	2.5	30.62
27	10	7.5	55	0.2	2.5	30.5
28	28	7.5	55	0.2	2.5	28.99
29	8	7.5	55	0.2	2.5	29.74
30	4	7.5	55	0.2	2.5	31.62

4.2.5.1 Response surface analysis for DH of Casein Hydrolysate using FlavourSEB

Degree of hydrolysis of Casein Hydrolysate using FlavourSEB under various conditions of independent variables is presented in Table 4.2.31. Results indicated that %DH ranged from 27 to 35.056. The minimum value (27%) was obtained for the two combinations of independent variables; one of which had pH 7.5, Temp 55°C, E/S 0.4 and Time 2.5h and the second combination had pH 7.5, Temp 55°C, E/S 0.2 and Time - 0.5 h. Data shows that the maximum %DH value was obtained only for one combination of independent variables comprising of pH 7.0, Temp 50°C, E/S 0.3 and Time 4 h.

The experimental design and results of CCD are presented in Table 4.2.32. For debittering of casein hydrolysates using FlavourSEB, the coefficient of determination (R^2) was 0.770, which indicated a positive correlation between the independent variables and response (%DH). The probability p-value was very low (0.0097), indicating the significance of the model. The ANOVA also showed that there was a non-significant ($P>0.05$) lack of fit, which further validated the model. The APVwas found to be 8.17.

To determine the effect of various independent variables singly or in combinations on response (DH), the coefficient of estimate of DH in terms of linear order (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) order and interaction (AB, AC, AD, BC, BD) of all the four independent parameters were studied. It was observed that except for E/S ratio, all the independent variables (pH, Temp and time) significantly ($P<0.05$) affected the DH. However, the interactive effect of combinations (AB, AC, AD, BC, BD and CD) of all four independent variables on the %DH was found to be non-significant ($P>0.05$). Similarly, significant effect was not observed on %DH response, when quadrant forms were studied.

4.2.5.1.1 Response surface plots and effects of factors on %DH of casein hydrolysates prepared using FlavourSEB

According to the model's regression analysis, the best explanatory model equation of %DH of casein hydrolysates as affected by different factors in terms of coded factors is as following

$$DH = + 31.99 - 0.96 * A - 0.79 * B + 0.036 * C + 1.18 * D + 0.62 * A * B - 0.38 * A * C - 0.32 * A * D - 0.42 * B * C - 0.51 * B * D - 0.32 * C * D - 0.36 * A^2 - 0.37 * B^2 + 0.15 * C^2 - 0.061 * D^2$$

Table 4.2.32 Coefficient of the full second order polynomial model for coded responses for Casein Hydrolysate, hydrolysed using FlavourSEB.

Source	**Sum of Squares**	**Df**	**Mean of Sum of Squares**	**F-Value**	**p-value Prob > F**
Model	81.55	14	5.83	3.95	0.0097
A-pH	25.94	1	25.94	15.98	0.0012
B-Temp	11.03	1	11.03	6.79	0.0199
C-E/S	1.88	1	1.88	1.16	0.2984 ^{ns}
D-Time	26.94	1	26.94	16.59	0.0010
AB	3.19	1	3.19	1.96	0.1814 ^{ns}
AC	1.76	1	1.76	1.08	0.3141 ^{ns}
AD	0.35	1	0.35	0.22	0.6471 ^{ns}
BC	3.09	1	3.09	1.90	0.1878 ^{ns}
BD	2.79	1	2.79	1.72	0.2096 ^{ns}
CD	1.53	1	1.53	0.94	0.3466 ^{ns}
A^2	0.025	1	0.025	0.015	0.9033 ^{ns}
B^2	2.38	1	2.38	1.47	0.2446 ^{ns}
C^2	0.61	1	0.61	0.38	0.5486 ^{ns}
D^2	0.68	1	0.68	.0.42	0.5267 ^{ns}
Residual	24.35	15	1.62		
Lack of Fit	20.42	10	2.04	2.59	0.1522 ^{ns}
Pure Error	3.94	5	0.79		
Cor Total	105.90	29			
Other statistics					
R-squared					0.7700
APV					8.175
Mean					29.83±1.27

significant(P<0.05); Highly significant (P<0.01); non-significant(P>0.1)

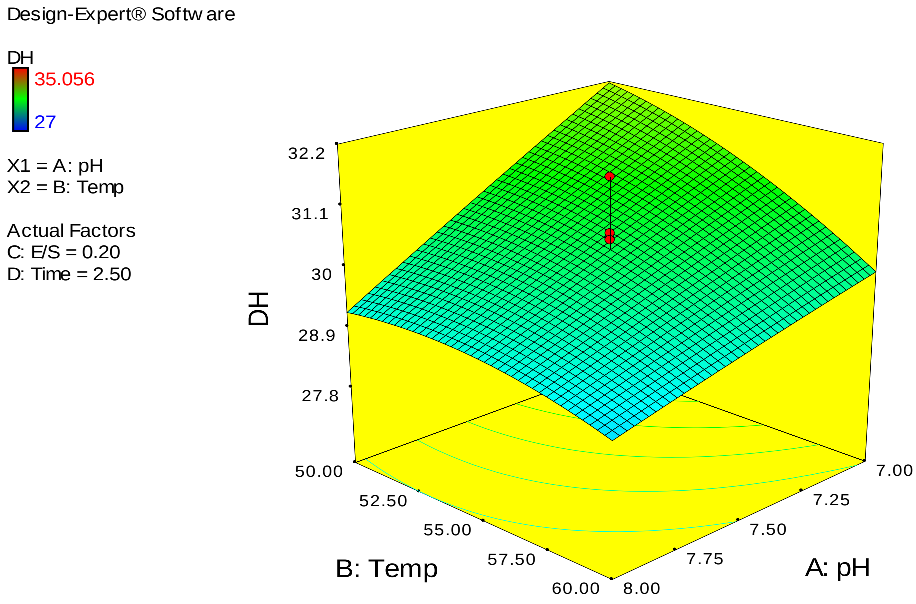

Fig 4.2.25 Response surface related to %DH value of Casein hydrolysate prepared using FlavourSEB as influenced by Temp and pH

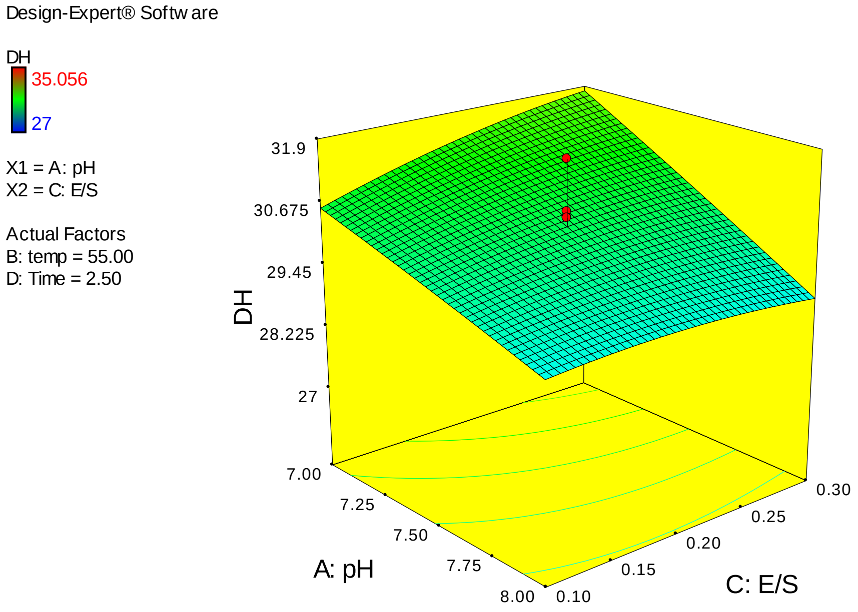

Fig 4.2.26 Response surface related to %DH value of Casein hydrolysate prepared using FlavourSEB as influenced by E/S and pH

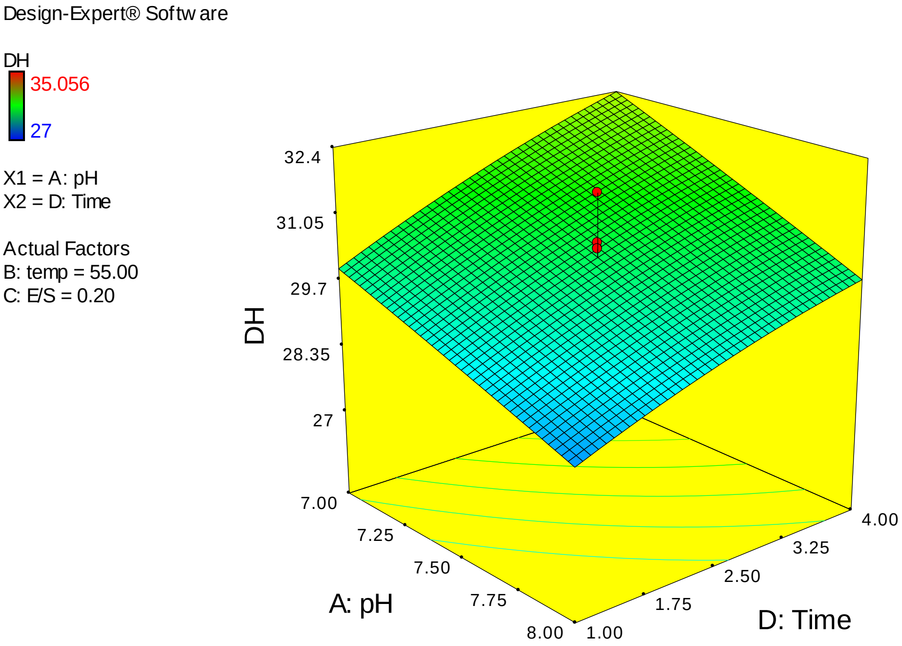

Fig 4.2.27 Response surface related to percentage %DH value of Casein hydrolysate prepared using FlavourSEB as influenced by Time and pH

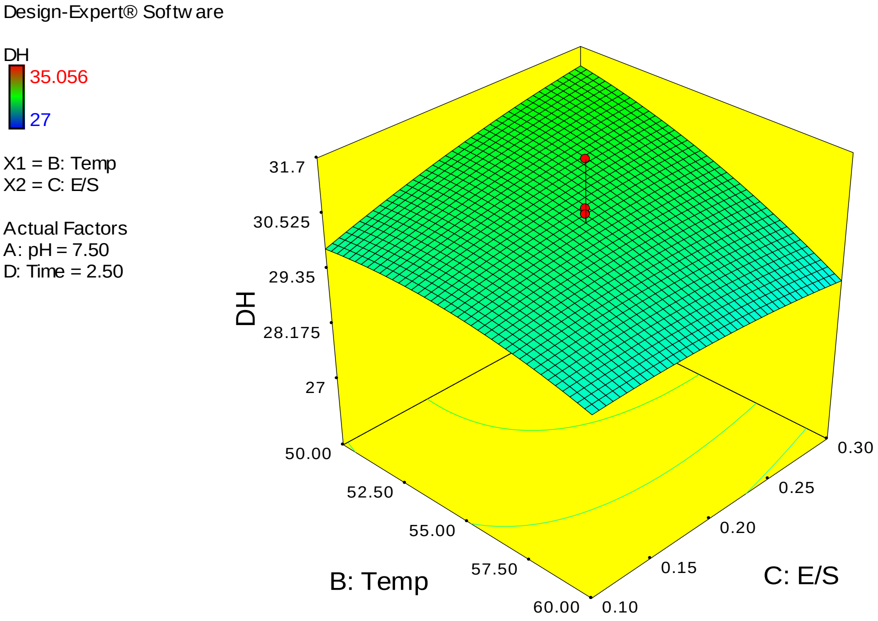

Fig 4.2.28 Response surface related to %DH value of Casein hydrolysate prepared using FlavourSEB as influenced by Temp and E/S

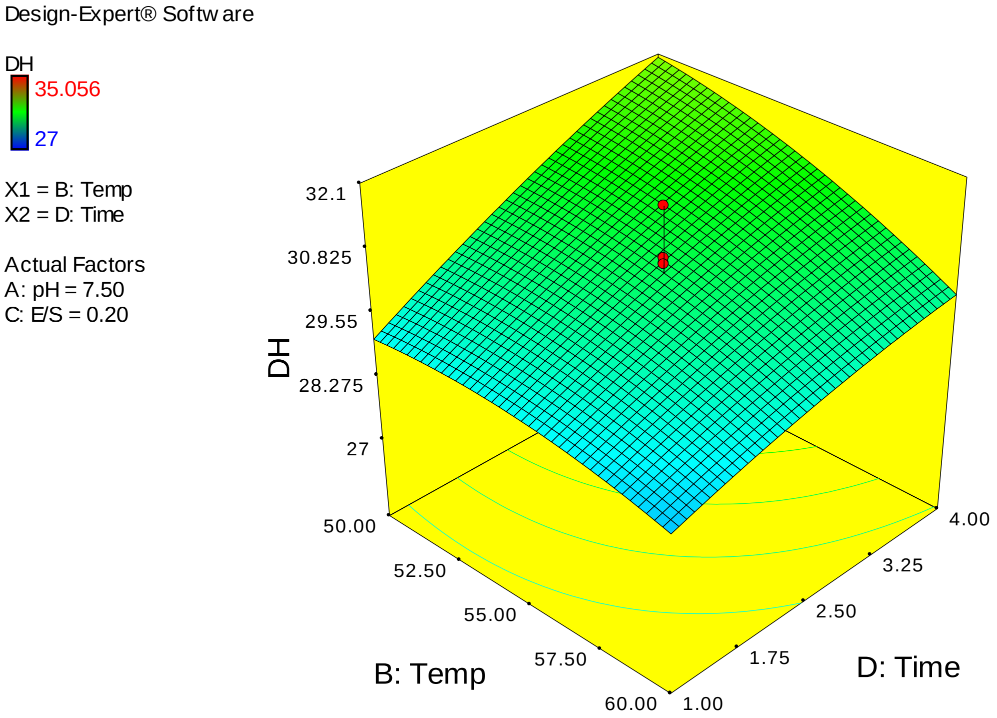

Fig 4.2.29 Response surface related to %DH value of Casein hydrolysate prepared using FlavourSEB as influenced by Temp and Time

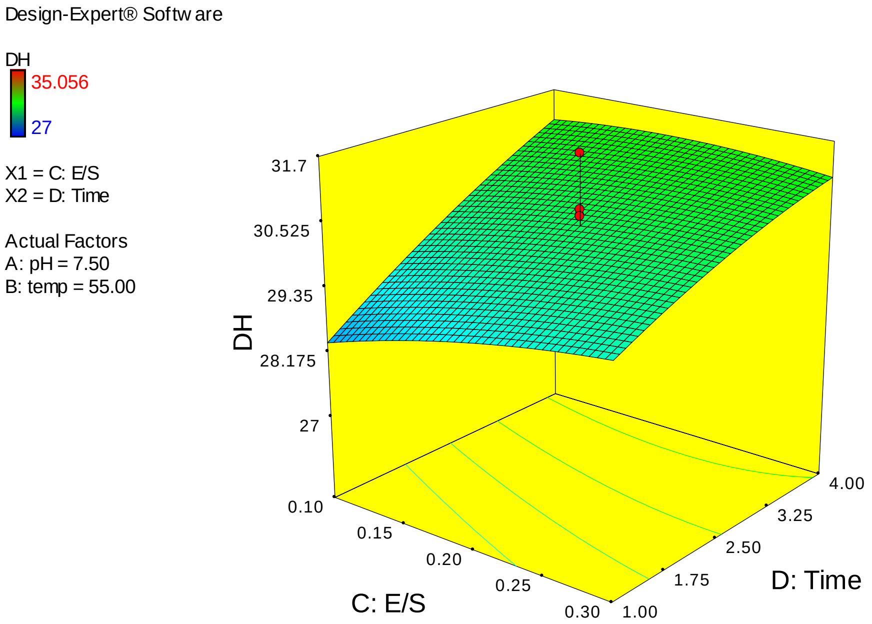

Fig 4.2.30 Response surface related to %DH value of Casein hydrolysate prepared using FlavourSEB as influenced by E/S and Time

Response surface graphs were developed to study the effect of independent variables (in combination) on %DH of sodium caseinate, debittered using FlavourSEB. Figures 4.2.25 to 4.2.30 show the 3D response surface graphs, for which interpretation is described below:

When E/S and time were kept at centre point 0.20 and 2.5 h, respectively (Fig. 4.2.25), a significant decrease in %DH was observed with an increase in pH. However, in case of Temperature, first a gradual increase was observed in %DH followed by a decrease with increase in Temperature. In another possible combination, when the Temperature and time were kept at centre point 55^{o}C and 2.5 h, respectively, correlation was not observed between the pH and %DH as well as between E/S and %DH (Fig. 4.2.26).

Response surface graph of %DH as affected by time and pH is shown in Fig. 4.2.27, which suggested that on keeping Temperature and E/S at the centre point 55^{o}C and 0.20, respectively, %DH increased significantly ($P<0.05$) on increasing the hydrolysis time. However, an inverse correlation was observed between pH and %DH.

On keeping pH and time constant (i.e. at centre point) 7.5 and 2.5 h, respectively, increase in Temperature did not significantly affect ($P>0.05$) %DH. Similar trend was observed between E/S and %DH, as shown in Fig. 4.2.28. When pH and E/S were kept at centre point 7.5 and 0.20, respectively, increase in Temperature did not significantly affect the %DH. However, hydrolysis showed a significant effect ($P<0.05$) and positive correlation on %DH (Fig. 4.2.29). Here, Temperature constant at 7.5 and 55°C, respectively, E/S and time showed a positive and significant association with DH.

Table 4.2.33 Criteria chosen for optimizing values of independent variables in functional Casein using Hydrolysate Alcalase hydrolysed using FlavourSEB

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	Minimize	7	8.	3
	Temp	Minimize	50	60	3
	E/S	Minimize	0.1	0.3	3
	Time	In range	1.00	4.00	3
Responses	DH	Maximize	27	35.056	3

For optimization, goal for factors as well as for response was selected. Three of the four factors viz. pH, Temp and E/S were kept 'Minimize' and time was kept 'In range'. The goal (Table 4.2.33) for DH was set 'Maximize'. Twenty nine different solutions were obtained out of which 8 solutions with different values for dependent and independent variables are presented in Table 4.2.34. The desirability of the solution ranged from a minimum of 0.899 to a maximum of 0.919. With intention to get maximum value of DH percentage with a high desirability value, solution 2 was shortlisted for comparing and finalising the formulation

Table 4.2.34 Suggested solutions and predicted dependent variable values of Casein Hydrolysate, hydrolysed using FlavourSEB

Name	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7.00	50.00	0.1	4	32.75	0.919
Solution 2	7.00	50.00	0.1	4	32.79	0.916
Solution 3	7.00	50.00	0.11	4	32.80	0.915
Solution 4	7.00	50.00	0.11	4	32.83	0.913
Solution 5	7.00	50.23	0.1	4	32.72	0.913
Solution 6	7.02	50.00	0.1	4	32.69	0.912
Solution 7	7.00	50.48	0.1	4	32.69	0.906
Solution 8	7.00	50.71	0.1	4	32.67	0.899

To confirm the applicability of the model designed, confirmation runs using the calculated levels of the variables were carried out in triplicates. Although this actual value was higher than the expected value, there was no significant difference ($P<0.05$) between them (Table 4.2.35). This result also indicated that the second-order polynomial model could be used to predict the %DH of sodium caseinate hydrolysed by FlavourSEB under different conditions.

From the results, it can be concluded that maximum degree of hydrolysis (32.79%) of sodium caseinate using FlavourSEB, was observed under the optimum conditions of pH 7.0, Temp. 50°C, E/S 0.1 and time 4 h. In other words, these values of independent variables can be considered optimum condition for the production of casein hydrolysates catalysed by FlavourSEB.

Table 4.2.35 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	32.75	33.08±1.191	0.4787 ^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Whey protein hydrolysate, hydrolysed using Trypsin; t-value was found to be non-significant at 1% level of significance; ^{ns} non-significant

4.2.6 Process optimization for debittering of Whey Protein Hydrolysate prepared using FlavourSEB

Table 4.2.36 shows various independent variables and their coded value. The central value of the coded levels of four independent variables i.e. pH, Temp, E/S and time coded as A, B, C and D were taken as 7.5, 55°C, 0.20 and 2.5 h, respectively. Results revealed that out of 30 experiments, which were run in randomised order, included 16 factorial, 8 axial and 6 centre points (Table 4.2.37). Degree of hydrolysis (%DH) was analysed as a response, which can be considered a dependant function of experimental and independent parameters (pH, Temp, E/S and time; A, B, C and D). Second order polynomials were developed by multiple regression technique for each of the response using a software package, Design Expert v7.0.0.

Table 4.2.36 Boundaries of the experimental domain and spacing of the compositional variables level.

Independent variables	Coded Value	Coded Levels				
		-2α	**-1**	**0**	**+1**	**+2α**
pH	A	6.5	7.0	7.5	8.0	8.5
Temp(°C)	B	45	50	55	60	65
E/S	C	0.00	0.10	0.20	0.30	0.40
Time	D	-0.5	1	2.5	4	5.5

$$Y_F = +\beta_{F0} + \beta_{F1}*A + \beta_{F2}*B + \beta_{F3}*C + \beta_{F4}*D + \beta_{F12}*A*B + \beta_{F13}*A*C + \beta_{F23}*B*C + \beta_{F14}*A*D + \beta_{F24}*B*D + \beta_{F34}*C*D + \beta_{F11}*A^2 + \beta_{F22}*B^2 + \beta_{F33}*C^2 + \beta_{F44}*D^2$$

Where β_{F0} is the intercept, β_{F1}, β_{F2}, β_{F3}, β_{F4} are the first order coefficients, β_{F12}, β_{F13}, β_{F23}, β_{F14}, β_{F24}, β_{F34} are the cross product coefficients and β_{F11}, β_{F22}, β_{F33} and β_{F44} are the second order coefficients.

Table 4.2.37 **Central Composite Rotatable Design (CCRD) consisting of thirty experiments for the study of four experimental factors expressed in coded units**

Std Run	Run Order	pH	Temp	E/S	Time (h)	Coefficients
1	16	7.0	50	0.10	1.0	Fact
2	2	8.0	50	0.10	1.0	Fact
3	18	7.0	60	0.10	1.0	Fact
4	24	8.0	60	0.10	1.0	Fact
5	9	7.0	50	0.30	1.0	Fact
6	19	8.0	50	0.30	1.0	Fact
7	4	7.0	60	0.30	1.0	Fact
8	22	8.0	60	0.30	1.0	Fact
9	10	7.0	50	0.10	4.0	Fact
10	12	8.0	50	0.10	4.0	Fact
11	23	7.0	60	0.10	4.0	Fact
12	17	8.0	60	0.10	4.0	Fact
13	21	7.0	50	0.30	4.0	Fact
14	28	8.0	50	0.30	4.0	Fact
15	5	7.0	60	0.30	4.0	Fact
16	14	8.0	60	0.30	4.0	Fact
17	29	6.5	55	0.20	2.5	Axial
18	27	8.5	55	0.20	2.5	Axial
19	1	7.5	45	0.20	2.5	Axial
20	15	7.5	65	0.20	2.5	Axial
21	11	7.5	55	0.00	2.5	Axial
22	20	7.5	55	0.40	2.5	Axial
23	8	7.5	55	0.20	-0.5	Axial
24	30	7.5	55	0.20	5.5	Axial
25	6	7.5	55	0.20	2.5	Center
26	3	7.5	55	0.20	2.5	Center
27	25	7.5	55	0.20	2.5	Center
28	7	7.5	55	0.20	2.5	Center
29	13	7.5	55	0.20	2.5	Center
30	26	7.5	55	0.20	2.5	Center

Table 4.2.38 Central Composite Design Matrix (CCDM) with the experimental data on dependent variables of Whey Protein Hydrolysate, hydrolysed using FlavourSEB

Std. Order	Run Order	Coefficients assessed by	pH (A)	Temp (B)	E/S ratio (C)	Time (D)	DH
1	16	Factorial	7.0	50	0.10	1.0	30.75
2	2	Factorial	8.0	50	0.10	1.0	29.75
3	18	Factorial	7.0	60	0.10	1.0	30.09
4	24	Factorial	8.0	60	0.10	1.0	29.14
5	9	Factorial	7.0	50	0.30	1.0	33.25
6	19	Factorial	8.0	50	0.30	1.0	29.26
7	4	Factorial	7.0	60	0.30	1.0	30.65
8	22	Factorial	8.0	60	0.30	1.0	29.00
9	10	Factorial	7.0	50	0.10	4.0	35. 32
10	12	Factorial	8.0	50	0.10	4.0	31.71
11	23	Factorial	7.0	60	0.10	4.0	32.49
12	17	Factorial	8.0	60	0.10	4.0	31.84
13	21	Factorial	7.0	50	0.30	4.0	36.44
14	28	Factorial	8.0	50	0.30	4.0	31.75
15	5	Factorial	7.0	60	0.30	4.0	31.16
16	14	Factorial	8.0	60	0.30	4.0	29.24
17	29	Axial	6.5	55	0.20	2.5	30.05
18	27	Axial	8.5	55	0.20	2.5	29.00
19	1	Axial	7.5	45	0.20	2.5	30
20	15	Axial	7.5	65	0.20	2.5	29.01
21	11	Axial	7.5	55	0.00	2.5	29
22	20	Axial	7.5	55	0.40	2.5	31.96
23	8	Axial	7.5	55	0.20	-0.5	29
24	30	Axial	7.5	55	0.20	5.5	32.71
25	6	Central	7.5	55	0.20	2.5	32.86
26	3	Central	7.5	55	0.20	2.5	30.23
27	25	Central	7.5	55	0.20	2.5	31.86
28	7	Central	7.5	55	0.20	2.5	31.75
29	13	Central	7.5	55	0.20	2.5	32.36
30	26	Central	7.5	55	0.20	2.5	32.84

4.2.6.1 Response Surface Analysis for DH of Whey Protein Hydrolysate debittered using FlavourSEB

Experimental data using Central Composite Design Matrix showed that among the 30 hydrolysates evaluated (Table 4.2.38), 8 experiments showed DH more than 32.00% and 22 showed DH in the range of 29-32%. It is also noteworthy that the highest results for the DH were obtained when FlavourSEB was used at pH of 7.0, Temperature of 50°C, E/S ratios of 0.30 and 4 h reaction time. However, lowest DH was observed under the conditions of 7.5 pH, 55°C, E/S of 0.20 ratio and 3 h reaction time.

The analysis of variance was performed to evaluate the significance of the model as given in Table 4.2.39. The analysis of variance demonstrated that the regression coefficient was significant (R^2=0.75), which meant there was 24% chance that a "model F-value" could occur due to noise. An adequate precision is a measure of the range of predicted response relative to its associated error or, in other words, a single-to-noise ratio, whose value above 4 is considered to be desirable (Canettieri *et al.*, 2007). The Adequate Precision value (APV) was found to be 7.524, which was significantly higher, indicating the higher prediction ability. ANOVA data showed that the F-value was 3.33, again supporting our assumption that the model is appropriate for optimizing process conditions.

ANOVA results for linear, quadratic and interaction of the 4 independent variables (A, B, C and D) on the response values (Y) is presented in Table 5.2.39. It is noted that except for E/S ratio, all other independent variables had a significant effect on DH (P<0.05). On the contrary, the interactive effect of independent variables was found to be non-significant on %DH (P>0.05). Analysis data revealed that the quadratic model in terms of A^2, B^2, C^2 and D^2 was non-significant (P>0.05). The lack-of-fit F value of 0.201 was non-significant (P>0.05), which implied that the model was fitted to all data. P value of the model was less than 0.05, implying again that the model was significant and could be used to optimize hydrolysis conditions.

4.2.6.1.1 Response surface plots and effects of factors on %DH of whey protein hydrolysates prepared using FlavourSEB

The polynomial model of the DH was regressed and shown as below (in terms of coded factors):

DH = + 31.99 - 0.86 * A - 0.69 * B + 0.23 * C + 1.06 * D + 0.51* A * B-0.38* A * C - 0.20 * A * D - 0.42 * B * C - 0.40 * B * D - 0.32 * C * D - 0.40 * A2 - 0.41 * B2 - 0.16 * C2 - 0.070 * D2

Table 4.2.39 Coefficient of the full second order polynomial model for coded responses for Whey Protein Alcalase Hydrolysate, hydrolysed using FlavourSEB.

Source	Sum of Squares	Df	Mean of Sum of Squares	F-Value	p-value Prob > F
Model	79.56	14	5.68	3.33	0.0136
pH (A)	17.66	1	17.66	10.34	0.0058
Temp (B)	11.45	1	11.45	6.71	0.0205 ns
E/S (C)	1.30	1	1.30	0.76	0.3962 ns
Time (D)	27.09	1	27.09	15.87	0.0012
AB	4.12	1	4.12	2.41	0.1411 ns
AC	2.28	1	2.28	1.34	0.2657 ns
AD	0.66	1	0.66	0.39	0.5428 ns
BC	2.79	1	2.79	1.63	0.2207 ns
BD	2.53	1	2.53	1.48	0.2422 ns
CD	1.67	1	1.67	0.98	0.3382 ns
A^2	4.43	1	4.43	2.59	0.1282 ns
B^2	4.55	1	4.55	2.67	0.1233 ns
C^2	0.74	1	0.74	0.43	0.5211 ns
D^2	0.14	1	0.14	0.079	0.7819 ns
Residual	25.61	15	1.71		
Lack of Fit	20.83	10	2.08	2.18	0.2019 ns
Pure Error	4.78	5	0.96		
Cor Total		29			
Other statistics					
R-squared					0.7565
APV					7.524
Mean					31.15±1.31

significant(P<0.05); Highly significant (P<0.01); non-significant(P>0.1)

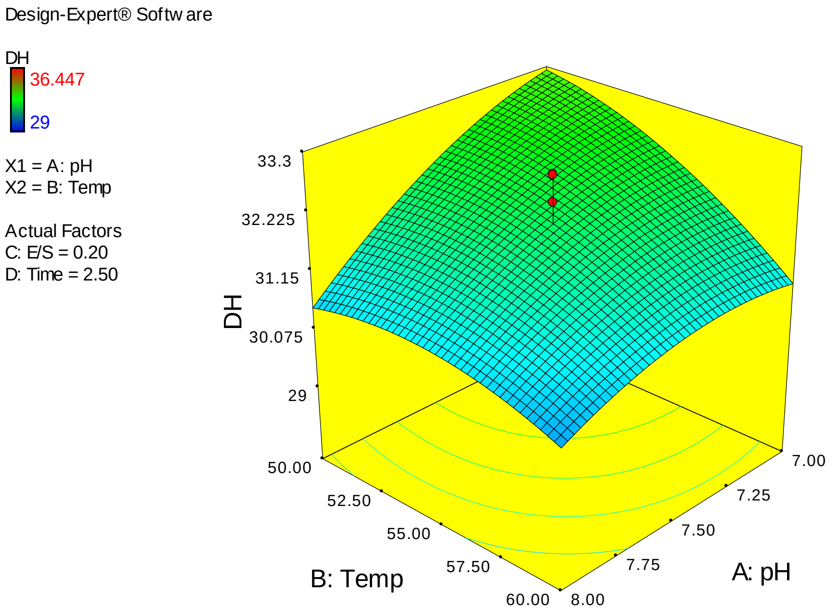

Fig 4.2.31 Response surface related to %DH value of Whey protein Hydrolysate debittered using FlavourSEB as influenced by Temp and pH

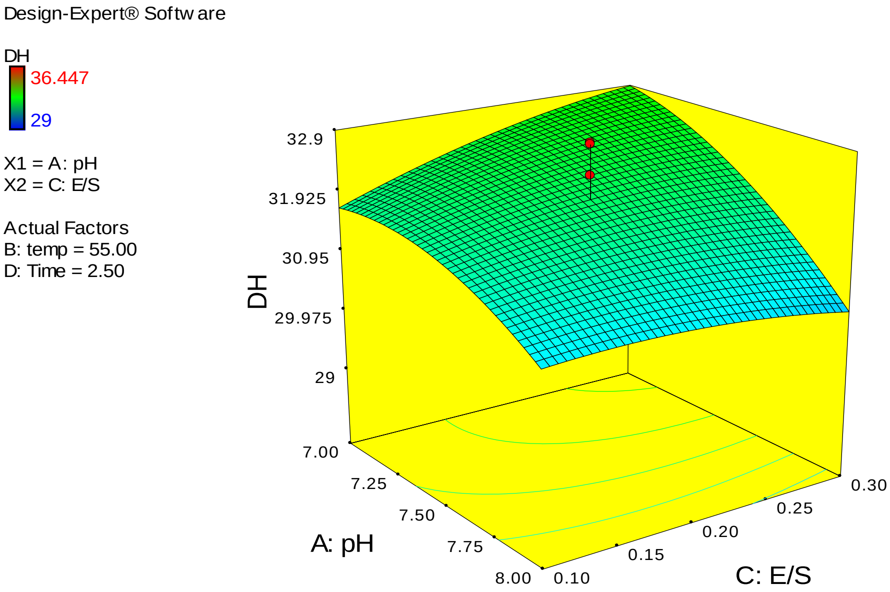

Fig 4.2.32 Response surface related to %DH value of Whey protein Hydrolysate debittered using FlavourSEB as influenced by E/S and pH

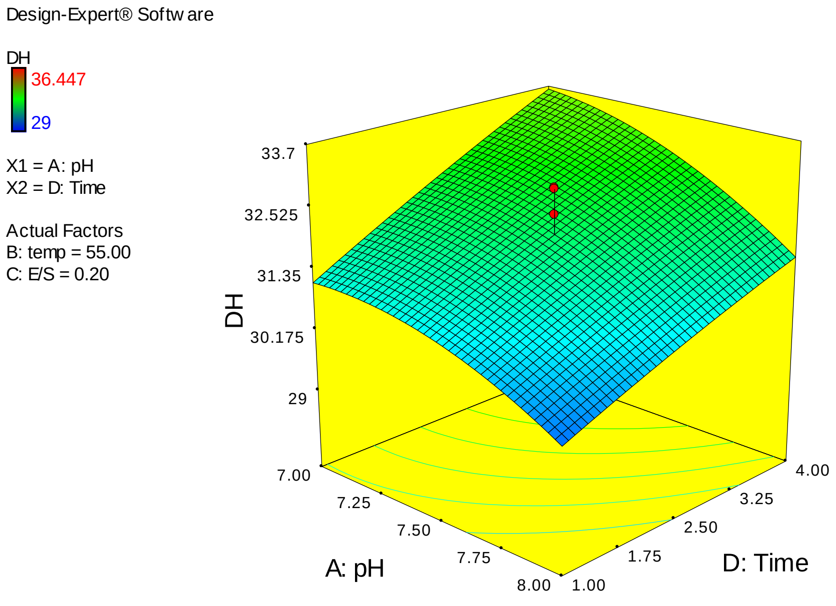

Fig 4.2.33 **Response surface related to %DH value of Whey protein Hydrolysate debittered using FlavourSEB as influenced by Time and pH**

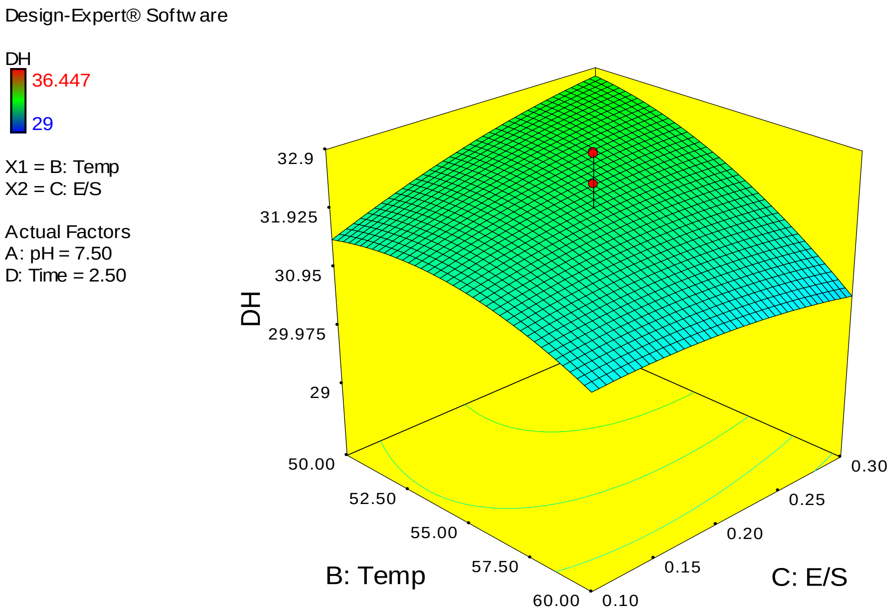

Fig 4.2.34 **Response surface related to %DH value of Whey protein Hydrolysate debittered using FlavourSEB as influenced by Temp and E/S.**

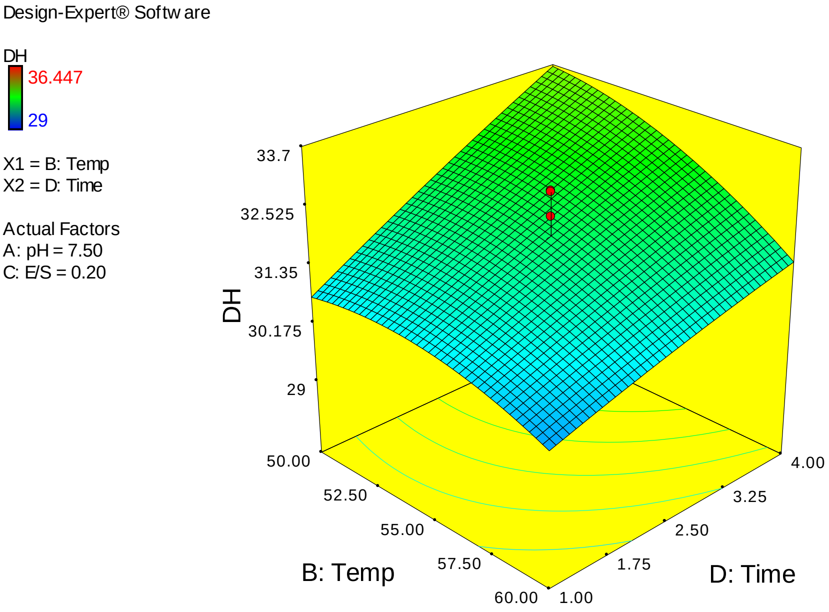

Fig 4.2.35 **Response surface related to %DH value of Whey protein Hydrolysate debittered using FlavourSEB as influenced by Temp and Time**

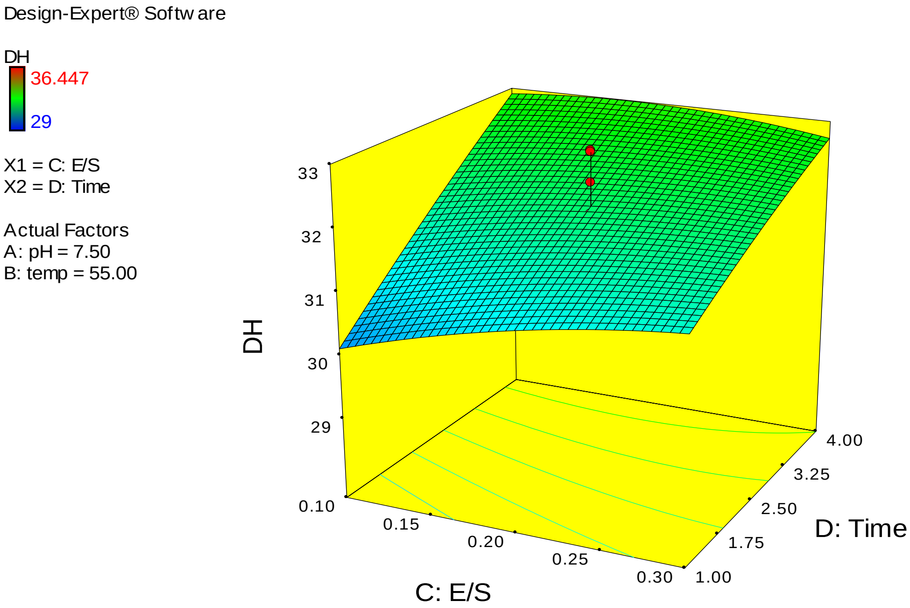

Fig 4.2.36 **Response surface related to %DH value of Whey protein Hydrolysate debittered using FlavourSEB as influenced by E/S and Time**

All of the response surface plots are shown in Fig. 4.2.31 to 4.2.36. Figure 4.2.31 represents the effects of pH (A) and Temperature (B), where the E/S ratio and reaction time were set at constant values of 0.20 and 2.5 h, respectively. It is clear from the said figure that the %DH first increased with decrease in the pH followed by a gradual decrease. Similar relationship was observed between temperature and %DH. The effect of pH and E/S ratio is shown in the response surface plot (Figure 4.2.32) where the Temperature and reaction time are set at constant values of 55°C and 2.5 h, respectively. It is clear from the figure that the %DH decreased with an increase in pH. On the contrary, enzyme concentration (E/S ratio) had no significant effect on %DH. Figure 4.2.33 demonstrated the effect of pH and reaction time, where the Temperature and E/S ratio were set at constant values of 55°C and 0.20, respectively. It shows that when reaction time is 4 h and pH is 7.0, the DH was maximum. Figures 4.1.34, 4.1.35 and 4.1.36 show, respectively, the effect of Temperature and E/S, Temperature and reaction time, E/S and reaction time on the response (DH). It can be concluded from the three figures that %DH was maximum under the conditions when the Temperature, E/S ratio and reaction time were 50°C, 0.20 and 4 h, respectively.

Table 4.2.40 Criteria chosen for optimizing values of independent variables in Whey Protein Hydrolysate debittered using FlavourSEB

Name		Goal	Lower limit	Upper limit	Importance
Factors	pH	Minimize	7	8	3
	Temp	Minimize	50	60	3
	E/S	Minimize	0.1	0.3	3
	Time	In range	1.00	4.00	3
Responses	DH	Maximize	29	36.447	3

For the validation of the optimized conditions for preparation of WPH using papain, the most desirable combination of independent variables was selected for further investigation. The selection was made using optimization command of the software package Design Expert® (version 7.0.0).

For the optimization of independent variables, three of the four factors viz. pH, Temp and E/S were kept at ‘Minimize’ and time was kept at ‘In range’. The goal (Table

4.2.40) for DH was set at ‘Maximize’. The desirability of the solutions obtained ranged from a minimum of 0.894 to a maximum of 0.903. On the basis of results, solution 1 was selected due to its highest value of %DH for comparing and finalizing the formulation.

Table 4.2.41 Suggested solutions and predicted dependent variable values of Whey Protein Hydrolysate debittered using FlavourSEB

Name	pH	Temp	E/S	Time	DH	Desirability
Solution 1	7	50.00	0.1	4.00	33.966	0.903
Solution 2	7.01	50.03	0.1	4.00	33.951	0.901
Solution 3	7	50.07	0.1	4.00	33.983	0.900
Solution 4	7.02	50.00	0.1	4.00	33.946	0.899
Solution 5	7	50.00	0.1	3.92	33.862	0.899
Solution 6	7	50.05	0.1	3.89	33.825	0.896
Solution 7	7.03	50.00	0.1	4.00	33.935	0.896
Solution 8	7.01	50.32	0.1	4.00	33.930	0.894

To know the adequacy of the predicted model, the tests were carried out at optimal conditions for pH, Temperature, E/S ratio and hydrolysis time in triplicate. The predicted response were experimentally verified. The suitability of the response surface equations was indicated by a comparison between the experimental value and the predicted data. The result revealed (Table 4.2.42) that the difference between the predicted and the actual values of independent variables was statistically non-significant. The hydrolytic conditions consisting pH 7, Temp 50°C, E/S 0.1 and time 4 h were finally selected for the preparation of whey protein hydrolysates, prepared using FlavourSEB with maximum degree of hydrolysis (33.966%).

Table 4.2.42 Comparison of actual and predicted values of independent variables

Dependent Variable	Predicted Score	Actual Score	t- Value
DH	33.96	35.20±1.169	1.234^{ns}

Predicted value as obtained from design expert v7.0.0 software package.

Actual mean values ± standard deviation (average of three trials) of optimized functional Whey protein hydrolysate, prepared using Trypsin; t-value was found to be non-significant at 1% level of significance; ^{ns} non-significant Casein Alcalase hydrolysate 29%DH

4.2.7 Optimized conditions for developing de-bittered casein hydrolysates and whey protein hydrolysates using Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB

In this way, conditions of independent varaibles were optimized for developing de-bittered casein hydrolysates and whey proteins hydrolysates using Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB, which are shown in Table 4.2.43. and 4.2.44. It is clear from the tables that Flavourzyme 1000L led to maximum %DH of casein as well as whey protein (alcalase catalysed). The developed de-bittered casein and whey protein hydrolysates were assessed for its bitterness on the basis of sensory evaluation.

Table 4.2.43. Optimized conditions for developing de-bittered casein hydrolysates

Enzyme used		**Optimized conditions for hydrolysis**			
	pH	**Temperature (°C)**	**E/S ratio**	**Time (h)**	**DH (%)**
Flavourzyme 500L	7	50	0.03	4	32.40
Flavourzyme 1000L	7	50	0.015	4	34.55
FlavourSEB	7	50	0.1	4	32.79

Table 4.2.44. Optimized conditions for developing de-bittered whey protein hydrolysates

Enzyme used		**Optimized conditions for hydrolysis**			
	pH	**Temperature (°C)**	**E/S ratio**	**Time (h)**	**DH (%)**
Flavourzyme 500L	7	50.0	0.03	4	33.77
Flavourzyme 1000L	7	50	0.015	4	35.23
FlavourSEB	7	50	0.1	4	33.96

4.2.8 Sensory evaluation of de-bittered casein and whey proteins hydrolysate developed using Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB

Samples of casein hydrolysate, optimized using Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB were analyzed for bitterness on the basis of 4-point intensity scale, where 1 indicates most bitter and 4 indicates least bitter. The results of the sensory evaluation are shown in Table 5.2.45 and 5.2.46. It is clear from the said table that casein hydrolysates de-bittered by Flavourzyme 1000L were found to be the lowest in terms of bitterness. There was significant difference ($P<0.05$) among the bitterness of Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB catalyzed casein hydrolysates. Casein hydrolysates debittered by using Flavourzyme 500L showed highest degree of bitterness followed by FlavourSEB and Flavourzyme 1000L. Similar trend was observed for whey protein hydrolysates.

Table 4.2.45 Bitterness intensity scores of casein hydrolysates on 4 point intensity scale

Parameters	Flavourzyme 500L	Flavourzyme 1000L	FlavourSEB
Bitterness **(DH %)**	2.9 ± 0.04^{a} (33.77)	3.9 ± 0.08^{b} (35.22)	3.6 ± 0.03^{c} (32.79)

Values with different small superscripts (a,b,c) differ significantly ($P<0.05$) within the rows.

Table 4.2.46 Bitterness intensity scores of whey protein hydrolysates on 4 point intensity scale

Parameters	Flavourzyme 500L	Flavourzyme 1000L	FlavourSEB
Bitterness **(DH %)**	3.1 ± 0.06^{a} (33.77)	3.8 ± 0.01^{b} (35.23)	3.4 ± 0.2^{c} (33.96)

Values with different small superscripts (a,b,c) differ significantly ($P<0.05$) within the rows.

Flavourzyme 1000L catalysed WPH exhibited the minimum bitterness among all the samples followed by FlavourSEB and Flavourzyme 500L (Table 4.2.46). In this way, Flavourzyme 1000L was finally selected as the best enzyme for the production of debittered casein as well as whey protein hydrolysates. The optimum conditions for developing debittered casein hydrolysates as well whey protein hydrolysates (alcalase catalysed) using Flavourzyme 1000L were pH 7.0, Temperature 50°C, E/S ratio of 0.015 and reaction time of 4 h. The developed casein and whey protein hydrolysates, debittered using Flavourzyme 1000L were further utilized for preparing flavoured dairy beverage.

4.3 Utilization of debittered casein and whey protein hydrolysates for preparation of flavoured milk

The developed casein and whey protein hydrolysates, debittered using Flavourzyme 1000L were further utilized for preparing flavoured dairy beverages. Sensory acceptability of milk fortified with debittered casein and whey protein hydrolysates was determined by evaluating the developed product for colour and appearance, taste, sweetness, mouthfeel and overall acceptability. Milk was warmed to 40-45°C before serving to sensory panel. The sensory scores for the milk fortified debittered casein hydrolysates have been depicted in Table 4.3.1. It is evident from the said table that there was no significant difference ($P<0.05$) in the colour and appearance, sweetness and mouthfeel scores amongst experimental samples as well as between control and experimental samples. However, scores for taste decreased significantly ($p<0.05$) in milk fortified with 7.5% of debittered casein hydrolysates. However, no significant difference ($P<0.05$) was observed among the taste scores of control and milk fortified with 2.5 and 5.0% casein hydrolysates. It can be noted here that even though significant difference was noted between control and milk fortified with 7.5% casein hydrolysates for attribute significant ($P<0.05$) difference was not observed with respect to the overall acceptability of all studied samples. It is interesting to note that there was no significant difference among all sensory attributes of control and test samples, when milk beverage was fortified with debittered WPH. Kumar (2011) developed an ergogenic beverage based on WPH fortified with functional ingredients. In his study, optimum DH (8.41%) was obtained using Flavourzyme under the conditions of 0.02% enzyme concentration and 30 min incubation time. Sensory evaluation showed better sensory scores for the ergogenic drink, except for flavour, as compared to control sample. The results obtained in his study are in accordance with the results of the present study.

Table 4.3.1 Mean sensory score of Flavoured Milk added with Casein Hydrolysate (debittered using Flavourzyme 1000L) at different levels

Sensory attributes	Level of addition of debittered casein hydrolysates in milk			
	Control	2.5%	5.0%	7.5%
Colour & Appearance	8.15 ± 0.08^a	8.13 ± 0.12^a	8.78 ± 0.18^a	8.03 ± 0.32^a
Taste	7.35 ± 0.11^a	7.22 ± 0.10^a	7.16 ± 0.11^a	6.59 ± 0.25^b
Sweetness	7.02 ± 0.08^a	7.01 ± 0.22^a	7.00 ± 0.13^a	6.83 ± 0.06^a
Mouthfeel	7.07 ± 0.08^a	6.90 ± 0.12^a	7.00 ± 0.34^a	6.78 ± 0.42^a
Overall Acceptability	7.36 ± 0.10^a	7.24 ± 0.29^a	7.19 ± 0.19^a	6.93 ± 0.10^a

*values with different superscripts in a row differs significantly at ($P<0.05$) from each other.

Table 4.3.2 Mean sensory score of floavoured milk beverage added with whey protein hydrolysate (debittered using Flavourzyme 1000L) at different levels

Sensory attributes	Level of addition of debittered whey protein hydrolysates in milk			
	Control	2.5%	5.0%	7.5%
Colour & Appearance	7.38 ± 0.09^a	7.27 ± 0.15^a	7.24 ± 0.19^a	7.21 ± 0.18^a
Taste	7.16 ± 0.08^a	7.07 ± 0.17^a	7.09 ± 0.29^a	7.21 ± 0.22^a
Sweetness	7.48 ± 0.46^a	7.46 ± 0.43^a	7.44 ± 0.47^a	7.41 ± 0.36^a
Mouthfeel	8.23 ± 0.42^a	8.02 ± 0.20^a	7.91 ± 0.41^a	7.83 ± 0.56^a
Overall Acceptability	8.06 ± 0.79^a	7.79 ± 1.02^a	7.76 ± 0.35^a	7.11 ± 0.45^a

*values with different superscripts in a row differs significantly ($P<0.05$) from each other

Similarly, Mann *et al.*, (2015) developed milk beverage using WPH. WPC was hydrolyzed using flavouzyme, alcalase and corolase PP. In their study, the hydrolysates produced were added to milk flavoured with strawberry and chocolate flavours. They concluded that WPH could be successfully incorporated at 2% level for formulating functional health drink; and sensory scores of WPH containing strawberry and chocolate flavoured milk was similar to that of control. Similar findings have been reported by Kumari *et al.,* (2013) who observed comparable sensory scores after addition on 1% flavourzyme and alcalase catalysed WPH in ice cream. In another study, Kankanamge *et al.,* (2015) prepared WPH suitable for beverage applications using flavourzyme, protease A, protease M, protease S, and trypsin.

Overall, it can be concluded that casein hydrolysates and whey protein hydrolysates developed using Alcalase and debittered using Flavourzyme 1000L under optimized conditions could be utilized successfully upto 7.5% by weight of beverage in developing strawberry flavoured milk beverage.

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Milk protein hydrolysates have many advantages over the intact proteins in terms of bioavailability, functional characteristics and biological properties. Endoproteases such as Alcalase, Papain and Trypsin are used to make protein hydrolysates. Small peptides and amino acids present in protein hydrolysates are absorbed more rapidly from the intestine than free amino acids or intact proteins. Nevertheless, casein- and whey-derived hydrolysates have not gained a wide popularity due to bitter taste. A bitter off-taste is common for all protein hydrolysates, but large differences occur depending on the protein source used. The extent of bitterness is correlated with the degree of hydrolysis, source protein, concentration of peptides and hydrophobic amino acid, etc. This bitter taste of hydrolysates represents a technical problem that limits its utilization in formulation of energy drinks, supplements, nutritious beverages, etc.

Bitterness can be reduced by solvent extraction, adsorption by activated carbons/ matrices, masking the off- or bitter flavour using microencapsulation technique, or by further hydrolysis using exoproteases such as Flavourzyme 500L, FlavorSEB-P, Prolyve 1000, etc. Debittering of casein and whey protein hydrolysates can be performed by using different exoproteases under optimized substrate: enzyme ratio, pH and temperature conditions. After debittering, protein hydrolysates could further be exploited to develop nutritional beverage, supplements, nutraceuticals, etc.

Keeping all these factors in mind, a systematic study was planned with the objective to standardize enzymatic hydrolysis conditions to develop casein and whey proteins hydrolysates using trypsin, alcalase and papain followed by debittering using endo- and exo-proteases (Flavourzyme 500L, Flavourzyme 1000L and FlavorSEB). Another objective of the study was the formulation of functional dairy beverage using developed debittered casein and whey protein hydrolysates. The major findings of the present study were as follows:

1) Four independent variables *viz.* temperature, reaction time, pH and enzyme to substrate (E/S) ratio were taken to measure one dependent variable i.e. response (degree of hydrolysis) using Central Composite Rotatable Design of Response Surface Methodology.

2) First of all, sodium caseinate and whey protein concentrates (WPC-80) were hydrolyzed by using trypsin, alcalase and papain enzymes under different conditions of pH, temperature, reaction time and E/S ratio. Values of different variables were selected by keeping in mind the optimum pH and temperature of different enzymes.

3) The optimum conditions for developing casein hydrolysates with maximum %DH using trypsin (16.79±1.27) were pH 7.0, temperature 60°C, E/S ratio 0.010 and reaction time 4 h. Alcalase produced maximum %DH (27.08±1.83) under pH 8.0, temperature 60°C, E/S ratio 0.004 and reaction time 4 h. Similarly, papain developed casein hydrolysates with maximum %DH (12.43±0.60) under the conditions of pH 7.0, temperature 50°C, E/S ratio 3.0 and reaction time 4 h.

4) In the same manner, optimum conditions were determined for whey protein hydrolysates using trypsin, alcalase and papain. The optimum conditions for developing whey protein hydrolysates with maximum %DH (17.42±1.38) using trypsin were pH 7.0, temperature 60°C, E/S ratio 0.010 and reaction time 4 h. Alcalase produced maximum %DH (29.03±1.93) under pH 8.0, temperature 60°C, E/S ratio 0.004 and reaction time 4 h. Similarly, papain developed casein hydrolysates with maximum %DH (13.19±0.38) under the conditions of pH 7.0, temperature 50°C, E/S ratio 3.0 and reaction time 4 h.

5) Data revealed that when sodium caseinate and whey protein hydrolysates were developed using above mentioned three enzymes under various conditions of independent factors, significant difference ($P<0.05$) was observed among the degree of hydrolysis (%DH). Alcalase showed the maximum %DH among all the casein hydrolysates as well as whey protein hydrolysates.

6) After optimizing the conditions for independent variables to obtain maximum degree of hydrolysis (optimized using Trypsin, Alcalase and Papain), samples of casein and whey protein hydrolysates were analyzed for bitterness on the basis of 4-point intensity scale, where 1 indicates most bitter and 4 indicates least bitter. There was significant difference ($P<0.05$) among the bitterness of alcalase, trypsin & papain catalyzed casein hydrolysates. Alcalase showed the highest degree of bitterness followed by Trypsin and Papain, respectively. Similar results were observed in case of whey protein hydrolysates. Alcalase catalyzed whey

protein hydrolysates showed the maximum bitterness among all the samples followed by trypsin and papain.

7) In this way, it is clear why alcalase catalysed hydrolysates, with maximum degree of hydrolysis, produced maximum bitterness in present study. The present work was planned to develop non-bitter protein hydrolysates with maximum degree of hydrolysis. Therefore, out of studied samples, casein as well as whey protein hydrolysates catalysed by alcalase were selected for further treatment i.e. de-bittering using different enzymes.

8) Alcalase catalyzed casein and whey protein hydrolysates were further treated with exopeptidases (Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB) to reduce bitterness (debittering). Again, conditions of independent varaibles were optimized for developing de-bittered casein and whey proteins hydrolysates.

9) The optimum conditions for developing casein hydrolysates with maximum %DH using Flavourzyme 500L (32.40%) were pH 7.0, temperature 50°C, E/S ratio 0.03 and reaction time 4 h. Flavourzyme 1000L produced maximum %DH (34.55%) under pH 7.0, temperature 50°C, E/S ratio 0.015 and reaction time 4 h. Similarly, FlavourSEB developed casein hydrolysates with maximum %DH (32.79) under the conditions of pH 7.0, temperature 50°C, E/S ratio 0.1 and reaction time 4 h.

10) In the same manner, optimum conditions were measured for whey protein hydrolysates. The optimum conditions for developing whey protein hydrolysates with maximum %DH (33.77) using Flavourzyme 500L were pH 7.0, temperature 50°C, E/S ratio 0.03 and reaction time 4 h. Flavourzyme 1000L produced maximum %DH (35.23) under pH 7.0, temperature 50°C, E/S ratio 0.015 and reaction time 4 h. Similarly, FlavourSEB developed whey protein hydrolysates with maximum %DH (33.96) under the conditions of pH 7.0, temperature 50°C, E/S ratio 0.1 and reaction time 4 h.

11) The study revealed that flavourzyme 1000L redulted in the maximum %DH of casein hydrolysates (34.55%) and whey protein hydrolysates (35.23%). The developed de-bittered casein and whey protein hydrolysates were evaluated for its bitterness on the basis of sensory evaluation.

12) Casein hydrolysates de-bittered by Flavourzyme 1000L were found to be the lowest in terms of bitterness. There was significant difference ($P<0.05$) among the bitterness produced by Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB catalyzed casein hydrolysates. Flavourzyme 500L showed highest degree of bitterness followed by FlavourSEB and Flavourzyme 1000L. Similar trend was observed for whey protein hydrolysates debittered by Flavourzyme 500L, Flavourzyme 1000L and FlavourSEB.

13) In this way, Flavourzyme 1000L was finally selected as the best enzyme for the production of debittered casein and whey protein hydrolysates. The optimum conditions for developing debittered casein hydrolysates as well whey protein hydrolysates (alcalase catalysed) using Flavourzyme 1000L were as pH 7.0, temperature 50°C, E/S ratio 0.015 and reaction time 4 h. Developed casein and whey protein hydrolysates, debittered by Flavourzyme 1000L were further utilized for preparing flavoured dairy beverage.

14) Sensory acceptability of flavoured milk fortified with debittered casein and whey protein hydrolysates (at the level of 2.5, 5.0 and 7.5%) was determined by evaluating the developed product for colour and appearance, taste, sweetness, mouth feel and overall acceptability. There was no significant difference ($P<0.05$) in colour and appearance, sweetness and mouth feel scores among the samples and control. However, scores for taste decreased significantly ($p<0.05$) in flavoured milk fortified with 7.5% debittered casein hydrolysates. Nevertheless, no significant ($P<0.05$) difference was observed among the overall acceptability of all studied samples (fortified with casein hydrolysates). It is interesting to note that there was no significant difference among all sensory attributes of control and test samples, when milk beverage was fortified with optimized and debittered whey protein hydrolysates, irrespective of the fortification level.

Overall, the study can be concluded that casein and whey protein hydrolysates developed by using Alcalase and debittered by using Flavourzyme 1000L under optimized conditions could be utilized successfully at the rate of 7.5% for manufacture of flavoured milk beverage.

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