SOLID STATE FERMENTATION OF APPLE POMACE: METHODS OF ETHANOL RECOVERY, PHYSICO-CHEMICAL EVALUATION AND ACCEPTABILITY OF ANIMAL FEED PRODUCED

THESIS

by

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Submitted in partial fulfilment of the requirements for the Degree of

MASTER OF SCIENCE

in

HORTICULTURE
(POSTHARVEST TECHNOLOGY)

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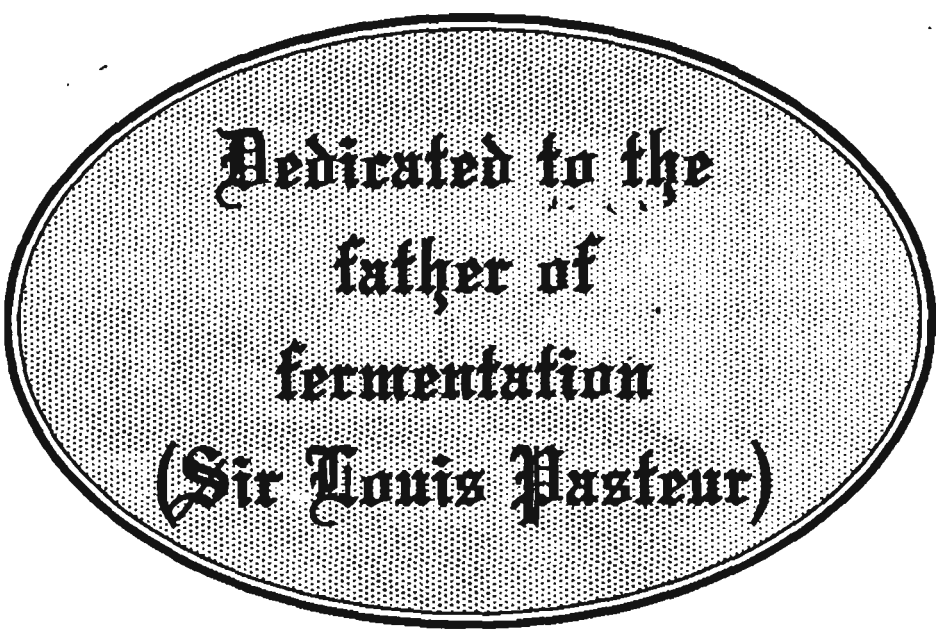
1997


Dedicated to the
father of
fermentation
(Sir Louis Pasteur)

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Solid state fermentation of apple pomace : Methods of ethanol recovery, physico-chemical evaluation and acceptability of animal feed produced "** submitted in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** in **HORTICULTURE (Postharvest Technology)** to Dr. Y.S. Parmar University of Horticulture and Forestry, Solan (H.P.) is a bonafide record of research work carried out by **Mr. Devarajan A.** (H-95-15-M) under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation has been fully acknowledged.

Place : Solan
Dated : 23rd December, 1997

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Major Advisor

CERTIFICATE-II

This is to certify that the thesis entitled **"Solid state fermentation of apple pomace : Methods of ethanol recovery, physico-chemical evaluation and acceptability of animal feed produced"** submitted by **Mr. Devarajan A.** (H-95-15-M) to Dr. Y.S. Parmar University of Horticulture and Forestry, Solan (H.P.), in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE in HORTICULTURE (Postharvest Technology)** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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ACKNOWLEDGEMENTS

It gives me immense pleasure and privilege to express my deep sense of gratitude and profound personal regard to the Chairman of my Advisory Committee, Dr. V.K. Joshi, Associate Professor, Department of Postharvest Technology for his judicious guidance, keen interest, constant encouragement and constructive criticism during the course of present studies. I shall always remain indebted to him for his personal affection and generosity bestowed upon me.

I am grateful to the respected members of my Advisory Committee, B.B. Lal Kaushal, Senior Hort. Tech. and Head (Department of Postharvest Technology), Dr. R.K. Goyal (Deptt. of Vegetable Crops), Dr. S.P. Dhall (Deptt. of Basic Sciences) and Dr. (Mrs.) Nivedita Sharma (Deptt. of Basic Sciences) for their help, encouragement and guidance.

Grateful thanks are extended to Dr. K.N. Gowal (CRI, Kasauli), Dr. Chander Sheikher (Deptt. of EAP), Dr. Ramesh Shirkot (H.P. Agro. Corp.), Dr. Kuldeep Gupta (Deptt. of SAT) and Sh. Villavan Kothai (Officer-in-Command, Kasauli) for their whole hearted help.

I am also thankful for the help rendered by Mrs. Radha Sharma, Prakash and other laboratory/office staff of the Department of Postharvest Technology.

I acknowledge with pleasure the timely help and pleasant company provided by my friends and seniors Thiru, Pradeep, Suresh, Laxuman, Ramesh, Brahma, Truyen, Ananta, Harsha, Kanshi, Das, Tandon, Akila, Priya, Bhawna, Monica, Meenakshi and others.

Above all, I am indebted to my parents, uncle Mr. Palanivel Gounder, Rangaswamy Gounder, Kandaswamy Gounder, Raja, brothers Manohar and Suresh, sister Rajalakshmi and other relatives who always encouraged and inspired for carrying out the studies with dedication.

I extend my cordial thanks to Mr. Rajinder, Mr. Jagdeep, Mr. J.S. Bansal and Mr. Asi of DPT Computer's for preparation and typing of manuscript.

Needless to say errors and omissions are mine.

Place : Solan

Date : December 23, 1997

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ABBREVIATIONS USED

ALT	Alanine amino transferase
AMS	Ammonium sulphate
AST	Aspartate amino transferase
°B	Degree brix
BUN	Blood urea nitrogen
CFU	Colony forming unit
DAHP	Diammonium hydrogen phosphate
DIG	Digestibility
ECI	Efficiency of conversion of ingested food
GR	Grwoth rate
M.A.	Malic acid
SCP	Single cell proteins
SSF	Solid state fermentation
TSS	Total soluble solid
WA	Weight of animals

CONTENTS

Chapter 1

INTRODUCTION

Apple (*Malus domestica* Borkh.) is the oldest fruit known to man and is grown extensively throughout the temperate zones of the world. Apple originated in South-Western Himalayan hills (Singh, 1995). Among the temperate fruits, apple ranks first in production with the estimated world production of about 98,155 thousand metric tonnes (Anon., 1995). In India, the apple production is about 1,238 thousand metric tonnes (Anon., 1995), and at present Himachal Pradesh alone contributes 288.538 thousand metric tonnes (Anon., 1997). Increase in fruit production accelerated the growth of fruit processing industries and apple processing industries is no exception to it. Apple fruit is converted into a number of processed products like concentrate, jam, jelly, squash, preserve, wine, cider, vermouth, etc. (Downing, 1989; Sharma and Joshi, 1996). Rapid industrialization in the field of agriculture and processing of agricultural produce is responsible for the generation of waste and by-products and consequently for creating environmental problems (Joshi and Joshi, 1990).

Apple pomace is a by-product of juice extraction and is generated in large quantities by apple processing industries ranging from 25-30 per cent of the fresh fruit utilized (Sargent, 1984; Wang and Thomas, 1989). In Himachal Pradesh, approximately 15,000 tonnes of culled apples are utilized for processing purpose (Anon., 1990) and generates about 3,500 tonnes of apple pomace, annually.

Apple pomace is a rich source of carbohydrates, vitamins, minerals, fibers, etc. and throwing of such a waste from the fruit processing industries into the river is more or less like throwing coins into the sea. At the same time, apple pomace has high level of BOD (Biological Oxygen Demand 240-19,000 mg/l) and is highly biodegradable in nature (Joshi and Joshi, 1990). Disposal of such a waste in the environment affects the aquatic life, ecosystem and creates foul smelling environment around the fruit processing industries. Thus, there is a strong need to devise techniques to utilize apple pomace in economical and effective way to avoid the environmental pollution.

Use of apple pomace in human food as jam, sauce, bakery products and incorporation in cookies upto 30 per cent have been attempted successfully (Kaushal and Joshi, 1995; Joshi and Kaushal, 1995; Joshi *et al.*, 1996). But the utilization of huge quantity of apple pomace for a single product may not be possible because of very large quantity of cookies which has to be produced to utilise the apple pomace. Perhaps it points out towards a need for production of more edible products of different types rather than restricting to a single product. Besides, the major obstacle associated with using apple pomace in human food is competition with the traditional high quality, inexpensive food ingredients (Kennedy, 1994), and maintenance of hygienic conditions. Pectin could be isolated from apple pomace (Downing, 1989). But the apple pomace pectin is not preferred due to its high methoxyl content which does not make a good jelly as compared to that made from citrus pectin (Olsen, 1934). This process was technically feasible but economically unjustified. High value products like aroma (Kennedy, 1994) or through fermentation in to citric acid or ethanol (Hang *et al.*, 1982; Gupta *et al.*, 1990; Joshi and Sandhu, 1994) could be produced from apple pomace as a single separate product of each fermentation. But the left-over material again has to be thrown generating more waste than it would have utilized, which will forfeit our objective of reducing the pollution level and complete utilization of the raw materials.

Apple pomace has also been utilized as an animal feed as such or after ensilage (Smock and Neubert, 1950). But apple pomace is not suitable as an animal feed, being low in digestible proteins (Rumsey, 1978). The quantity of protein can be increased by addition of non-protein nitrogen to apple pomace but it led to birth of dead or weak calves (Fotenote, *et al.*, 1977). This problem can be overcome through fermentation and by manufacturing as high protein animal feed. But the most promising method for complete utilization of apple pomace may be through fermentation with yeasts, separating out ethanol and using the left-over protein rich material after drying as an animal feed. Studies on concomitant production of ethanol and animal feed from apple pomace have also been reported (Sandhu and Joshi, 1997b). However, till now no method has been developed to separate out ethanol from fermented apple pomace with applicability on industrial scale. Besides, no report is available on effect of feeding of fermented apple pomace to animals. However, separation of ethanol from pulpy fermented tapioca (Jaleel *et al.*, 1991) and

feeding of single cell proteins to animals (Yacoub *et al.*, 1990) have been quite encouraging. Therefore, studies on utilization of apple pomace were carried out with the following objectives:

i) To optimise the concentration of additives for solid state fermentation of apple pomace with different yeast spp. as such or as sequential interactive co-culture.

ii) To determine changes in apple pomace fermented by solid state fermentation for some important compositional parameters.

iii) To find out the physico-chemical characteristics of fermented apple pomace produced after process optimization.

iv) To select a suitable method for ethanol separation from the fermented apple pomace

v) To find out the acceptability of animal feed produced from apple pomace to the rats and study the effect of feeding on rats.

Chapter 2

REVIEW OF LITERATURE

Apple pomace which is a left-over by-product of juice extraction, at present constitutes a waste. Apple processing industries generate as much as 25% of the original fresh fruit processed as pomace which is being biodegradable causes environmental problems, besides loss of precious resources. Research carried out on various aspects of utilization of apple pomace has been reviewed in the chapter and described under the following headings :

2.1 APPLE POMACE PRODUCTION, ITS COMPOSITION AND NUTRITIVE VALUE

2.1.1 Apple pomace production

It is estimated that the annual production of apple world over is about 48,155 thousand metric tons and in India apple production is about 1,238 thousand metric tons (Table 2.1). In Himachal alone, the apple production is about 2,88,538 tons (Anon, 1997) of which approximately 15,000 of culled apples were utilized for processing purpose (Anon, 1990) which generated about 3,500 tones of apple pomace annually.

Table 2.1. Production of apple pomace in world, India and H.P.

Year	World (000MT)	India (000MT)	Himachal (000MT)
1989-1990	40,760	978	394.87
1990-1991	40,010	1,020	342.07
1991-1992	43,326	1,113	301.73
1992-1993	39,307	1,200	279.05
1993-1994	48,155	1,238	294.73
1994-1995	-	-	122.78
1995-1996	-	-	276.68
1996-1997	-	-	288.54

Source: FAO(1995)

2.1.2 Composition and nutritive value of apple pomace

The proximate composition of apple pomace is (Table 2.2) clearly showed that the apple pomace is a rich source of carbohydrate having about 9.5-22.0% of fermentable sugar depending upon the variety processed (Smock and Neubert, 1950). High fermentable sugar content makes it a good substrate for fermentation while its low protein content indicates its unsuitability for animal feed (Hang *et al.*, 1981). The amount of initial sugar content depends upon the variety of apple processed, the processing conditions used and the amount of filter aids added (Hang *et al.*, 1982). Since it has high moisture content it can be easily decomposed by microorganisms. Being rich in carbohydrates, acids, fibers, vitamin-c, and minerals its disposal as a waste in the environment is a huge loss of precious natural resources (Hang and Walter, 1989; Joshi and Joshi, 1990).

Table 2.2. Proximate chemical composition of apple pomace

Constituent	Wet wt. basis	Dry wt. basis
Moisture (%)	66.4-78.2	3.97-5.4
Acidity(%)	-	2.60
Total Carbohydtrates (%)	9.50-22.0	48.0-62.0
Glucose (%)	6.1	22.7
Fructose (%)	13.6	23.6
Sucrose (%)	-	1.8
Vitamin-C (mg/100g)	-	18.5
Proteins (%)	1.0-1.8	4.45-5.67
Crude fiber (%)	4.3-10.5	4.70
Fat (Ether extract) (%)	0.8-1.40	3.90
Pectins (%)	1.5-2.5	3.50
Minerals		
Potassium (%)		0.95
Calcium (%)		0.06
Sodium (%)		0.20
Magnesium (%)		0.02
Copper (mg/l)		1.10
Zinc (mg/l)		15.00
Manganese(mg/l)		9.00
Iron (mg/l)		230.00
Calorific value (kcal/100g)		295.00

Source: Johr *et al.* (1960); Garg *et al.* (1994); Kennedy (1994); Joshi and Kaushal (1995) and Rahmat *et al.* (1995).

2.2 DISPOSAL PROBLEM OF APPLE POMACE

The primary source of solid residues from apple processing operations are sorting, cutting, slicing, peeling, pulping and processing. Apple pomace has high level of BOD/COD (Biochemical Oxygen Demand/Chemical Oxygen Demand) and is highly bio-degradable. Its direct disposal to the environment poses serious problem (Joshi and Joshi, 1990). The BOD of different wastes (kg/kkg) are washing 0.09, peeling (mechanical peeling) 0.16, slicing 2.49, deaeration 2.21, cooking 0.14, cooling 0.02, transportation 0.02, and cleanup 1.90 (Downing, 1989). According to **"The Water Prevention and Control of Pollution Act 1974** and **The Air Prevention and Control of Pollution Act 1981** laws (GOI) as amended to **"The Environment Protection Act 1986"** wherein the provision for heavy punishment (fine of Rs 1 lakh and/or imprisonment for 5-7 years) has been made (Bhalerao and Mulmuley, 1989) for discharging of such waste in the environment. It indicates an urgent need for the utilization of pomace to reduce pollution level, economizes the cost of finished product and complete utilization of raw material.

2.3 OPTIONS FOR THE UTILIZATION OF APPLE POMACE

Considerable research has been directed through-out the world to utilize the apple pomace which is detailed in Table 2.3. But there is a lack of economically viable use of apple pomace and successful technique for this purpose has still to be investigated for the alternative uses of these products in the long run before the apple processing industries are forced to act by local body or government regulation to dispose-off apple pomace.

Table 2.3. Options for the utilization of apple pomace

By-products and process developed	References
Edible products	
Soft drinks	Johr *et al.* (1960) and Shah and Masoodi (1994)
Chutney	Lal *et al.* (1980)
Cookies	Kaushal and Joshi (1995); Dauel (1986) and Wang and Thomas (1989)
Jam	Joshi and Kaushal(1995)
Sauce	Joshi *et al.* (1996)

By-products and process developed	References
Leather	Narotam (1992)
Toffees	Narotam (1992)
Bread	Narotam (1992) and Chen *et al.* (1988)
Fermented edible products	
Cider	Johr *et al.*(1960); Barwal (1990) and Masuda*et al.*(1964)
Beer	Johr *et al.* (1960)
Brandy	Amemiya *et al.* (1964)
Vinegar	Hang *et al.* (1982) and Sharma (1983)
Pectin recovery	Downing (1989); Olsen (1934); Jain *et al.* (1984); Sharma *et al.* (1985) and Renard *et al.* (1991).
Fiber extract	Moris (1985); Garg *et al.* (1994); Walter *et al.* (1977 and 1985).
Animal feed (unfermented)	Smock and Neubert (1950) and Shah and Masoodi (1994)
Fermented products	
Additives	
Citric acid	Hang and Woodams (1984 and 1986)
Enzymes production	Bhalla and Joshi (1993) and Hours *et al.* (1988)
Single cell proteins	Ballamy (1962); Reese *et al.* (1972) and Ghose (1977)
Animal feed	Hang *et al.*(1981); Joshi and Sandhu (1994); Hang *et al.* (1982); Hodge *et al.* (1990); Rahmat *et al.* (1995); Hang (1988); Joshi and Sandhu (1996b); Bhalla and Joshi (1994); Smock and Nuebert (1950) and Fotenote *et al.* (1977) and Sandhu and Joshi (1997b)
Ethanol	Hang *et al.* (1982); Jarosz (1988); Hang *et al.* (1981); Joshi and Sandhu (1996a); Gupta *et al.* (1990); Joshi and Sandhu (1994); Ngadi and Correia (1992) and Joshi and Sandhu (1996a)

By-products and process developed	References
Miscellaneous	
Charcoal	Walter and Sherman (1976) and Sharma(1983)
Pigments	Sandhu and Joshi (1997a)
Fertilizer	Stapleton (1982)
Medium	Chong (1992)
Oil	Shaw and Bain (1993)
Aroma	Sharma and Joshi (1996) and Bomben and Gaudagni (1973)

2.4 EDIBLE PRODUCTS

2.4.1 Soft drinks

Johr *et al.* (1960) produced highly acceptable soft drinks by raising the TSS and acidity of the apple pomace extract to 12°B and 0.4% respectively, followed by carbonation, fortification with Vit-C, bottling and pasteurization. Shah and Masoodi (1994) prepared highly acceptable products such as RTS and squash. RTS incorporated with 10% apple pomace pulp and squash incorporated with 35% apple pomace pulp was found to quite acceptable.

2.4.2 Chutney

Lal *et al.* (1980) suggested that apple pomace could also be used for the preparation of chutney.

2.4.3 Cookies

Kaushal and Joshi (1995) reported that good quality cookies can be prepared by incorporating apple pomace powder in dough at the rate of 30%. Addition of apple pomace of more than 30% deteriorated the quality of cookies and no drastic change was obtained upto 6

months storage of apple pomace. Dauel (1986) also prepared high fiber bakery products by incorporating apple pomace into cookies and granular bars and muffins. Wang and Thomas (1989) used apple pomace as a source of dietary fiber and sugar in bakery products and the sensory evaluation demonstrated that the experimental muffins and cookies with 50% w/w and 10% w/w of plain wheat bran substituted by powdered apple pomace were more acceptable than the control bran muffins and control moon cookies.

2.4.4 Jam

Apple pomace pulp was prepared in the 1:3 ratio of pomace and water. The jam prepared with the ratio of 1:1.25 pulp and sugar was found to be the best in quality and it could be stored up to 6 months (Joshi and Kaushal, 1995).

2.4.5 Sauce

Apple pomace sauce was prepared by raising the TSS of pulp to 20°B which was prepared from apple pomace collected from the processing plant. The sauce remained highly acceptable for the whole storage period of 6 months at room temperature (Joshi *et al.*, 1996). Apple pomace acts as a hypocholesterolemic agent and can be used in preparation of soups and sauces. The major difficulty associated with using apple pomace in human food is the competition with traditional, high quality and inexpensive food ingredients (Kennedy 1994).

2.4.6 Apple pomace leather

Leather of acceptable quality was prepared by adding 10 per cent sugar alongwith citric acid @ 0.2% and sodium-benzoate at the rate of 200 ppm in the apple pomace pulp followed by drying in a mechanical dehydrator for 10-12 hours at 60±2°C (Narotam, 1992).

2.4.7 Apple pomace toffees

Sugar and apple pomace pulp in the ratio of 1:1.5 alongwith glucose 1.5%, skimmed milk 3%, vegetable ghee 3% and citric acid were mixed and heated to the desired consistency (80-82°B) followed by drying for 2 hours (Narotam, 1992).

2.4.8 Bread

Narotam (1992) observed that the apple pomace is not suitable for the preparation of bread. Pectin was found to be the limiting factor in the development of dough for bread making. However, the leavening activity of yeast could not be enhanced with the addition of additives to any significant level. Chen *et al.* (1988) also reported that poor baking properties of apple fiber bread due to the interaction of fiber and gluten.

2.4.9 Fermented edible products

2.4.9.1 Cider

An acceptable low alcoholic beverage (3-5%) was prepared by adding colour, citric acid, SO_2 and raising the TSS from 4 to 12°B by adding sugar. Other steps including the fermentation were similar to the production of cider from apple juice and further improvement was made by carbonating drinks (Johr *et al.*, 1960). Another attempt was also made for the preparation of cider by combining distillates from fermented apple pomace with fermented apple juice (Barwal, 1990). Masuda *et al.* (1964) reviewed the possibilities for the production of apple cider from apple pomace.

2.4.9.2 Beer

Organoleptically acceptable beer was produced by addition of hops to the fermented apple pomace liquid which is prepared as described above in cider (Johr *et al.*, 1960).

2.4.9.3 Brandy

Amemiya *et al.* (1964) reported that a good quality brandy can be prepared from apple pomace.

2.4.9.4 Vinegar

Hang *et al.* (1982) suggested that alcohol produced from solid state fermentation of apple pomace could be used as a base for the vinegar production. Apple pomace is mixed with molasses in the ratio of 2:1 and fortified with 0.05% ammonium sulphate for alcoholic fermentation which is carried out for 4 days followed by acetic acid fermentation by acetic acid bacteria. Vinegar produced by this method was found to be physico-chemically and organoleptically acceptable (Sharma, 1983).

2.4.10 Pectin recovery

Pectin could be isolated from dried apple pomace using dilute acetic solution of pH 1.6-3.0 followed by precipitation with ethyl alcohol or aluminium trichloride (Downing, 1989). The pectin isolated from this method was found to be superior and the precipitates washed with acidified alcohol yielded high methoxyl pectins. But the jellies made from this pectins are elastic require less acid and depended more for the textural properties on pH compared to the jellies made from citrus pectins (Olsen, 1934). Jain *et al.* (1984) isolated pectin from apple pomace of Golden Delicious variety and mixed (Golden Delicious, Red Delicious and Royal Delicious) variety using 0.05N HCL with yields of 17.86 and 17% respectively. Sharma *et al.* (1985) extracted pectin from four varieties of apple pomace (Golden Delicious, Red Delicious Royal Delicious and Red Gold) with 0.05N HCL. Among the 4 varieties, Golden Delicious pomace was promising with respect to pectin yield and jelly grade. Renard *et al.* (1991) extracted pectic substances from apple cell walls successively by solublizing with 30% uronic acid with dilute NaOH and hot acid which leads to the extraction of only limited amount of pectin. Pectin extraction from apple pomace has been extensively evaluated and a process was designed and evaluated by New Zealand institute of Industrial Research and Development, in 1982. This process was technically feasible but economically unjustified (Kennedy, 1994).

2.4.11 Fiber extraction

Moris (1985) reported that a low moisture powder apple fiber product was made from apple cell wall materials and apple fibers have been produced by Tree Top Inc Slah Wash. This

product has a dietary content of 56.1%. Apple crude fiber is constituted by the structural non-nutritive component of apple tissue in which cellulose and lignin are the main fractions (Garg *et al.*, 1994). Walter *et al.*(1977, 1985) reported that when apple pomace is subjected to weak alkaline degradation, the insoluble alpha cellulose remained in the apple pomace. The solvent extracted fraction was made up of pectic substances, pentosans and was free from cellulose. The solvent extracted fraction was found to have be more water dispersability and colloidal functional. Kennedy (1994) also suggested that apple pomace to be a good bulking agent, can be added directly to baked goods is a good fiber source (the fiber can be separated from the apple pomace prior to addition).

2.4.12 Animal feed

Smock and Nuebert (1950) suggested that apple pomace as an animal feed could be fed to the cattle as fresh silage or dried apple pomace. Shah and Masoodi (1994) partially replaced fresh apple pomace @ 10.2 kg/ day/ animal with a fodder in a herd of 70 milching cows of Holstein Friesian breed significantly which reduced the cost of fodder without having any detrimental effect on health and milk yield of the animals.

Rumsey (1978) suggested that apple pomace is not suitable as an animal feed as it has poor content of digestible proteins. The low protein content could be increased by addition of non-protein nitrogen. Singh and Narang (1992) observed that apple pomace is an energy source and feasible as a supplement for ruminants feed when supplemented with urea. But feeding of non-protein nitrogen to pregnant cows reportedly gave birth to dead or weak calves (Fotenote *et al.*, 1977).

2.5 FERMENTED PRODUCTS

2.5.1 Additives

2.5.1.1 Citric acid

Hang and Woodams (1984) carried out solid state fermentation of apple pomace with five different citric acid producing fungal strains viz., *Aspergillus niger* (2001), *Aspergillus niger* (2270), *Aspergillus niger* (599), *Aspergillus niger* (328) and *Aspergillus niger* (567). Addition

of methanol at the rate of 4 per cent enhanced the citric acid production by all the strains and *Aspergillus niger (567)* gives the maximum citric acid production when grown on apple pomace at different temperature in the presence of 4% of methanol *Aspergillus niger* produced considerably more citric acid in 5 days at 30°C than either at 25 or 35°C (Hang and Woodams, 1986).

2.5.1.2 Enzyme production

Bhalla and Joshi (1993) produced cellulase and xylanase enzyme by solid and liquid state fermentation of dried and pectin extracted apple pomace with *Trichoderma viride* and *Aspergillus niger*. The maximum production of cellulase (5 units) and xylanase (4.2 units) was obtained by *Trichoderma viride* and *Aspergillus niger*, respectively in dried apple pomace. Hours *et al.* (1988) suggested that the use of apple pomace as a raw material for pectinase production in solid state culture using *Aspergillus foetidus*. Enzyme activity up to 1062 units/g was obtained in 48 hours. Characterization of the enzymatic pool showed it to be adequate for practical purpose.

2.5.2 Single cell proteins

Single cell protein (SCP) could be produced from apple pomace for cultivation of micro-organisms for the use of human or animal protein supplements in the form of cell or protein extract (Ballamy, 1962, Reese *et al.*, 1972 and Ghose, 1977). The high nucleic acid content is a major obstacle to wide acceptability of SCP products (Viikari and Linko, 1977). Most adults who consumed large amount of SCP develop high concentration of uric acid in their blood serum and urine (Edozien *et al.*, 1970).

2.6 FERMENTED ANIMAL FEED

2.6.1 Micro-organisms

Different micro-organisms have been employed to produce animal feed by fermentation as per details given in the Table 2.4.

Table 2.4. Details of microorganisms, nutrients added and the protein content of animal feed prepared by solid state fermentation of apple pomace

Micro-organisms	Nutrients added (%)	Crude protein (%)	Soluble protein (%)	References
Saccharomyces cerevisiae	-	9.0		Hang *et al.* (1982)
Saccharomyces cerevisiae	AMS 1.8	16.8	4.4	Joshi & Sandhu (1996b)
Candida utilis	AMS 1.8	18.5	3.4	-do-
Candida utilis	AMS 0.9	18.0	-	Hang (1988)
Candida utilis	AMS 1.0	-	7.5	Rahmat *et al.* (1995)
Torula utilis	AMS 1.8	15.0	3.1	Joshi & Sandhu (1996b)
Kloeckera apiculata	AMS 1.0	-	7.5	Rahmat *et al.* (1995)
Candida utilis + *Aspergillus niger*	Czapek's medium	20.0		Bhalla and Joshi (1994)

AMS - Ammonium sulphate

2.6.2 Production technology

It had been suggested that apple pomace because of its physical nature is not readily amenable to micro-organisms in submerged microbial fermentation. The solid state fermentation (SSF) process has been used for decomposition of substrate, enrichment of substrate production, production of SCP and other related products of microbial origin (Chahal *et al.*, 1981; Hang *et al.*, 1981; Sethi and Grainger, 1981; Zadrazil, 1981; Khanna and Gupta, 1986). Kennedy (1994) suggested that the single cell protein production from apple pomace has an advantage that the whole product (SCP+apple pomace) can be dried and sold as a high protein animal feed.

2.6.2.1 Moisture

Hodge *et al.* (1990) fermented apple pomace with *Kloeckera apiculata* (a natural isolate from the natural microflora of the substrate) and showed the greatest activity in the SSF, with the activity of other strains in decreasing order viz *Candida utilis, Saccharomyces cerevisiae, Saccharomyces lipolytica* and *Schizosaccharomyces pombe*. The maximum cell concentration was obtained at 70-80% of moisture level.

2.6.2.2 Nitrogen source

Joshi and Sandhu (1994) reported that addition of ammonium sulphate affected the ethanol and crude protein contents in concentrations dependent manner, both being maximum at 1.8 and 2.5 concentration of ammonium sulphate. Rahmat *et al.* (1995) carried out solid state fermentation of apple pomace with *Kloeckera apiculata* and *Candida utilis* with 1% ammonium sulphate in jars which are held horizontally on the perimeter of an 0.75m vertical wooden disk and rotating at 2 rev/min. The fermented apple pomace was found to be increased in crude protein content from 4.8% to 13.1%. Addition of both AMS and AMS + $ZnSO_4$ increased the crude protein content as compared to control although AMS alone was maximum in crude protein and soluble protein contents compared to AMS + $ZnSO_4$. The increase in AMS concentration enhanced the crude protein content upto 2.5% after which it got stabilized. The increase in crude protein content decreased the soluble protein content (Sandhu and Joshi, 1997b).

2.6.2.3 Co-culture

Co-culture system of cellulolytic molds and yeasts in liquid and solid state fermentation has been reported (Bhalla and Joshi, 1994). Among the various combinations tried *Aspergillus niger* with *Candida utilis* give the maximum protein content of 20 and 17% of dried and pectin extracted apple pomace. However, there is no report on sequential interactive co-culture of yeast species and changes produced in the fermented apple pomace by SSF.

2.6.2.4 Composition of fermented apple pomace

Hang *et al.* (1982) reported that solid state fermentation of apple pomace with *Saccharomyces cerevisiae* at 30°C for 96 hrs increased the protein content to 9.0%. Solid state fermentation of apple pomace with *Candida utilis* increased 2.5 fold in protein content, 3.4 fold in niacin, 10 fold pantothenic acid and 1.5 fold thiamine (Hang, 1988). Joshi and Sandhu (1996b) fermented apple pomace for 96 hrs in solid state fermentation. The dried left-over material after ethanol separation was found to have an increase of 3 times in crude protein, 1.5 times in fat and 2 times in vitamin-C in the presence of 1.8% ammonium sulphate. Among the three yeasts tried *Candida utilis* gave maximum crude proteins, *Saccharomyces cerevisiae* gave maximum soluble

2.7 Ethanol

2.7.1 Solid state fermentation

Both liquid and SSF have been used for production of ethanol from apple pomace. SSF is preferred best due to higher yield but has one difficulty of ethanol extraction from the solid materials. Solid state fermentation refers to the cultivation of micro-organisms in the absence of free water (Hang *et al.*, 1982). or near absence of free water (Pandey and Radhakrishnan,1990).

2.7.2 Micro-organisms

Different microorganisms have been used for the production of ethanol, predominantly yeast belonging to *Saccharomyces cerevisiae* has been employed in most of the studies. Different micro-organisms used with their ethanol yield and fermentation efficiency have been listed in the Table 2.5.

Table 2.5. The various microorganisms used by different authors with ethanol yield and fermentation efficiency

Micro-organisms	Ethanol yield (%)	Fermentation efficiency (%)	References
Saccharomyces cerevisiae	2.86-4.31	70.0-94.0	Hang *et al.*(1982)
Saccharomyces cerevisiae	3.7-5.4	44.5-64.9	Gupta *et al.* (1990)
Saccharomyces diasticus	3.6-5.7	43.3-68.5	-do-
Saccharomyces cerevisiae	3.92-4.3	60.0-68.0	Joshi *et al.* (1995)
Candida utilis	3.71-4.59	57.0-69.0	-do-
Torula utilis	3.93-4.10	59.0-62.0	-do-

Hang *et al.* (1981) produced about 43 gms of ethanol/kg of apple pomace by a Montrachet strain *Saccharomyces cerevisiae* with a fermentation efficiency of 89% at 30° in

24 hrs. Jarosz (1988) collected apple pomace from three factories and fermented at 30°c for 72 hrs with or without addition of inoculum. The natural microflora induced the fermentation but addition of yeast @ of 0.% accelerated the fermentation and brought to the 78.9% of the theoretical yield of ethanol. Sandhu and Joshi (1997) reported that natural fermentation of apple pomace was inferior to the yeast inoculated fermentation for ethanol, crude and soluble proteins and the production of ethanol in natural fermentation was almost half than that of *Saccharomyces cerevisiae* fermented apple pomace.

2.7.3 Techniques

Hang *et al.* (1982) developed a solid state fermentation system of apple pomace with *Saccharomyces cerevisiae* at 30°C in 96 hrs producing 43g ethanol/ kg of apple pomace and the ethanol was separated out by vacuum evaporation with a separation efficiency of 99%. Joshi *et al.* (1995) provided partial aseptic and anaerobic condition to the solid state fermentation of apple pomace by addition of SO_2 and they found that the concentration of SO_2 upto 200 ppm increased the ethanol content by *Saccharomyces cerevisiae* and 150 ppm for *Candida utilis* and *Torula utilis*

2.7.4 Factors affecting solid state fermentation

2.7.4.1 Moisture and pH

Ngadi and Correia (1992) reported that when the apple pomace was fermented at 77% and 85% of moisture level yields 19.26 and 18.10% of ethanol on dry basis. The original pH and the initial moisture content of apple pomace was found to be suitable for ethanol production, decreasing the pH reduced alcohol content and by increasing the moisture content ethanol content was decreased (Joshi and Sandhu 1994).

2.7.4.2 Time and temperature

Hang *et al.* (1982) conducted SSF of apple pomace for 96 hours and found that apple pomace inoculated without pasteurization or autoclaving with *Saccharomyces cerevisiae* fermented rapidly and had an ethanol content of more than 4% in 24 hours at 30°C, Whereas, the control

samples contained a very trace amount of ethanol. However, Jarosz (1988) reported that solid state fermentation of apple pomace with *Saccharomyces cerevisiae* at 30°C for 72 hours brings out the theoretical yield of ethanol to 78.9 per cent. Joshi and Sandhu (1994) also observed that increase in fermentation time increased the ethanol production upto 96 hrs at 30°C and no further increase in ethanol yield took place after this period by any of the three yeasts tried viz. *Saccharomyces cerevisiae*, *Candida utilis* and *Torula utilis*.

2.7.4.3 Nitrogen source

Gupta *et al.* (1990) found that the addition of nitrogen, phosphate and trace elements to the solid state fermentation of apple pomace with *Saccharomyces diasticus* increased the fermentation efficiency to 67.7, 68.5 and 68.8% respectively as compared to the control fermentation efficiency of about 43.8%. Joshi and Sandhu (1994) optimized several factors relevant to solid state fermentation of apple pomace for ethanol production. Among the different nitrogen source tried ammonium sulphate gave highest ethanol production and *Saccharomyces cerevisiae* gave better response to it as compared to *Candida utilis* and *Torula utilis*. Addition of 0.4% of ammonium sulphate increased the ethanol yield. The combined effect of AMS and $ZnSO_4$, however was detrimental to the ethanol production but AMS alone gave better ethanol yield. The effect was more visible in SSF by *Saccharomyces cerevisiae* than *Candida utilis* and *Torula utilis* (Sandhu and Joshi, 1997b).

2.7.4.4 Bio-reactor technology

Ngadi and Correia (1992) conducted solid state fermentation in a 40L capacity stainless steel horizontal batch bio-reactor with three sampling ports, a pressure relief valve and a water bath to maintain a constant temperature of 30°C. The bio-reactor mixing speed at 2 and 20 rpm yields 10.79 and 10.27% of ethanol, respectively.

2.7.4.5 Ethanol recovery

Hang *et al.* (1982) recovered ethanol after fermentation of apple pomace with *Saccharomyces cerevisiae* at 30° for 96 hrs by Bucchi evaporator with a separation efficiency of

99% and the ethanol content in the final distillate was 13%. Ethanol recovered from fermented fibrous tapioca pulp by hydraulic press at three stages by inter stage addition of water led to 79.68% separation efficiency but the concentration of ethanol in the pooled extract was nearly 60.53%. The lower concentration of ethanol was obtained by percolation technique (Jaleel *et al.*, 1992). However, there is no information with respect to apple pomace for ethanol recovery method, especially that which could be used at an industrial scale.

2.7.4.6 Composition of distillate

Hang *et al.* (1982) reported that the apple pomace fermented at 30 °c for 96 hrs followed by distillation with a bucchi evaporator yielded 0.029, 4.1, 0.0003, 0.01 and 0.011% of methyl, ethyl, n-propyl, isobutyl and isoamyl alcohols respectively. Joshi and Sandhu (1996a) reported that all the yeast fermented apple pomace distillates were found to contain methyl, butyl alcohols and aldehyde. *Saccharomyces cerevisiae* gave the highest ethanol and esters, *Torula utilis* high propyl alcohol,*Candida utilis* gave highest amyl alcohol, volatile acidity and lower pH. *Saccharomyces cerevisiae* fermented distillate had more desirable characteristics and has potential for conversion into potable alcohol.

2.8 MISCELLANEOUS USES OF APPLE POMACE

2.8.1 Pigments

Sandhu and Joshi (1997a) standardized several factors for the production of pigments by *Rhodotorula*. The optimum conditions were 0.3% ammonium sulphate, original pH incubation for 72 hrs at 30±1°C. The pigment had higher solubility in hexane followed by acetone but was having poor solubility in water and HCl. However, addition of $ZnSO_4$ completely inhibited the pigment production.

2.8.2 Fertilizer

Apple pomace could be used as fertilizer for damaged soils (Stapleton 1982) as an amendment in container growing media for silverleaf dog wood, *cuonymus* and *Andora juniper* (Chong, 1992).

2.8.3 Bio-gas

Apple pomace can be used to produce bio-gas by anaerobic digestion which can then be used as a fuel or burnt directly to produce energy, overseas both the options have been successfully used. But it is felt that such options should be tried when there is no alternative as apple pomace being nutritive should get preference for food or feed (Kennedy, 1994).

2.8.4 Oil

Apple pips contain oil and the oil can be extracted and used in cosmetic industry as a speciality oil. Upto 600 tones of pips oil can be produced per annum in New Zealand. Although, these quantities are dependent on collection of seeds from a variety of producers for processing (Shaw and Bain, 1993).

2.8.5 Aroma

The manufacture of aroma compounds from apple pomace are a special case, because aromas and flavours are one of the few high value products that can be made from apple pomace. Flavouring compounds by extracting with liquid CO_2 then fractioned at the different temperature obtaining flavourless fraction and intensively flavoured fraction. Flavour extracted by the this procedure are reported to have broad flavour spectrum than those obtained by distillation (Sharma and Joshi, 1996). Bomben and Guadagni (1973) have also used apple pomace to prepare apple flavour food thickener and texture modifier for use in various products.

MATERIALS AND METHODS

The present studies on **"Solid State Fermentation of Apple Pomace : Methods of Ethanol Recovery, Physico-chemical Evaluation and Acceptability of Animal Feed Produced"** were undertaken in the Department of Postharvest Technology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) during 1996-97. The various materials used and methods/ techniques employed for analyses have been described herein under the following sub-headings :

3.1 MATERIALS

3.2 EXPERIMENTAL LAYOUT

3.3 ANALYSES

3.1 MATERIALS

3.1.1 Collection of apple pomace

Apple pomace was collected from HPMC Fruit Processing Plant, Parwanoo, Solan (H.P.) directly from plant outlet and was brought to lab in 60 liter capacity plastic barrels within 2 hours of collection or was prepared in the laboratory, where ever needed sulfur dioxide was added at the rate of 300 ppm at the time of collection to avoid natural fermentation during transportation. It was dried in a mechanical dehydrator at 60±1°C for 8 hours (Plate 1) and packed either in polythene bags or properly sealed drums for subsequent studies.

3.1.2 The yeasts

The culture *Saccharomyces cerevisiae* UCD (595) used was originally procured from the Department of Enology and Viticulture, California, Davis, U.S.A. While that of *Schizosaccharomyces pombe* was from IIHR, Bangalore and *Candida utilis* and *Torula utilis*

Plate 1 Mechanical dehydration of apple pomace

were obtained from National Chemical Laboratory, Pune and *Kloeckera* spp. was isolated from the normal microflora of the apple pomace, identification procedure used were those of Lodder (1971). The cultures were maintained yeast malt extract slants. The composition of the broth used for the active culture preparation for SSF is shown in Table 3.1. To make solid medium or slants agar-agar at the rate of 20 gm per liter was used. The medium was sterilized as per the standard method.

Table 3.1. Composition of broth used for culturing yeast

Constituents	Concentration (g) per 250 ml
Yeast extract	0.75
Beef extract	0.75
Peptone	1.25
Glucose	2.50

3.1.3 Sugar and nitrogen source

Molasses, used as sugar source in SSF of apple pomace, was procured locally while that of ammonium sulphate (AMS) and di-ammonium hydrogen phosphate (DAHP) was manufactured by Emerck India Ltd., Bombay (India). The composition of molasses (based on analysis is shown in table 3.2.

Table 3.2 Composition of molasses

Total soluble solids (°B)	70.00
Reducing sugars (%)	51.10
Total sugars (%)	58.70
Nitrogen (%)	0.42
Titratable acidity (%) (as malic acid)	2.85

3.1.4 Rats

The rats *Rattus rattus* (Rotan strain) was procured from Central Research Institute, Kasauli, Solan (H.P.) of uniform weight and same age group. They were maintained in the laboratory using humane conditions while used in evaluation of animal feed in animal.

3.1.5 Constituents for reformulation of feed

The jaggery used in reformulation of feed was procured locally, ground nut oil was manufactured by National Dairy Development Board, Anand (India), salt was manufactured by M/s DCW Home Products Limited, Madras (India), mixed flavours(Pineapple, kewra and Vanilla) were manufactured by M/s Arun Chemical Industries, New Delhi, (India).

3.1.6 Animal feed

The standard rat animal feed was made by and procured from H.P. Agro-industries, Parwanoo, Solan (H.P.), which was used as a standard feed (control) in apple pomace animal feed evaluation in animal. The composition of standard rat animal feed used in this experiment is shown in Table 3.3.

Table 3.3 Composition of standard rat animal feed

Constituents	Dry weight basis (%)
Crude protein	21.0
Ether extract	5.0
Crude fiber	4.0
Ash	8.0
Calcium	1.0
Phosphorus	0.6
Nitrogen free extract	53.0
Metabolisable energy (k cal/kg)	3600.0

Source : H.P. Agro-industries Limited, Parwanoo

3.2 EXPERIMENTAL LAY OUT

3.2.1a Experiment I (A)

Effect of addition of molasses and nitrogen source (DAHP and AMS) on solid state fermentation of apple pomace

Molasses at two concentrations with two different nitrogen sources (DAHP and AMS) at two concentrations were tried. Prior to fermentation, molasses and apple pomace were analysed for different physico-chemical characteristics.

Solid state fermentation

The dried apple pomace was reconstituted by addition of water and the apple pomace water ratio was kept at 1:4. The reconstituted apple pomace (300 g) alongwith molasses and

Table 3.4 Details of treatment of solid state fermentation of apple pomace with different concentration level of molasses and nitrogen source

Treatments	Nitrogen sources (%)	Molasses (%)	Yeast spp. used in each treatment
T_1			1. *Saccharomyces cerevisiae*
i) Control	-	-	2. *Candida utilis*
ii) Control	-	-	3. *Torula utilis*
T_2			4. *Schizosaccharomyces pombe*
i)	1.8 DAHP	5	5. *Kloeckera* spp.
ii)	1.8 AMS	5	
T_3			
i)	1.8 DAHP	10	
ii)	1.8 AMS	10	
T_4			
i)	2.0 DAHP	5	
ii)	2.0 AMS	5	
T_5			
i)	2.0 DAHP	10	
ii)	2.0 AMS	10	

AMS - Ammonium sulphate
DAHP - Diammonium hydrogen phosphate

Treatments = 5x2; Yeast spp. = 5; Replications = 2

nitrogen sources was sterilized at 121°C for 15 minutes in 750 ml beer bottles. The fermentation was initiated by addition of active cultures of different yeasts @ 5 per cent and fermentation was carried out at 25±1°C for 96 hours with 8-10 times of intermittent rotation (clockwise and anticlockwise) per day (Plate 2). When there was no loss in TSS and reducing sugar, the fermentation was considered as complete. During fermentation, the apple pomace was analysed for TSS, reducing sugar, pH and acidity at an interval of 24 hours. The fermented apple pomace was dried in a mechanical dehydrator at 60±1 °c for 8 hours and thereafter ground into powder and was packed in polythene bags. The dried apple pomace was analysed for different parameters. The details of treatments of SSF of apple pomace are given in Table 3.4.

3.2.1b Experiment I (B)

Sequential culture

After screening on the basis of high ethanol and crude protein content, the concentration of molasses and nitrogen source (molasses 10% and nitrogen source 1.8% concentration) was selected from Experiment I (A). The fermentation conditions were similar as those of experiment I (A) except that was fermented with the separate yeasts for 96 hours, followed by fermentation with respective sequential (second) yeast for 120 hours by addition of 5 per cent of active yeast cultures (9-15 x 10^8 cells/ml). During fermentation, apple pomace was analysed for TSS, reducing sugar, ethanol, pH and acidity at an interval of 48 hours for 216 hours. The details of treatments of sequential culture system of solid state fermentation of apple pomace are given in Table 3.5. The fermented apple pomace was dried in a mechanical dehydrator at 60± 1°C for 8 hours and thereafter, ground into powder and was packed in polythene bags. The dried apple pomace was analysed for different parameters.

Plate 2 **Solid state fermentation of apple pomace by *Saccharomyces cerevisiae* with different treatments**

Table 3.5. Details of treatments of sequential culture system in solid state fermentation of apple pomace

Treatments	Initial culture		Seqential culture
I_1S_1	*Saccharomyces cerevisiae*	+	*Saccharomyces cerevisiae*
I_1S_2	*Saccharomyces cerevisiae*	+	*Candida utilis*
I_1S_3	*Saccharomyces cerevisiae*	+	*Torula utilis*
I_1S_4	*Saccharomyces cerevisiae*	+	*Schizosaccharomyces pombe*
I_1S_5	*Saccharomyces cerevisiae*	+	*Kloeckera spp.*
I_2S_1	*Candida utilis*	+	*Saccharomyces cerevisiae*
I_2S_2	*Candida utilis*	+	*Candida utilis*
I_2S_3	*Candida utilis*	+	*Torula utilis*
I_2S_4	*Candida utilis*	+	*Schizosaccharomyces pombe*
I_2S_5	*Candida utilis*	+	*Kloeckera spp.*
I_3S_1	*Torula utilis*	+	*Saccharomyces cerevisiae*
I_3S_2	*Torula utilis*	+	*Candida utilis*
I_3S_3	*Torula utilis*	+	*Torula utilis*
I_3S_4	*Torula utilis*	+	*Schizosaccharomyces pombe*
I_3S_5	*Torula utilis*	+	*Kloeckera spp.*
I_4S_1	*Schizosaccharomyces pombe*	+	*Saccharomyces cerevisiae*
I_4S_2	*Schizosaccharomyces pombe*	+	*Candida utilis*
I_4S_3	*Schizosaccharomyces pombe*	+	*Torula utilis*
I_4S_4	*Schizosaccharomyces pombe*	+	*Schizosaccharomyces pombe*
I_4S_5	*Schizosaccharomyces pombe*	+	*Kloeckera spp.*
I_5S_1	*Kloeckera spp.*	+	*Saccharomyces cerevisiae*
I_5S_2	*Kloeckera spp.*	+	*Candida utilis*
I_5S_3	*Kloeckera spp.*	+	*Torula utilis*
I_5S_4	*Kloeckera spp.*	+	*Schizosaccharomyces pombe*
I_5S_5	*Kloeckera spp.*	+	*Kloeckera spp.*

I	=	Initial Cultures
S	=	Sequential Cultures
Treatments	=	5 x 5
Replications	=	2

3.2.2 Experiment II: Recovery of ethanol

The best two treatments (*Saccharomyces cerevisiae* (I_1S_1); *Candida utilis* and *kloeckera* spp. (I_2S_5) were selected from experiment I(B) on the basis of high ethanol and crude protein content. Fermentation procedures were similar as those of Experiment I (B) but the fermentation was carried out in 5 litre conical flasks with 3 kg of reconsituted apple pomace. Ethanol in the

fermented apple pomace was extracted by four different methods. The detailed procedures of different methods of ethanol recovery aredescribed below:

Method I: Hydraulic Pressing

Fermented apple pomace (1.5 kg) was transferred to 5 L conical flask. Hot water (750 ml) at 50°C was added and the flask alongwith contents was allowed to stand for 30 minutes, followed by pressing. To the second press, the same quantity of water was added, followed by pressing. The pooled extract of both were collected and 1/3 of the initial content of material was distilled (1 L). The base of the distillate was added back to the fermented apple pomace prior to drying (Plate 3).

Method-II Direct Distillation

The fermented apple pomace (1.5 kg) was transferred to 5 L conical flask and the water and fermented apple pomace ratio was kept 1:1. The flask was directly kept on the heater, followed by distillation and 1/3 of the total volume was distilled (1 L). The distillation arrangement is shown in Plate 4.

Method-III: Steam Distillation

Fermented apple pomace (1.5 kg) was transferred to 5 L conical flask and the same quantity of water was added. The mouth of the flask was fitted with a cork having a steam inlet going upto the bottom of the flask. Steam was generated in an autoclave upto 25 lb and thereafter the steam was released into the flask. From the top outlet of flask steam was collected. The steam distillation arrangement is shown in Plate 5.

Method IV: Vacuum Distillation

Hot water (175 ml) at 50°C was added to the fermented apple pomace (175 g). The material was allowed to stand for 30 minutes, and it was transferred to vacuum distillation flask which was kept on water bath. Water bath temperature was maintained constantly at 78°C. The distillation set used is shown in Plate.6.

Plate 3 Ethanol separation by hydraulic pressing of fermented apple pomace

Plate 4 Ethanol separation by direct distillation of fermented apple pomace

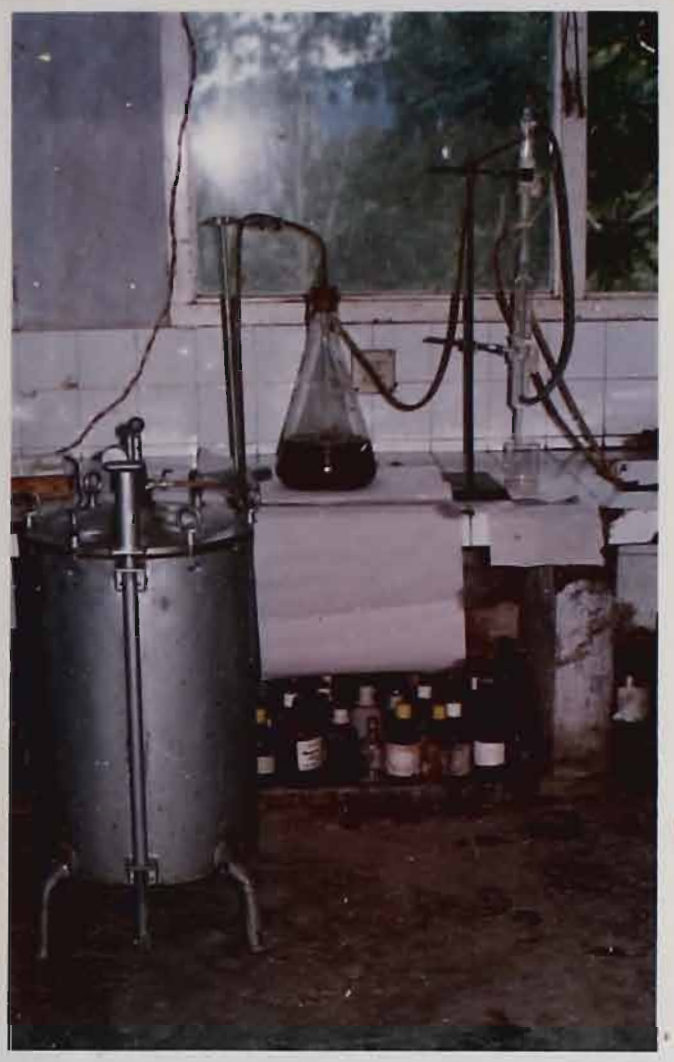


Plate 5 Ethanol separation by steam distillation of fermented apple pomace

Plate 6 Ethanol separation by vacuum distillation of fermented apple pomace

Distillates of each treatment was analysed for alcohol content. The left over material was dried, ground, packed in polythene bags and analysed for different physico-chemical parameters. Details of treatments are as shown below:

Treatments = 4 x 2

Replications = 2

3.2.3 Preparation and evaluation of animal feed in white rats

3.2.3.1 Experiment III(A) : Preparation of animal feed

After screening for ethanol recovery from the fermented apple pomace the steam distillation method was selected as the best method on the basis of highest separation efficiency and minimum nutritional loss. The yeast spp. *Candida utilis* and *Kloeckera* spp. as sequential interactive co-culture was selected on the basis of highest crude and soluble protein contents.

The dried apple pomace was reconstituted by adding water and the apple pomace water ratio kept 1:4. The reconstituted apple pomace (50Kg) was sterilized at 121°C for 15 minutes along with 1.8% AMS and 10% molasses and then transferred to 60 liters capacity barrel provided with air lock to avoid contamination. Fermentation was carried out in plastic barrel with *Candida utilis* for 96 hours followed by *Kloeckera* spp. for 120 hours with intermittent rotation. After completion of fermentation, apple pomace was dried in a mechanical dehydrator at 60°C for 8 hours and was ground and packed in polythene bags. The powdered apple pomace was used for subsequent studies on evaluation of animal feed in rats.

3.2.3.2 Experiment III(b) : Evaluation of feed in animals

The albino rats, *Rattus rattus* (Rotan strain) procured from Central Research Laboratory, Kasauli, Solan (H.P.) were tested for the suitability of the animal feed (before and after reconstitution) prepared, in both 'no choice' and 'choice' experimental setups in the laboratory, following the method described by Malhi and Shiekher (1988).

i) Apple pomace either fermented or unfermented or its incorporation into the standard rat feed (in the ratio of 1:1) were fed to rats for 2 weeks for no choice study and 6 days for choice study. During feeding trial weight of feed intake and faecal matter were recorded

at 24 hours interval in both choice and no choice study while weight of animals were taken at weekly intervals in no choice study. Animals were divided into groups for treatments and were provided with different items as described below in Table 3.6.

No choice study

For no choice study special type of individual cages were used as shown in Plate 7.

Table 3.6 Details of different types of animal feed (treatments) given to rats (no choice)

Treatments	Type of feed
T_1	Standard rat feed
T_2	100% fermented apple pomace
T_3	100% unfermented apple pomace
T_4	1:1 ratio of fermented apple pomace and standard rat feed
T_5	1:1 ratio of unfermented apple pomace and standard rat feed

Treatments = 5
Replications = 2 (Four rats in each replications)

Choice

Choice experiment was conducted in plus maze by placing 3 food items at one time (one in each arm) and water in 4th arm. The order of placement of foods and water was changed each

Table 3.7 Details of treatments of different types of feeds to animal (choice) experiment

Treatments	Types of feed (choice)	
T_1	i)	Standard rat feed
	ii)	100% fermented
	iii)	100% unfermented
T_2	i)	Standard rat feed
	ii)	1:1 ratio of fermented apple pomace and standard rat feed
	iii)	1:1 ratio of unfermented apple pomace and standard rat feed

Treatments = 2; Replications = 2 (Five rats in each replications)

Plate 7 Cages used for animal feed trial (No choice). with white rats

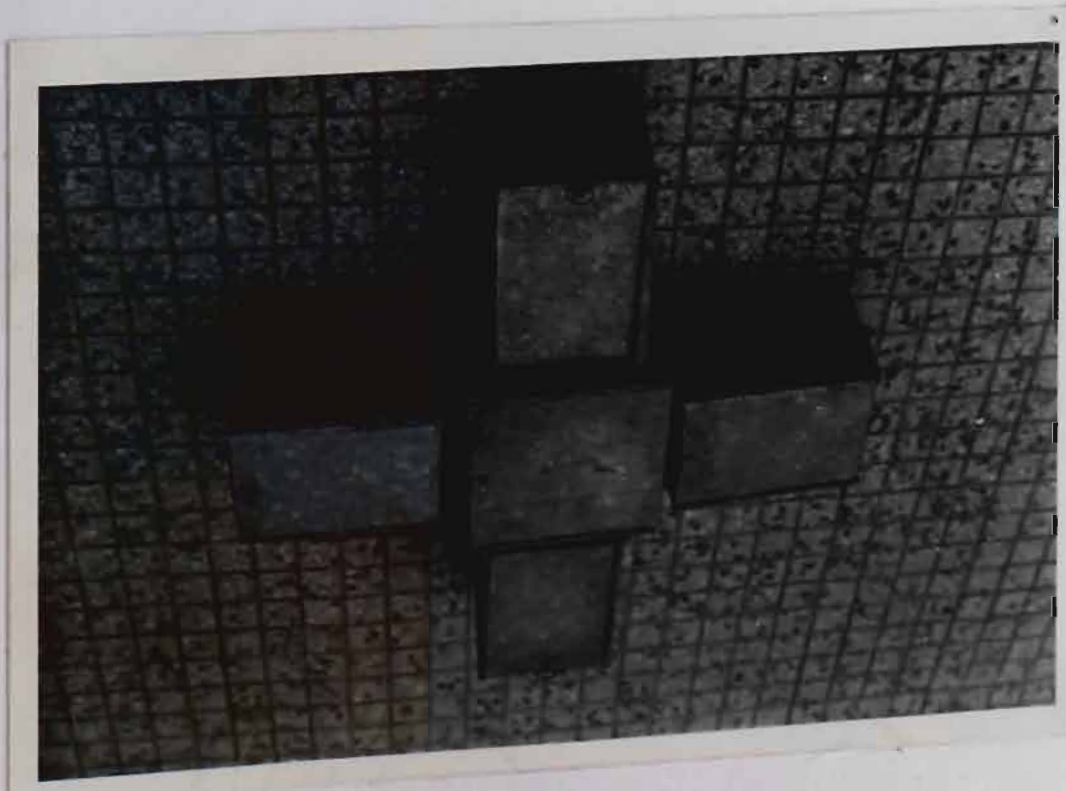


Plate 8 Cages used for animal feed trial (choice) with white rats

day during the experiment (Plate 8). Water was provided *ad lib* and each test was run for 5 days. Details of treatments of different types of feeds to animal (choice) are presented in Table 3.7.

ii) The apple pomace after reconstitution was fed to the rats for a period of 2 weeks in no choice study and for 6 days in choice study. During feeding trial weight of feed intake and faecal mater were taken at 24 hours of interval during both choice and no choice study. While weight of animals were taken at weekly intervals in no choice study. Animals were divided into groups for treatments and were provided with different feeds as described in Table 3.8 and 3.9. The feeding trial cages were remained as explained earlier.

No choice study

Table 3.8 Details of different types of animal feed (treatments) after reconstitution given to rats (no choice)

Treatments	Type of feed
T_1	Standard rat feed
T_2	Fermented apple pomace + 2% ground nut oil + 10% jaggery + 0.1% mixed flavour + 1% salt
T_3	Fermented apple pomace + 10% jaggery + 0.1% mixed flavour + 2% salt
T_4	Fermented apple pomace + 2% ground nut oil + 0.1% mixed flavour + 1% salt
T_5	Unfermented apple pomace + 2% ground nut oil + 10% jaggery + 0.1% mixed flavour + 1% salt
T_6	Unfermented apple pomace + 10% jaggery + 0.1% mixed flavour + 1% salt
T_7	Unfermented apple pomace + 2% ground nut oil + 0.1% mixed flavour + 1% salt

Treatments = 7
Replications = 2 (Four rats in each replications)

Choice study

Table 3.9 Details of treatments of different type of feeds given to rats after reconstitution of apple pomace (choice study)

Treatments	Types of feed (choice)
T_1	i) Standard rat feed ii) Fermented apple pomace + 2% ground nut oil + 10% jaggery + 0.1% mixed flavour + 1% salt iii) Fermented apple pomace + 10% jaggery + 0.1% mixed flavour + 1% salt
T_2	i) Standard rat feed ii) Fermented apple pomace + 2% ground nut oil + 0.1% mixed flavour + 1% salt iii) Unfermented apple pomace + 2% ground nut oil + 10% jaggery + 0.1% mixed flavour + 1% salt
T_3	i) Standard rat feed ii) Unfermented apple pomace + 10% jaggery + 0.1% mixed flavour + 1% salt iii) Unfermented apple pomace + 2% ground nut oil + 0.1% mixed flavour + 1% salt

Treatments = 3

Replications = 2 (Five rats in each replications)

3.3 ANALYSES

3.3.1 Physico-chemical characteristics

The various physico chemical characteristics of apple pomace were analysed for the following parameters during and after fermentation on dry and/or wet weight basis.

Ethyl alcohol

Ethyl alcohol during and after fermentation was determined by colourimetric method (Caputi et al., 1968). One g of sample with 30 ml of distilled water was distilled into 25 ml potassium dichromate solution. The optical densities of samples as well as standard were taken at a wave length of 600 nm in a colourimeter against a blank. The amount of ethanol present in the samples was determined from the standard curve, prepared similarly.

Ascorbic acid

Ascorbic acid content was determined as per A.O.A.C. method (1980), using 2, 6-dichlorophenol-indophenol dye. The sample was extracted in HPO_3 solution and titrated with the dye to a pink end point.

Calculations

$$\text{Ascorbic acid (mg/100 g)} = \frac{\text{Titre x Dye factor x Vol. made up x 100}}{\text{Aliquot of extract taken} \times \text{Weight. of sample taken for estimation}}$$

Total soluble solids

Total soluble solids (TSS) were measured (°B) using Erma hand refractometer (0 to 32°B). The results were expressed as degree brix (°B). The readings were corrected by applying the correction factor for the temperature variations (A.O.A.C., 1980).

Titratable acidity

Titratable acidity was determined by titrating a known aliquot of the sample (solid materials were diluted to 10 times) against N/10 NaOH solution using phenolphthalein, as an indicator. The titratable acidity was calculated and expressed as per cent malic acid (A.O.A.C., 1980) as detailed below:

$$\% \text{ Titratable acidity (malic acid)} = \frac{V \times N \times 67 \times 100}{v \times 100}$$

where, V = Volume of N/10 NaOH used for titration
N = Normality of NaOH solution
v = Volume of sample taken

pH

The pH was taken with ELTOP - 3030 pH meter. The solid materials were diluted to 10 times. Prior to pH measurement, the instrument was calibrated with buffer solutions of pH 4 and 7 (Ranganna, 1986).

Sugars

The total and reducing sugars were measured by Lane and Eynon's volumetric method (A.O.A.C., 1980) by titrating against Fehling solutions (before and after hydrolysis). The total sugars and reducing sugars were expressed as per cent.

Caculations

$$\text{Reducing sugars } (\%) = \frac{\text{Factor} \times \text{dilution} \times 100}{\text{Titre} \times \text{weight of sample taken}}$$

Crude proteins

Crude proteins were estimated by the micro-kjeldahl method (Ranganna, 1986). Samples (4-6 g) were digested for 2 hours with 25 ml conc. H_2SO_4 and 2 g of digestion mixture (2.5 g SeO_2, 100 g K_2SO_4 and 20 g $CuSO_4$), cooled and volume was made up to 100 ml. Ten milliliters of this aliquot was distilled with excess of 30 per cent NaOH and liberated ammonia was collected quantitatively in 2 per cent boric acid solution, containing a few drops of mixed (methyl red and bromocresol green) indicator. The boric acid solution was titrated against standardized 0.01 N HCl and protein content was calculated using the equation given below:

$$\text{Nitrogen(\%)} = \frac{(\text{Sample Titre} - \text{blank Titre}) \times \text{Normality of HCl} \times 14 \times \text{Volume made up of digest}}{\text{Aliquot of digest taken} \times \text{Weight of sample taken} \times 1000} \times 100$$

$$\text{Crude protein (\%)} = \text{Nitrogen (\%)} \times 6.25$$

Soluble proteins

The extraction of soluble proteins was made according to the method adopted by Lowry *et al.* (1951). 500 mg of dried and ground sample was taken for protein extraction. The protein was extracted in 19.5 ml of phosphate buffer (pH 7.5). The sediments were removed by centrifuging at 4500 rpm. The supernatant was used for protein estimation. After 30 minutes of colour development, transmittance of the resultant blue colour solution was read at 660 nm in colourimeter. The amount of protein was calculated with the help of a standard curve prepared from the Bovine Serum Albumin (BSA) as reference protein and expressed as percentage on dry weight basis.

Fat

It was determined by extracting 2-5 g of sample in petroleum ether (b.p.35-45°C) using a Soxhlet extraction apparatus (Ranganna, 1986). Extraction of sample was done for 16 hours or longer on a water bath. Thereafter, ether was removed by evaporation, the extracted fat was weighed and percentage of ether-soluble material on dry weight basis was calculated as:

$$\text{Per cent crude fat} = \frac{\text{Weight of ether soluble material}}{\text{Weight of sample}} \times 100$$

Moisture

Infrared moisture meter was used to determine the moisture (Ranganna, 1986). 1 g of sample was weighed by adjusting the balance indicator to vernier zero. Infrared bulb was switched on and the sample was dried at a temperature of 75±1°C by adjusting the lamp height to 9 cm. As the drying proceeded, balance indicator moved upward. it was again brought to vernier zero with

the help of adjusting knob. When the sample had fully dried, the balance indicator stopped completely. The per cent moisture loss was directly read on the scale to get the final reading.

Crude fiber

Crude fiber was determined by AOAC method (1980). Appropriate weight of the sample (5 g) was boiled with 200 ml of 1.25 per cent H_2SO_4 for 30 minutes, cooled, filtered and residue washed with hot water. Then added 200 ml of boiling hot 1.25 per cent NaOH to the residue, boiled for another 30 minutes, cooled, filtered and residue washed. It was then ignited at 600°C for 20 minutes in an electric muffle furnace.

Calculations

$$\text{Crude fiber (\%)} = \frac{\text{Loss in weight}}{\text{Weight of sample taken}} \times 100$$

Yeast count

Total yeast count was taken by standard plate count method (Harrigen and McCanca, 1966). The composition of media is shown in the table 3.10

Table 3.10 Composition of media used for yeast count

Constituents	Concentration (g/l)
Yeast extract	3
Beef extract	3
Peptone	5
Glucose	10
Agar-Agar	20

3.3.2 Evaluation of animal feed in rats

Consumption index, growth rate, utilization and digestibility indices of rats

Methods for calculation of consumption index, growth rate, efficiency of conversion of ingested food and digestibility of rats has been described by Waldbauer (1968). The calculations were made as per the following procedure.

i) Consumption index

From the data of weight of food taken, mean weight of animals during feeding period and duration of experiment, the consumption index was calculated as below:

$$\text{Consumption index} = \frac{\text{Weight of food eaten}}{\text{Mean weight of animal during feeding period} \times \text{Duration of experiment}}$$

ii) Growth rate

The growth rate of animals were calculated from the data of duration of feeding, weight gained by animals and mean weight of animals during feeding trial as below:

$$\text{Growth rate} = \frac{\text{Weight gained by animals}}{\text{Duration of feeding period} \times \text{Mean weight of animal during feeding}}$$

iii) Efficiency of conversion of ingested food

From the data of weight gained by animals and weight of food ingested the efficiency of conversion of ingested food material were calculated as below:

$$\text{Efficiency of conversion of ingested food} = \frac{\text{Weight gained by animals}}{\text{Weight of food ingested}} \times 100$$

iv) **Digestibility**

The digestibility of animals were calculated from the data of food ingested and faecal matter as below:

$$\text{Digestibility} = \frac{\text{Food ingested - Faecal matter}}{\text{Food ingested}} \times 100$$

3.3.3 Biochemical analysis of blood sample

The blood samples were collected from the rat sacrificing them after anesthesia. The different biochemical characteristics of rat serum was analysed as described below:

Total protein

Total protein content in blood serum was determined by Beiurett method (Henry et al., 1974). Analytical kits used were obtained from Reckon diagnostic Pvt. Ltd., Baroda (India). Three solutions viz. blank (2.5 ml total protein reagent (TPR) + distilled water 0.05 ml), standard (2.5 ml total protein nitrogen reagent + 0.05 ml standard protein reagent) and test solution (2.5 ml TPR + sample 0.05 ml sample) were taken in 3 separate test tubes. Absorbancec of test and standard were read at 540 nm against a blank in a digital spectrophotometer. The total protein content was calculated as below:

$$\text{Total protein content (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 6$$

Blood urea nitrogen (BUN)

Blood urea nitrogen content in blood serum was calculated by End point dam method (Henry *et al.*, 1974). Analytical kits used were obtained from J. Mitra and Co. Ltd., New Delhi

(India). Three test tubes each containing 1.5 ml + 1.5 ml + 2 ml of urea colour reagent A, B and C were taken. In case of blank, 50 ul distilled water, standard 50 ul urea standard (40 mg/dl) and test solution 50 ul sample were added. Optical density of each solutions were measured at 520 nm in a digital spectrophotometer.

$$\text{Concentration of urea} = \frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times 40 \text{ mg/dl}$$

BUN = Urea concentration x 0.467 mg/100 ml

Glucose content

Glucose content of blood serum was determined by GOD /BOD method (Trinder, 1969 and Tietz, 1976). Kits used were obtained from M/s Span Diagnostic Ltd. Surat (India). 1.5 ml of working glucose reagent and distilled water were taken in 3 test tubes. Blank was kept as such while that of standard 0.02 ml of glucose standard 100/mg 100 ml and test solution 0.02 ml of serum was added, O.D. was measured at 510 nm in a digital spectrophotometer.

$$\text{Serum glucose} = \frac{\text{O.D. test - O.D. blank}}{\text{O.D. standard - O.D. blank}} \times 100$$

Enzymes

Alanine amino transferase (ALT)

ALT in the blood serum was calculated by 2,4-DNPH method (Reitman and Frankel, 1957). Kits were obtained from Span Diagnostics Pvt. Ltd. Surat (India). 0.5 ml of buffer alanine -α- KG substrate pH 7.4 was taken and incubated at 37°C for 5 minutes. 0.1 ml of serum was added to it and incubated at 37°C for 30 minutes followed by adding 0.5 ml of DNPH colour reagent incubated at room temperature for 20 minutes. Optical density was measured at 505 nm in a digital spectrophotometer. and the ALT concentration was measured by comparing with standard curve which was prepared in the similar way with pyruvate standard.

Aspartate aminotransferase (AST)

AST content of blood serum was determined by 2, 4 DNPH method (Reitman and Frankel, 1957). Kits were manufactured by Span Diagnostics Pvt. Ltd. Surat (India). 0.5 ml of buffer Aspartate - α- KG substrate, pH 7.4 was taken in test tube and it was incubated at 37°C for 5 minutes. 0.1 ml of serum was added to it and incubated 37°C for 60 minutes followed by 0.5 ml of DNPH, colour reagent was added and incubated at room temperature for 20 minutes. O.D. was measured at 5.05 nm in a digital spectrophotometer and the SGOT concentration was measured by comparing with standard curve which was prepared in the similar way with pyruvate standard.

3.4 STASTISTICAL ANALYSIS

The data of quantitative estimation of various physico-chemical characteristics of apple pomace and the data of evaluation of animal feed trials were analysed by complete randomised block design or factorial (CRD) as described by Cochran and Cox (1963).

RESULTS AND DISCUSSION

The results obtained in the present studies on **"Solid State Fermentation of Apple Pomace - Methods of Ethanol Recovery, Physico-chemical Evaluation and Acceptability of Animal Feed Produced"** are presented in this chapter aspect wise as follows:

4.1 PHYSICO-CHEMICAL CHARACTERISTICS OF APPLE POMACE

4.2 OPTIMIZATION OF MOLASSES AND NITROGEN SOURCE CONCENTRATION FOR SOLID STATE FERMENTATION OF APPLE POMACE

4.3 EFFECT OF SEQUENTIAL INTERACTIVE CO-CULTURE ON SSF OF APPLE POMACE

4.4 STANDARDIZATION OF METHOD FOR ETHANOL RECOVERY FROM FERMENTED APPLE POMACE

4.5 ACCEPTABILITY AND EVALUATION OF ANIMAL FEED PRODUCED IN RATS

4.1 PHYSICO-CHEMICAL CHARACTERISTICS OF APPLE POMACE

The physico-chemical composition of apple pomace (Table 4.1) shows that it has high moisture content on fresh weight basis and high reducing and total sugars, titratable acidity, crude fiber and ascorbic acid content but low protein content on dry weight basis. The pH ranged from 3.83-3.98. The values were in the range as reported earlier (Joshi and Sandhu, 1996b; Hang *et al.*, 1982; Rahmat *et al.*, 1995). The carbohydrate content of apple pomace ranged from 9.5-22 per cent on fresh weight basis. However, total soluble sugars of dried apple pomace varied from 48.1-62 per cent in different batches (Rahmat *et al.*, 1995). Differences in carbohydrate content of apple pomace discussed earlier could be related to the variety of apple processed, the processing

Table 4.1. Physico-chemical characteristics of apple pomace

Characteristics	Mean±S.E.
Moisture (%)*	80.13±1.010
Reducing sugars (%)**	40.47±0.155
Total sugars (%)**	48.06±0.035
TSS (°B)**	57.85±0.050
Titratable acidity (%)**	3.28±0.025
pH**	3.90±0.070
Soluble proteins (%)**	3.29±0.080
Crude proteins (%)**	5.19±0.085
Ascorbic acid (mg/100 ml)**	16.25±0.280
Crude fiber (%)**	15.65±0.965
Crude fat (%)**	3.49±0.285

S.E. Standard error
* On wet weight basis
** On dry weight basis

conditions used and the amount of press aids added (Hang *et al.*, 1982). High moisture content makes the apple pomace susceptible to microbial degradation. Both crude and soluble proteins are very low in apple pomace (Rumsey, 1978; Hang *et al.*, 1982; Hang and Walter, 1989). Because of low protein content of apple pomace as such it is a poor animal feed (Hang, 1988). Based upon the composition of apple pomace presented here it was concluded that apple pomace is a good source of fermentable sugars and holds promise for its use in ethanol and animal feed production, through Solid state fermentation by yeasts. Since, low protein content of apple pomace is a big obstacle for its use as an animal food, it could be improved after recovery of value added product like ethanol produced through fermentation.

4.2 OPTIMIZATION OF MOLASSES AND NITROGEN SOURCE CONCENTRATION FOR SOLID STATE FERMENTATION OF APPLE POMACE

4.2.1 Solid state fermentation of apple pomace with different yeasts

The solid state fermentation was conducted with different yeasts *Saccharomyces cerevisiae, Candida utilis, Torula utilis, Schizosaccharomyces pombe and Kloeckera spp.* and monitored for various parameters. The results obtained are discussed here.

Effect of addition of molasses and nitrogen source on solid state fermentation of apple pomace

Rate of fermentation

Effect of treatment : The results obtained on mean rate of fermentation during SSF of apple pomace are presented in Table 4.2.1. The treatments which have been framed by adding different quantities of molasses and nitrogen source had significantly statistically increased fermentation rates as compared to the control. However, there was no significant difference for this parameter between the two nitrogen sources (DAHP and AMS). In between the treatments, the average rate of fermentation varied from 1.179 to 3.212 (fall °B/24 hrs). The highest average fermentation rate 3.212 (fall °B/24 hrs) was observed in 10 per cent molasses and 1.8 per cent nitrogen source blended apple pomace followed by apple pomace blended with 10 per cent molasses and

Table . 4.2.1. Effect of treatments and yeast species on mean rate of fermentation (fall °B/24 hrs) during SSF of apple pomace

Yeast spp. / Treatments	*Saccharomyces cerevisiae*	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
T_1 (Control)	1.331	1.112	1.112	1.150	1.187	1.179
T_2 (1.8% NS + 5% Molasses)	3.281	2.475	2.562	2.475	2.781	2.715
T_3 (1.8% NS + 10% molasses)	3.550	2.987	3.150	3.025	3.350	3.212
T_4 (2.0% NS + 5% molasses)	3.244	2.450	2.550	2.462	2.781	2.697
T_5 (2.0%NS+10% molasses)	3.487	2.987	2.987	3.000	3.250	3.142
Mean	2.979	2.402	2.472	2.422	2.670	

Nitrogen source	DAHP	AMS
Mean	2.590	2.588
CD (0.05)	ns	

Effect	CD (0.05)
Treatments	0.008
Yeast spp.	0.008
Treatment x Yeast spp.	0.018

DAHP - Diammonium hydrogen phosphate
AMS - Ammonium sulphate
NS - Nitrogen source
ns - Non-significant

2.0 per cent nitrogen source. The lowest fermentation rate was observed in control. The increase in fermentation rate due to addition of nitrogen source is understandable due to availability of more nitrogen for the growth of micro-organisms. These results are similar to those reported earlier (Gupta *et al.*, 1990; Sandhu and Joshi, 1997b). Increase in molasses concentration expectedly made available more sugar and accordingly enhanced the fermentation rate.

Effect of yeast spp.: Amongst the yeasts spp., *Saccharomyces cerevisiae* had the highest statistically significantly average rate of fermentation (2.979 fall °B/24 hr) followed by *Kloeckera* spp. (2.670 fall °B/24 hr). The lowest average fermentation rate was observed in *Candida utilis* fermented apple pomace. The interaction between treatment and yeast species was found to be significant. Within the interactions, apple pomace blended with 10 per cent molasses and 1.8 per cent of nitrogen source with *Saccharomyces cerevisiae* fermented apple pomace had the highest average rate of fermentation followed by *Saccharomyces cerevisiae* with 10 per cent molasses and 2 per cent nitrogen source. The lowest average rate of fermentation was observed in *Candida utilis* and *Torula utilis* fermented apple pomace with control treatment. Highest fermentation rate observed in *Saccharomyces cerevisiae* is apparently due to its high fermentability in sugar substrates (Lodder, 1970) while that of *Kloeckera* spp. could be attributed due to better adaptation being natural microflora of the substrate (Hodge *et al.*, 1990).

Total soluble solids (TSS) and ethanol

Effect of treatment : The data relating to change in TSS during solid state fermentation of apple pomace are depicted in Fig. 4.2.1 and 4.2.2. Clearly, addition of molasses increased the TSS at initial stage and as expected the TSS continued to decline throughout the fermentation period and the difference between the different treatments could be easily observed. It is also clear from the figure that the apple pomace blended with 10 per cent molasses and 1.8 per cent of nitrogen source (DAHP or AMS) had reduced more TSS as compared to the control. Addition of molasses and nitrogen source increased the availability of both sugar and nitrogen for the growth of micro-organisms. The differences in TSS content of different treatments at the end of the fermentation are due to the addition of molasses which might have increased the soluble solids other than

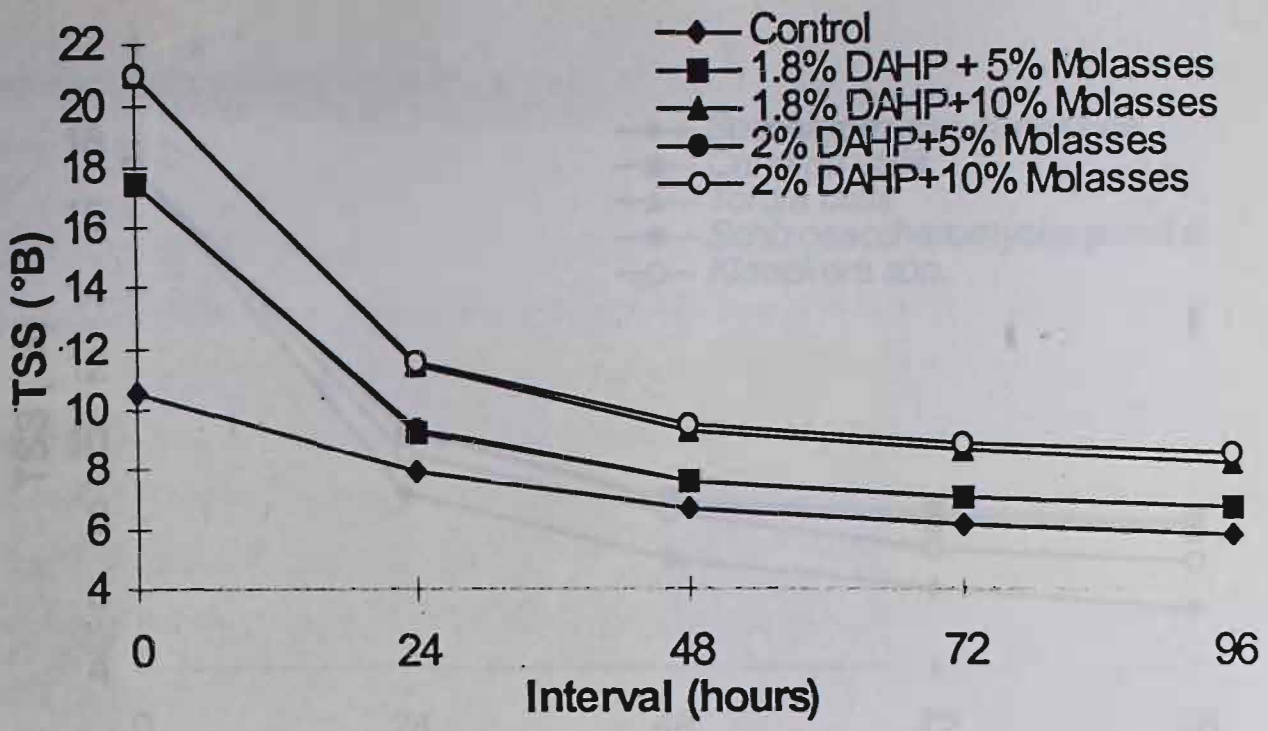


Fig. 4.2.1. Effect of treatments on TSS (°B) during solid state fermentation of apple pomace with DAHP

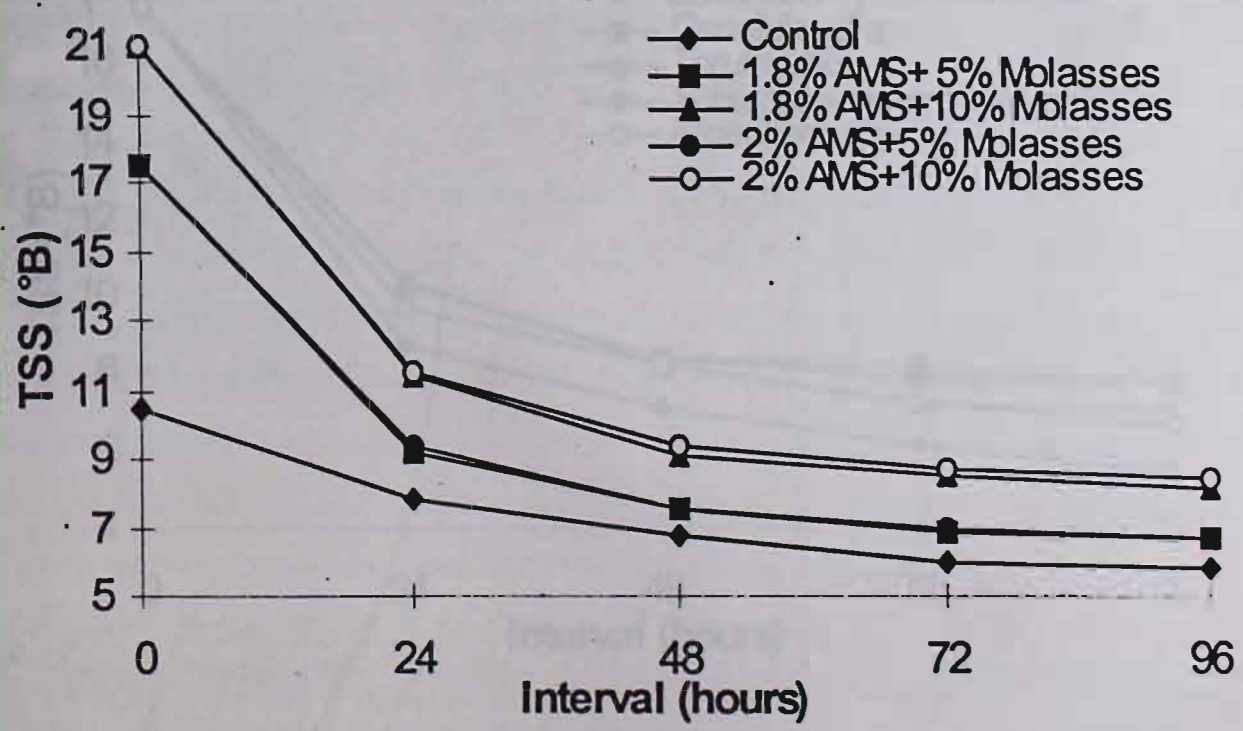


Fig. 4.2.2. Effect of treatments on TSS (°B) during solid state fermentation of apple pomace with AMS

fermentable sugars. Similar trend was observed in TSS consumption in the SSF with either of nitrogen sources.

Effect of yeast spp.: With respect to yeast spp. (Fig. 4.2.3 and 4.2.4) *Saccharomyces cerevisiae* reduced more TSS followed by Kloeckera spp. The lowest reduction in TSS was obtained in *Candida utilis* fermented apple pomace. Reduction of more TSS by *Saccharomyces cerevisiae* and *Kloeckera* spp. is attributed to higher fermentability and adaptation as explained earlier for rate of fermentation. The trends of TSS consumption by different yeasts were similar in the SSF of apple pomace with DAHP and AMS.

Ethanol

Effect of treatment: The ethanol content measured at 48 and 96 hrs are depicted in Fig. 4.2.5 and 4.2.6. Virtually, the highest ethanol was produced throughout the fermentation in apple pomace blended with 10 per cent molasses and 1.8 per cent of nitrogen source and the lowest ethanol was produced in control. Similar ethanol production trend was observed in the SSF of apple pomace with both the nitrogen sources. Addition of molasses and nitrogen source has increased the fermentable sugar and available nitrogen content for the microbial growth and consequently increased ethanol production. However, the ethanol production by addition of either of nitrogen source was similar.

The results obtained on average ethanol content of the fermented apple pomace after 96 hrs of SSF results are presented in the Table 4.2.2. It is revealed that the addition of molasses and nitrogen source significantly (statistically) increased the ethanol production as compared to control. The average highest ethanol concentration (6.015% v/v) was produced in apple pomace ameliorated with 10 per cent molasses and 1.8 per cent of nitrogen source. The lowest average ethanol concentration (2.161% v/v) was produced in the control. There was no significant difference between the two nitrogen sources (DAHP or AMS) for this parameter.

Effect of yeast spp.: Among the yeast spp. (Fig. 4.2.7 and 4.2.8) *Saccharomyces cerevisiae* had the highest ethanol production followed by *Kloeckera* spp. both at 48 and 96 hrs of SSF of apple pomace. The lowest ethanol content was produced by *Torula utilis*. Production of highest

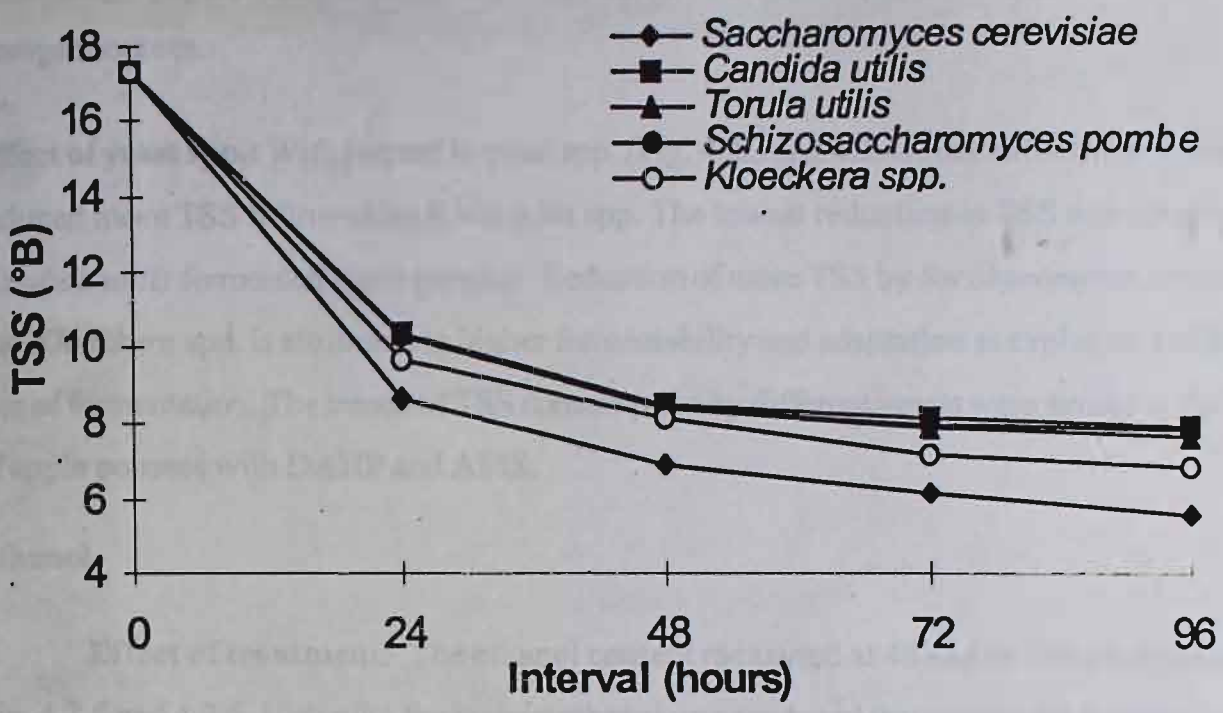


Fig. 4.2.3. Effect of different yeasts on TSS (°B) during solid state fermentation of apple pomace with DAHP

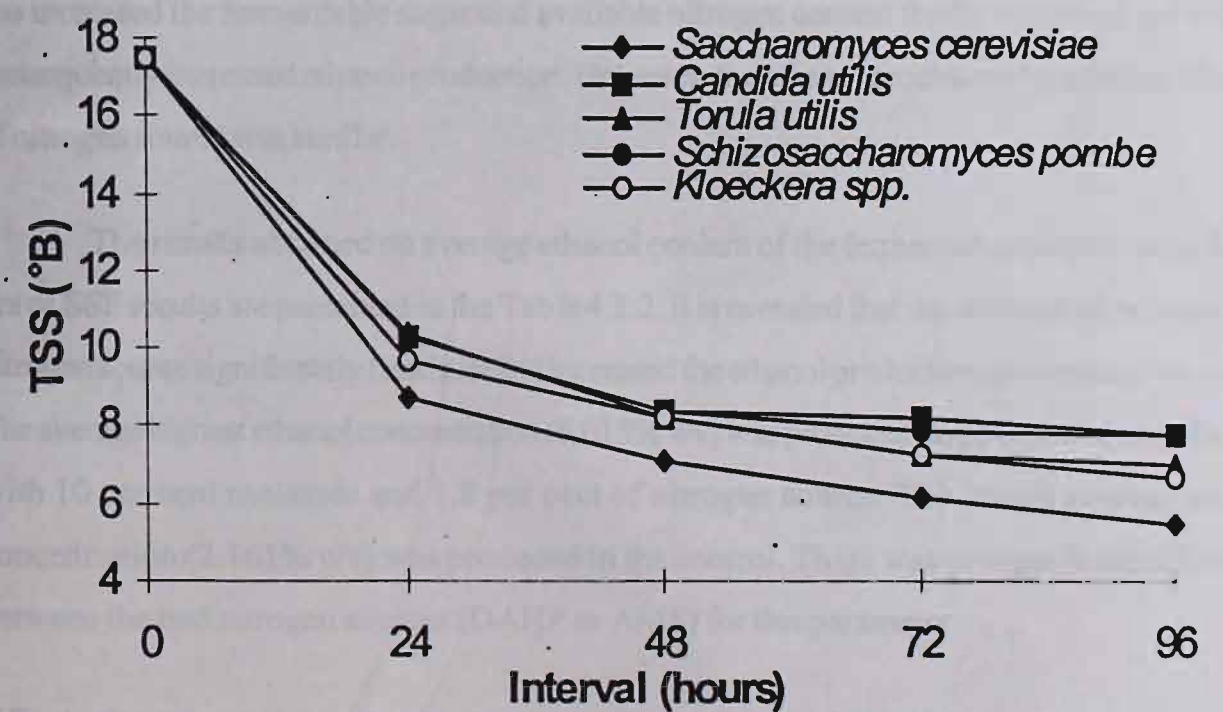


Fig.4.2.4. Effect of different yeasts on TSS (°B) during solid state fermentation of apple pomace with AMS

Table 4.2.2. Effect of treatments and yeast species on mean ethanol content of apple pomace after 96 hrs of SSF

Yeast spp. / Treatments	*Saccharomyces cerevisiae*	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
T_1 (Control)	2.260	2.057	2.090	2.217	2.180	2.161
T_2 (1.8% NS + 5% Molasses)	5.347	4.782	4.452	4.372	4.842	4.759
T_3 (1.8% NS + 10% molasses)	6.375	6.130	5.717	5.740	6.115	6.015
T_4 (2.0% NS + 5% molasses)	5.737	4.972	4.512	4.612	5.052	4.977
T_5 (2.0%NS+10% molasses)	6.287	5.950	5.707	5.620	6.097	5.932
Mean	5.201	4.778	4.496	4.512	4.857	

Nitrogen source	DAHP	AMS
Mean	4.771	4.767
CD (0.05)	ns	

Effect	CD (0.05)
Treatments	0.05
Yeast spp.	0.05
Treatment x Yeast spp.	0.10

DAHP - Diammonium hydrogen phosphate
AMS - Ammonium sulphate
NS - Nitrogen source
ns - Non-significant

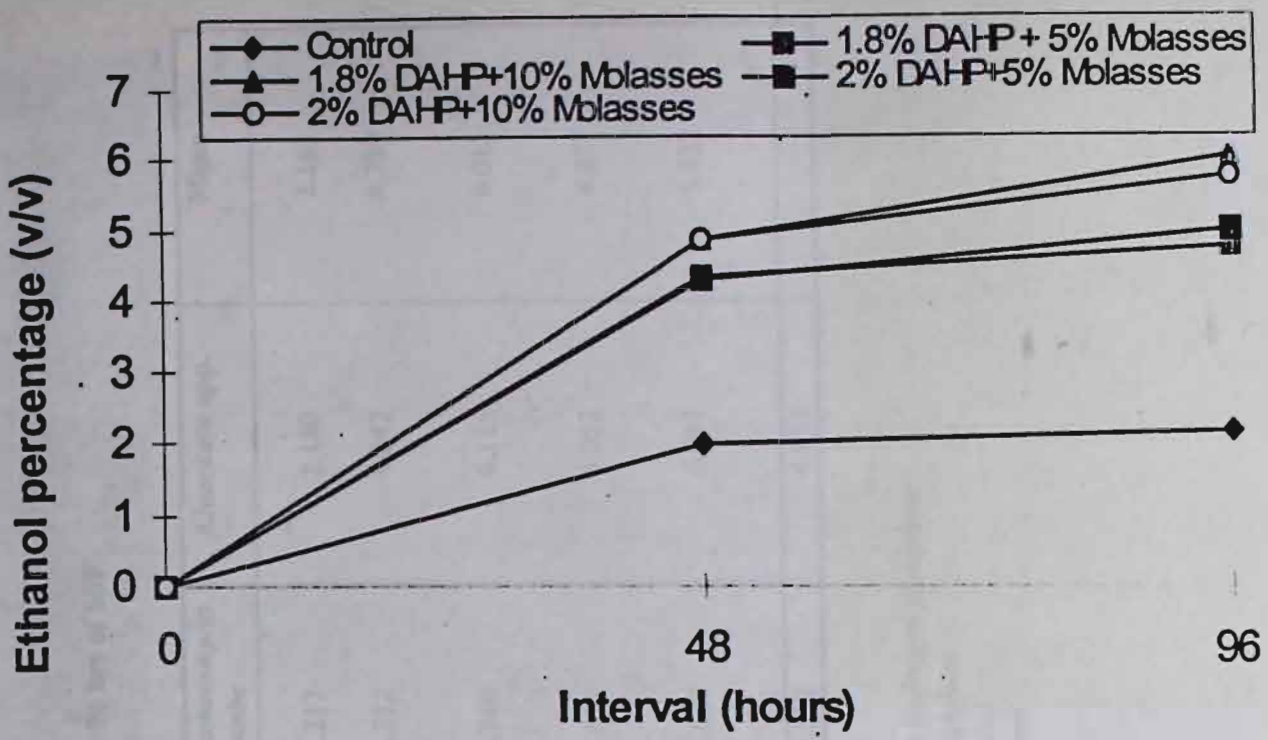


Fig. 4.2.5. Effect of treatments on ethanol content (%v/v) during solid state fermentation of apple pomace with DAHP

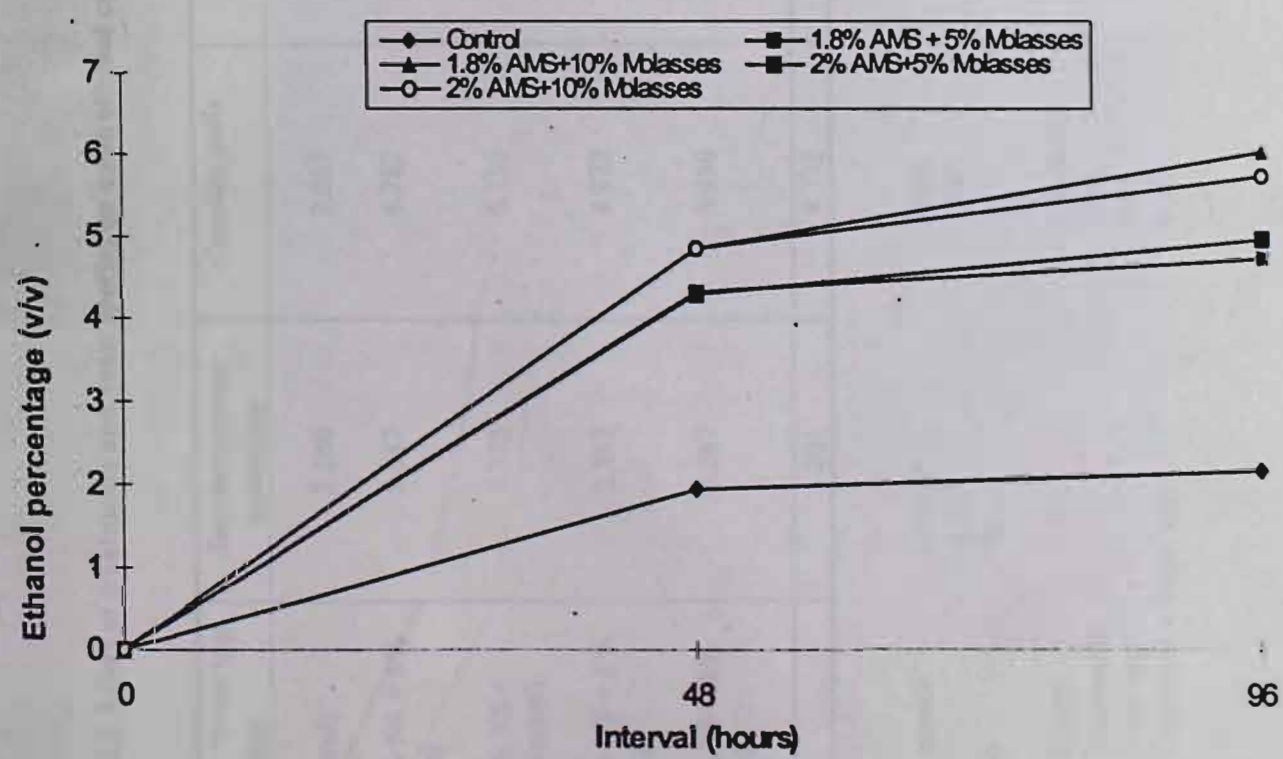


Fig. 4.2.6. Effect of treatments on ethanol content (%v/v) during solid state fermentation of apple pomace with AMS

ethanol by *Saccharomyces cerevisiae* is due to highest fermentability and by *Kloeckera* spp. due to better adoption being a natural microflora of the substrate as described earlier. Since the objective is to produce highest possible ethanol in SSF these yeasts hold promise for use in SSF of apple pomace. Similar characteristics of yeasts have also been reported earlier (Sandhu and Joshi, 1997b; Hodge *et al.* 1990). Addition of both either of nitrogen source (DAHP or AMS) ethanol production in a similar manner.

With respect to yeast spp. after 96 hrs of SSF of apple pomace (Table 4.2.2) *Saccharomyces cerevisiae* produced average highest ethanol (5.20% v/v) in the fermented apple pomace followed by *Kloeckera* spp. (4.86% v/v) fermented apple pomace. The lowest ethanol was produced by *Torula utilis* (4.496% v/v) fermented apple pomace. The interaction between the treatments and yeast species was found to be significant. Amongst the interactions, apple pomace blended with 10 per cent molasses and 1.8 per cent nitrogen source and fermented with *Saccharomyces cerevisiae* had the highest ethanol content followed by apple pomace blended with 10 per cent molasses and 2 per cent nitrogen source with the same yeast species. The lowest ethanol production was observed in the control treatment with *Candida utilis* fermented apple pomace.

Out of the various treatments tried, apple pomace blended with 10% molasses and 1.8% of nitrogen source (either (DAHP or AMS) with *Saccharomyces cerevisiae* followed by *Kloeckera* spp. proved to be the best treatment for SSF of apple pomace. The results discussed so far indicate that the increase in fermentation time decreased TSS and increased the ethanol content upto 96 hrs. But the highest reduction of TSS and production of ethanol rates were observed within 24 hrs. Throughout the fermentation period, apple pomace blended with 10 per cent of molasses and 1.8 per cent either of nitrogen source (DAHP or AMS) recorded higher reduction in TSS and highest ethanol production with *Saccharomyces cerevisiae*.

Titratable Acidity and pH

Effect of treatment: The changes in titratable acidity during fermentation by different yeasts treatments (Fig 4.2.9 and 4.2.10) clearly show that the addition of molasses increased the titratable

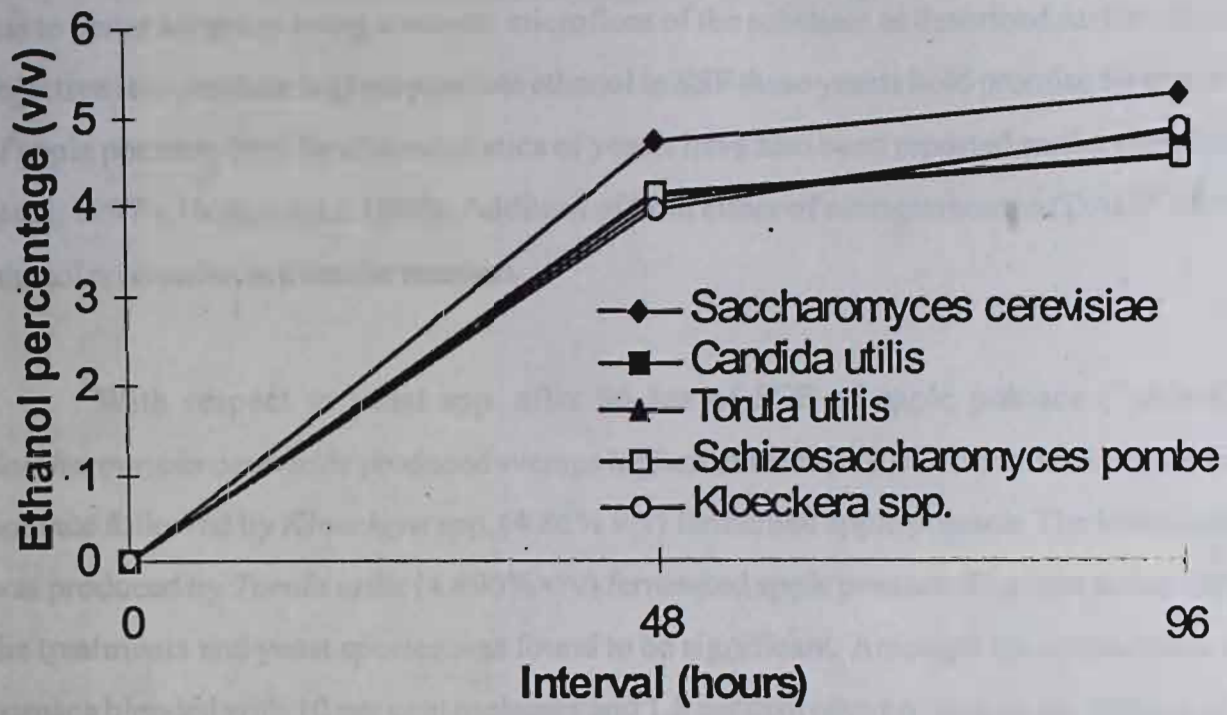


Fig. 4.2.7.Effect of different yeasts on ethanol (%v/v) content during soiid state fermentation of apple pomace with DAHP

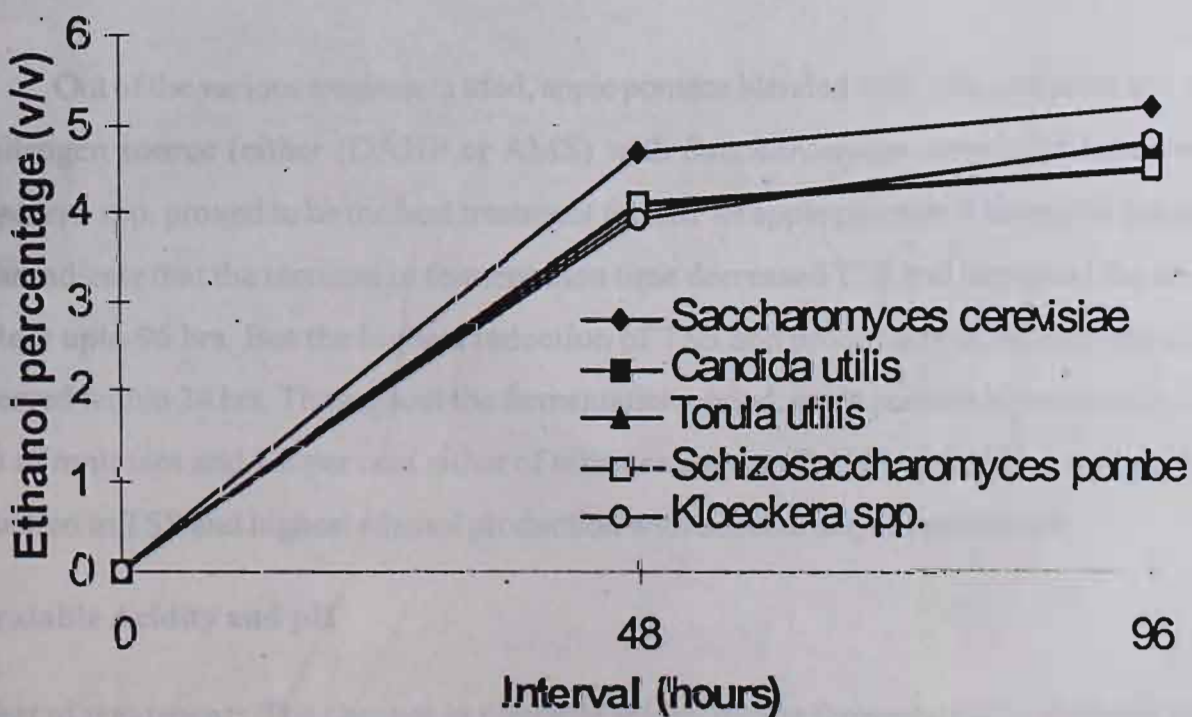


Fig.4.2.8. Effect of different yeasts on ethanol (% v/v) content during solid state fermentation of apple pomace with AMS

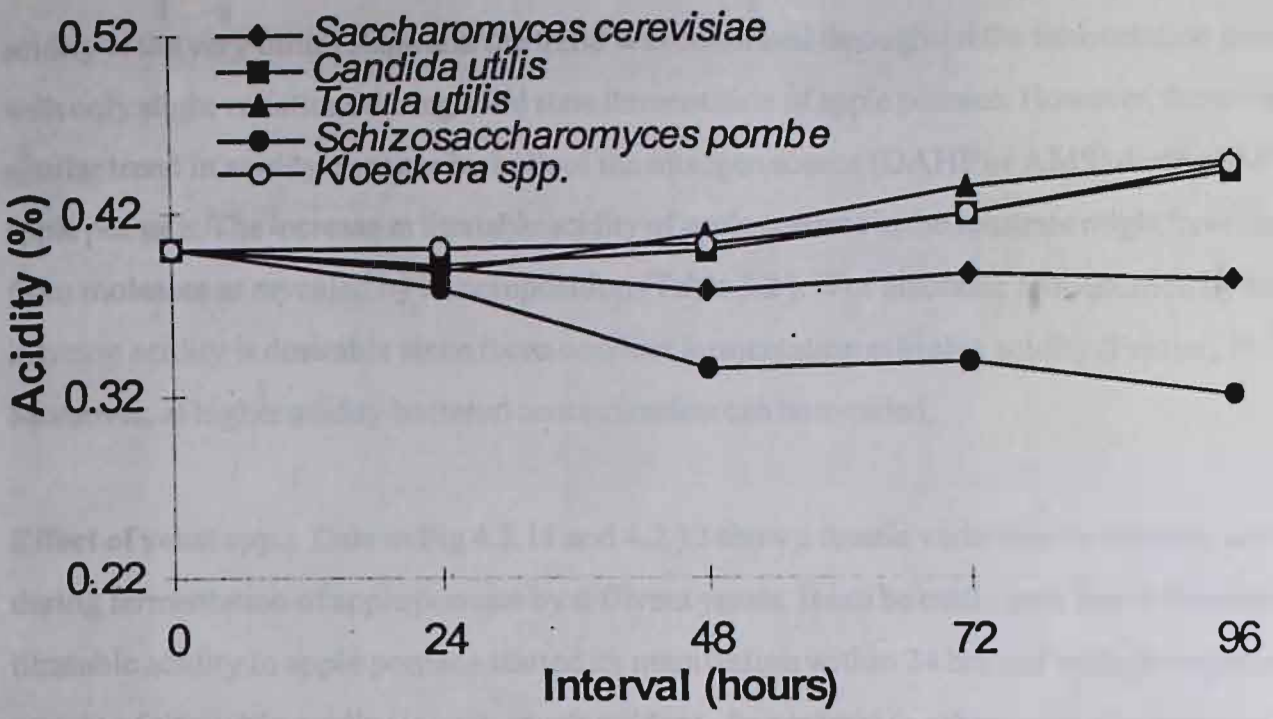


Fig.4.2.11. Effect of yeasts on titratable acidity (as malic acid) during solid state fermentation of apple pomace with DAHP

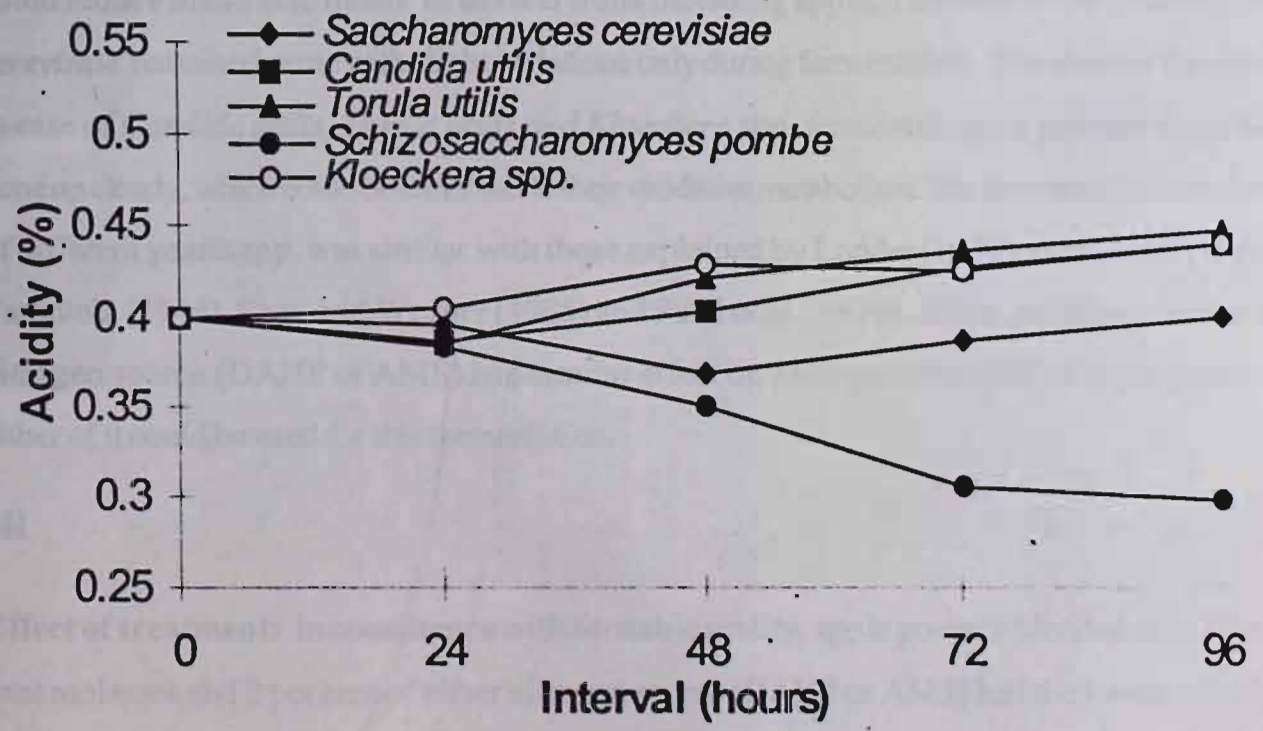


Fig. 4.2.12. Effect of yeasts on titratable acidity (as malic acid) during solid state fermentation of apple pomace with AMS

acidity at the very initial stage and the trend was continued throughout the fermentation period with only slight variations during solid state fermentation of apple pomace. However, there was a similar trend in acidity changes by both of the nitrogen source (DAHP or AMS) during SSF of apple pomace. The increase in titratable acidity of apple pomace in the substrate might have come from molasses as revealed by its composition (Table 3.2). For alcoholic fermentation by yeast increase acidity is desirable since it can conduct fermentation at higher acidity (Frazier, 1979). Moreover, at higher acidity bacterial contamination can be avoided.

Effect of yeast spp.: Data in Fig 4.2.11 and 4.2.12 show a drastic variations in titratable acidity during fermentation of apple pomace by different yeasts. It can be easily seen that differences in titratable acidity in apple pomace started its manifestion within 24 hrs and wide divergence in curves of titratable acidity is very much evident. In contrast to other yeast, the curve of the *Schizosaccharomyces pombe* fermented apple pomace showed a decrease from the initial acidity stage and continued to decrease upto 96 hrs. It is understandable due to its ability for conversion of malic acid into ethanol. Azad *et al.* (1986) also reported that *Schizosaccharomyces pombe* could reduce malic acid musts of several fruits including apple. The trend of *Saccharomyces cerevisiae* remained same with slight variations only during fermentation. The slope of the curve in case of *Candida utilis, Torula utilis and Kloeckera* spp. fermented apple pomace slope had gone up clearly, which could be attributed to their oxidative metabolism. The fermentation behaviour of different yeasts spp. was similar with those explained by Lodder (1978), Azad *et al.* (1986), Tarantola (1946), Shay and Wegner (1985) and Patel *et al.* (1979). Since, addition of either of nitrogen source (DAHP or AMS) had similar effect on acidity during SSF of apple pomace, either of it could be used for this fermentation.

pH

Effect of treatment: In consistence with titratable acidity, apple pomace blended with 10 per cent molasses and 2 per cent of either nitrogen source (DAHP or AMS) had the lowest pH (Fig 4.2.13 and 4.2.14) and the highest was recorded in the control. However, compared to the difference in acidity, the variations in pH values of different treatments were inappreciable. There was only slight difference in pH of DAHP and AMS blended apple pomace. This might be due

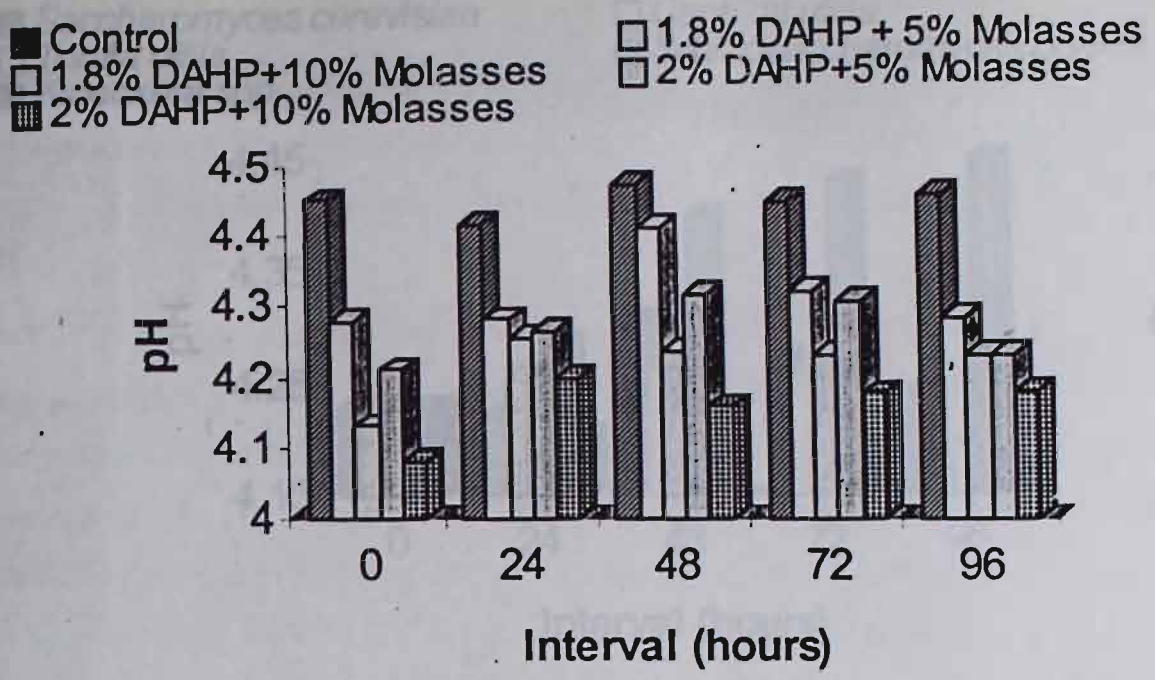


Fig. 4.2.13. Effect of treatments on pH during solid state fermentation of apple pomace with DAHP

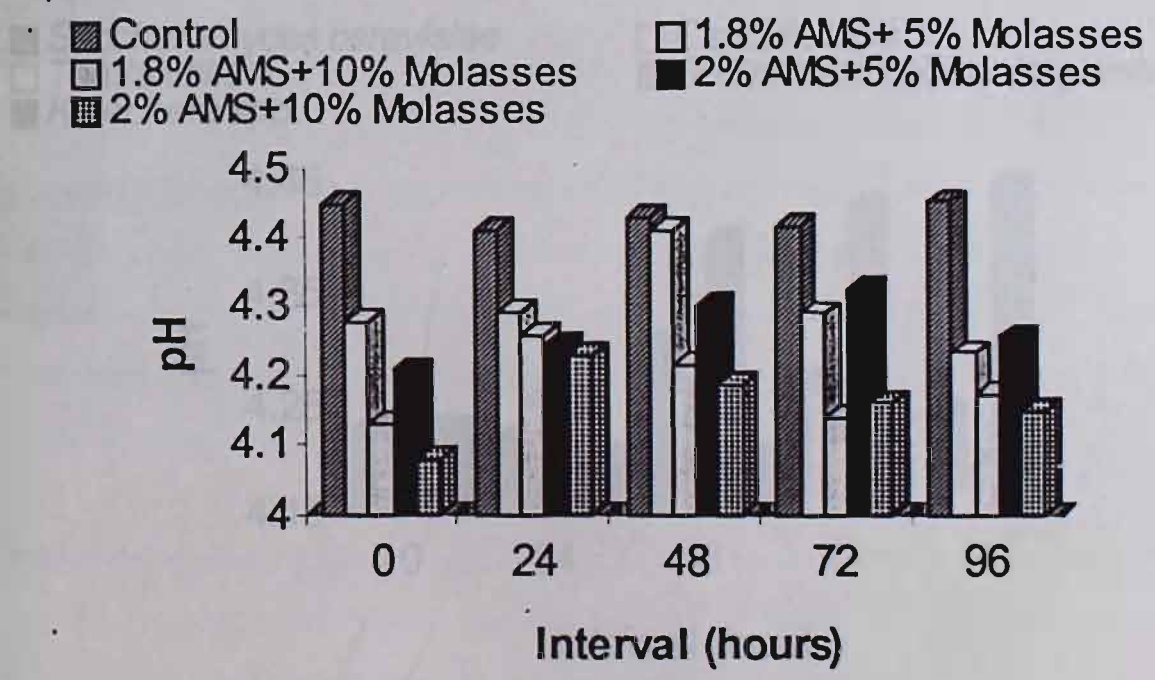


Fig. 4.2.14.Effect of treatments on pH during solid state fermentation of apple pomace with AMS

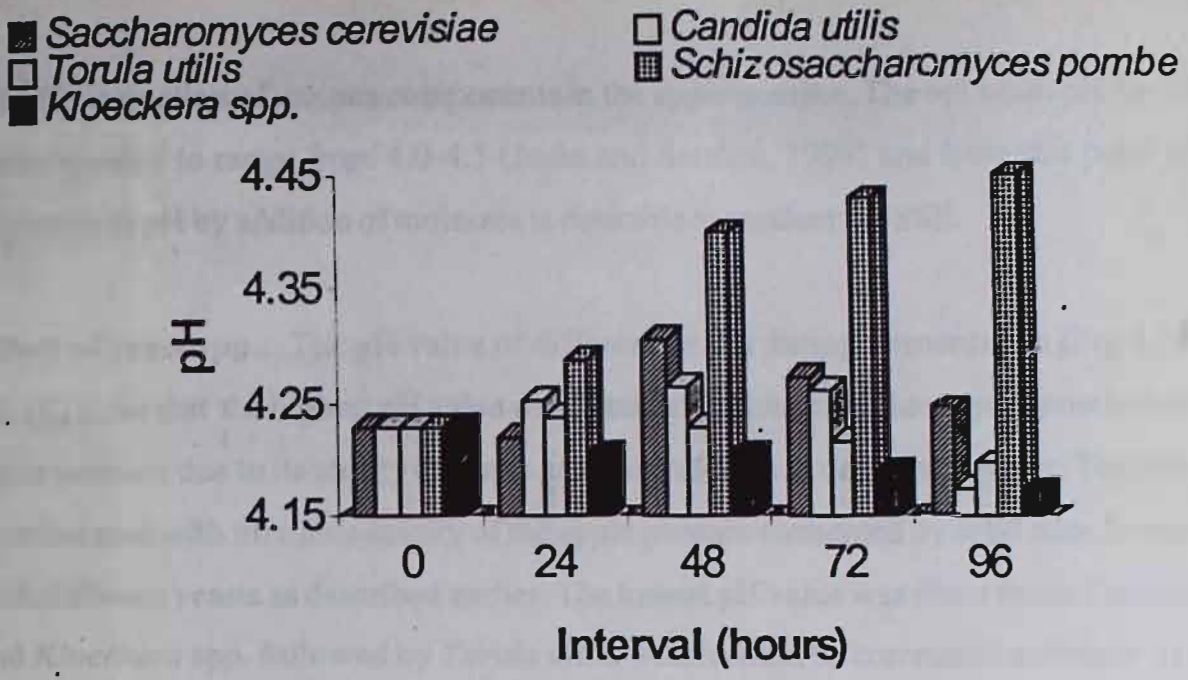


Fig. 4.2.15.Effect of different yeasts on pH during solid state fermentation of apple pomace with DAHP

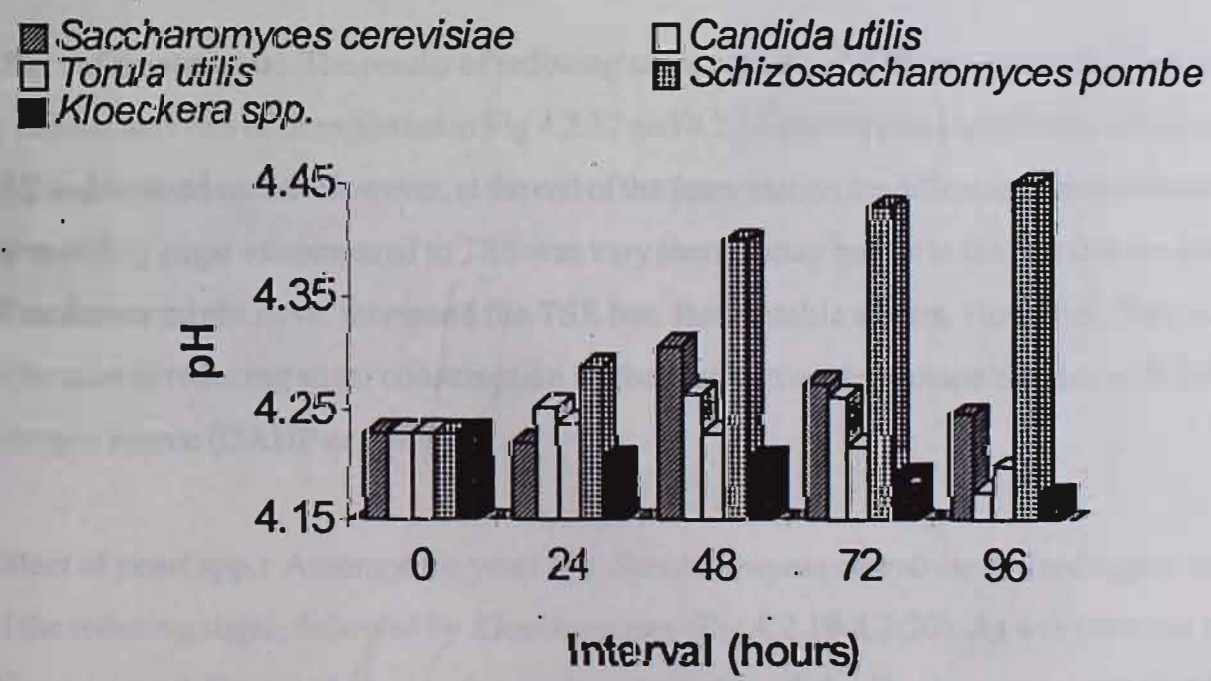


Fig. 4.2.16. Effect of different yeasts on pH during solid state fermentation of apple pomace with AMS

to buffering action of various components in the apple pomace. The optimum pH for SSF has been reported to range from 4.0-4.5 (Joshi and Sandhu, 1994) and from this point of view reduction in pH by addition of molasses is desirable to conduct the SSF.

Effect of yeast spp.: The pH value of different yeasts during fermentation (Fig 4.2.15 and 4.2.16) show that the highest pH value was obtained in *Schizosaccharomyces pombe* fermented apple pomace due to its ability of malic acid degradation as described earlier. The pH values corroborated with titratable acidity of the apple pomace fermented by solid state fermentation with different yeasts as described earlier. The lowest pH value was observed in *Candida utilis* and *Kloeckera* spp. followed by *Torula utilis* which could be correlated with their oxidative fermentation pattern which resulting in the production of acid which would naturally lower down the pH. *Saccharomyces cerevisiae* had the same initial pH value with only a slight variation during the fermentation period. There was similar trend in pH values of apple pomace blended with either of nitrogen source (DAHP or AMS) and is desirable since their addition would not affect the fermentation adversely.

Reducing sugars

Effect of treatments: The results of reducing sugar content of different treatments determined at various intervals of time plotted in Fig 4.2.17 and 4.2.18 show a trend which was similar to that TSS as discussed earlier. However, at the end of the fermentation the difference between treatments for reducing sugar as compared to TSS was vary narrow may be due to the fact that the addition of molasses might have increased the TSS but, fermentable sugars. However, there was no difference in reducing sugar consumption by the yeasts in apple pomace blended with either of nitrogen source (DAHP or AMS).

Effect of yeast spp.: Amongst the yeast spp. *Saccharomyces cerevisiae* utilized higher amount of the reducing sugar, followed by *Kloeckera spp.* (Fig 4.2.19-4.2.20). As was the trend earlier there was no difference between the apple pomace blended with nitrogen source (DAHP or AMS).

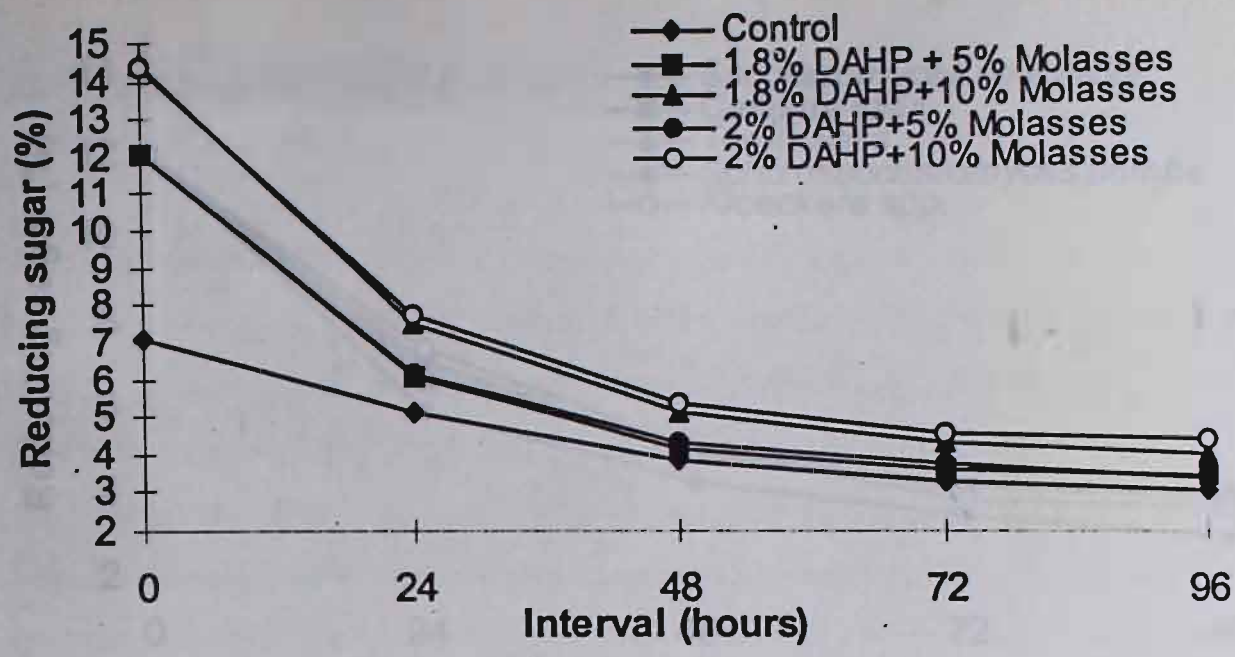


Fig. 4.2.17. Effect of treatments on reducing sugars (%) during solid state fermentation of apple pomace with DAHP

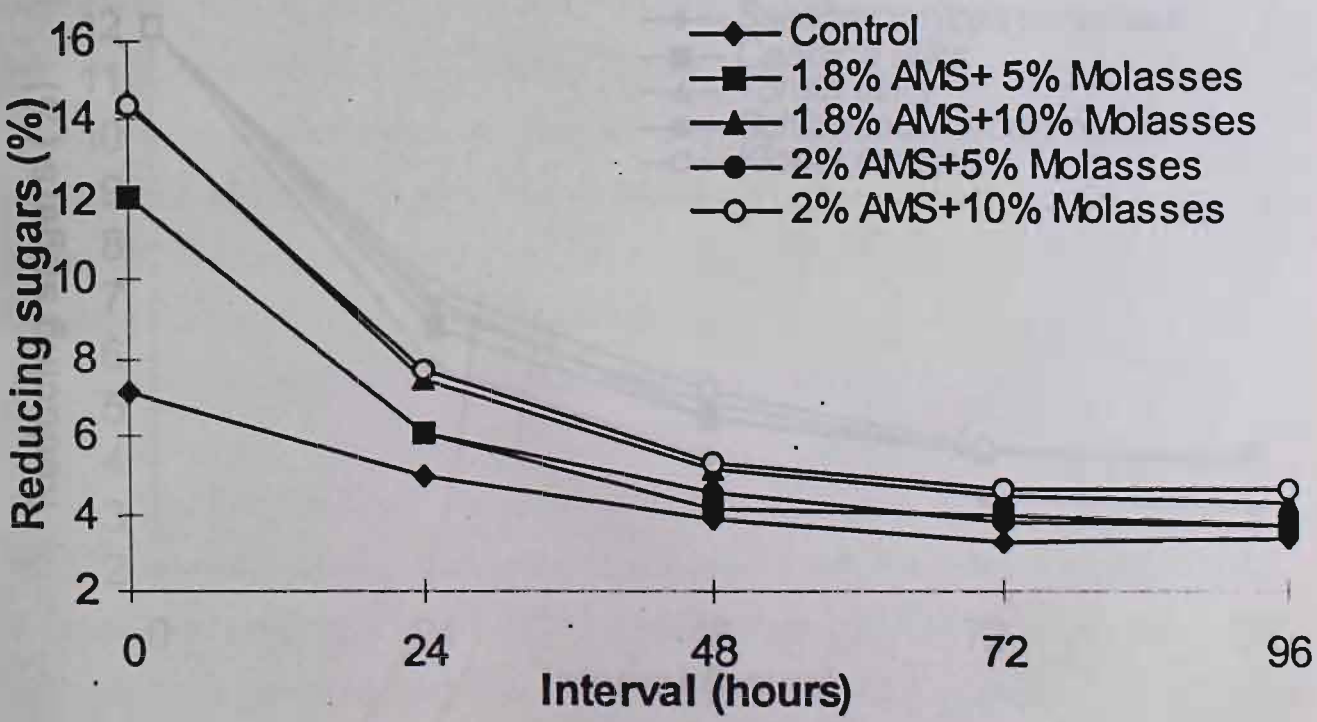


Fig. 4.2.18. Effect of treatments on reducing sugars (%) during solid state fermentation of apple pomace with AMS

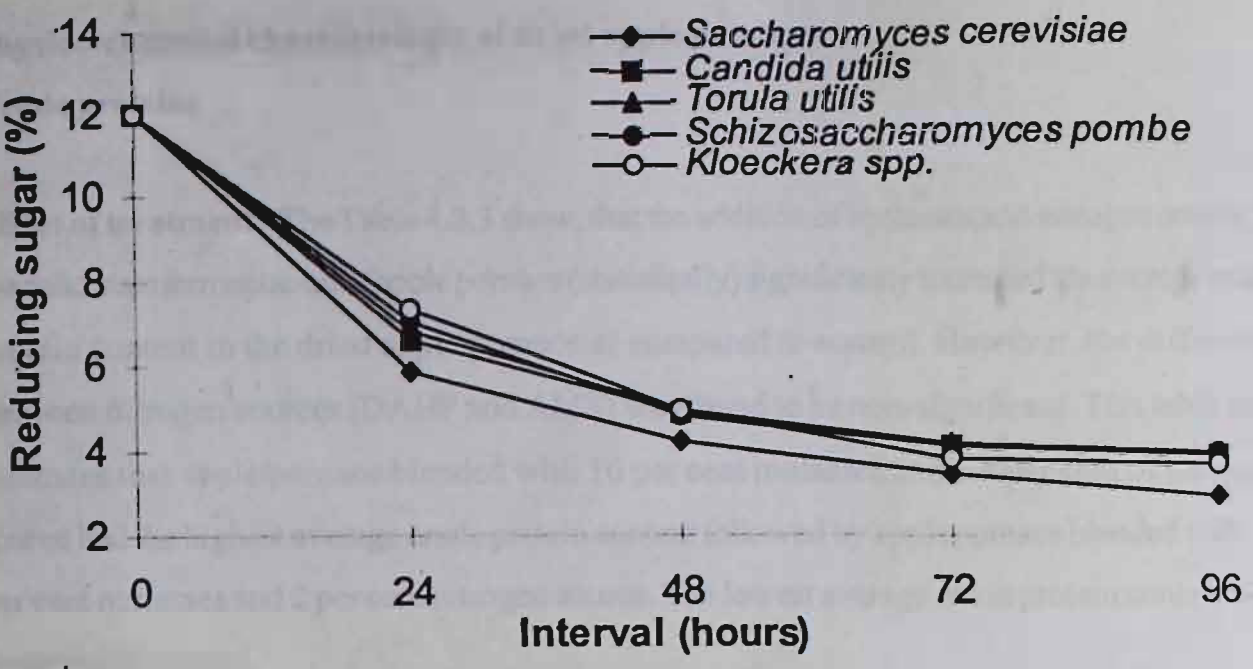


Fig.4.2.19. Effect of different yeasts on reducing sugars (%) during solid state fermentation of apple pomace with DAHP

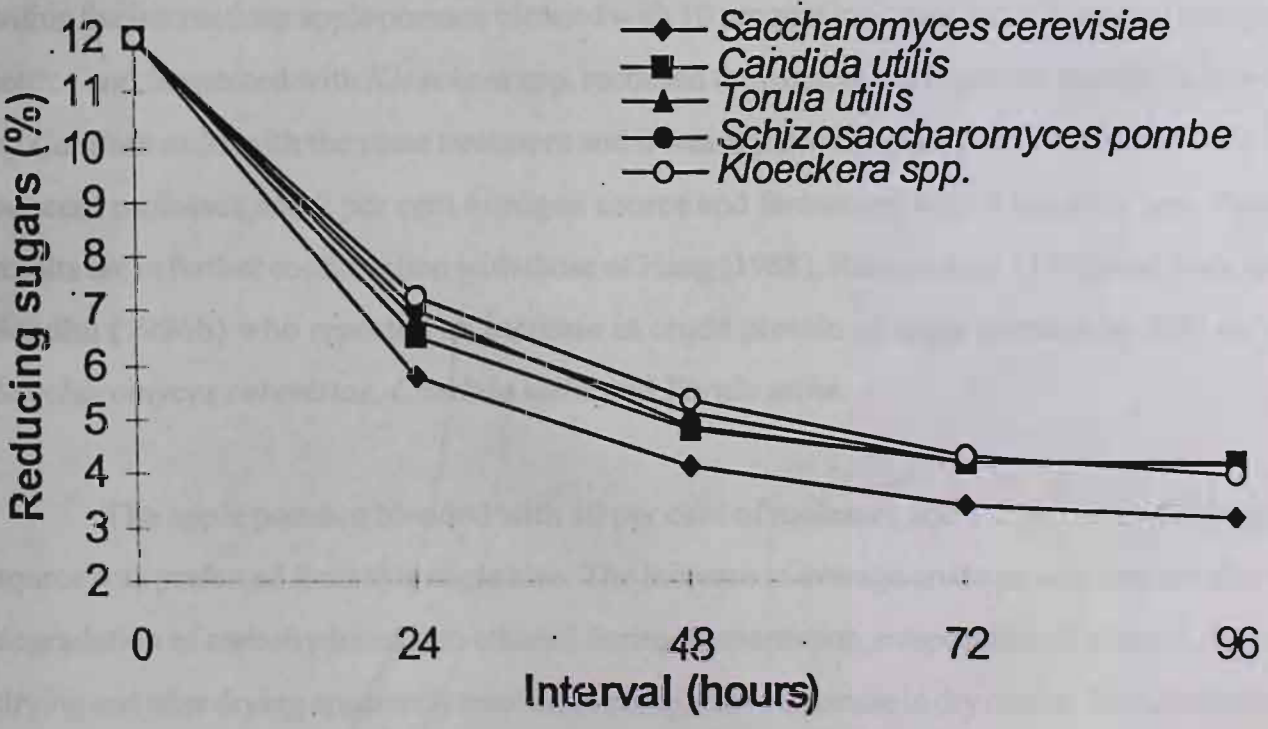


Fig. 4.2.20. Effect of different yeasts on reducing sugars (%) during solid state fermentation of apple pomace with AMS

Physico-chemical characteristics of dried apple pomace

Crude proteins

Effect of treatment: The Table 4.2.3 show, that the addition of molasses and nitrogen source to the solid state fermentation of apple pomace (statistically) significantly increased the average crude protein content in the dried apple pomace as compared to control. However, the difference between nitrogen sources (DAHP and AMS) was found to be non-significant. This table also indicates that apple pomace blended with 10 per cent molasses and 1.8 per cent of nitrogen source had the highest average crude protein content followed by apple pomace blended with 10 per cent molasses and 2 per cent nitrogen source. The lowest average crude protein content was observed in control.

Effect of yeast spp.: Amongst the yeast spp., *Kloeckera* spp. had the highest average crude protein content followed by *Candida utilis* fermented and dried apple pomace. The lowest average crude protein content was observed in *Schizosaccharomyces pombe* fermented and dried apple pomace. The interaction between treatments and yeast species was found to be significant and within the interactions apple pomace blended with 10 per cent molasses and 1.8 per cent nitrogen source and fermented with *Kloeckera* spp. recorded the highest crude protein content followed by *Candida utilis* with the same treatment and it was at par with apple pomace blended with 10 per cent molasses and 2 per cent nitrogen source and fermented with *Kloeckera* spp. These results are in further confirmation with those of Hang (1988), Rahmat *et al.* (1995) and Joshi and Sandhu (1996b) who reported an increase in crude protein of apple pomace by SSF using *Saccharomyces cerevisiae, Candida utilis* and *Torula utilis*.

The apple pomace blended with 10 per cent of molasses and 1.8 per cent of nitrogen source was preferred from this angle also. The increase in average crude protein content due to degradation of carbohydrates into ethanol during fermentation, evaporation of ethanol, during drying and after drying apparently resulted in comparative decrease in dry matter. Thus, reduction in dry matter considerably increased their constituents including nitrogen resulting in an increase in crude protein contents as reported earlier (Hang, 1988; Joshi and Sandhu, 1996b; Rahmat,

Table 4.2.3. Effect of treatments and yeast species on mean crude protein content of fermented and dried apple pomace

Yeast spp. / Treatments	*Saccharomyces cerevisiae*	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
T_1 (Control)	5.757	5.792	5.740	5.667	5.860	5.763
T_2 (1.8% NS + 5% Molasses)	18.35	20.43	17.52	17.78	21.60	19.14
T_3 (1.8% NS + 10% molasses)	23.62	25.41	23.47	22.29	26.37	24.23
T_4 (2.0% NS + 5% molasses)	20.01	20.93	18.32	17.54	21.42	19.64
T_5 (2.0%NS+10% molasses)	22.51	24.66	22.47	20.66	25.36	23.13
Mean	18.05	19.45	17.50	16.79	20.12	

Nitrogen source	DAHP	AMS
Mean	18.39	18.38
CD (0.05)	ns	

DAHP - Diammonium hydrogen phosphate
AMS - Ammonium sulphate
NS - Nitrogen source
ns - Non-significant

Effect	CD (0.05)
Treatments	0.14
Yeast spp.	0.14
Treatment x Yeast spp.	0.31

1995). Some contribution to the nitrogen levels might also be the result of yeast growth in the apple pomace which is known to be a good source of protein (Gastaineu *et al.*, 1979). In the past microbial fermentations have been used to upgrade the protein content of foods such as those rich in carbohydrates (Raimbault *et al.*, 1985) and therefore, it could become a powerful tool to upgrade the nutrition level of apple pomace as well as observed earlier also (Bhalla and Joshi, 1994; Hang, 1988).

Reducing sugar

Effect of treatment: Treatment (Table 4.2.4) revealed that the addition of molasses and nitrogen source statistically significantly enhanced the residual sugar content in the dried apple pomace as compared to control. In between the treatments the reducing sugar content of dried apple pomace ranged from 12.19 to 17.44 per cent. The highest reducing sugar content was observed in apple pomace blended with 10 per cent molasses and 2 per cent of nitrogen source. The lowest reducing and total sugar content was observed in control. The difference between the nitrogen sources (DAHP or AMS) was found to be non-significant.

Effect of yeast: Regarding yeast spp *Saccharomyces cerevisiae* had the lowest reducing sugars content followed by *Kloeckera* spp. in the dried and fermented apple pomace due to the higher fermentability. Highest reducing sugar content was observed in *Schizosaccharomyces pombe* fermented apple pomace while that of *Candida utilis* and *Torula utilis* fermented and dried apple pomace were at par. The interaction between the treatments and yeast species was found to be significant. Within the interactions, apple pomace blended with 5 per cent molasses and 2.0 per cent of nitrogen source and *Saccharomyces cerevisiae* fermented apple pomace recorded the lowest average reducing sugar content and the highest was observed in apple pomace blended with 10 per cent molasses and 2 per cent nitrogen source and fermented with *Kloeckera* spp. and it was at par with apple pomace blended with 1.8 per cent nitrogen source and 10 per cent molasses and fermented with *Schizosaccharomyces pombe.* The higher quantities of reducing sugar content in fermented dried apple pomace compared to the control is due to presence of residual sugar at the end of the fermentation, which increased further due to drying.

Table 4.2.4. Effect of treatments and yeast species on mean reducing sugar content of fermented and dried apple pomace

Yeast spp. / Treatments	*Saccharomyces cerevisiae*	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
T_1 (Control)	11.10	12.46	13.21	11.91	12.27	12.19
T_2 (1.8% NS + 5% Molasses)	9.82	15.22	14.40	15.44	13.08	13.59
T_3 (1.8% NS + 10% molasses)	13.84	16.49	17.04	18.57	15.32	16.25
T_4 (2.0% NS + 5% molasses)	9.32	15.59	14.66	15.27	13.07	13.58
T_5 (2.0%NS+10% molasses)	14.19	17.76	18.16	18.35	18.73	17.44
Mean	11.66	15.51	15.49	15.91	14.49	

Nitrogen source	DAHP	AMS
Mean	14.60	14.62
CD (0.05)	ns	

DAHP - Diammonium hydrogen phosphate
AMS - Ammonium sulphate
NS - Nitrogen source
ns - Non-significant

Effect	CD (0.05)
Treatments	0.11
Yeast spp.	0.11
Treatment x Yeast spp.	0.25

Based upon the parameters discussed in this section reduction in TSS, rate of fermentation, ethanol content in the fermented apple pomace and crude protein content of the fermented dried apple pomace, the apple pomace mixed with 10 per cent of molasses and 1.8 per cent of nitrogen source performed well amongst the treatments. Out of the yeast spp. tried *Saccharomyces cerevisiae* was the best with respect to all the parameters discussed except for crude protein content in the fermented dried apple pomace where *Kloeckera* ranked the highest.

Increase in crude protein in apple pomace alongwith the ethanol production by fermentation appear to be right approach for utilization of this waste fermentatively. However, these results suggest that in a single yeast fermentation both the highest crude protein content of dried apple pomace as well as highest ethanol is as concomitant production of ethanol and animal feed might not be feasible. May be their interactive co-culture may improve one or other parameters i.e. ethanol and crude protein to the extent to serve as animal feed or sources of ethanol production economically. But there is no report available on sequential interactive co-culture of yeast spp. in solid state fermentation of apple pomace. However, Lammel *et al.* (1979) suggested the two stage continuous fermentation which would cultivate *Saccharomyces fibulgier* in the first stage and the second stage with *Candida utilis* would be predominant. The best treatments (10% molasses with 1.8% AMS) was selected on the basis of high ethanol and crude protein content for further interactive co-culture experiment

4.3 EFFECT OF SEQUENTIAL INTERACTIVE CO-CULTURE SYSTEM ON SSF APPLE POMACE

Total soluble solids: The data relating to change in TSS of different treatments are depicted in Fig. 4.3.1. As expected the TSS declined upto 96 hrs by all the yeasts and after addition of sequential culture there was no appreciable decrease in TSS content in all the cases. The maximum reduction in TSS was observed in *Saccharomyces cerevisiae* with its sequential cultures, followed by *Kloeckera* spp. and its sequential cultures. It indicates that the available nutrients except the metabolites of first fermentation (ethyl alcohol) and sugar in the form of TSS have been completely utilized by the initial cultures.

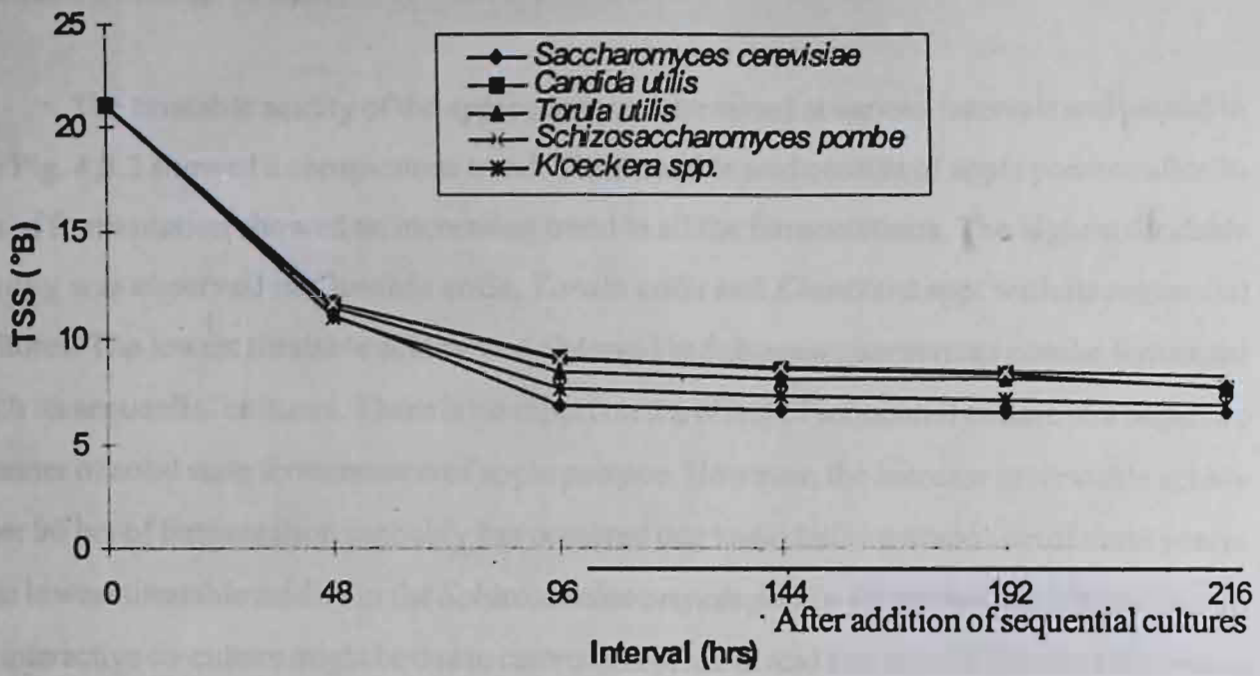


Fig. 4.3.1. Effect of sequential interactive co-cultures on TSS (°B) during SSF of apple pomace

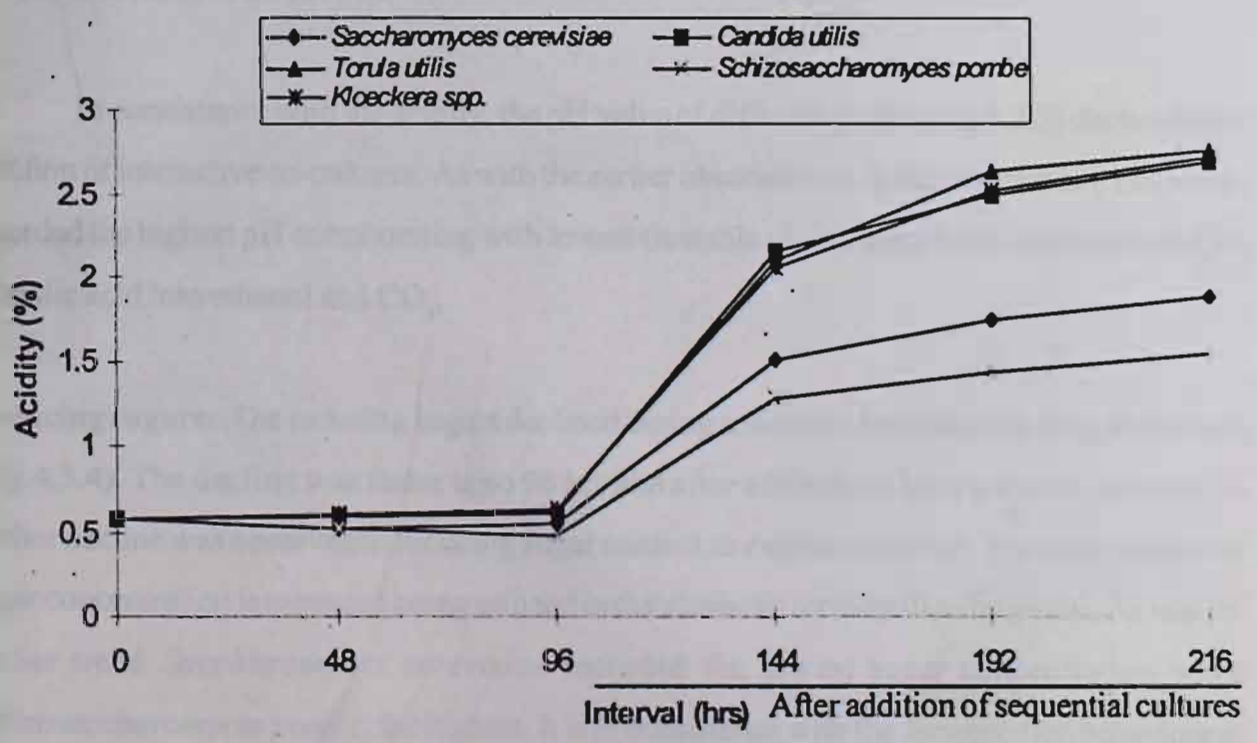


Fig. 4.3.2. Effect of sequential interactive co-cultures on acidity (% as acetic acid) during SSF of apple pomace

Titratable acidity and pH

The titratable acidity of the apple pomace determined at various intervals and plotted in the Fig. 4.3.2 showed a conspicuous trend. The titratable acid content of apple pomace after 96 hrs of fermentation showed an increasing trend in all the fermentations. The highest titratable acidity was observed in *Candida utilis*, *Torula utilis* and *Kloeckera* spp. with its sequential cultures. The lowest titratable acidity was observed in *Schizosaccharomyces pombe* fermented with its sequential cultures. There is no report on the effect of sequential culture in a sequence manner of solid state fermentation of apple pomace. However, the increase in titratable acidity after 96 hrs of fermentation probably has occurred due to oxidative metabolism of these yeasts. The lowest titratable acidity in the *Schizosaccharomyces pombe* fermented apple pomace with its interactive co-culture might be due to conversion of malic acid into ethanol therefore decreasing the acidity. The fermentation behaviour of different yeasts are their inherent characteristics (Lodder, 1978) depending upon the enzymes systems of that particular species. The fermentation behaviour of all the yeast were similar with those of Azad *et al.* (1986), Tarantola (1946), Shay and Wegner (1985) and Patel *et al.* (1979).

In consistence with the acidity, the pH value of different yeasts (Fig 4.3.3) declined after addition of interactive co-cultures. As with the earlier observations, *Schizosaccharomyces pombe* recorded the highest pH corroborating with lowest titratable acidity apparently due to metabolism of malic acid into ethanol and CO_2.

Reducing sugars: The reducing sugars declined during solid state fermentation of apple pomace (Fig 4.3.4). The decline was faster upto 96 hrs and after addition of interactive co-culture, no further decline was observed in reducing sugar content as explained earlier. The reduction in the sugar concentration is expected being utilized in the alcoholic fermentation by yeasts. As was the earlier trend *Saccharomyces cerevisiae* recorded the lowest sugar concentration while *Schizosaccharomyces pombe*, the highest. It is in consistence with the fermentation behaviour of the respective yeasts. The stabilization of curve indicates that virtually the sugar utilization had ceased or indirectly it indicates the non-availability of fermentable sugar in the medium.

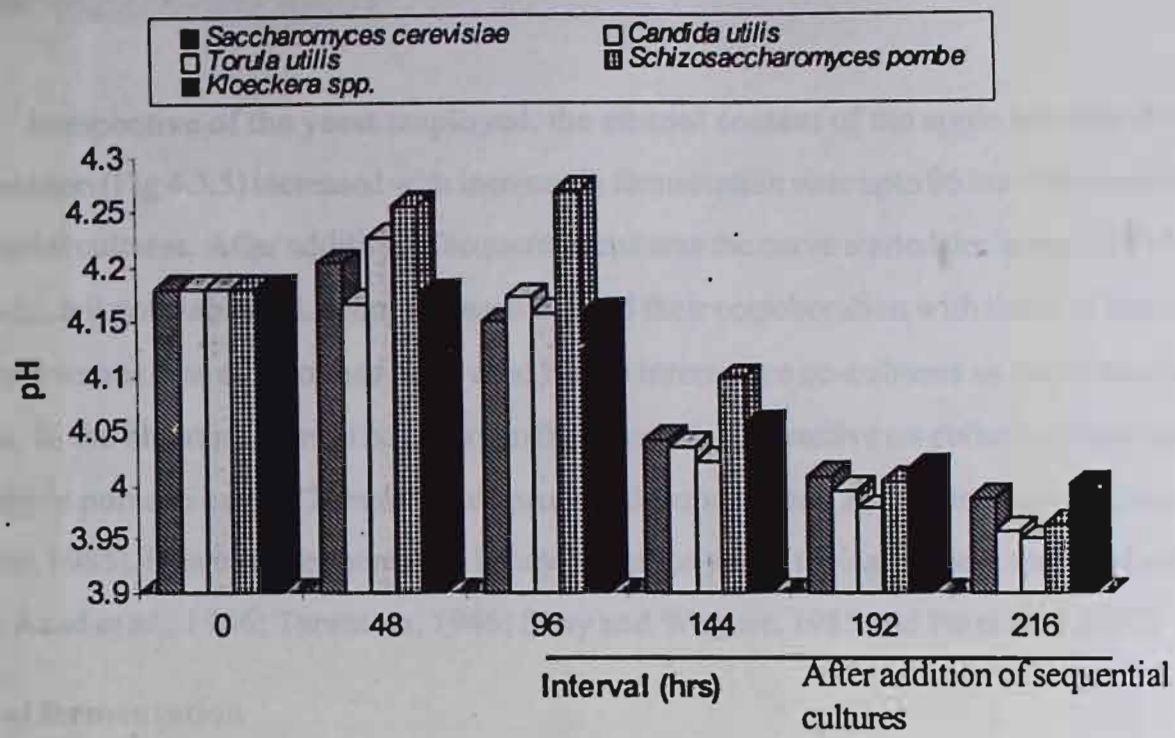


Fig. 4.3.3. Effect of sequential interactive co-cultures on pH during SSF of apple pomace

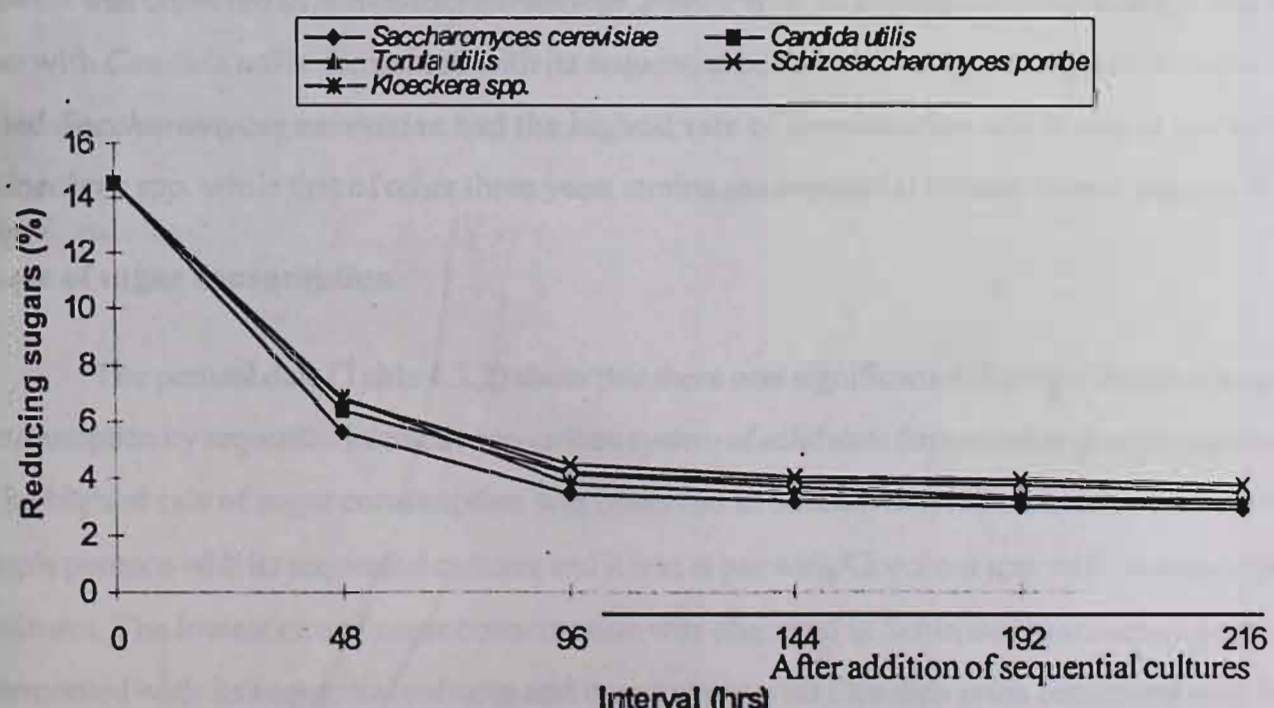


Fig. 4.3.4. Effect of sequential interactive co-cultures on reducing sugars (%) during SSF of apple pomace

Ethanol

Irrespective of the yeast employed, the ethanol content of the apple pomace during fermentation (Fig 4.3.5) increased with increase in fermentation time upto 96 hrs of fermentation with initial cultures. After addition of sequential cultures the curve started declining till 216 hrs after which it got stabilized. From these results and their corroboration with those of titratable acidity it seems that ethanol has been used by the interactive co-cultures as a sole source of carbon. In the literature there is no report on the sequential interactive co-culture of these yeasts with apple pomace except *Torula* yeast, produced from ethanol as carbon source (Shay and Wegner, 1985). However, fermentation behaviour of the yeasts typical of these species (Lodder, 1978; Azad *et al.*, 1986; Tarantola, 1946; Shay and Wegner, 1985 and Patel *et al.*1997)

Rate of fermentation

The results show that (Table 4.3.1) there was a significant difference in rate of fermentation of SSF of apple pomace by sequential interactive co-culture system. But the interaction between initial and sequential culture was found to be non-significant. Significantly highest rate of fermentation was observed in *Saccharomyces cerevisiae* with its sequential cultures and the lowest was observed in *Schizosaccharomyces pombe* with its sequential cultures and it was at par with *Candida utilis* fermented with its sequential cultures. Among the sequential cultures tried *Saccharomyces cerevisiae* had the highest rate of fermentation and it was at par with *Kloeckera* spp. while that of other three yeast strains as sequential culture were at par with one another.

Rate of sugar consumption

The perusal data (Table 4.3.2) show that there was significant difference in rate of sugar consumption by sequential interactive co-culture system of solid state fermentation of apple pomace. The highest rate of sugar consumption was observed in *Saccharomyces cerevisiae* fermented apple pomace with its sequential cultures and it was at par with *Kloeckera* spp. with its sequential cultures. The lowest rate of sugar consumption was observed in *Schizosaccharomyces pombe* fermented with its sequential cultures and it was at par with *Candida utilis* fermented with its sequential cultures. Among the sequential cultures *Saccharomyces cerevisiae* had the highest

Table 4.3.1. **Effect of sequential interactive co-culture yeast fermentation on rate of fermentation (°B fall/24 hrs) during solid state fermentation of apple pomace**

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	1.640	1.610	1.620	1.600	1.630	**1.620**
Candida utilis	1.510	1.358	1.400	1.389	1.430	**1.417**
Torula utilis	1.467	1.430	1.415	1.456	1.500	**1.454**
Schizosaccharomyces pombe	1.456	1.410	1.372	1.356	1.440	**1.407**
Kloeckera spp.	1.525	1.498	1.503	1.544	1.589	**1.532**
Mean	1.520	1.461	1.462	1.469	1.518	

CD(0.05)

Initial culture	=	0.032
Sequential culture	=	0.032
Initial culture x Sequential culture	=	NS

Table 4.3.2. **Effect of sequential interactive co-culture yeast fermentation on rate of sugar consumption (%/24 hrs) during solid state fermentation of apple pomace**

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	1.315	1.284	1.276	1.259	1.296	**1.286**
Candida utilis	1.291	1.011	1.133	1.005	1.164	**1.121**
Torula utilis	1.125	1.370	1.130	1.109	1.243	**1.195**
Schizosaccharomyces pombe	1.127	1.187	1.062	1.042	1.124	**1.109**
Kloeckera spp.	1.275	1.250	1.276	1.245	1.289	**1.267**
Mean	1.227	1.220	1.176	1.132	1.223	

CD(0.05)

Initial culture	=	0.052
Sequential culture	=	0.052
Initial culture x Sequential culture	=	NS

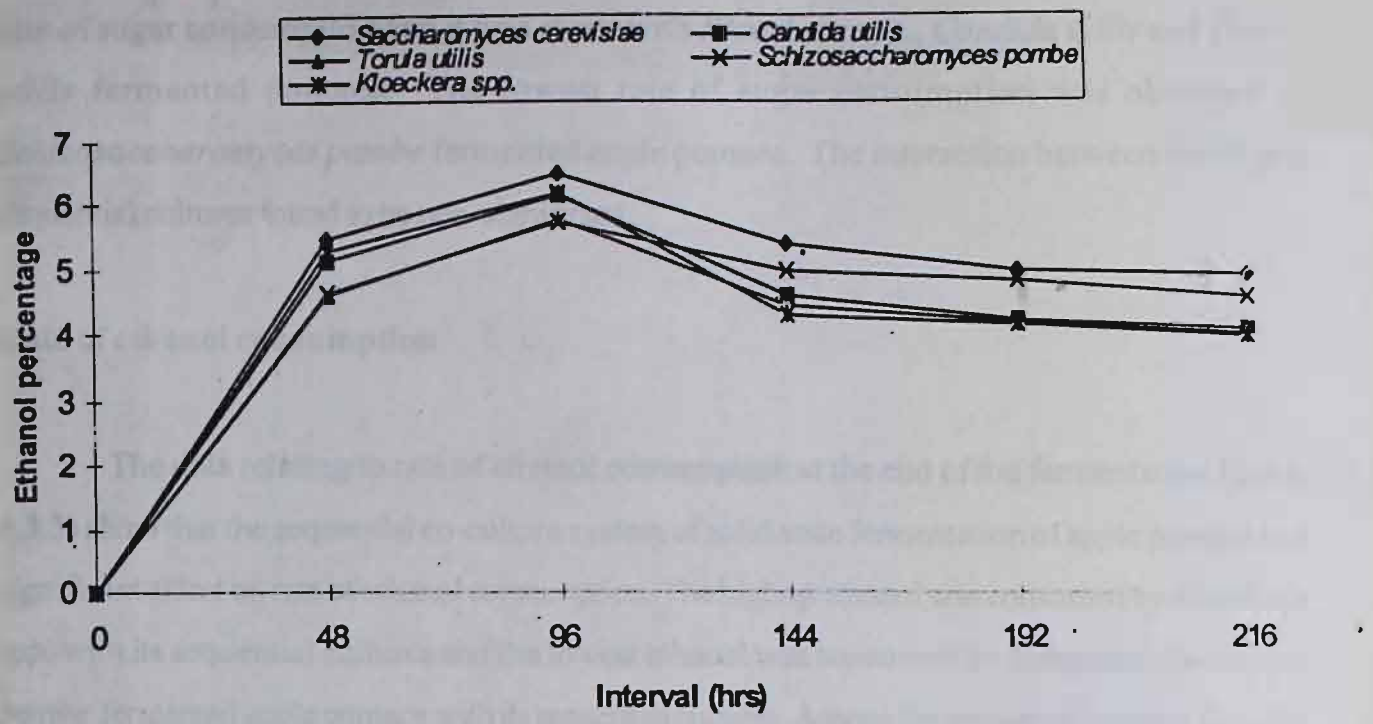


Fig. 4.3.5. Effect of sequential interactive co-cultures on ethanol (% v/v) production during SSF of apple pomace

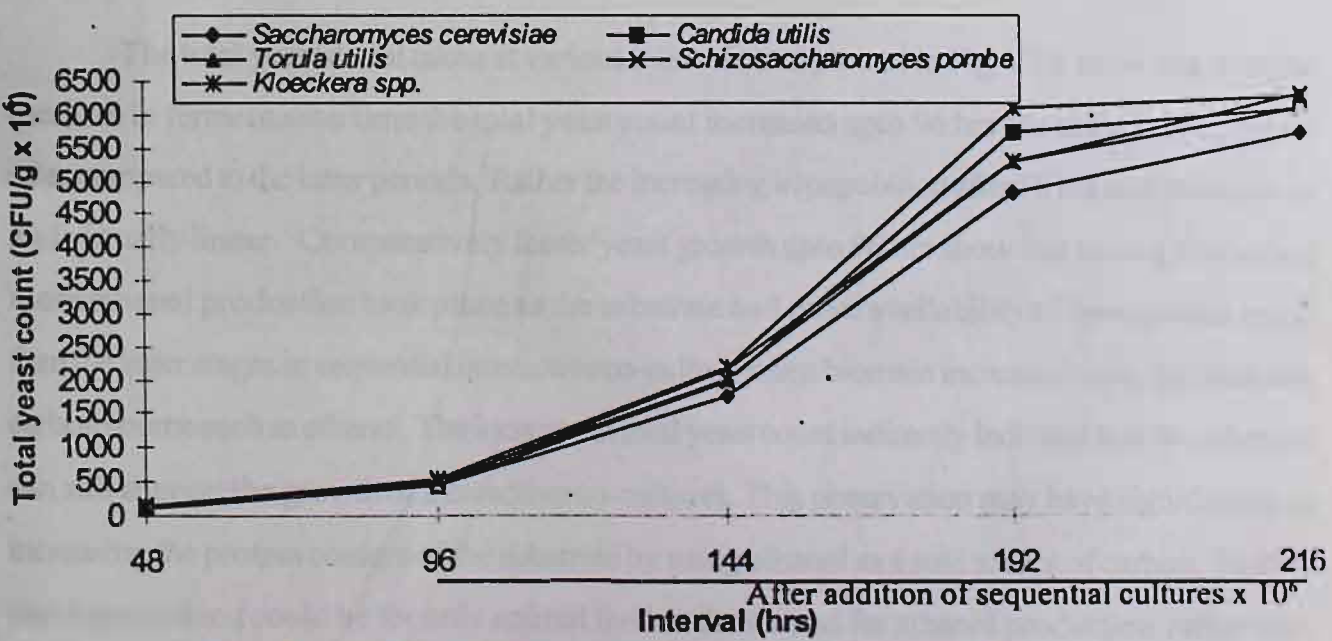


Fig. 4.3.6. Effect of sequential interactive co-culture on total yeast count (CFU/g x 10^6) during SSF of apple pomace

rate of sugar consumption and it was at par with *Kloeckera* spp., *Candida utilis* and *Torula utilis* fermented pomace. The lowest rate of sugar consumption was observed in *Schizosaccharomyces pombe* fermented apple pomace. The interaction between initial and sequential cultures found to be non-significant.

Rate of ethanol consumption

The data relating to rate of ethanol consumption at the end of the fermentation (Table 4.3.3) show that the sequential co-culture system of solid state fermentation of apple pomace had significant effect on rate of ethanol consumption. The highest ethanol was consumed by *Kloeckera* spp. with its sequential cultures and the lowest ethanol was consumed by *Schizosaccharomyces pombe* fermented apple pomace with its sequential cultures. Among the sequential cultures *Candida utilis* consumed highest ethanol while lowest ethanol was consumed by *Schizosaccharomyces pombe*. The interaction between initial and sequential culture was found to be significant. These results indicate that consumption of ethanol may increase the total yeast count where the possibilities of enhancement of nutrients (Proteins, crude fat) in the substrate exists (Shay and Wegner, 1985).

Total yeast count

The total yeast count taken at various intervals and plotted in Fig 4.3.6 show that with the increase in fermentation time the total yeast count increased upto 96 hrs at comparatively lower rates compared to the latter periods. Rather the increasing in population after 96 hrs was tremendous and virtually linear. Comparatively lesser yeast growth upto 96 hrs show that during this period more ethanol production took place as the substrate had more availability of fermentable sugar than the latter stages in sequential interactive co-culture when biomass increased using the available carbon source such as ethanol. The increase in total yeast count indirectly indicates that the substrate can still support the growth of interactive co-cultures. This observation may have significance in increasing the protein content of the substrate by using ethanol as a sole source of carbon. Further one fermentation could be for only animal feed or the second for ethanol production rather than production of ethanol and animal feed, concomitantly. The growth of *Candida utilis* and

Table 4.3.3. Effect of sequential interactive co-culture yeast fermentation on rate of ethanol consumption (%/24 hrs) during solid state fermentation of apple pomace

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	0.000	0.567	0.549	0.000	0.532	**0.3296**
Candida utilis	0.523	0.117	0.451	0.501	0.481	**0.4146**
Torula utilis	0.385	0.380	0.183	0.437	0.389	**0.3548**
Schizosaccharomyces pombe	0.000	0.544	0.338	0.000	0.412	**0.2588**
Kloeckera spp.	0.503	0.608	0.511	0.456	0.152	**0.4460**
Mean	0.2822	0.4432	0.4064	0.2788	0.3932	

CD(0.05)

Initial culture	=	0.0073
Sequential culture	=	0.0073
Initial culture x Sequential culture	=	0.0120

Kloeckera spp. is shown in Plate 9. Bhalla and Joshi (1994) used the similar approach but with cellulolytic molds and yeasts as interactive co-culture for protein enrichment of apple pomace. The yeast to yeast interactive co-culture approach was taken, as many yeast specially *Saccharomyces cerevisiae* and *Candida utilis* are well established for food/feed production (Reed and Peppler, 1973). Moreover, unlike fungi, the yeast are also a source of fats and vitamins besides proteins (Angola *et al.* 1989).

Ethanol

The ethanol content determined at the end of the fermentation of sequential interactive co-culture (Table 4.3.4) showed significant difference in the final ethanol content of the fermented apple pomace. The highest ethanol content was observed in *Saccharomyces cerevisiae* with its sequential cultures. The lowest ethanol content was observed in *Kloeckera spp.* and it was at par with *Torula utilis* with its sequential culture fermented apple pomace. The interaction between initial and sequential culture was found to be significant. Amongst the sequential yeast culture the highest ethanol was found in *Saccharomyces cerevisiae* and the lowest ethanol was found in *Candida utilis* fermented apple pomace. Within the interactions *Saccharomyces cerevisiae* alone had the highest ethanol content indicating that its suitability for ethanol production from the fermented apple pomace.

Physico-chemical characteristics of dried apple pomace

Crude proteins

The crude protein content determined in the fermented dried apple pomace (Table 4.3.5) show that the addition of sequential active co-cultures had significantly increased the crude protein content in all the treatments as compared to control. Further, the highest crude protein content was observed in *Candida utilis* and its interactive co-cultures and it was at par with *Kloeckera* spp. with its interactive co-cultures. The lowest crude protein content was observed in *Schizosaccharomyces pombe* with its interactive co-cultures. Among the sequential cultures *Kloeckera* spp. gave the highest crude protein content, followed by *Candida utilis* while the

Table 4.3.4. **Effect of sequential interactive co-culture yeast fermentation on ethanol content of the fermented apple pomace after 216 hrs of fermentation**

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	6.700	3.615	3.720	6.530	3.805	**4.874**
Candida utilis	3.495	5.540	3.870	3.665	3.760	**4.066**
Torula utilis	3.830	3.850	4.830	3.575	3.800	**3.977**
Schizosaccharomyces pombe	6.245	3.000	4.015	5.765	3.650	**4.535**
Kloeckera spp.	3.650	3.160	3.600	3.910	5.430	**3.950**
Mean	4.784	3.833	4.007	4.689	4.089	

CD(0.05)

Initial culture = 0.039
Sequential culture = 0.039
Initial culture x Sequential culture = 0.063

Table 4.3.5. Effect of sequential interactive co-culture yeast fermentation on crude proteins (%) of dried apple pomace

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	25.75	29.49	27.75	25.79	28.65	**27.49**
Candida utilis	28.72	27.76	28.29	27.65	29.50	**28.39**
Toryla utilis	28.05	28.60	26.35	28.00	28.60	**27.92**
Schizosaccharomyces pombe	27.16	26.91	27.62	25.68	27.35	**26.95**
Kloeckera spp.	26.88	29.40	27.69	27.05	29.72	**28.15**
Mean	27.31	28.43	27.54	26.84	28.77	

CD(0.05)

Initial culture	=	0.29
Sequential culture	=	0.29
Initial culture x Sequential culture	=	0.64

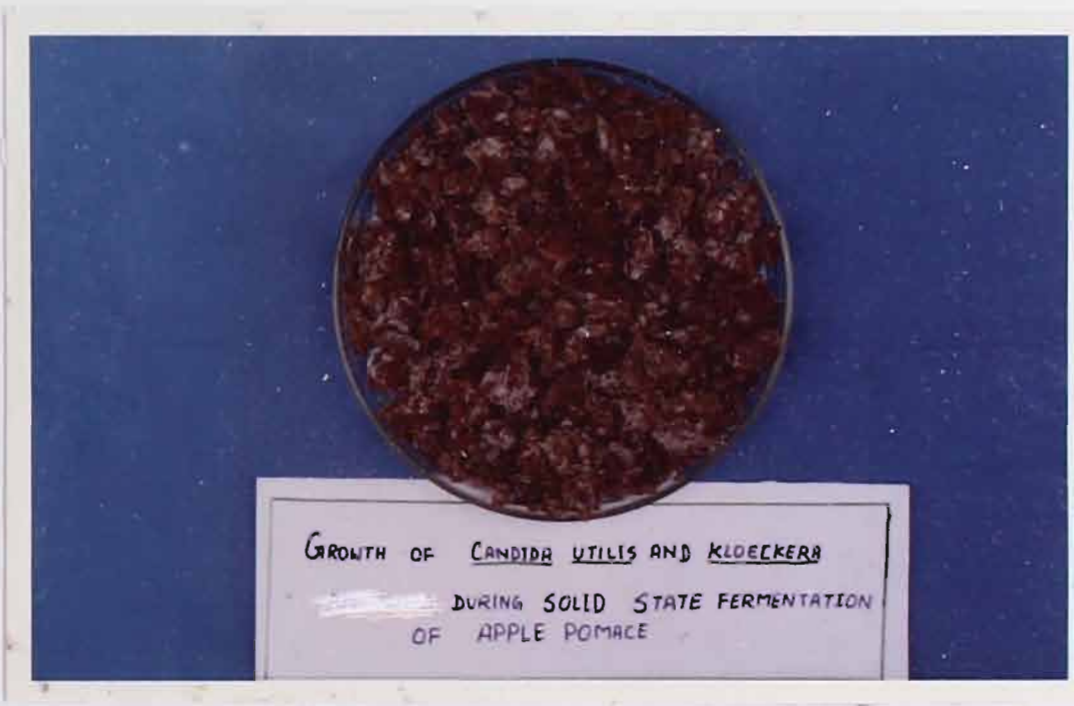


Plate 9 Grwoth of *Candida utilis* and *Kloeckera* spp. as sequential interactive co-culture during SSF

lowest was observed in *Schizosaccharomyces pombe*. The interactions between initial and sequential cultures were also found to be significant. Within the interactions *Kloeckera spp.* with *Kloeckera* spp. had the highest crude protein content and it was at par with *Candida* and *Kloeckera* spp., *Kloeckera* and *Candida utilis, Saccharomyces cerevisiae* and *Candida utilis* interactions. The increase in crude protein content can partly be attributed to degradation of carbohydrates, drying and synthesis of proteins by the yeast as indicated by its population in the apple pomace. Since, the yeast cell remained in the apple pomace after fermentation these might have contributed to the protein content of fermented apple pomace also. There is no report available on the effect of interactive co-cultures of yeast except cellulolytic mold and yeast as co-culture in solid state fermentation of apple pomace (Bhalla and Joshi, 1991). However, similar results as increase in crude protein contents were also reported by Hang (1988), Joshi and Sandhu (1996b) and Rahmat *et al.* (1995). Considering the absolute values of crude proteins produced by sequential and that produced in single culture there is a definite improvement to the extent of 5 per cent. The results clearly indicate the potential of sequential culture in increasing the crude protein content of apple pomace.

Soluble proteins

Table 4.3.6 reveals the soluble protein content of the fermented dried apple pomace. It is clear from the Table that addition of sequential interactive co-cultures had significantly, increased the soluble protein contents of the fermented dried apple pomace. The highest soluble protein content was observed in *Candida utilis* with its interactive co-cultures. The lowest soluble protein content was observed in *Torula utilis* with its sequential culture fermented apple pomace. Amongst the sequential cultures the *Saccharomyces cerevisiae* had the highest soluble protein content and it was at par with *Schizosaccharomyces pombe*. The lowest soluble protein content was observed in *Candida utilis* and it was at par with *Torula utilis and Kloeckera spp.*

The interactions between initial and sequential cultures was found to be significant. Within the interactions, the highest soluble protein content was observed in *Candida utilis* and *Kloeckera* spp. sequential interactive co-cultures and it was at par with *Candida utilis* and

Table 4.3.6. Effect of sequential interactive co-culture yeast fermentation on soluble proteins (%) of dried apple pomace

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	5.520	7.425	6.445	7.075	6.900	**6.673**
Candida utilis	7.365	5.170	6.005	8.035	8.345	**6.984**
Torula utilis	6.120	6.845	5.490	6.900	5.955	**6.262**
Schizosaccharomyces pombe	7.600	5.955	7.785	6.115	5.480	**6.587**
Kloeckera spp.	7.805	6.185	5.955	6.235	5.955	**6.427**
Mean	6.882	6.316	6.336	6.872	6.527	

CD(0.05)

Initial culture	=	0.225
Sequential culture	=	0.225
Initial culture x Sequential culture	=	0.504

Schizosaccharomyces pombe sequential interactive co-cultures. The highest soluble protein content in *Candida utilis* and *Kloeckera* spp. is considered the best interactive co-culture system for animal feed production.

The increase in soluble protein content might be due to synthesis of proteins by yeasts during fermentation. Similar results were also reported in solid state fermentation of apple pomace with *Candida utilis* and *Kloeckera* spp. separately by Rahmat *et al.* (1995). Moreover, fermentation with initial cultures had produced ethanol and when the sequential cultures are added might have utilized the ethanol as a sole source of carbon for their growth apparently increasing the soluble protein. There is no report available on the effect of interactive co-cultures between the yeasts on soluble proteins, however production of single cell protein from *Torula utilis* from ethanol as a carbon source has been reported by Shay and Wegner (1985), indicating that in our study the loss of ethanol represents the amount used in increasing the yeast population as shown by the results on alcohol reduction and yeast count.

Reducing sugar content

The perusal of data in Table 4.3.7 indicates that the addition of sequential interactive co-cultures had significant effect on reducing sugar content of fermented apple pomace. The highest reducing sugar content was observed in *Schizosaccharomyces pombe* fermented apple pomace with its sequential cultures. The lowest sugar content was observed in *Saccharomyces cerevisiae* fermented apple pomace with its sequential cultures. Amongst the sequential cultures alone, *Schizosaccharomyces pombe* fermented apple pomace had the highest reducing sugar content and it was at par with *Candida utilis* fermented apple pomace. The lowest reducing sugar content was observed in *Saccharomyces cerevisiae* fermented apple pomace. The reason for highest reducing sugar content in *Schizosaccharomyces pombe* might the result of its poor fermentability while lowest reducing sugar in *Saccharomyces cerevisiae* might be due to higher fermentability during solid state fermentation of apple pomace as explained earlier.

Based upon the ethanol, crude and soluble protein content of the sequential interactive co-culture system of solid state fermentation of apple pomace *Saccharomyces cerevisiae* alone

Table 4.3.7. Effect of sequential interactive co-culture yeast fermentation on reducing sugars (%) of dried apple pomace

Initial culture	Sequential culture					
	Saccharomyces cerevisiae	*Candida utilis*	*Torula utilis*	*Schizosaccharomyces pombe*	*Kloeckera* spp.	Mean
Saccharomyces cerevisiae	10.41	11.32	11.56	12.71	11.42	11.49
Candida utilis	11.74	14.80	13.75	14.86	12.73	13.58
Torula utilis	14.10	15.33	14.00	14.04	13.46	14.19
Schizosaccharomyces pombe	13.71	15.45	16.55	17.43	15.26	15.68
Kloeckera spp.	12.35	13.62	12.54	13.70	12.14	12.87
Mean	12.46	14.11	13.68	14.55	13.00	

CD(0.05)

Initial culture	=	0.47
Sequential culture	=	0.47
Initial culture x Sequential culture	=	0.74

gave the highest ethanol production. In case of highest crude and soluble protein content *Candida utilis* as initial culture and *Kloeckera* spp. as sequential interactive co-culture was the best method for animal feed production. Unlike the single culture where animal feed and ethanol can be recovered concomitantly the sequential culture gives a definite advantage for intended purpose i.e. either animal feed or ethanol production efficiently. So both the methods were selected for further experiments on ethanol recovery and nutritional evaluation of left-over dried apple pomace for the suitability of production of ethanol and/or high value animal feed.

4.4 ETHANOL RECOVERY BY DIFFERENT METHODS

The results obtained on the mass flow and the separation of ethanol from fermented apple pomace by four different methods are shown in Table 4.4.1. Differences in the initial ethanol content exhibited by the table reflect the differences in the two treatment at 0 hrs for ethanol content. It is also evident that complete alcohol contents are not removed in the first press, as shown by the alcohol levels in the first and second press. Further, the final ethyl alcohol content in the distillates were according to present in the fermented apple pomace after SSF.

The average separation efficiency of two treatments by four different methods varied from 73.155 to 99.45 per cent. The highest separation efficiency was obtained by steam distillation and the lowest in hot water extraction and basket press followed by distillation which may be due to incomplete leaching out of ethanol molecules through pressing. The results on hot water extraction and hydraulic pressing followed by distillation are in confirmation with those obtained by Jaleel *et al.* (1991) on fiberous tapioca fermentation. However, higher separation efficiency of ethanol has been obtained in our study which could be attributed to the fine pulpy material i.e. apple pomace, used in our study rather than fibrous material used by Jaleel *et al.* (1991) which might have resulted in more leaching of ethanol in our study than that reported by him. The highest ethanol by steam distillation in the present investigation might be due to the complete trapping out of ethanol molecules from the fermented apple pomace particles in the form of vapours due to uniform high temperature. There is no report on the separation efficiency of ethanol from apple pomace using different methods except separation of ethanol by vacuum distillation by Hang *et al.* (1982) which also confirmed our findings.

Table 4.4.1. Effect of different methods on mass flow and ethanol separation

Methods	Initial athanol (%)	Ethanol extraction (%)		Final ethanol (%)	Separation efficiency (%)
		1st press	2nd press		
E_1					
Y_1	6.59 (98.85)	4.055 (58.13)	1.14 (14.72)	7.14 (71.40)	72.22
Y_2	3.64 (54.60)	2.47 (32.94)	0.70 (8.27)	4.00 (40.04)	74.08
E_2					
Y_1	6.64 (99.67)	NA	NA	9.81 (98.10)	98.42
Y_2	3.56 (53.47)	NA	NA	5.19 (51.90)	97.07
E_3					
Y_1	6.61 (99.67)	NA	NA	9.84 (98.40)	99.24
Y_2	3.54 (52.44)	NA	NA	5.22 (52.20)	99.66
E_4					
Y_1	6.60 (99.07)	NA	NA	9.66 (96.60)	97.55
Y_2	3.64 (54.60)	NA	NA	5.26 (52.60)	96.43

Initial weight of sample taken for ethanol separation = 1.5 kg *Final volume of distillate* = 1 litre **CD(0.05) 2.857**

E_1 = Hot water extraction and hydraulic pressing followed by distillation; E_2 = Direct distillation; E_3 = Steam distillation; E_4 = Vacuum distillation

Y_1=*Saccharomyces cerevisiae* fermented apple pomace; Y_2 = *Candida utilis* and *Kloeckera* spp. as interactive co-culture fermented apple pomace

Values indicated in the parentheses are quantity of alcohol as absolute alcohol (ml)

NA = Not applicable

Physico-chemical characteristics of fermented dried apple pomace

The fermented apple pomace of different treatments after extraction of ethanol by different methods as discussed under 4.3 was dried separately and analyzed for different parameters. The results obtained are described here.

Moisture : Table 4.4.2 shows the moisture content of ethanol extracted dried apple pomace by four different methods. It is clear from the table that extraction of ethanol by different methods did not have significant effect on moisture content of dried apple pomace. Since, similar time, temperature and drying methods were employed for all the treatment it might be the reason for non-significant differences.

Reducing Sugar: The reducing sugar content of dried apple pomace after extraction of ethanol by different methods (Table 4.4.2) was significant effects by different methods of ethanol separation. The apple pomace dried without ethanol recovery (control) recorded the highest reducing sugar content followed by steam distillation method, whereas the lowest reducing sugar content was observed in hot water extraction followed by distillation and without adding back the base of the distillate. The reason behind this observation apparently is the leaching out of nutrients while the apple pomace in hydraulic press. However, when the base of the distillate was added back before drying it increased the reducing sugar content to a greater extent compared to the control. The reason for lower reducing sugar content in all the treatments than control by all methods except that without adding back the distillate base might be the loss of sugar during browning reactions of sugar and with nitrogenous components resulting in loss of both the components (Meyer, 1978).

Total sugars: The perusal of data (Table 4.4.2) reveals that the dried apple pomace from the steam distillation treatment had significantly highest total sugar content, though, less than the control. Differences might be due to the loss of sugar in browning reaction during distillation as explained earlier. The lowest total sugar content was observed in hydraulic pressing and when the base of distillate was not added back to the ethanol extracted apple pomace before drying. It is obviously due to the leaching of sugar in hot water extraction method.

Table 4.4.2. Effect of different methods of ethanol separation on physico-chemical characteristics of dried apple pomace

Methods	Moisture (%)			Reducing sugars (%)			Total sugars (%)			Titratable acidity (%) as malic acid			Crude proteins (%)			Soluble proteins (%)		
	S. c.	*C.u.+ Kl.*	Mean	*S. c.*	*C.u.+ Kl.*	Mean	*S. c.*	*C.u.+ Kl.*	Mean	*S. c.*	*C.u.+ Kl.*	Mean	*S. c.*	*C.u.+ Kl.*	Mean	*S. c.*	*C.u.+ Kl.*	Mean
T_1 Control	5.05	5.05	5.05	9.80	10.97	10.39	10.57	11.66	11.11	3.52	3.35	3.43	25.61	29.50	27.55	5.58	8.41	6.99
T_2 DD	5.05	5.00	5.02	9.36	10.22	9.79	9.39	10.50	9.95	3.34	3.19	3.27	25.39	29.03	27.21	5.22	8.38	6.80
T_3 BPWB	4.92	4.95	4.94	4.80	5.63	5.22	5.40	6.04	5.72	1.30	1.49	1.39	14.43	17.41	15.92	2.66	4.25	3.45
T_4 BPAB	5.05	5.05	5.05	8.35	8.51	8.43	9.54	10.56	10.05	3.28	2.96	3.12	25.05	28.73	26.89	5.48	8.39	6.94
T_5 VD	5.15	5.05	5.10	9.24	10.40	9.82	10.49	11.47	10.98	3.43	3.16	3.29	25.41	29.32	27.36	5.53	8.41	6.97
T_6 SD	5.00	5.05	5.02	9.46	10.44	9.95	10.53	11.59	11.06	3.455	3.28	3.37	25.48	29.48	27.48	5.568	8.41	6.99
Mean	5.04	5.02		8.50	9.36		9.32	10.3		3.05	2.90		23.56	27.24		5.01	7.71	

CD (0.05)

Treatment =	NS	0.06	0.08	0.11	0.092	0.047
Yeast	NS	0.03	0.05	0.07	0.04	0.03
Treat x Yeast	NS	0.08	0.12	0.16	0.10	0.01

T_1 = Without ethanol separation
T_2 = Direct distillation
T_3 = Basket press without added back
T_4 = Basket press after added back
T_5 = Vacuum distillation
T_6 = Steam distillation
S.C = *Saccharomyces cerevisiae*
C.u+Kl = *Candida utilis+ Kloeckera* spp. fermented apple pomace as sequential interactive co-culture

Titratable acidity: It is evident from the Table 4.4.2 that the highest acidity was observed in control and it was at par apple pomace obtained after extraction of ethanol by steam distillation. The direct distilled and vacuum distillated were at par with each other. The lowest titratable acidity was observed in hot water extraction followed by distillation and without adding back the base of the distillate before drying.

Crude and Soluble proteins: The data (Table 4.4.2) also reveal that there was a minimum loss in crude and soluble proteins of fermented apple pomace when the ethanol was separated by steam distillation. The maximum loss of nutrient was observed when the base of distillate was not added back to the apple pomace before drying after hot water extraction.

Based upon the highest separation efficiency and minimum nutrition loss, steam distillation was selected as the best method for separation of ethanol from the fermented apple pomace. The separation efficiency of direct distillation and vacuum distillation were comparable with that of steam distillation. However, steam distillation was found to be more rapid in ethanol separation than any other method tried. Moreover, employing of either direct distillation or vacuum distillation will add additional cost of production at industrial scale level. Hence, it can be concluded that steam distillation has the potential for ethanol recovery from the fermented apple pomace, at industrial scale for commercial exploitation.

4.5 EVALUATION OF ANIMAL FEED PRODUCED BY SSF WITH WHITE RATS

4.5.1 Compositional evaluation of animal feed

After screening for ethanol recovery of two treatments by four different methods, the apple pomace fermented with *Candida utilis* and *Kloeckera* spp. as sequential interactive co-culture followed by ethanol separation by steam distillation combination was selected on the basis of highest ethanol separation efficiency and high crude and soluble protein content. The composition of apple pomace prepared by above mentioned method is described in Table 4.5.1.

SSF of apple pomace reduced the TSS, reducing and total sugar content, however, the concentration of residual sugar in the fermented apple pomace remained sufficient to be used as

Table 4.5.1. Physico-chemical characteristics of fermented apple pomace

Characteristics	Mean±S.E.
Moisture (%)	5.25±0.05
Reducing sugars (%)	12.60±0.025
Total sugars (%)	13.47±0.05
TSS (°B)	28.6±0.05
Titratable acidity (%)	3.59±0.01
pH	3.45±0.04
Soluble proteins (%)	8.47±0.115
Crude proteins (%)	29.60±0.008
Ascorbic acid (mg/100 ml)	35.21±0.43
Crude fiber (%)	20.89±0.68
Crude fat (%)	10.99±0.14

an animal feed. besides, the higher content of TSS than sugar is due to presence of other soluble materials. The fermented apple pomace contained almost 5.5 times more crude protein than the unfermented apple pomace (Table 4.1 and 4.5.1). Apple pomace has been described as a low protein and high carbohydrate feed with low palatability and rumen digestibility (Sharma *et al.*, 1989; Hang, 1988; Rumsey, 1978). However, the low protein content can be over come through adding non-protein nitrogen materials. But, such type of feed when fed to pregnant cows resulted in giving birth to dead or weak calves (Fotenote *et al.*, 1977) Thus protein enhancement through fermentation is a better way of improving the protein content of apple pomace. Moreover, SSF apple pomace by sequential interactive co-culture system increased the soluble protein content 2.55 times, crude fat 3.13 times and vitamin-C content 2 times is more attractive from the nutritional point of view. The acidity and pH of the fermented and unfermented apple pomace remained almost same with slight variations. A considerable amount of increase in crude fiber content was also observed in fermented apple pomace. The increase in crude protein, crude fat and vitamin-C content might due to the degradation of carbohydrates during fermentation and evaporation of metabolites during drying resulting in reduction in dry matter, consequently increasing the concentration of other components. The increase in soluble protein and crude fat might be due to synthesis by yeast during fermentation. There is no report on composition of apple pomace fermented in SSF by sequential interactive co-culture system and affect of addition of molasses. However, these results in general are confirmation with those of Hang (1988); Rahmat *et al.* (1995) and Joshi and Sandhu (1996b). But, in contrast to these results in enhancement of crude protein content by 1.7 times, crude fat content 1.2 times and soluble protein content 1.4 times were more in our study which may be attributed to the effect of addition of molasses and sequential interactive co-culture. From the nutrition point of view, the sequential interactive co-culture appear to be quite advantageous.

4.5.2 Evaluation of animal feed in rats

i) Feed intake study : (No choice)

Fig 4.5.1 shows the intake of different types of feed viz. fermented apple pomace, unfermented apple pomace and the feed comprising of standard rat feed and either fermented or unfermented apple pomace in the ratio of 1:1. The feed intake per 100 g of body weight of

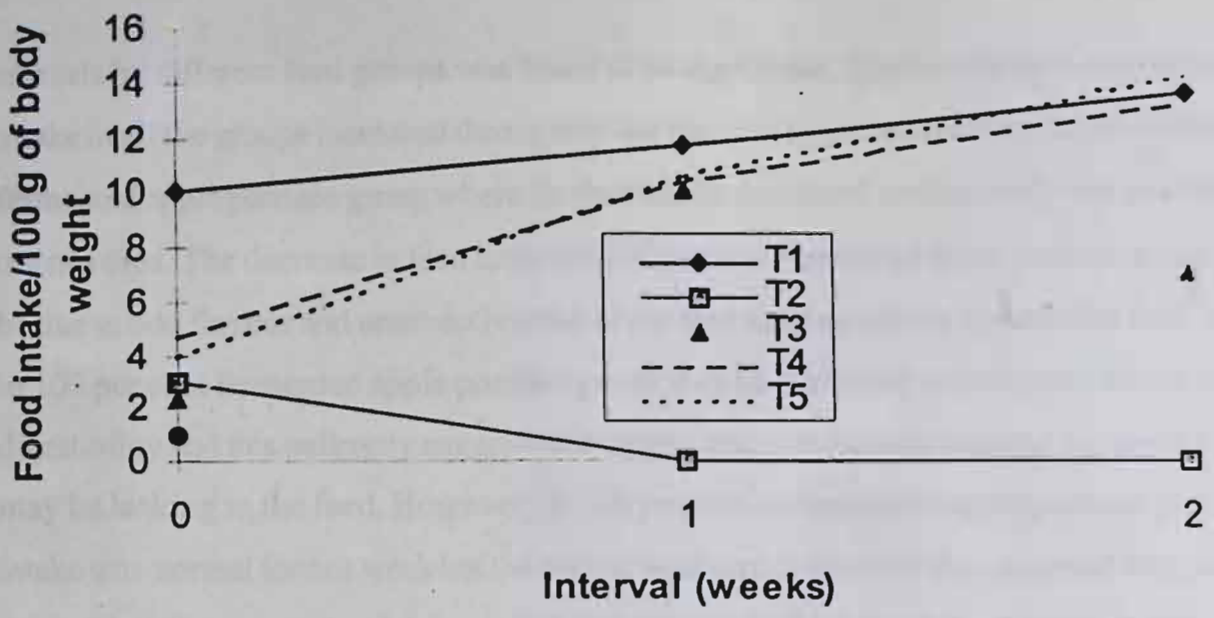
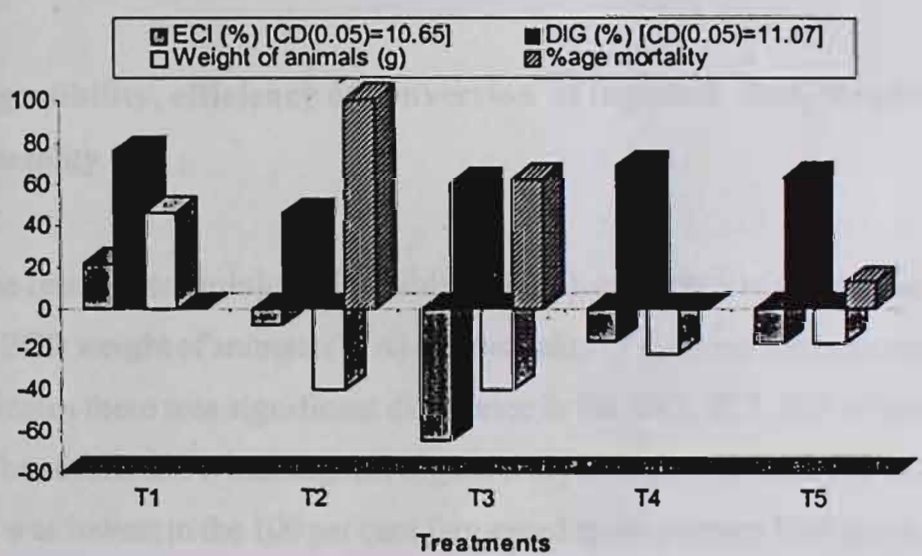


Fig. 4.5.1. Different types of food intake (no choice) per 100g of body weight of animal.



ECI - Efficiency of conversion of ingested food
DIG - Digestibility

Fig. 4.5.2. Effect of different feeds on efficiency of conversion of digested feed (%), digestibility (%) and weight of animals (g) of white rats

T1 : Standard rat animal feed T2 : 100% fermented apple pomace
T3 : 100% unfermented apple pomace
T4 : 1:1 standard rat feed and 100% fermented apple pomace
T5 : 1:1 standard rat feed and 100% unfermented apple pomace

animals by different feed groups was found to be significant. The results indicate that the feed intake in all the groups increased throughout the trial (for two weeks) except in the 100 per cent fermented apple pomace group where the feed intake decreased continuously and as a result all the rats died. The decrease in feed intake of 100 per cent fermented apple pomace group might be due to odd flavour and unattractiveness of the feed as compared to standard rat feed. Deaths in 100 per cent fermented apple pomace group may be attributed to starvation and decreased digestibility and this indirectly suggests that certain essential nutrient required for survival of rats may be lacking in the feed. However, in 100 per cent unfermented apple pomace group feed intake was normal for one week but the animal were unable to utilize the consumed feed probably due to high fiber content and this resulted in decreased feed intake after one week. A reference to the standard diet of rat (Table 3.3) indicates higher crude fiber content in the fermented apple pomace and it substantiates our hypothesis further.

ii) Digestibility, efficiency of conversion of ingested feed, weight of animals and mortality

The results pertaining to digestibility (DIG), efficiency of conversion of ingested feed materials (ECI) weight of animals (WA) and mortality of different feed groups of rat groups (Fig 4.5.2) indicates there was significant difference in the DIG, ECI, WA of animals of different groups. The results show that highest digestibility of feed was observed in standard rat feed, whereas it was lowest in the 100 per cent fermented apple pomace feed group. The digestibility in other groups, unfermented apple pomace, the feed comprising of standard rat feed and either fermented or unfermented apple pomace mixed in the ratio of 1:1 were almost comparable but, it was lower than the standard rat feed. The decrease in digestibility might be due to high crude fiber content as described earlier. Moreover, the lowest digestibility in the fermented apple pomace might be due to loss of fermentable organic matter during fermentation. Similar results have also been reported thereby indicating decrease in digestibility of potato processing waste in ruminants (Arora *et al.*, 1995) and also in fermented orange peels and grape distillery stalks (Nicoloni *et al.*, 1993).

The studies further show that the ECI in all feed groups were negative except in the standard rat feed group. The reason seems to be the less availability of essential nutrients required for basal metabolism which might have resulted in negative ECI, hence loss of body weight.

iii) Mortality

No death was observed in standard rat feed and the feed comprising of standard rat feed and fermented apple pomace in the ratio of 1:1. Whereas the highest (100%) mortality was observed in 100% fermented apple pomace group which might be due to starvation and indigestibility as described earlier (Plate 10). A lower mortality (62.5%) was observed in unfermented apple pomace group, and the feed comprising unfermented apple pomace and the standard rat feed in the ratio of 1:1 (12.5%) this mortality can be due to the inability of the animal to utilize the ingested feed was evident in the post-mortem examination (Plate 11 and 12).

iv) Growth rate and consumption index

The results obtained on growth rate and consumption index during animal feed trial (depicted Fig 4.5.3) show that the there was significant difference in the consumption index and growth rate of different feed groups. The highest consumption index was in standard rat feed group and it was followed by the group feed comprising of standard rat feed either fermented or unfermented apple pomace feed in the ratio of 1:1. The lowest consumption index was observed in fermented apple pomace group which might be due to its poor acceptability as described earlier.

From the results discussed above it is clear that the growth rates were negative in all the feed groups except the standard rat feed group. The reason may be attributed to decrease of ECI in these groups.

4.5.3 Feed acceptability studies (choice feed)

Fig 4.5.4 shows the acceptability of standard rat feed, fermented apple pomace, unfermented apple pomace and the feed comprising of 1:1 ratio of either fermented or unfermented apple pomace in the choice study. It is clear from the figure that the standard rat feed was more

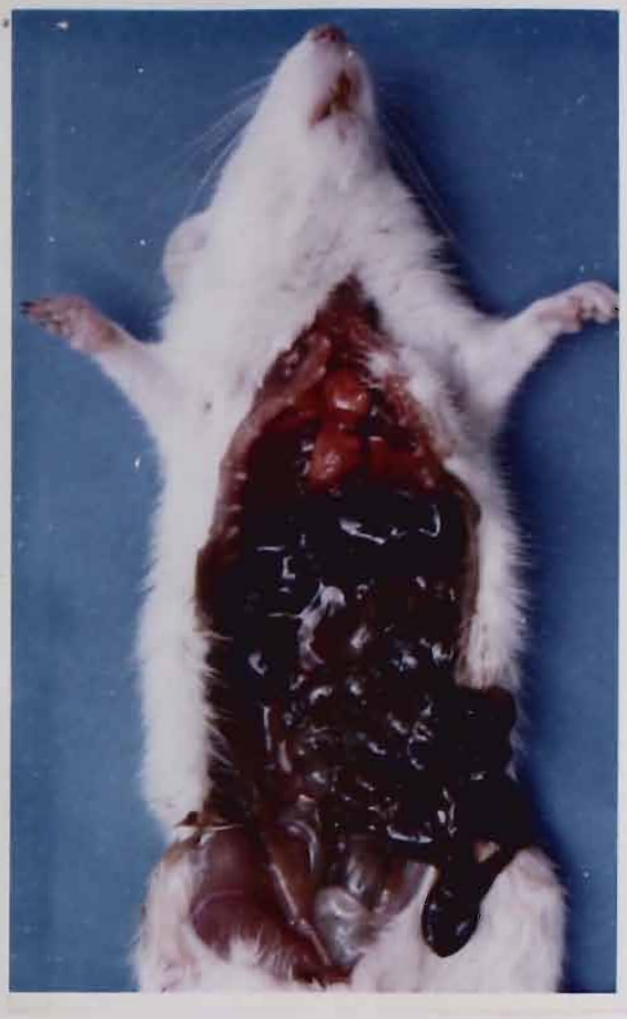


Plate 10 Empty intestine and undigested food material in stomach of white rats fed with 100% fermented apple pomace

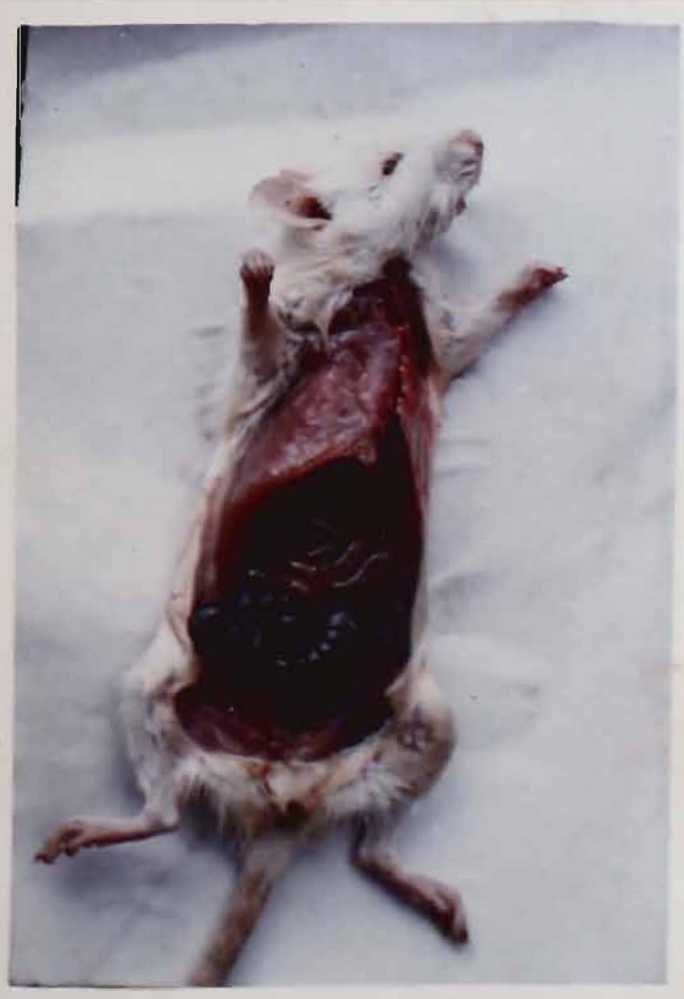


Plate 11 Undigested food material filled in stomach of white rats fed with 100% unfermented apple pomace

Plate 12 Undigested food material filled in stomach of white rats fed with 1:1 ratio of standard rat feed and unfermented apple pomace

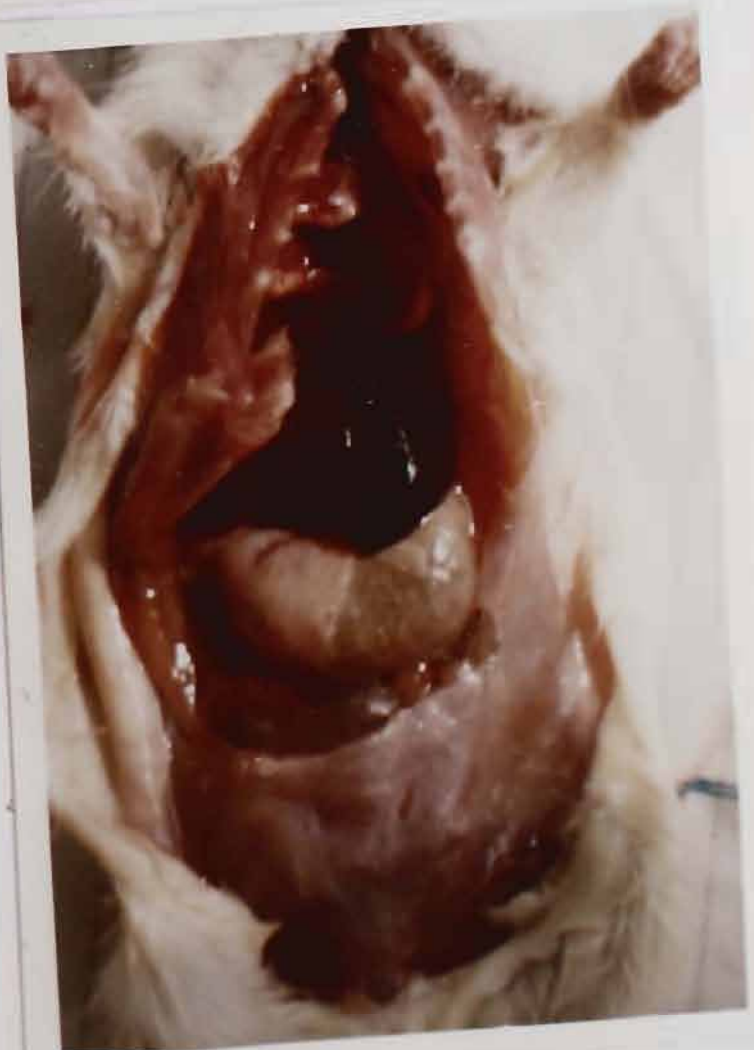


Plate 13 Digested food material in stomach with liver damage of white rats fed with reconstituted fermented apple pomace comprising of 10% jaggery, 2% groundnut oil, 0.01% mixed flavour and 1% salt

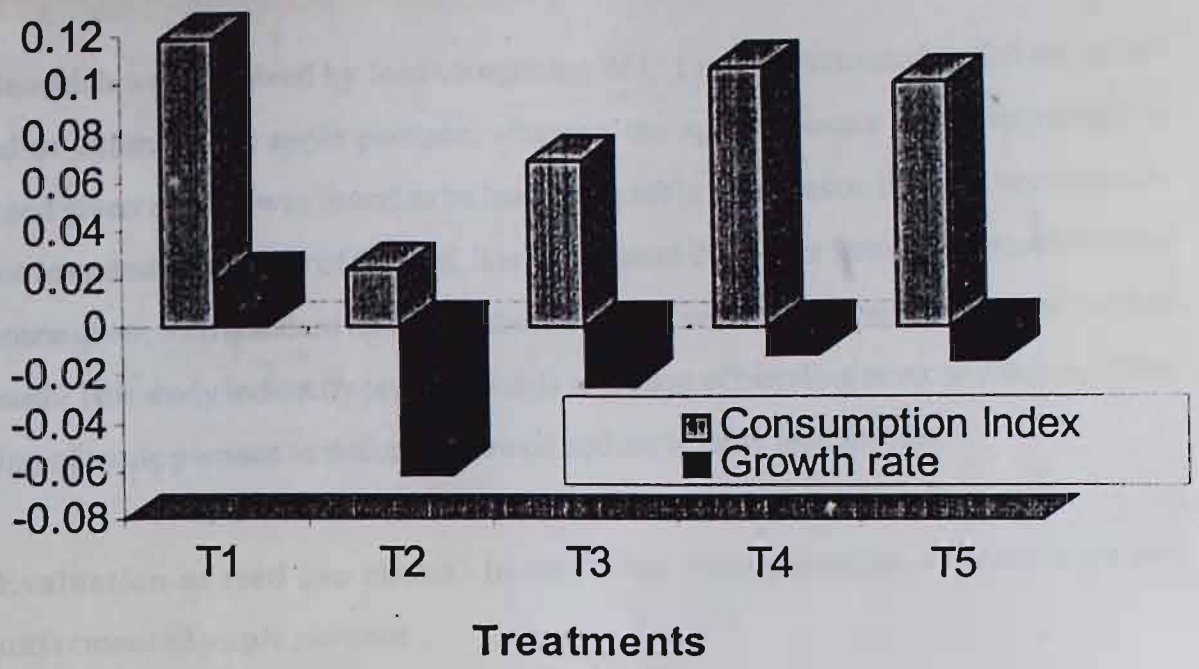


Fig. 4.5.3. Effect of different feeds on growth rate and consumption index of white rats

CD(0.05) : Consumption Index = 0.019; Growth rate = 0.01

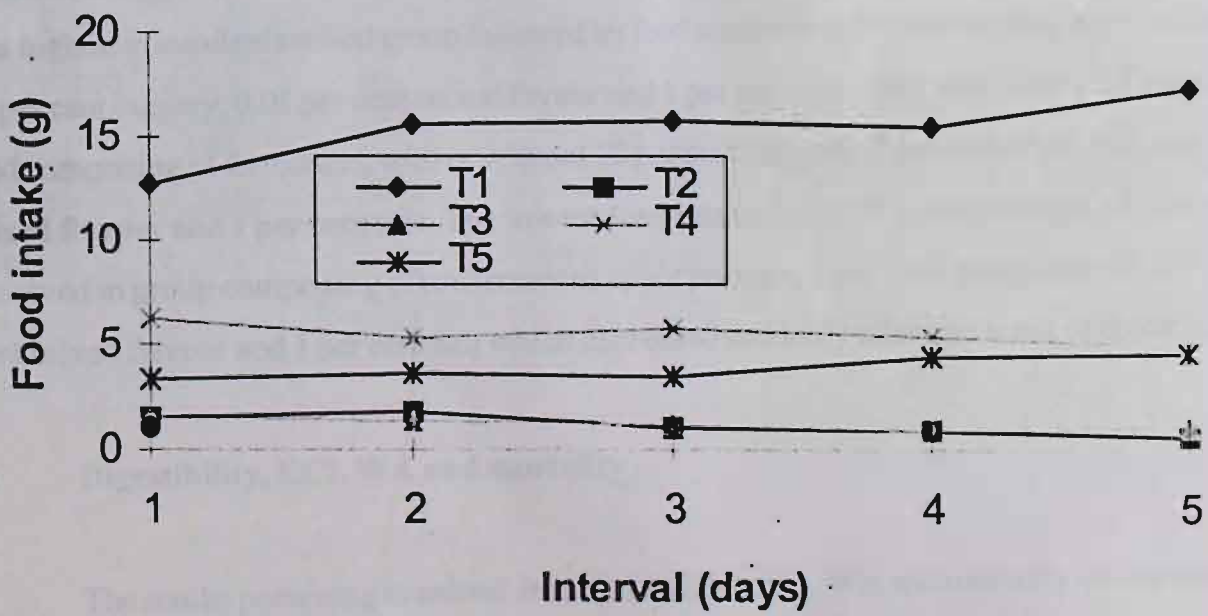


Fig. 4.5.4. Different types of food intake (g) (choice)

T1 : Standard rat animal feed
T2 : 100% fermented apple pomace
T3 : 100% unfermented apple pomace
T4 : 1:1 standard rat feed and 100% fermented apple pomace
T5 : 1:1 standard rat feed and 100% unfermented apple pomace

acceptable which was followed by feed comprising of 1: 1 ratio of standard rat fed and either fermented or unfermented apple pomace, whereas, the apple pomace either fermented or unfermented given as such was found to be least acceptable. The reason for least acceptability may be due to undesirable odour of the feed. It is also evident that either fermented or unfermented apple pomace given with standard rat feed in the ratio of 1:1 was more acceptabililty than the feed given as such. This study indirectly points towards some sort of blending or reconstitution of feed comprising of apple pomace to make a balanced and attractable feed for rats.

4.5.4 Evaluation of feed (no choice) in rats after reconstitution of fermented and unfermented apple pomace

i) Feed intake study (No choice)

The apple pomace both fermented and unfermented after reconstitution was fed to rats and their results are (Fig. 4.5.5) the feed intake (per 100 g of body of weight of animals) of different feed groups was found to be significant. The feed intake/100 g of body weight of rats, was highest in standard rat feed group followed by feed comprising of unfermented, apple pomace, 10 per cent jaggery, 0.01 per cent mixed favour and 1 per cent salt and it was closely followed by feed comprising of fermented, with or without 10 per cent jaggery, 2 per cent of oil, 0.01 per cent mixed flavour and 1 per cent salt. The lowest feed intake (per 100 g body weight of rats) was observed in group comprising of unfermented apple pomace, 2 per cent groundnut oil, 0.01 per cent mixed flavour and 1 per cent salt which decreased suddenly after one week of feeding.

ii) Digestibility, ECI, WA and mortality

The results pertaining to animal feed trial on DIG, ECI, WA and mortality are depicted in Fig. 4.5.6. The digestibility and ECI in different feed groups was found to be non significant, however, weight of animals was found to be significant. Regarding digestibility and ECI of the reconstituted feed comprising of fermented and unfermented apple pomace were comparable to the standard rat feed. These results indicate that the reconstituted feed comprising of apple pomace

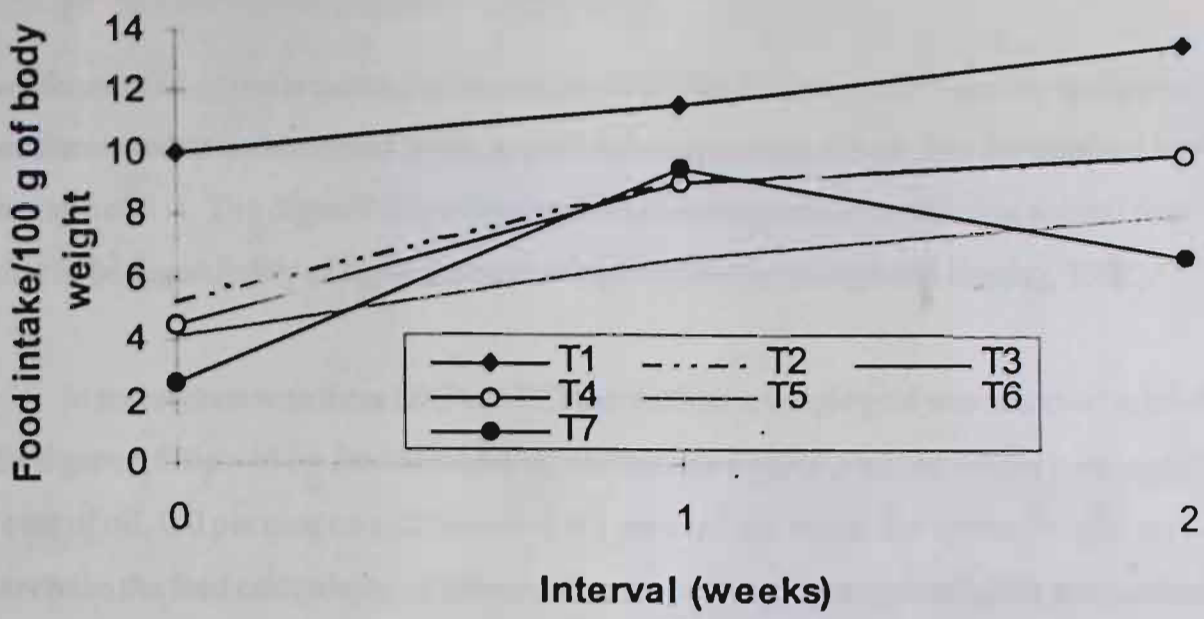
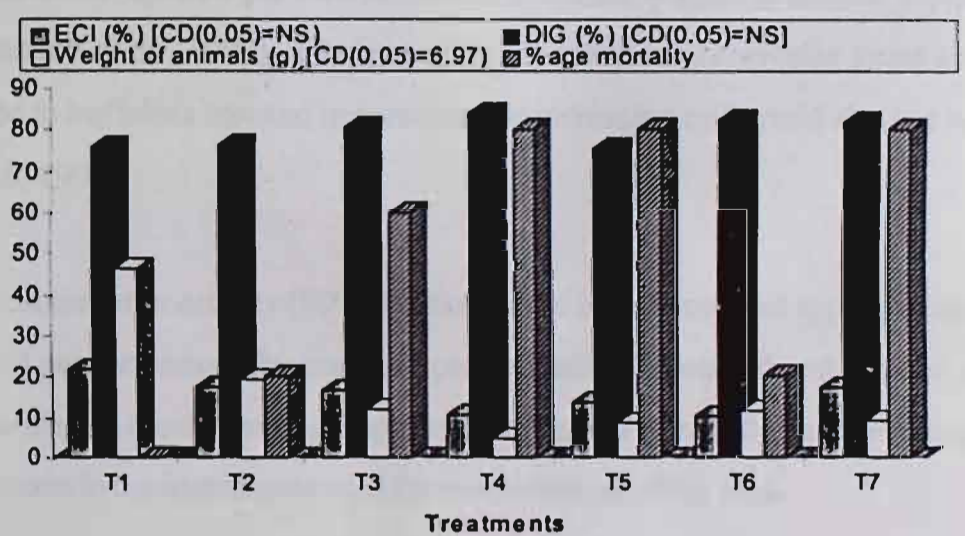


Fig. 4.5.5. Different types of food intake (no choice) per 100g of body weight of animal.



ECI - Efficiency of conversion of inested food
DIG - Digestibility

Fig. 4.5.6. Effect of different feeding on efficiency of conversion of digested feed (%), digestibility (%) and weight of animals (g) of white rats

T1 : Standard rat animal feed
T2 : Fermented AP + 2% ground nut oil + 10% jaggery + 0.01% mixed flavour + 1% salt
T3 : Fermented AP + 10% jaggery + 0.01% mixed flavour + 1% salt
T4 : Fermented AP + 2% ground nut oil + 0.01% mixed flavour + 1% salt
T5 : Unfermented AP + 2% ground nut oil + 10% jaggery + 0.01% mixed flavour + 1% salt
T6: Unfermented AP + 10% jaggery + 0.01% mixed flavour + 1% salt
T7: Unfermented AP + 2% ground nut oil + 0.01% mixed flavour + 1% salt

either fermented or unfermented gives better results on DIG and ECI than the apple pomace either fermented or unfermented given as such or incorporation of both into the standard rat feed in the ratio of 1:1. The digestibility of reconstituted apple pomace containing animal feed was similar to be digestibility of apple pomace as reported earlier (Singh and Narang, 1992).

In accordance with these DIG and ECI the maximum weight gain was observed in standard rat feed group followed by feed comprising of fermented apple pomace, 10 per cent jaggery, 2 per cent of oil, 0.0 per cent mixed flavour and 1 per cent salt while the lowest weight gain was observed in the feed comprising of fermented apple pomace, 2 per cent oil, 0.01 per cent mixed flavour and 1 per cent salt. These observations indicate that addition of jaggery is a must in the reconstitution of fermented apple pomace for feeding the rats. Similarly, the effect of feeding of SCP as replacement upto 50 per cent in feed on the body weight of Karadi lambs have been reported (Yacoub *et al.*, 1990). Feeding of *Saccharomyces cerevisiae* yeast alongwith the media was fed to buffaloes resulted in considerable increasing milk yield also has been reported (Kumar *et al.*, 1992).

The maximum mortality (80%) was observed in unfermented apple pomace with 2 per cent of oil, 0.01 per cent mixed flavour and 1 per cent salt. The detailed post-mortem examination (PME) of rats died in the different groups revealed generalized oedema which might be due to some hepatotoxin in the ingredients used for reconstitution of the feed.

ii) Consumption index and growth rate

The consumption index and growth rate calculated at the end of the trial or depicted in Fig 4.5.7. Consumption index in different feed groups was found to non-significant and growth rate in different feed groups was found to be significant. The highest growth rate was observed in control feed fed group followed by the feed comprising of fermented apple pomace, with or without jaggery with 2 per cent ground nut oil, 0.01 per cent mixed flavour and 1 per cent salt. The lowest growth rate was observed in unfermented apple pomace with 10 per cent jaggery and ground oil, 0.01 per cent mixed flavour and 1 per cent salt.

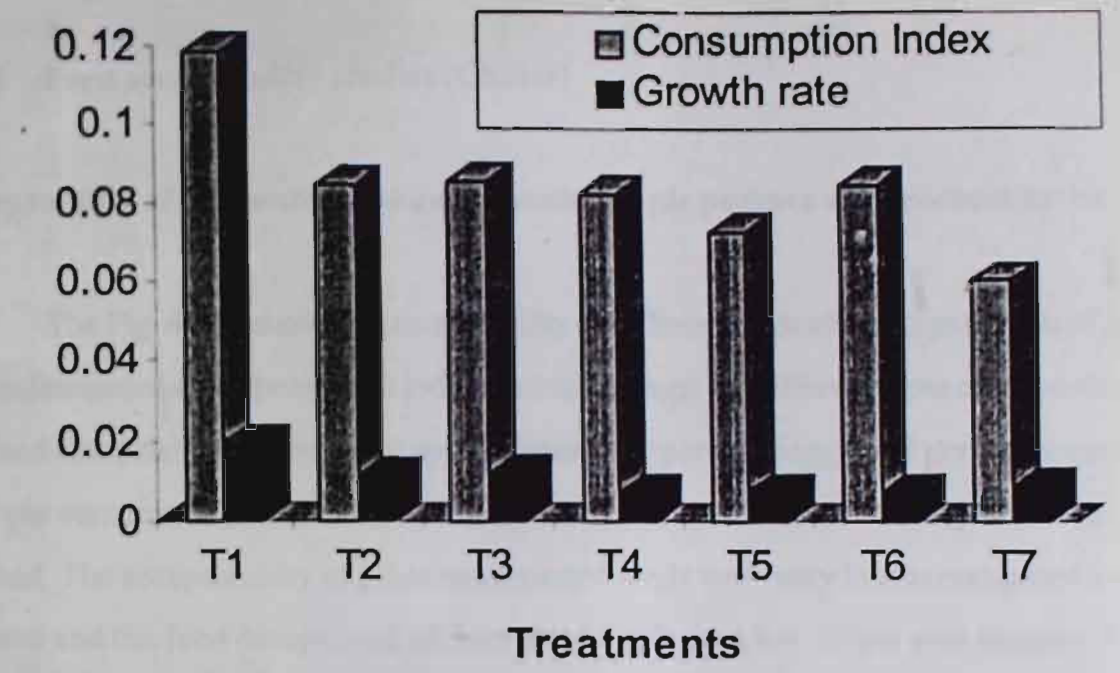


Fig. 4.5.7. Effect of different feeding on growth rate and consumption index of white rats

CD(0.05) : Consumption Index = NS; Growth rate = 0.005

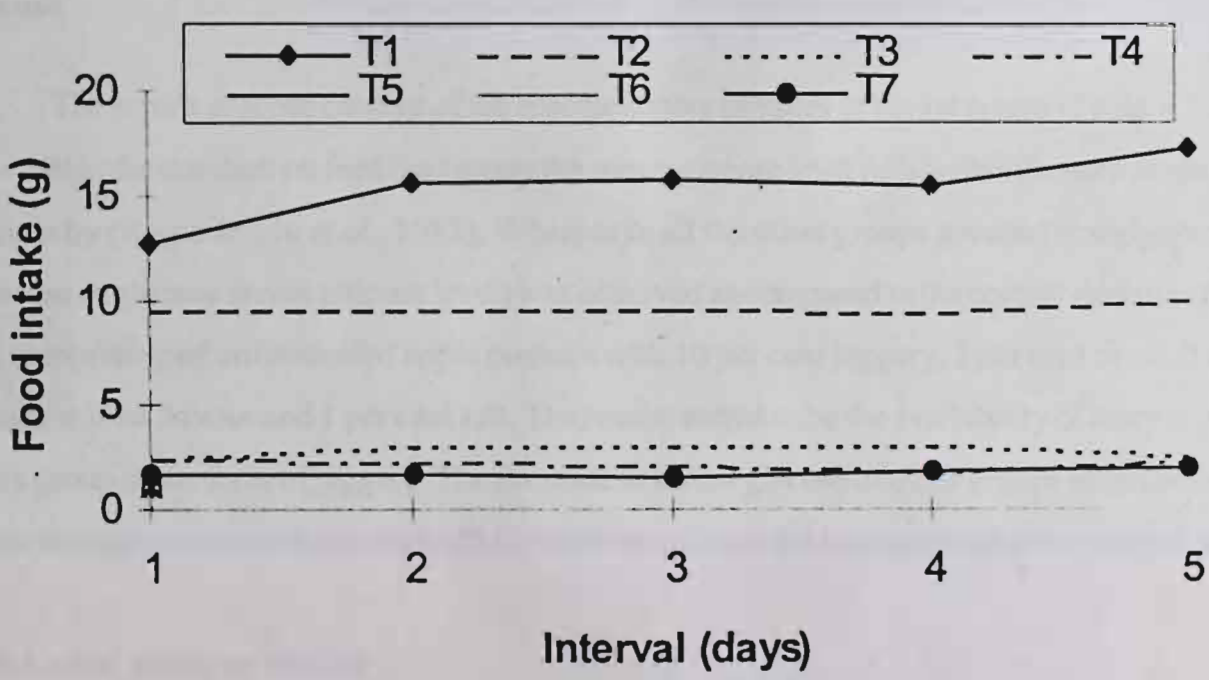


Fig. 4.5.8. Different types of food intake (g) (choice)

T1 : Standard rat animal feed
T2 : Fermented AP + 2% ground nut oil + 10% jaggery + 0.01% mixed flavour + 1% salt
T3 : Fermented AP + 10% jaggery + 0.01% mixed flavour + 1% salt
T4 : Fermented AP + 2% ground nut oil + 0.01% mixed flavour + 1% salt
T5 : Unfermented AP + 2% ground nut oil + 10% jaggery + 0.01% mixed flavour + 1% salt
T6: Unfermented AP + 10% jaggery + 0.01% mixed flavour + 1% salt
T7: Unfermented AP + 2% ground nut oil + 0.01% mixed flavour + 1% salt

4.5.5 Feed acceptability studies (Choice)

Acceptability of fermented and unfermented apple pomace after reconstitution

The Fig. 4.5.8 shows the acceptability of different feeds after reconstitution of fermented and unfermented apple pomace. It indicates that amongst the different types of reconstituted feed, the feed comprising of fermented apple pomace, 10 per cent jaggery, 2 per cent groundnut oil, 0.01 per cent mixed flavour and 1 per cent salt was found to be most acceptable after standard rat feed. The acceptability of other reconsituted feeds were very less as compared to standard rat feed and the feed comprising of fermented apple pomace, 10 per cent jaggery, 2 per cent groundnut oil, 0.01 per cent mixed flavour and 1 per cent salt. The difference amongst the reconstituted feeds was however, marginal.

4.5.6 Blood biochemical analysis

Glucose

The serum glucose content of the representative samples of the rat serum (Table 4.5.2) show that in the standard rat feed feed group the serum glucose level falls within the normal range as given by (Raguramulu *et al.*, 1983). Whereas in all the other groups groups hypoglycemia (decrease in glucose serum glucose level) was observed as compared to the control except in the feed comprising of unfermented apple pomace with 10 per cent jaggery, 2 per cent of oil, 0.01 per cent mixed flavour and 1 per cent salt. The reason seems to be the availability of more sugar in this group in the form of jaggery. The decrease in serum glucose of other groups might be due to liver damage as it was evident on the PME which would have led to impaired glucose metabolism.

Blood urea nitrogen (BUN)

BUN levels of rat serum in all treatments including standard rat feed fed group were within the range of the BUN level of normal rat serum (Table 4.5.2). However, a non-specific increase in BUN in the group feed comprising of unfermented apple pomace with or without jaggery 0.01 per cent mixed flavour and 1 per cent salt and unfermented apple pomace with 2 per

Table 4.5.2 **Serum biochemical changes of rat**

Treatments	Glucose (mg/100 ml)	Blood urea nitrogen (mg/100 ml)	Total proteins (g/100 ml)	A.L.T. (I.U.)	A.S. T.(I.U.)
T_0	50-135	5-29	4.7-8.2	46-81	18-30
T_1	52.00	14.01	6.50	50.00	40.00
T_2	20.00	22.40	7.08	304.00	170.00
T_3	15.00	18.68	7.10	380.00	184.00
T_4	15.00	18.68	7.50	230.00	97.00
T_5	50.00	25.68	8.02	236.00	170.00
T_6	25.00	42.96	7.40	270.00	60.00
T_7	15.00	41.00	7.46	260.00	160.00

T_0 = Normal rat blood serum ranges

T_1 = Standard rat animal feed

T_2 = Fermented apple pomace + 2% ground nut oil + 10 % jaggery + 0.01% mixed flavour + 1% salt

T_3 = Fermented apple pomace + 10 % jaggery + 0.01% mixed flavour + 1% salt

T_4 = Fermented apple pomace + 2% ground nut oil + 0.01% mixed flavour + 1% salt

T_5 = Unfermented apple pomace + 2% ground nut oil + 10 % jaggery + 0.01% mixed flavour + 1% salt

T_6 = Unfermented apple pomace + 10 % jaggery + 0.01% mixed flavour + 1% salt

T_7 = Unfermented apple pomace + 2% ground nut oil + 0.01% mixed flavour + 1% salt

A.L.T. = Alanine amino transferase

A.S.T. = Aspartate amino transferase

cent ground oil, 0.01 per cent mixed flavour and 1 per cent salt. Increase in BUN level indicates kidney damage but in our study there no kidney damage was observed on post-mortem examination.

Total proteins

The results pertaining to total protein of rat serum (Table 4.5.2) show that the total protein levels in all the treatments were within the normal range was given by Raguramulu *et al.* (1983). Normally, the decrease in serum total protein is observed in chronic liver damage, whereas, in our study there was acute liver damage resulting in sudden death.

ALT and AST

The results relating to the serum AST and ALT levels in the different groups (Table 4.5.2) show that the levels in the standard rat feed fed groups were within the normal range. However, in all the other treatments ALT and AST levels were higher which might be due to liver damage which was evident on PME. So the increased activity of ALT and AST in the serum of rats could be attributed to liver damage (Plate 13, 14 and 15) at cellular level (Dratman and Lahorn, 1978) and also due to increased plasma membrane permeability(Ramazzatto and Carlin, 1978) as a result these enzymes are liberated in the rat serum. .

The blood biochemical studies reveal presence of some toxic substance in the feed which led to sudden liver damage, decrease in glucose level in serum and increased in serum ALT and AST values as explained earlier. These results corroborated with PME findings as explained earlier.

The preliminary studies on rats with apple pomace show that either fermented or unfermented apple pomace as such is not suitable as a feed for the rats. However, there was increased in feed intake per 100 g body weight of animals in either fermented or unfermented apple pomace incorporated into the standard rat feed in the ratio of 1:1. Whereas, the results pertaining to GR, ECI, WA were found to be negative. From the overall results it is concluded

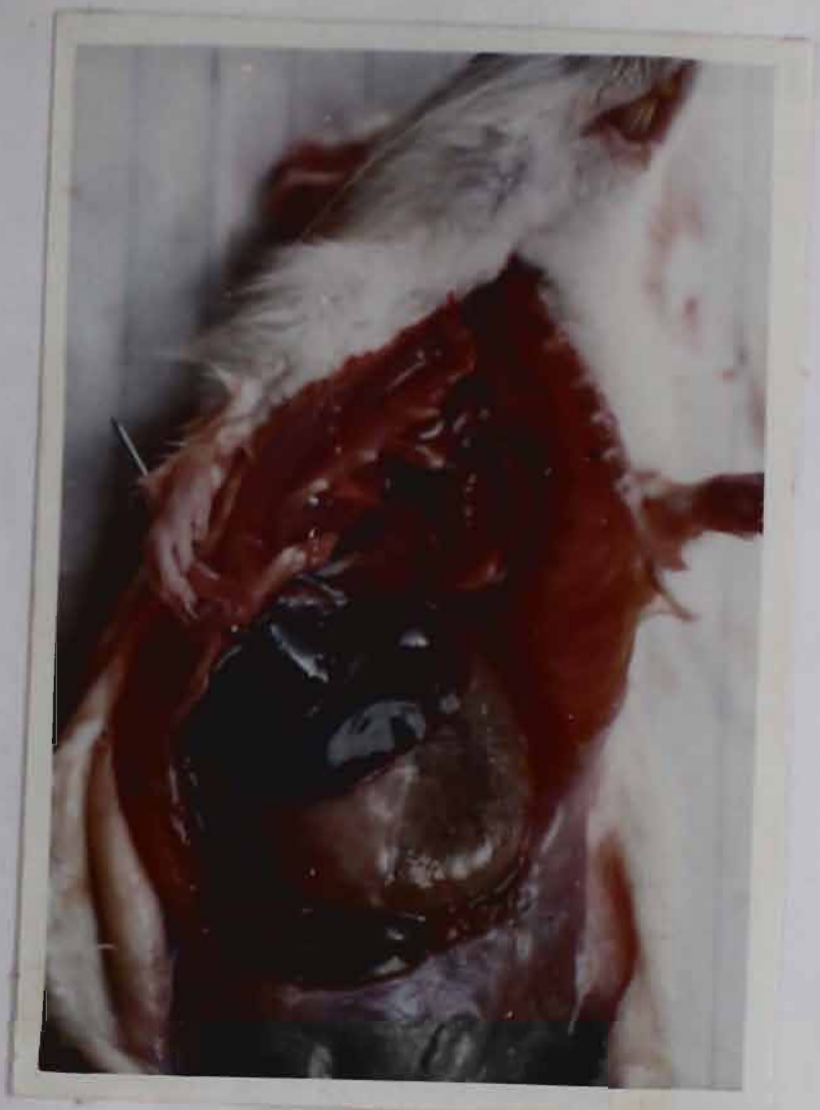


Plate 14 **Digested food material in stomach with liver damage of white rats fed with reconstituted fermented apple pomace comprising of 10% jaggery, 0.01% mixed flavour and 1% salt**

that the apple pomace either fermented or unfermented could be made more acceptable after reconstitution.

The results pertaining to DIG, ECI, WA and GR of reconstituted feed comprising of apple pomace either fermented or unfermented indicate that it was more or less comparable with the standard rat feed. Amongst the reconstituted feeds, feed comprising of fermented apple pomace, 10 per cent jaggery, 2 per cent of oil, 0.01 per cent mixed flavour and 1 per cent salt gives the best results.

Nevertheless, based on these results it can be concluded that the reconstituted feed has been brought to the acceptability of that of level of standard rat feed but further work is required to study the long term effects.

SUMMARY AND CONCLUSION

Investigations on "**Solid State Fermentation of Apple Pomace: Methods of Ethanol Recovery, Physicochemical Evaluation and Acceptability of Animal Feed Produced**", was carried out to standardize the methodology for production of ethanol and animal feed from apple pomace with molasses, along with two different nitrogen sources using five different yeasts viz. *Saccharomyces cerevisiae*, *Candida utilis*, *Torula utilis*, *Schizosaccharomyces pombe* and *Kloeckera* spp. Another approach which was successfully attempted was the sequential interactive co-culture with the conditions optimized. Four different methods were employed for the recovery of ethanol. The ethanol extracted dried apple pomace was evaluated in rats for their acceptability and effect on the growth parameters. Based on the results following conclusions could be drawn:

A. EFFECT OF ADDITION OF MOLASSES AND NITROGEN SOURCE IN SSF OF APPLE POMACE

1. The highest rate of fermentation and consequently highest ethanol production was recorded in apple pomace blended with 10 per cent molasses and 1.8 per cent of either of nitrogen source (DAHP or AMS). Addition of molasses and nitrogen source had affected all the physico-chemical characteristics viz. TSS, acidity, pH and reducing and total sugars.

2. There was no significant difference between the nitrogen source with respect to the fermetability by SSF of apple pomace, but based on the cost, AMS was selected.

3. The highest amount of crude protein was also observed in apple pomace fermented with 10 per cent molasses and 1.8 per cent of either of nitrogen source (DAHP or AMS).

4. Amongst the yeast species tried *Saccharomyces cerevisiae* fermented apple pomace had the highest ethanol content, whereas, the highest crude protein content was observed in *Kloeckera* spp. fermented apple pomace.

5. The apple pomace treated with 10 per cent molasses and 1.8 per cent of either of nitrogen source (DAHP or AMS) treatment was selected as the best treatment on the basis of highest ethanol and crude protein content in the fermented and fermented dried apple pomace respectively.

6. In case of yeast spp. selection for concomitant production of ethanol and animal feed none of the yeast alone had given both the parameters at the highest level i.e. ethanol and crude protein which was achieved through sequential interactive co-culture yeast.

B. SEQUENTIAL INTERACTIVE CO-CULTURE OF YEASTS IN SSF OF APPLE POMACE

7. Ethanol content in the sequential interactive co-culture system of fermentation with 10 per cent molasses and 1.8 per cent of AMS has increased upto 96 hrs of fermentation and after addition of sequential culture, the ethanol was consumed by sequential cultures except in *Saccharomyces cerevisiae and Schizosaccharomyces pombe*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* interactions.

8. The consumption of ethanol by sequential cultures resulted in and increase of biomass production and consequently considerable increase in crude and soluble proteins.

9. Out of different yeast spp. interactions, *Saccharomyces cerevisiae* alone had the highest rate of fermentation and accordingly highest ethanol production. Besides, highest crude and soluble protein was observed in *Candida utilis* and *Kloeckera* spp., as sequential interactive co-culture.

C. ETHANOL RECOVERY

10. The two treatments with highest ethanol by *Saccharomyces cerevisiae* and crude and soluble protein content by *Candida utilis and Kloeckera* spp as sequential interactive co-culture were selected for ethanol recovery by four different methods.

11. Amongst the different methods employed for ethanol recovery, steam distillation method gave the highest separation efficiency while lowest separation efficiency was found in hydraulic pressing (Hot water extraction followed by distillation).

12. Evaluation of dried apple pomace after recovery of ethanol by four different methods indicated that steam distillation method resulted in minimum nutritional loss viz. crude and soluble proteins and reducing and total sugars. The maximum nutritional loss was observed in hydraulic pressing.

D. ANIMAL FEED STUDY

13. After extraction of ethanol the dried apple pomace was evaluated in white rats as an animal feed.

14. From the animal feed (choice) acceptability study it was revealed that standard rat feed had highest acceptability followed by 1.1 ratio of standard rat feed and fermented apple pomace. The fermented or unfermented apple pomace as such as an animal feed was found to be least acceptable.

15. Evaluation of feed in rats (no choice) indicated that either fermented or unfermented apple pomace as such is not suitable as an animal feed for rats due to their negative growth parameters.

16. From the evaluation of reconstituted feed (no choice) it was concluded that fermented apple pomace with 10 per cent jaggery, 2 per cent ground nut oil, 0.01 per cent mixed flavour and 1 per cent salt gave the best results as regards the growth parameters of rats are concerned.

17. Out of different types of the reconstituted feeds the fermented apple pomace with 10 per cent jaggery, 0.01 per cent mixed flavour, 2 per cent ground nut oil and 1 per cent salt was found to be most acceptable. It also concluded that reconstituted apple pomace was more acceptable to rats than the either fermented or unfermented apple pomace as such.

18. The post-mortem examination of rats, but died after consumption of reconstituted feed containing either fermented or unfermented apple pomace revealed the cause of death to liver damage which may probably due to some toxins in the additives used for reconstitution of feed.

19. The analysis of blood sample of rats, fed with reconstituted feed for various biochemical parameters indicated liver damage which was evident in the form of increasing ALT and AST serum levels.

20. The overall animal feed trial study indicated that more elaborated feed studies with different animal spp. for extended period with different formulations based on apple pomace is required.

From the overall results by optimizing molasses (10%) and AMS (1.8%) with five different yeasts it is concluded that the highest ethanol production was obtained in apple pomace fermented with *Saccharomyces cerevisiae*. However, the low protein content of apple pomace could be increased to a higher level through SSF with *Candida utilis* and *Kloeckera* spp. as sequential interactive co-culture. Highest separation efficiency of ethanol with minimum nutritional loss was obtained through steam distillation, which also has potential for commercial exploitation. The dried apple pomace after extraction of ethanol was made into more attractive feed by reconstituting it with 10% jaggery, 2% ground nut oil, 0.01% mixed flavour and 1% salt. However, the effect of feeding of reconstituted apple pomace on animal metabolism and utilization as feed need more elaborated studies with different animals.

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THESIS ABSTRACT

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Year of Award of Degree	:	2002
No. of pages in Thesis	:	80+xv
No. of words in Abstract	:	342

ABSTRACT

The quest of generating renewable energy sources has naturally focused on plant biomass and its effective utilization through advances in technology development. This underutilized plant waste, in the shape of forest and agricultural biomass generated through annual cycles in nature may pose a serious threat to the environment. Cellulose and hemicellulose (mainly xylan) are the renewable carbohydrates present in all the plants and comprise more than 60% of all plant biomass. They can serve as the potential substrates for the commercial utilities viz., alcohol, fuel, food/feed, fertilizers, chemicals and single cell proteins via enzymatic hydrolysis with microbial cellulases and xylanases.

Fourty five cellulolytic and xylanolytic bacteria were isolated and screened from degrading wood and forest soils of Solan (H.P.). Among these, best cellulase and xylanase producers with cellulase activity of 2.36 EU and 2.04 EU and xylanase activity of 0.567 EU and 0.767 EU, respectively, were identified as *Bacillus coagulans* and *Bacillus licheniformis*. They were able to grow well under co-culture conditions. The forest residues selected for biodegradation were *Pinus*, *Quercus*, *Robinia* and *Cedrus*. In order to achieve enhanced biodegradation, various physico-chemical pretreatments were given to lignocellulosic residue i.e. grinding, NaOH, NH_3, dil H_2SO_4, dil HNO_3, $H_2O_2 + M_n^{++}$ and H_2O_2. The biodegradation of pretreated waste was carried out *in vitro* with co-culture under Submerged Fermentation (SmF) and Solid State Fermentation (SSF).

The results revealed that SSF using tap water was superior over SmF as showing maximum percent increase in Biodegradation Index of 174.90 in dil H_2SO_4 pretreated *Robinia* wood. However all the pre-treatments have been found effective in increasing biodegradation. When tap water was replaced with Basal Salt Medium the maximum per cent increase in BI ranged upto 140.97 in dil HNO3 pretreated *Robinia* over tap water. When BSM was further modified with additional supplements, the biodegradation enhanced much more. This increase was recorded upto 802.240% over tap water. Further Moisture level was optimised for forest waste degradation and it was observed that different moisture : substrate ratio is suitable for different substrates i.e. for hardwoods (1:3.0) and softwoods (1:2.0).

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