

**PROCESS OPTIMIZATION FOR ETHANOL
PRODUCTION FROM SWEET SORGHUM JUICE
USING *Saccharomyces cerevisiae***

Thesis

**Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of**

**MASTER OF SCIENCE
in
MICROBIOLOGY
(Minor Subject: Biotechnology)**

**By
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CERTIFICATE I

This is to certify that thesis entitled “**Process optimization of sweet sorghum juice for ethanol production using *Saccharomyces cerevisiae***” submitted for the degree of **M.Sc** in the subject of **Microbiology** (Minor subject: **Biotechnology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Jatminder Kaur** (L-2010-BS-238-M) under my supervision and no part of this thesis has been submitted for any other degree.

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

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CERTIFICATE II

This is to certify that thesis entitled “**Process optimization of sweet sorghum juice for ethanol production using *Saccharomyces cerevisiae***” submitted by **Jatminder Kaur** (L-2010-BS-238-M) to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Microbiology** (Minor subject: **Biotechnology**) has been approved by the Student’s Advisory Committee along with Head of the Department after an oral examination on the same.

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ABSTRACT

Ethanol is the most consumed biofuel in the world. Sweet sorghum [*Sorghum bicolor* (L.) Moench], a C₄ Gramineous crop which has sugar rich stalks having good potential as an alternative feed stock for ethanol production. In present study the sweet sorghum juice of variety CSV19SS was procured and was being analyzed for storage stability and its fermentation potential. Sweet sorghum juice was found to be stable under refrigeration conditions for about 75 days without any significant changes in the sugar profile and acidity of the juice. Two *Saccharomyces cerevisiae* strains namely *S. cerevisiae* strain-35 and *S. cerevisiae* strain NRRL Y-2034 were used for fermentation of sweet sorghum juice. *S. cerevisiae* strain-35 was found to be more efficient yielding 8.6% ethanol with fermentation efficiency of 86.69% as compared to *S. cerevisiae* strain NRRL Y-2034 yielding 8.4% ethanol with fermentation efficiency of 86.34%. Optimization of cultural parameters for maximum ethanol production and fermentation efficiency was done with the help of statistical software Statgraphics Centurion XVI.I using Response Surface Methodology. Various optimizing parameters were inoculum concentration, agitation rate and temperature and the resultant responses were ethanol content, total acidity and pH, using sugarcane and sweet sorghum juice inoculated separately by *Saccharomyces cerevisiae* strain NRRL Y-2034 and *Saccharomyces cerevisiae* strain-35. Production of ethanol from sweet sorghum juice and sugarcane juice (COJ-89) was compared under optimized conditions. Sweet sorghum juice inoculated with *S. cerevisiae* NRRL Y-2034 yielded 8.85% (v/v) ethanol with fermentation efficiency of 87.35% under optimized conditions of temperature (30°C), agitation rate (50 rpm) and inoculum size (7.5% v/v). The sugarcane juice inoculated with *S. cerevisiae* NRRL Y-2034 yielded 10.14% (v/v) ethanol under optimized conditions of temperature (30°C), agitation rate (100 rpm) and inoculum size (5% v/v) with fermentation efficiency of 97.67%. When sweet sorghum juice inoculated with *S. cerevisiae* Strain-35 8.47% (v/v) ethanol was produced under optimized conditions of temperature (30°C), agitation rate (50 rpm) and inoculum size (5% v/v) with fermentation efficiency of 83.56%. However in sugarcane higher ethanol production was observed (11.23% v/v) with fermentation efficiency of 95.62% under optimized conditions of temperature (30°C), agitation rate (100 rpm) and inoculum size (7.5% v/v). Sweet sorghum juice and sugarcane juices are good substrates for ethanol production. However, lifecycle assessment of both the crops has an edge towards the utilization of sweet sorghum juice for ethanol production.

Keywords: Sweet sorghum juice, ethanol, fermentation efficiency, *Saccharomyces cerevisiae*.

Signature of Major Advisor

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CHAPTER-I

INTRODUCTION

Non-renewable resources like petroleum cannot be produced, or generated on a scale which can sustain its consumption rate. Once depleted there will not be sufficient availability for future needs. Petroleum provides the single largest fraction of the world's energy, accounting for about 37% of the total energy used. Energy required for economic development has come mainly from fossil fuels extracted from deposits that took million of years to form. A large portion of petroleum is used in transportation, and the transportation sector is almost totally dependent on petroleum, particularly for powering personal vehicles and trucks. Uncertainty about future supplies and environmental concerns related to increase of atmospheric carbon dioxide levels has increased the interest in biofuel sector. Corresponding climate change consequences has motivated an interest in biofuels as they are considered renewable and carbon neutral. Soaring prices of fuel also lead to a renewed interest in the search of alternative sources especially liquid transportation fuels and chemicals to support the pace of growth. Biofuels like ethanol and liquid hydrogen etc. provides suitable alternative due to the limited oil reserves and concern about climate change from green house gas emissions.

Biofuels are liquid fuels derived from plant materials which are entering the market at a fast pace. Biofuels provided 1.8% of the world's transport fuel in 2008. Ethanol fuel is the most common biofuel worldwide and widely used in Brazil and USA. Ethanol is also known as ethyl alcohol, drinking alcohol or grain alcohol which is flammable, colourless chemical compound having a pleasant odour. Ethanol is an alcohol made by fermenting the sugar components derived from wheat, corn, sugar beets, sugarcane and molasses etc. Ethanol can be used in vehicles in its pure form, but is usually used as a gasoline additive to increase the octane number and improve vehicle emissions. It is a versatile solvent, miscible with water and many organic solvents, including acetic acid, acetone, benzene, carbon tetrachloride, chloroform, diethyl ether and toluene. It is also miscible with light aliphatic hydrocarbons such as pentane and hexane and also with aliphatic chlorides such as trichloroethane and trichloroethylene. The pH of 100% ethanol is 7.33 compared to 7.00 of pure water, thus hydroxyl group of ethanol is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar weight.

Crop plants are one of the best sources of renewable energy which can be used as feedstock for biofuel production. However, as these crops are majorly grown for food and feed, their use for fuel production will not meet the energy requirements. Bioethanol is an alcohol made by fermentation of carbohydrates present in sugars or starchy crops such as corn or sugarcane. Cellulosic biomass, derived from non-food sources such as crop residue and

grasses, is also being exploited as a feedstock for ethanol production. In India, also 10% blending of ethanol petroleum has been made mandatory in vehicles.

Ethanol is not a new fuel. In the 1850s, ethanol was a major lighting fuel. During the Civil War, a liquor tax was placed on ethanol to raise money for the war. The tax increased the price of ethanol so much that it could no longer compete with other fuels such as kerosene in lighting devices. Ethanol production declined sharply because of this tax and production levels did not begin to recover until the tax was repealed in 1906. As a transportation fuel, ethanol can be used as a total or partial replacement for gasoline containing ten percent ethanol (E10) is used in many urban areas. All vehicles that run on gasoline can use E10 without making changes to their engines. Over 99 percent of the ethanol produced in the United States is mixed with gasoline to make E-10. E85 is 85 percent ethanol and 15 percent gasoline, used mainly in the Midwest (especially Minnesota and Illinois) and South. Vehicles are not modified to run on E85; they are specially manufactured as flexible fuel vehicles (FFV). Flexible Fuel Vehicles can use any mixture of ethanol and gasoline up to E85. There are about 146,000 cars and trucks using E85. Most of these are fleet vehicles. Using ethanol means that we use a little bit less oil (a nonrenewable fuel) to make gasoline. Unlike gasoline, ethanol is nontoxic (safe to handle) and biodegradable; it quickly breaks down into harmless substances if spilled. When small amounts of ethanol are added to gasoline, usually less than 10 percent, they are advantageous. Ethanol reduces carbon monoxide and other toxic pollution from the tail pipes of vehicles, making the air cleaner. It keeps engines running smoothly without the need for lead or other chemical additives.

Domestic production and use of ethanol for fuel can solve the problems of energy crisis and hunger. It will also reduce dependence on foreign oil. The importance of the bioethanol production increased many fold with the realization that air and underground water pollution can be reduced by replacing lead or MTBE (Methyl-tert-butyl ether) of petroleum to bioethanol. MTBE is known as fuel oxygenate (Fischer *et al* 2005) and used as a fuel additive to raise the octane number (standard measure of the performance of a motor). However it is highly soluble in water and is a possible human carcinogen (Belpoggi *et al* 1995). Therefore, ethanol (an oxygenous biomass fuel) is considered as a predominant alternative to MTBE for its biodegradable nature, low toxicity, persistence and regenerative characteristic (Cassada *et al* 2000).

Therefore, a strong need exists for efficient ethanol production with low cost raw materials. Bioethanol have been produced on industrial scale by fermentation of sugars derived from wheat, corn, sugar beets and sugarcane juice etc. Nearly all fuel ethanol is produced by fermentation of corn glucose in America or sugarcane sucrose in Brazil.

Sweet sorghum is a revolutionary agricultural crop which has proven potential to help world's fuel dependency, pollution, deforestation, housing and food supply problems. Sweet

sorghum [*Sorghum bicolor* (L.) Moench], a C₄ Gramineous crop which has sugar rich stalks. It has a very good potential as an alternative feed stock for ethanol production. It is the only crop that provides grain and sugar rich stem and can be used for sugar, alcohol, syrup, jaggery, fodder, fuel, bedding, roofing, fencing, paper and chewing purposes. It is easier and less expensive to cultivate than corn, need fewer pesticides and fertilizers. Due to remarkably short growth cycle, two crops can be grown per year in warm climates. It has higher tolerance to salt and drought comparing to sugarcane and corn that are currently being used for biofuel production. In addition high carbohydrate content and high fermentable sugar content in sweet sorghum stalk makes it suitable for fermentation to ethanol.

Ethanol fermentation also referred to as alcoholic fermentation, is a biological process in which sugars such as glucose, fructose and sucrose are converted into ethanol (C₂H₅OH), carbon dioxide and energy. As yeast performs this conversion in the absence of oxygen, ethanol fermentation is classified as anaerobic. Ethanol fermentation occurs in the production of alcoholic beverages and ethanol fuel. Ethanol is produced through the fermentation of agricultural products such as sugarcane, corn, wheat, sugarbeet and cassava. Sweet sorghum juices usually contain approximately 16-18% fermentable sugar, which can be directly fermented into ethanol by yeast at room temperature. Fast degradation of fermentable sugars at the room temperature is the main technical challenge for using sweet sorghum juices as a feedstock for ethanol production. As much as 20% of the fermentable sugars are lost in three days at room temperature because of activities of contaminating bacteria, which lead to significant increase in bacterial count and decrease in pH values. However, no significant changes in pH value, sugar contents and sugar profiles are observed in juices stored in refrigerator (Xiaorong *et al* 2010).

Saccharomyces cerevisiae is a model eukaryotic organism, often used in research because it is easy to manipulate and culture, and is comparatively similar in structure to human cells. This yeast is also widely used in industrial applications to manufacture enzymes and proteins for beer, wine and bread, and because it metabolizes glucose to ethanol, is also the most commonly used microorganism for ethanol production which utilizes hexoses of the feed and convert them into ethanol. Pentose fermenting yeasts are being explored for utilizing 5-C carbohydrates available in biomass. Various physicochemical parameters like incubation temperature, pH, agitation rate and additives etc. affect ethanol production to a large extent. The temperature also affects the growth and productivity of the cells. As *S. cerevisiae* approach their optimum growth temperature of 30°C, their growth rate approximately doubles for every 10°C the temperature increases. Past the optimal growth temperature, the growth rate slows and metabolism may become faster than the diffusion rate within the bioreactor, making the diffusion of nutrients the rate limiting step. The pH inside the bioreactor affects the enzymes inside the cells and changes the rates of reaction. Yeast cells have evolved so

that they can thrive in more acidic environments than many competing organisms. As yeast cells consume their nitrogen source, hydrogen ions are released decreasing the pH of the solution. Buffers are often used to maintain the solution within the desired pH range. The amount of dissolved oxygen in the fermentation broth has major implications for the reactions that occur in yeast. When oxygen is present, respiration occurs converting simple sugars to cell mass and carbon dioxide and water as waste. Under some conditions, ethanol may be consumed by yeast cells.

Yeast can also tolerate high ethanol concentrations under appropriate conditions. Temperature can affect sensitivity of yeast to ethanol concentration, growth rate and rate of fermentation. The ability of yeast cells to convert sugar into carbon dioxide and alcohol is due to enzymes. Several enzymes are involved in each step in the process. High concentrations of alcohol actually destroy enzymes and kill the yeast cell. Different strains of yeast can tolerate different concentrations of alcohol. Brewers yeast cannot withstand much beyond 5 or 6% alcohol by volume. Wine yeast is more tolerant at a range of 10-15% especially cultured strains of yeast with the correct environment can withstand alcohol levels up to 21%. A lot of work is being done for the development of cost effective technology for ethanol production from sweet sorghum juice and efficient strains are being developed (Gokhale *et al* 1983; Karhumma *et al* 2006; Chu and Le 2007; Ban *et al* 2008).

Sweet sorghum juice is assumed to be converted to ethanol at 85% theoretical efficiency, or 54.4 l ethanol per 100 kg fresh stalk yield. Potential ethanol yield from the fiber is more difficult to predict (Rains *et al* 1993). The emerging enzymatic hydrolysis technology has not been proven on a commercial scale (Taherzadeh and Karimi 2008). One ton of sweet sorghum stalks has the potential to yield 74l of 200-proof alcohol (Smith and Frederiksen 2000). Sweet sorghum costs 93.96/- to produce a gallon of ethanol, compared with 118.26/- for sugarcane and 114.48/- for corn. It also compares well on energy balance with 8 units of energy produced for every unit of energy invested in its cultivation and production, compared with 8.3 units of sugarcane and 1.8 units for corn. On the other hand, only 0.8 unit of energy is produced in fossil fuel production for every unit invested. In the United States, the main feedstock for the production of ethanol is currently corn. Approximately 2.8 gallons (10.584 liters) of ethanol are produced from one bushel of corn (0.42 liter per kilogram). While much of the corn turns into ethanol, some of the corn also yields by-products such as DDGS (distillers dried grains with solubles) that can be used as feed for livestock. A bushel of corn produces about 18 pounds of DDGS (320 kilograms of DDGS per metric ton of maize). Although most of the fermentation plants have been built in corn-producing regions. Thus sweet sorghum also has an advantage over corn because the grain of sweet sorghum is not used in ethanol production, hence it does not have any impact on food prices and food security. Ethanol production from sweet sorghum is carbon neutral. The carbon

dioxide fixed during the growing cycle offsets the carbon dioxide produced during crop production, processing and ethanol utilization. Therefore, it is considered as the most suitable crop for ethanol production for biofuel compared to other crops such as corn or sugarcane.

Response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables in which a sequence of designed experiments are used to obtain an optimal response. Reports on optimization of process parameters using Response Surface Methodology for ethanol production from sweet sorghum and sugarcane juice are not available in the literature. Therefore the present work was aimed at maximizing ethanol production from sweet sorghum juice and sugarcane juice using *S. cerevisiae* strain NRRL Y-2034 and *S. cerevisiae* strain-35.

The objectives of present investigation were as follows:

- Optimization of fermentation parameters for ethanol production from sweet sorghum juice.
- Comparison of ethanol productivity of juices from sweet sorghum and sugarcane.

CHAPTER-II

REVIEW OF LITERATURE

Fossil fuel is the primary source of transport fuel in the world is depleting dramatically to meet the ever increasing energy demands globally. Climate change caused by carbon emissions from fossil fuels and energy security concerns are two major driving forces of utilizing alternative energy sources (Rubin 2008). Ethanol production as an alternative energy resource has been a subject of great interest since oil crisis of the 1970s (Tao 2005). By 1905, ethanol was emerging as the fuel of choice for automobiles among engineers and motorists, opinion being heavily swayed by fears about oil scarcity, rising gasoline prices, and the monopolistic practices of standard oil. Ethanol has excellent fuel properties for spark ignition of internal combustion engines. Characteristics like high octane number, high heat of vaporization, low photochemical reactivity in the atmosphere, and less smog formation compared to gasoline, makes ethanol much efficient than other fuels. Also it is less volatile than gasoline has a very low toxicity level and is readily degradable in water and soils (Bailey 1996).

Sorghum can be classified as sweet, grain, and forage types (Almodares *et al* 2008). Sweet sorghum is a C₄ crop of the grass family belonging to genus *Sorghum bicolor* L with high photosynthetic efficiency. Sweet sorghum like grain sorghum produces 3-7 t/ha of grains (Almodares and Mostafafi 2006). But the importance of sweet sorghum is not from its seed, but from its stalk, which contains high sugar content. It can produce 54-69 tones of stalk per hectare (Almodares *et al* 2008c). The sugar content in the sweet sorghum varies from 14.32 to 22.85% in different varieties (Almodares and Sepahi 1996). Besides having rapid growth, high sugar accumulation (Almodares *et al* 1994), sweet sorghum has wider adaptability (Reddy *et al* 2005) from sub-tropical and temperate regions of the world and is water efficient. It has many good characteristics such as drought resistance water logging tolerance, salinity resistance and with a high yield of biomass etc (Tesso *et al* 2005). Thus, sweet sorghum play an important role in promoting the development of agricultural production (Almodares and Hadi 2009), livestock husbandry (Fazaeli *et al* 2006), energy sources (Nahvi *et al* 1994), refining sugar and paper making etc.

According to the kind of sugar in stalk it can be divided into saccharin type and syrup type sweet sorghum (Anglani 1998). Saccharin type sweet sorghum which mainly contains sucrose can be used for refining crystal sugar. Sweet sorghum contains large quantities of fermentable sugars and total phenolics in stem juice. The fermentable sugars such as glucose, fructose, and sucrose can be used for ethanol production. Sweet sorghum stalks are used for producing biofuel by squeezing the juice and then fermenting the juice into ethanol. The juice of the stalk extracted by the squeezer contains approximately 84% water and 14% sugars as

glucose, fructose, sucrose and others as starch, amino acid, crude protein, phosphorous and mineral element in minor quantities. The sterile and concentrated juice with 16-18°Brix has potential to produce ethanol in good quantity. The juice is converted into ethanol by the yeast *Saccharomyces cerevisiae*. Sugar content in sugarcane is 10-12%, in sugar beet 15-18%, whereas in sweet sorghum is 7-12% (Almodares and Hadi 2009). Ethanol production directly from juice also varies in sugarcane (3000-5000 l/ha), sugar beet (5000-6000l/ha) and sweet sorghum 3000l/ha. Syrup type sweet sorghum, which mainly contains glucose can be used for producing syrup. Glucose, Sucrose, fructose, maltose, xylose and invert sugars are present (Almodares *et al* 2008c). However, mannose, galactose and arabinose are not detected in sweet sorghum juice. Carbohydrates in the stalk (sucrose and invert sugar) are suitable for ethanol production because these carbohydrates are easily converted to ethanol. Although ethanol can be produced from sweet sorghum grains but it needs more process for converting its starch to glucose and then later can be converted to ethanol (Jacques *et al* 1999).

Table 2.1 Microorganisms used in ethanol production from sweet sorghum

S. No.	Microbes used in fermentation of	Ethanol yield	References
Juice			
	<i>Saccharomyces cerevisiae</i> CFTR01 and SG	0.39-0.48(g/g consumed sugar)	Ratnavathi <i>et al</i> (2010)
	Fermax yeast (<i>Saccharomyces cerevisiae</i>)	77.07-79.58(g/l)	Kundiyana <i>et al</i> (2010)
	SuperStart yeast <i>Saccharomyces cerevisiae</i>	73.18-76.95(g/l)	Kundiyana <i>et al</i> (2010)
	<i>Saccharomyces cerevisiae</i> TISTR 5048	0.42-0.48g/g	Laopaiboon <i>et al</i> (2007)
	<i>Saccharomyces</i> strains	29-87% (sugar conversion efficiency)	Bulawayo <i>et al</i> (1996)
	<i>Saccharomyces cerevisiae</i> (Nanyang)	91.61%	Liu <i>et al</i> (2008)
	<i>Saccharomyces cerevisiae</i>	0.42-0.45 (g/g sugar) (80-90%)	Sipos <i>et al</i> (2009)
Bagasse			
	<i>Saccharomyces cerevisiae</i> 424(LNH-ST)	EtOH conc. 42.3g/l (EtOH yield:82.4-96.9%)	Li <i>et al</i> (2010)
	<i>Pachysolen tannophilus</i> , <i>Saccharomyces cerevisiae</i>	0.147g/g dry material	Ban <i>et al</i> (2008)

The most commonly used organism for ethanol production is the yeast *Saccharomyces cerevisiae* which is known to generate high ethanol yields apart from being more resistant to fermentation inhibitors. So far the research is only focused on the fermentation of hexose sugars which is because many organisms utilizing them naturally exists in nature. However, the pentose degrading microbes (except from the bacterium *Zymomonas mobilis* which can use xylose and ferment it to alcohol) (Zhang *et al* 1995) were not explored and the research on pentose fermenting organisms is required for more economical utilization of lignocellulosic feedstocks and to increase overall ethanol yield. Chohnan *et al* (2011) produced ethanol from three varieties of sweet sorghum using repeated batch fermentation. *Saccharomyces cerevisiae* cells could be recycled in 16 cycles of the fermentation process with good ethanol yields. This technique gives scope for using a broader range of sweet sorghum varieties for ethanol production. To reduce the high investment cost in ethanol production from sweet sorghum juice Kundiyana *et al* (2010) investigated the field production of ethanol with an objective to determine whether the fermentation process can occur without process control.

2.1 Production of Ethanol from Sweet Sorghum Juice

Sweet sorghum [*Sorghum bicolor* L. Moench] is the best multipurpose crop for simultaneous production of sugary juice from its stalk for making ethanol, syrup, or jaggery. Sweet sorghum has also been certified by an international agency to be a carbon neutral crop. The amount of carbon dioxide that sweet sorghum fixes is equal to the amount it emits during the entire process of crop growth, its conversion to ethanol and final combustion of ethanol. Kresovich and Henderlong (1984) reported on the feasibility of sorghum for ethanol production. Smith *et al* (1987) and Smith and Buxton (1993) stated that the sweet sorghum juice was a good substrate for the production of alcohol by yeasts.

Now a days, demand for ethanol as free octane fuel has increased steadily; therefore sorghum juice performance is always with high percentage of simple sugars which can be directly acted upon by yeast to produce alcohol. India's first plan for commercial production of ethanol has been implemented jointly by the Hyderabad based International Crops Research Institute for Semi-Arid Tropics and Rusni Distilleries.

Sweet sorghum is considered as a potential alternative crop for energy and in industries because it can yield biomass and fermentable sugars. The large amount of fermentable sugars accompanied by profuse mineral elements make the sweet sorghum juice a good and economic substrate for ethanol production. The production of fuel ethanol by fermentation requires the ability to produce high ethanol concentrations rapidly while maintaining good yields. Rapid formation and high alcohol levels are desirable to minimize capital costs and energy required for distillation, while good yields are necessary for process economics. The substrate is the main cost component for industrial ethanol production and it

is essential that ethanol production should be carried out with cheap substrates such as sweet sorghum (Yu *et al* 2009).

Sweet sorghum is a type of sorghum that has high concentration of soluble sugars in plant sap or juice. Sweet sorghum is a reasonable feedstock for ethanol production and could make a larger contribution to the nation's fuel requirements. Currently feedstocks for commercial ethanol production are corn grains ($\approx 95\%$) and sorghum grains ($\approx 4\%$) (Wu *et al* 2010).

Sweet sorghum is attractive for bioethanol production because of its high fermentable sugars (sucrose, glucose, and fructose) and very high yield of green biomass (20-30 dry tons/ha). Of the (20-30 dry tons /ha) of biomass approximately 40-45% are fermentable sugars and starch which is equivalent to more than 200 bushes/ acre of corn yield. If all fermentable sugars in sweet sorghum are converted to ethanol, potential ethanol yield could be 600-650 gal/acre. Normal pressing can recover only $\approx 50\%$ of the total sugars in the sorghum stalk (Bryan *et al* 1985). The presence of reducing sugars in sweet sorghum prevents crystallization and sweet sorghum cultivars have 90% fermentation efficiency. These desirable agricultural characteristics make sweet sorghum a promising alternative feedstock for fuel ethanol production in the Southern United States (Gibbons *et al* 1986).

Bioethanol is a "largest volume organic solvent" produced by fermentation which has gained immense importance in recent years as a chemical feedstock, fuel supplement and gasoline extender (Bajaj *et al* 2001). Indian distilleries have an installed capacity of over 9.73 billion liters of ethanol with average annual production of 0.9-1.0 million liters (Sharma and Tauro 1986). In India, ethanol is produced in about 200 distilleries by fermentation of dilute cane molasses employing *Saccharomyces cerevisiae* in a traditional batch process primarily for portable purpose (Kaur and Kocher 2002).

2.1.1 Uses of Ethanol

Ethanol offers a wide range of applicability like solvent, raw material for pharmaceuticals and alcoholic beverages especially wines, brandies, whiskies, country liquors and rum (Jacob 2007). An efficient ethanol production requires four components: fermentable carbohydrate, efficient yeast strain, few nutrients and suitable environmental conditions.

In order to produce ethanol economically, optimization of various physiochemical parameters of fermentation are important which include sugar level of fermentation medium, temperature, pH, ethanol tolerance of the yeast, fermentation efficiencies of the yeast strains and product recovery. Various raw materials like sweet sorghum (Bulawyo *et al* 1996), sugarcane juice and molasses (Morimura *et al* 1997) etc. have been used. It is thus desired to appropriately improve the fermentation process right from the step of inoculation to achieve better efficiency in terms of time and cost economy.

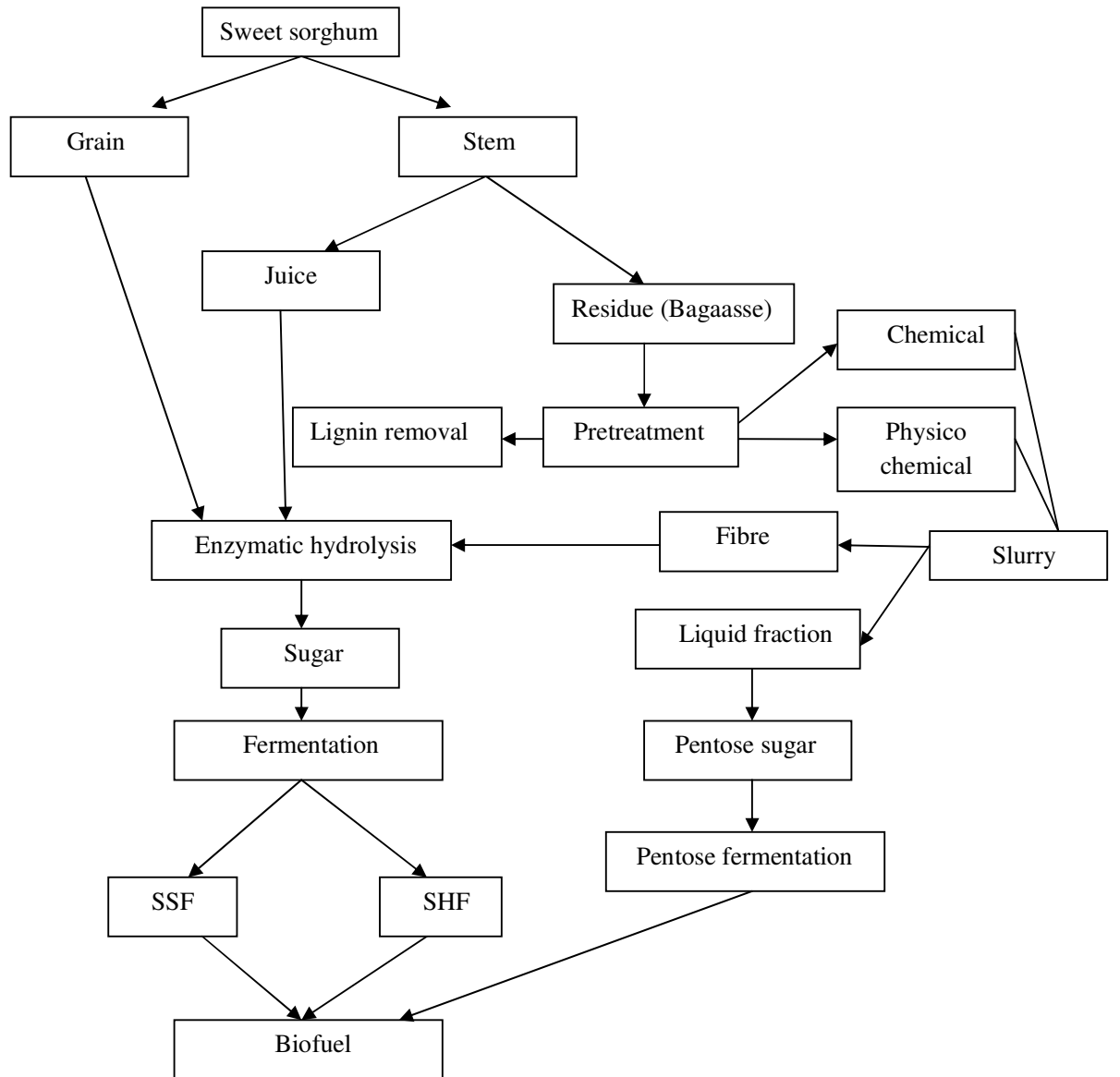


Figure 2.1 Bio ethanol production from Sweet Sorghum (Ratnavathi *et al* (2011))

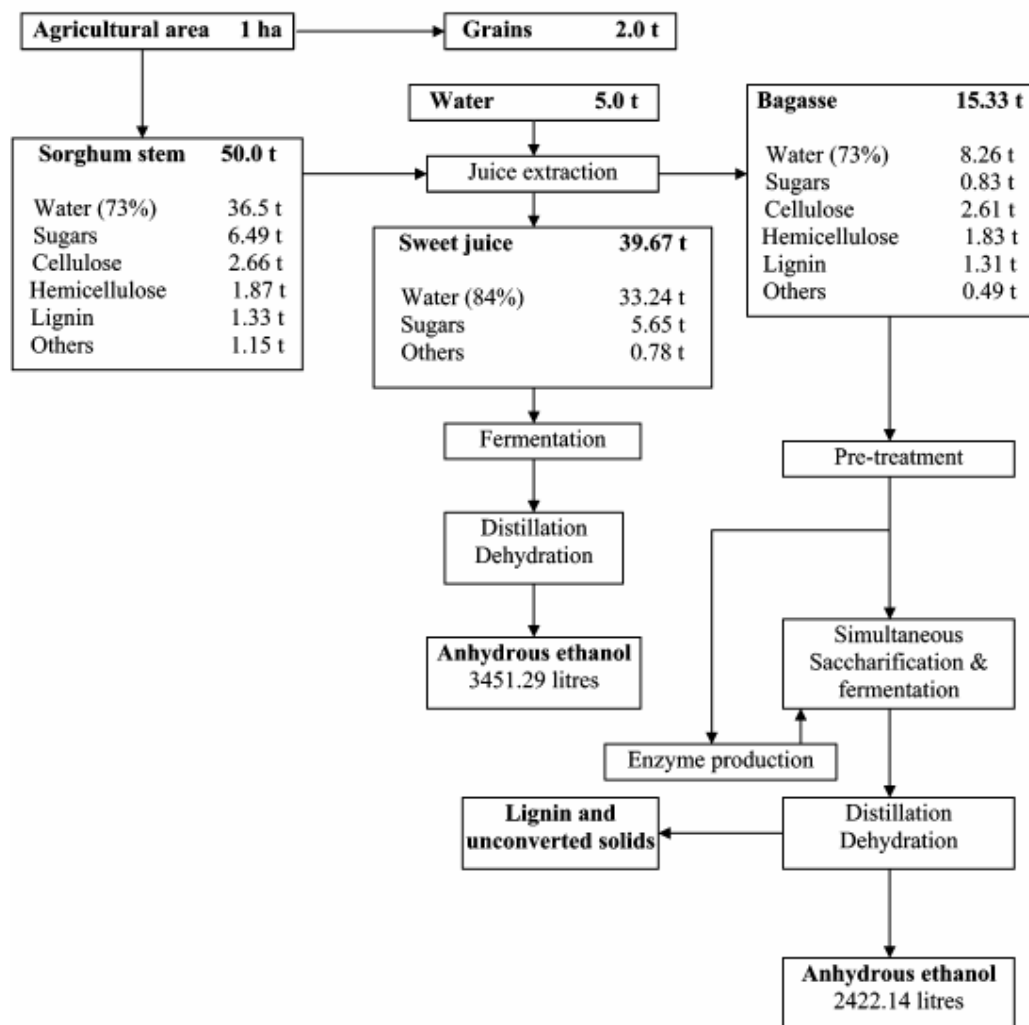


Figure 2.2 Mass balances of sweet sorghum juice extraction and ethanol production (Prasad *et al* 2007)

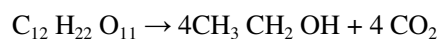
Adams (1990) defined the term “fermentation” as a form of energy yielding microbial metabolism in which an organic substrate, usually carbohydrate is incompletely oxidized, and an organic carbohydrate acts as an electron acceptor. Certain species of yeast most importantly *Saccharomyces cerevisiae* (Baker’s yeast) metabolize sugars in absence of oxygen, producing ethanol and CO₂. Ethanol fermentation results in four major products: additional yeast cells, ethanol, carbon dioxide, and heat. Yeast strains are major components of fermentative ethanol production. They differ widely in their capacity and rate of ethanol production.

Saccharomyces cerevisiae is the most commonly used organism for alcoholic fermentation of molasses (Bauer and pretorious 2000). Day and Sarkar (1982) evaluated performance of several yeast strains in ethanol fermentation of sweet sorghum juices and reported that ethanol productivity varied significantly among different yeast strains: ethanol

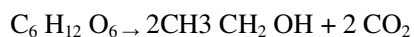
yields differed among juice batches. Karunakaran *et al* (1986) compared four strains of *Zymomonas mobilis* and found 15% sugar and pH 7.0 to be optimum for strains ATCC 10988 and ATCC 12526. Rein *et al* (1989) reported 17.9% to 41.1% fermentation efficiencies for unheated raw juices while higher than 90% for heated juices (30 minutes at 60 or 85°C). Uma and Polase (1990) isolated *S. cerevisiae* from palm wine which produced increased amounts of ethanol in yeast extract peptone dextrose medium. Different yeast species differ in their fermenting abilities and alcohol production efficiency.

Laluce *et al* (1993) studied the thermotolerance of wild yeast strains which were selected in the presence of high temperature, high sugar, and added alcohol during fermentation of sugar syrup. Kaur (1995) studied the alcoholic fermentation at higher substrate concentration and inoculum size and observed that 20 % of total sugars were fermented optimally with inoculum having cell population of 1×10^7 cells/ml with fermentation efficiency of 88%. Studies on ethanol production from sweet sorghum juice have been carried out at NARI to screen yeast from various sources for their potential to convert sweet stem sorghum juice to ethanol. Out of the 16 strains screened, NCIM 3319 was found to be best suited for fermentation. It gave average fermentation efficiencies of 90%, which was completed between 48 and 72 hrs. The juice contains an average of 10-11% (w/w) total fermentable sugars and alcohol yields are about 6% (v/v).

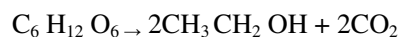
Sree *et al* (2000) reported four osmotolerant, thermotolerant, flocculating fermenting yeast strains isolated from soil samples. Bajaj *et al* (2001) screened yeast isolates from natural sources and selected isolate number: SBS13 and SBS14 which produced ethanol comparable to that of industrial strain of *Saccharomyces cerevisiae* and *Zymomonas mobilis* for ethanol production from molasses and found *Saccharomyces cerevisiae* to be more alcohol tolerant, producing high amount of ethanol at sugar concentration above 15%. Sweet sorghum is attractive for bioethanol production because of its high fermentable sugars (sucrose, glucose and fructose).



Sucrose Ethanol



Glucose Ethanol



Fructose Ethanol

Okunowo *et al* (2005) examined different yeast strains on orange juice where the fermentation efficiency varied between 48.05-99.46% with *S. cerevisiae* and *Saccharomyces carlsbergensis*. Appropriate inoculum size not only determines product efficiency of the alcohol fermentation plant but also helps prevent contamination by unwanted microorganisms. Farm scale fermentation processes using shredded sweet sorghum in solid

phase fermentation (Gibbons *et al* 1986) and sweet sorghum juice in liquid batch fermentation (Kundiya *et al* 2006) have been developed and tested. Laopaiboon *et al* (2007) supplemented sweet sorghum juice with 0.5% ammonium sulphate as a substrate for ethanol production by *Saccharomyces cerevisiae* and reported ethanol concentration produced, yield and productivity were 1007g/l, 0,42g/g and 1.67g/l/h, respectively with initial cell and sugar concentration of 1×10^8 cells/ml and 24⁰Brix respectively. Fed batch fermentation had higher conversion efficiency than batch fermentation (Laopaiboon *et al* 2007), and application of immobilized yeast in a fluidized bed reactor not only shortened fermentation time significantly but also increased conversion efficiency (Liu *et al* 2008). Liu and Shen (2008) optimized the main factors on bioethanol fermentation from stalk juice of sweet sorghum by immobilized *Saccharomyces cerevisiae* such as fermentation temperature, agitation rate, and particles stuffing rate and pH were 37°C, 200 rpm, 25% and 5.0 respectively. The experiments were performed in shaking flasks showed that ethanol yield and CO₂ weight loss rate were 98.07% and 1.020g/l respectively. The conclusion in the result would be beneficial for application of ethanol fermentation by immobilized *Saccharomyces cerevisiae* from stalk of sweet sorghum.

2.2 Production of Ethanol from Sugarcane Juice

Life cycle analysis of ethanol production using sugarcane as raw material has shown a positive energy balance and savings on GHG emissions and concerns about the fuel versus food debate also have been put in perspective avoiding unnecessary discussions. Countries employing sugarcane for sugar production use molasses in annexed plants as raw material for ethanol production. However there are also in operation in Brazil autonomous installations where ethanol is produced directly from harvested cane. In this installations juice still is extracted by pressing and then fermented with yeasts, usually in fed batch mode.

Contrary to the bioethanol demand, the sugar market is saturated, and the sudden price instabilities and oscillations are caused by economic factors and short term shortages that occur at random due to natural disasters like hurricanes and drought periods which affect sugar plantations worldwide.

Brazil is the world's largest sugarcane ethanol producer and a pioneer in using ethanol as a motor fuel. In 2010, Brazilian ethanol production reached 27.4 billion liters (7.2 billion gallons). Most of this production is absorbed by the domestic market where it is sold as either pure ethanol fuel or blended with gasoline. All gasoline sold in Brazil includes a blend of 18 to 25 percent ethanol. However, sugarcane ethanol's popularity really took off in 2003 with the introduction of flex fuel vehicles that run on either gasoline or pure ethanol. More than 90 percent of new cars sold today in Brazil are flex fuel due to consumer demand, and these vehicles now make up about half of the country's entire light vehicle fleet – a remarkable accomplishment in less than a decade.

As a C₄ photosynthetic species, sugar cane presents very high biomass productivity, amounting to 80-120 ton/ha. year with an industrial ethanol production of 8,000 litres/ha, higher when compared to 3,000 litres/ha from maize. Sugarcane is usually harvested according to parameters fixed by the sugar industry, in other words, when sugarcane shows the maximum sucrose content. As yeast is capable of fermenting not only sucrose but also hexoses into ethanol, the proper development stage to harvest sugarcane directed only for ethanol production might be different than the one for sugar production.

S. cerevisiae strains show a preference for glucose over other hexoses as a source of carbon and energy and its uptake by the cell is mediated by a family of hexose transporters which also transport fructose, although glucose is first depleted from the fermentation medium. This discrepancy in uptake rates has been shown to be strain dependent, and is more pronounced at the end of any batch fermentation (Berthels *et al* 2004; Verstrepen *et al* 2004; Perez *et al* 2005). In fact in wine fermentations, yeasts are forced to use fructose which is not preferred in conditions of nitrogen starvation and high ethanol concentrations which induce microbial stress and promote what is known in the trade as stuck fermentations (Guillaume *et al* 2007).

The first step in sucrose utilization by ethanolic yeasts is its complete hydrolysis into glucose and fructose by an extracellular invertase. Invertase is encoded by a family of SUC genes which also generate an intracellular constitutive invertase. This membrane-bound enzyme breaks down sucrose into its hexose components during active sucrose transport directly into the cell, which is another uptake mechanism present in ethanolic yeasts (Stambuk *et al* 2000; Badotti *et al* 2008). Fructose on the other hand, the amount originally in the cane particles and that produced during fermentation by sucrose hydrolysis, accumulated in the medium due to the impairment of the fructose transport system, hence more fructose was analytically detected at the end of the fermentation than the fructose quantity contained in the initial pulverized mature cane samples. Similar results have been recently published for the ethanolic fermentation of sweet sorghum juice (Wu *et al* 2010).

The thermotolerant yeast strain isolated from sugarcane juice through enrichment technique was identified as a strain of *Pichia kudriavzevii* (Issatchenkiaorientalis) through molecular characterization. The *P. kudriavzevii* cells adapted to galactose medium produced about 30% more ethanol from sugarcane juice than the non-adapted cells. The recycled cells could be used for four successive cycles without a significant drop in ethanol production (Dhaliwal *et al* 2011).

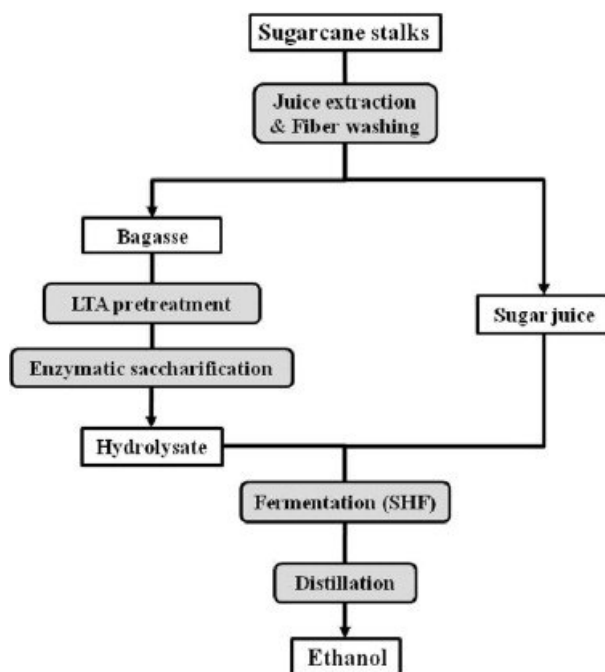


Fig 2.3 Production of ethanol from sugarcane stalks (Wu *et al* 2011)

Table 2.2 Comparison of the juice of sweet sorghum and sugarcane (Ratnavathi *et al*, 2011)

Sr. No.	Character	Sweet Sorghum	Sugarcane
1	Appearance	Juice is thick and turbid	Juice is clear and transparent
2	Contents	High starch and chlorophyll	Has no starch
3	Density	High	Low almost equal to water
4	Sugars	Sucrose and high amount of reducing sugar (2-4%)	Contains more sucrose and less reducing sugar (0.5-1%)
5	Invertase activity	High invertase	No invertase
6	Time of sugar accumulation	Starts after flowering	Sucrose accumulates at early stage
7	Purity of juice	70-90%	90-98%

2.3 Raw Materials

Various raw materials like (i) sugar bearing materials (sugarcane, molasses, sweet sorghum etc); (ii) starchy materials (corn, cassava, potato, grains) and (iii) lignocellulosics (agricultural crop residues, wood, etc.) can be used for ethanol production. Of these, sugar bearing materials are the easiest to process, since yeasts ferment sugars directly, while the other carbohydrates must be first hydrolyzed to sugar using current commercial technology before they can be fermented to yield ethanol.

i) Sugar bearing materials: Sugarcane is considered as the most suitable raw material for ethanol production as it envisages conversion of sugar into ethanol through EMP pathway. A ton of sugarcane with an average sugar concentration of 12.5% gives about 70 liters of ethanol through direct fermentation of juice (Chahal, 1982). Brazilian ethanol industry is based predominantly on cane juice molasses which is the by-product of sugar production from cane and every ton of sugar produced gives about 90 liters of molasses containing 50-55% of fermentable sugars mainly sucrose, glucose and fructose and about 280 liters of ethanol can be produced per ton of molasses (Lindman and Rocchiccioli, 1979). Available sugar percentage in sweet sorghum was significant and positively associated with percentage; °Brix; reducing sugar content; and stalk weight (Audilakshmi *et al* 2010).

ii) Starchy materials: The main starchy materials that have been tried for ethanol production are cassava, corn and potato. Grains of wheat, barley, sorghum oats, rice can also be used depending on their availability. The feedstock is ground into fine powder and starch is then hydrolyzed into fermentable sugars using amylases, liquefying enzymes. Fermentable sugars are converted to ethanol through yeast fermentation either in a batch or continuous process. However, lot of variations in the starch hydrolysis process has been reported upon the hydrolyzing enzymes (Butler and Crombil, 1980; Yang *et al* 1981).

iii) Lignocellulosics: Plant residues are the most abundant renewable source on earth amounting to about 50 billion tons/year (Goldstein, 1981). Notable among the lignocellulosics are various agricultural, agro-industrial and domestic refuse. The crop residues contain 30-45% cellulose; 16-27% hemicellulose; 3-13% lignin and 3.6-7.2% protein on dry weight basis (Slonekar, 1976). Ethanol production from cellulosic substrates involves pretreatment of lignocellulosics, hydrolysis into sugars and subsequent fermentation of these sugars into ethanol. For a material to be suitable for large scale ethanol production, it must be available at low cost and in reasonably uniform quality on a year-round basis.

2.4 Chemical Composition of Sweet Sorghum

Sweet sorghum is ideal as a “food and fuel” crop and has been noted for its potential as an energy crop for India. Overall out of many ‘new crops’ that are currently investigated as potential raw materials for energy and industry, sweet sorghum seems to be most promising one (Gosse 1996). Sweet sorghum is a high biomass and sugar yielding crop. It contains high

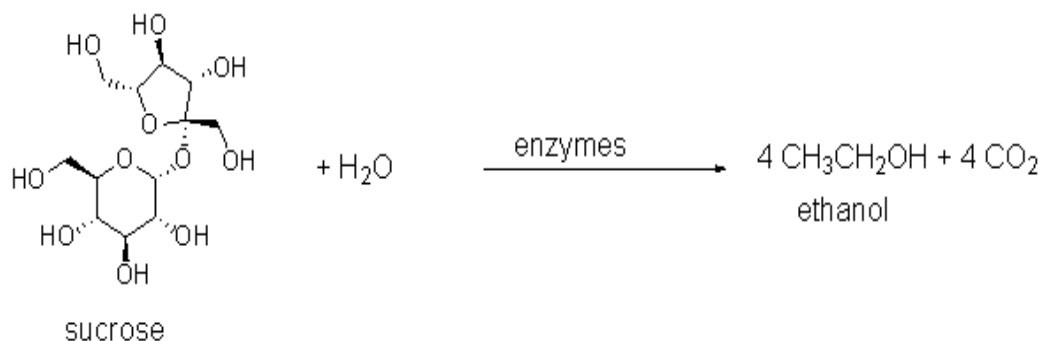
amount of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicelluloses), thus has been considered as an important source for production of bioethanol (Mamma *et al* 1995).

In addition to fermentative sugar, other kinds of sugars are also found in the stem juice of sweet sorghum. The sterile and concentrated juice with 16-18°brix has potential to produce ethanol in good quantity. Graansounau *et al* (2005) reported that sweet sorghum juice contains 85% sucrose, 9% glucose and 6% fructose on an average and only sucrose is readily converted to white sugar. Bhoyar and Thakare (2009) conducted experiment with ten cultivars of sweet sorghum to know the composition of stalk juice. They found reducing sugars, non reducing sugars and total sugars in range of 1.16- 2.48%, 9.92-15.94% and 11.5-17.4% respectively. Hunsigi *et al* (2010) conducted field trials and found that highest sugar content was in RSSV 56 (14.2%) followed by NSSV 254 (13.2%). The reducing sugar content varied from 1.05 to 2.50%. Yu *et al* (2009) reported the components of Rio variety such as glucose (10g/kg), fructose (8g/kg), and sucrose (69g/kg) in sweet sorghum juice.

2.4.1 Sucrose

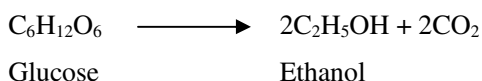
Sucrose is a widely existed disaccharide in natural world and is a non- reducing sugar. It is found in all photosynthetic plants, but it is more in cane and beet so popularly called as cane or beet sugar. It is the organic compound commonly known as table sugar and sometimes called as saccharose. Sucrose play an important role in plant physiology, it is not only the main product of photosynthesis but also the main form of storage and accumulation of carbohydrates. Sucrose is white odorless, crystalline powder with a sweet taste. It is best known for nutritional role. The molecule is a disaccharide composed of monosaccharide glucose and fructose with the molecular formula $C_{12}H_{22}O_{11}$. The mixture of glucose and fructose formed from sucrose hydrolysis is called invert sugar. It comes from plant sources sugarcane, sugar beets, date palm, sorghum and sugar maple. Sucrose is obtained by extraction of these crops with hot water; concentration of the extract gives syrups, from which solid sucrose can be crystallized. A 25° B sucrose solution has 25 grams of sucrose per 100 grams of liquid; or to put it another way, 25 grams of sucrose sugar and 75 grams of water exist in the 100 grams of solution.

Sucrose is a natural and economical sweetener. It is most versatile of all the sweeteners, performing many useful functions in a range of foods. It acts as a food for yeast in baking and in brewing beer. Sucrose used as a starting material in the fermentative production of ethanol, butanol, glycerol, citric and levulinic acids. Sucrose is present in stalk juice of sweet sorghum as a major fermentable sugar. One molecule of sucrose on fermentation yields four molecules each of ethanol and CO_2 as shown below:-



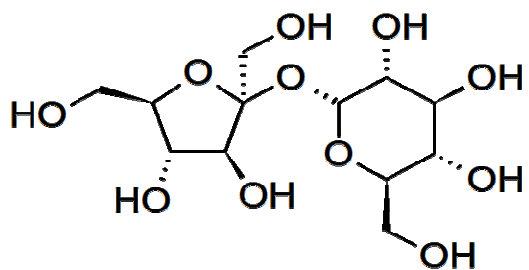
2.4.2 Glucose

Glucose is also known as D-glucose or dextrose. It is a simple monosaccharide found in plants. Glucose is one of the main products of photosynthesis and fuels for cellular respiration. All forms of glucose are colorless and easily soluble in water, acetic acid and several other solvents. In animals and fungi, glucose results from the breakdown of glycogen, a process known as glycogenolysis. Glucose is a type of sugar the body uses for energy. The stem juice of sweet sorghum is rich in fermentative sugar and is a desirable alcoholic fermentation material. Sweet sorghum is a C₄ crop with lower CO₂ compensation point, higher light saturation point and weak photorespiration, and consequently has higher biological yield. Glucose is a substrate of respiration and also a component of sucrose, starch and cellulose. As a reducing sugar, glucose can be fermented by *Saccharomyces*. In fermentation, acetic aldehyde and CO₂ are produced through decarboxylation of pyruvic acid formed from the dehydrogenation of glucose then acetic aldehyde is dehydrogenated and alcohol is produced. The whole process is under anaerobic and enzymatic conditions which are known as alcohol producing fermentation.



2.4.3 Fructose

Fructose is a hexose with reductive character. Fructose or fruit sugar is a simple monosaccharide found in many foods. It is one of the three important monosaccharides along with glucose and galactose. Fructose is a colorless crystal with strong hygroscopicity. It is sweetest among the sugars with a saccharinity of 1.15 to 1.5 times as much as glucose. So, it can be used as nutrient and as preserving agent. It is first transformed into fructophosphate ester, and then enter EMP pathway. The sucrose synzyme existing in high plants can use uridine diphosphate glucose (UDPG) as the offering of glucose to form sucrose with fructose.



2.5 Effect of Physicochemical Parameters on Ethanol Production

2.5.1 Yeast variety

Yeasts as a group of micro-organisms has been quantitatively and commercially exploited as a fermentative species needed to carry out alcoholic fermentation and this has urged many scientists to study the factors governing its growth, survival and biological activities in different ecosystems (Heard and Fleet 1986). Among several yeasts *Saccharomyces cerevisiae* and *S bayanus var. uvarum* are the most important species present during fermentation process (Pretorius 2000, Querol and Fleet 2006).

Saccharomyces cerevisiae is most frequently and traditionally used microorganism for fermenting ethanol from starch-based residues at industrial scales. It has few advantages such as its wide public acceptance, high fermentation rate and high ethanol tolerance that make it a good candidate for fermentation processes. *S. cerevisiae* is unable to efficiently utilize xylose as the sole carbon source or ferment it to ethanol. However Chu and Lee (2007) reported that the xylose-fermenting *S. cerevisiae* have been isolated which convert xylose to ethanol at efficiency close to its theoretical value of 0.51g g⁻¹(). Gokhale *et al* (1983) isolated a *Saccharomyces* species which exhibited 70% higher specific ethanol productivity than that of *Saccharomyces uvarum* ATCC 26602. The latest recombinant *S. cerevisiae* (TMB 3400) has been shown to successfully ferment both xylose and arabinose in addition to glucose (Karhumma *et al* 2006).

Karimi *et al* (2006) reported highest ethanol and glycerol yields by anaerobic SSF of the pre-treated rice straw with *M. indicus*. Ban *et al* (2008) reported ethanol production from the residues of sweet sorghum. In order to attain a higher ethanol yield and faster ethanol fermentation rate, orthogonal experiments of ethanol fermentation with immobilized yeast from stalk juice of sweet sorghum were carried out in the shaking flasks to investigate the effect of main factors, namely, fermentation temperature, agitation rate, particles stuffing rate and pH on the ethanol yield and CO₂ weight loss rate (Lie and Shen 2007).

Leplace *et al* (1991) studied combined alcoholic fermentation of D-xylose and D-glucose by strain of *Pichia stipitis*, *Candida slehatae*, *Saccharomyces cerevisiae*, and *Zymomonas mobilis* with respect to sugar and produced ethanol tolerance. A *Zymomonas* strain was compared with a *Saccharomyces* specie for the fermentation of sugarcane molasses. Both gave similar final ethanol concentrations but fermentation was slower with Z.

mobilis than with *S. cerevisiae* species (Murphy *et al* 1988). Sterilized and unsterilized juices are now under consideration for checking of ethanol yield by *Saccharomyces cerevisiae* as this can tolerate high ethanol concentrations. Kundiyanana *et al* (2010), reported sugar extraction and storability are two serious problems that have limited sweet sorghum as a potential energy crop. They also studied the influence of temperature, pH, and yeast on in-field production of ethanol from unsterilized sweet sorghum juice.

Liu and Shen (2007) reported optimized conditions for ethanol production from sweet sorghum juice using immobilized yeast cells. According to Graham (2001), a known taxa of yeast should comprise starter culture for juice fermentation. In *S. cerevisiae* production, high sugar concentrations and high specific growth rates triggers alcoholic fermentation even under fully aerobic conditions (Petrik *et al* 1983).

2.5.2 Effect of Temperature

Temperature is one of the most important parameters for the development of alcoholic fermentation because it affects both the kinetics of the process in terms of duration, rate of fermentation and the final quality of ethanol. The temperature determines how *Saccharomyces* strains develop and how effectively they ferment. Some strains perform better at high temperatures and others at low temperatures. The temperature of fermentation can affect the development of different *Saccharomyces* strains (Ribereau *et al* 2000).

Temperature can affect the sensitivity of yeasts to alcohol concentration, growth rate, rate of fermentation, viability, length of lag phase, enzyme and membrane function etc. The temperature of fermentation can affect the development of different *Saccharomyces* strains, the yield of ethanol, other fermentation byproducts and final composition of ethanol are also related to temperature (Torija *et al* 2003). The growth rate of yeast cells is strongly influenced by fermentation temperature. This is particularly evident during exponential phase. At warmer temperature (> 20°C), yeast cells experience a rapid decline in viability at the end of the fermentation. At cooler temperature, cell growth is retarded but viability is enhanced as low temperature prolongs the lag phase of fermentation and slows the rate of fermentation. Excessively high temperature disrupt enzyme and membrane functions, resulting in struck fermentation (Sener *et al* 2007). Fermentation is reported to cease at 30°C with 342 g/l sugar in the medium before whole of the sugar fermented while 25-30°C caused a negative effect on survival of *S.cerevisiae* (Strehalaiano and Goma 1983).

Fermentation temperatures greatly impact yeast growth, fermentation rate and production of volatile compounds. Fermentation rate increase with temperature up to a maximum value (commonly >29C), after which premature cessation is probable due to elevated ethanol toxicity (D'Amato *et al* 2006). Low temperature fermentation altered nitrogen transport and metabolism. Thus, this may hamper coordination between carbon and nitrogen metabolisms (Beltran *et al* 2007).

The effect of fermentation temperature (18°C and 25° C) on kinetics and yield parameters of ethanol fermentation by *S. cerevisiae* Zymaflore VL1 and *S. cerevisiae* Uvaferm CM was examined using the white Emire grape. Growth of both yeast species varied according to temperature (18 and 25° C) suggesting the kinectic and yield parameters were both temperature dependent (Srner *et al* 2007). Growth rates for all species increased with temperature. For most strains, maximum cell biomass increased until 15°C, after which no change in biomass production occurred, depending on the strains (Charoenchai *et al* 1998).

A lower temperature modified the cellular lipid composition of yeast, increasing the degree of unsaturation at the beginning of the fermentation and decreasing the chain length as fermentation progressed .Medium chain fatty acids, mainly dodecanoic acid, present in the cell phospholipids were reported by Beltran *et al* (2008). Fermentations that were performed at the lower temperature also emphasized this feature. The fermentation conducted at a controlled temperature below 32 °C will result in an increased yield and will also provide the very high gravity fermentation.

2.5.3 Effect of pH

The pH of the growth medium is another important parameter for the successful progress of fermentation because it influences yeast growth as well as ethanol formation. It is reported the pH range of 3.8-4.2 provides the conditions for *Saccharomyces cerevisiae* to be dominant species of the fermentation, and also for inhibition of wild yeasts. The lower the pH with increasing temperature the greater the effects found on rate of fermentation and rate of yeast growth (Ough *et al* 1991).

There have been many reports on the effects of pH and sulfur dioxide on the fermentation characteristics of yeast immobilized on different supports (Ton *et al* 2008). The maximum specific growth rate of immobilized yeast was 1.17-1.39 times more than that of free yeast cells when pH was adjusted to 3.5-4.5. Besides, the sugar uptake rate and ethanol production rate of the immobilized yeast were 1.04-1.41 times and 1.05-1.35, times respectively, more than those of the free yeast. However, the ethanol production yield of the immobilized yeast was similar or slightly lower than that of the free yeast with low initial pH or high sulfur dioxide content. Application of immobilized yeast produced ethanol with low pH and volatile acids content which led to an improvement in physicochemical, microbiological and sensory quality of the final product (Ton *et al* 2010).

2.5.4 Substrate concentration

Yeast cells sense the amount and quality of external nutrients through multiple interconnected signaling networks, which allow them to adjust their metabolism, transcriptional profile and development program to adapt readily and appropriately to changing nutritional states (Zaman *et al* 2008). The highly interconnected signaling networks provide the cell with a environment that the cell can interpret the information to any

nutritional condition. In a fermentative process, the variable used to establish the process kinetic has been the variation of biomass (synthesis), variation of substrate content, product (metabolite), oxygen consumed or CO₂ evolved, heat evolved etc. Among these variables, biomass synthesis during the process and substrate consumption determines the fermentation process (Panday *et al* 2001).

Kocher *et al* (2006) found 20% sugar to be optimum for ethanol production from sugarcane juice. Charoenchai *et al* (1998) observed that by increasing the sugar concentration of juice from 200g/l to 300g/l, the growth rate and final cell biomass decreased with no evidence of non-*Saccharomyces* species growing faster than *Saccharomyces*. Devine and Slaughter (1980) studied the effect of medium composition on ethanol production. Panchal and Stewart (1980) reported that when the substrate concentration is increased beyond 25% the effect of osmotic pressure becomes pronounced which seriously affects fermentation efficiency and leads to decreased ethanol production. High substrate concentrations have been shown to inhibit yeast growth and fermentation performance as a result of high osmotic pressure and low water activity.

2.6 Optimization of Cultural Conditions using Response Surface Methodology

Response Surface Methodology or RSM is a collection of mathematical and statistical techniques that are useful for modelling and analysis of problem in which response is influenced by several variables and the objective is to optimize the response. This technique provides the means of attaining optimum operating conditions of complete systems. It is a powerful and an efficient mathematical tool applied for optimization of any process, e.g. media components on fermentation (Adinarayana and Elliaiah 2002; Park *et al* 2002). It gives information about the interactions between various variables, process optimization and gives multiple responses at the same time. Statistical modelling is done to develop an appropriate model between the responses y (performance function) and independent variables x (decision variables).

In general the relationship is:

$$y = \emptyset(x_1, x_2 \dots\dots\dots, x_n) + \varepsilon$$

Where the form of the true response function \emptyset is unknown and perhaps very complicated one, another mathematical simpler function f must be found to approximate \emptyset and describe the system and ε is the term that represent other source of variability or error on the response. Often assuming it to have normal distribution with mean zero and variance σ^2 , then the new function $y' = f(x_1, x_2 \dots\dots\dots, x_n)$. This estimates y' rather than true value of y .

The most commonly used expression is polynomial of first or second degree is given as:

$$y' = \beta_0 + \sum_{i=1}^n \beta_i x_i + \varepsilon$$

$$y' = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_i x_i^2 + \sum_{i=1}^{n-1} \sum_{j=1}^{n-1} \beta_{ij} x_i x_j + \varepsilon$$

Where β_0 , β_i , β_{ij} are the constant coefficients usually determined by least square method and ε is the error involved in estimating the coefficient β from the experimental data. After the coefficients have been determined by taking the first partial derivatives of above equations equating them to zero and solving the system of n equations.

$$\partial y' / \partial x_i = 0 \quad (i = 1, 2, \dots, n)$$

n = total number of variables

Yu *et al* (2009) reported the maximum ethanol production rate (119.12 g/l/h) in sweet sorghum juice using immobilized *Saccharomyces cerevisiae* was observed for a medium consisting of 0.77 g/l phosphorus, 2.15 g/l nitrogen, and pH 6.39. Under optimized condition, the ethanol fermentation rate was 122.85 g/l/h using Response Surface Methodology.

Wang *et al* (2013) reported that under the recommended SSF condition of the temperature at 37 °C, yeast concentration at 1.4 g/l, enzyme loading at 25 FPU/g-glucan and bagasse solid concentration at 10%, the ethanol yield, concentration and production rate were 89.4%, 38 g/l and 1.28 g/l/h, respectively. The sweet sorghum bagasse solid concentration had significant effects on the ethanol concentration and ethanol production rate. The optimum bagasse solid concentration for the ethanol yield was 7%. Velmurugan and Muthukumar (2012) observed 92.11% of theoretical reducing sugar yield experimentally in sugarcane bagasse.

2.7 Ethanol Tolerance

Under appropriate environmental and nutritional conditions, *S. cerevisiae* can produce and tolerate high ethanol concentration (Thomas *et al* 1996; Bafrencova *et al* 1999). Yeast strains of *S. cerevisiae* have been traditionally used in baking, brewing and wine making industries and were extensively studied in recent years for fuel ethanol production, in which yeast cells are subject to a plethora of adverse conditions including N₂ limitation, high temperature, ethanol inhibition, the osmotic stress from substrate sugars and so on. The capacity to tolerate various stresses is one of the important criteria to elect superior strains for efficient ethanol fermentation. The key stress factors that the baker's and brewing yeast encounter have been previously reviewed (Attfield, 1997; Gibson *et al* 2007). Besides the common stress conditions such as ethanol toxicity and osmotic pressure, yeast strains for fuel ethanol production are also expected to tolerate high temperature since ethanol fermentation

under relatively low temperature cannot be operated in summer (or when environmental temperature is high) with the regular cooling water. Toxic inhibitors are released from the pretreatment of lignocellulosic fuel ethanol production (Klinkle *et al* 2004; Liu, 2006; Fischer *et al* 2008).

Understanding the mechanism of yeast stress tolerance under various adverse and environmental conditions will contribute to breeding robust strains for efficient ethanol fermentation, which is of particular importance for producing fuel ethanol. Using DNA microarray analysis for the comparison of sake brewing and laboratory yeast strains, tryptophan biosynthesis genes were identified and demonstrated to be related to ethanol tolerance (Hirasawa *et al* 2007). Ethanol not only inhibits cell growth but also represses glucose transport (Salmon 1989) thereby leads to defect in yeast metabolism and energy supply. Therefore the tolerance of sugar transporters to ethanol inhibition is an important feature to consider when selecting strains for efficient ethanol fermentation (Santos *et al* 2008)

In view of the high titers of ethanol towards the end of each fermentation cycle 8-12 % (v/v) the alcohol is one of the major stress factors that act upon yeast. The main target of ethanol is considered to be the cytoplasmic membrane of yeast cells (Thomas *et al* 1978; Alexandre *et al* 2001). The membrane fluidity, which is related to its lipid composition, is profoundly altered in the presence of ethanol and as a result, membrane permeability to some ions (especially ions H⁺) is significantly affected. As ions enters the cell, there is a dissipation of the electro-chemical gradient across the membrane, which in turns affects formation and maintenance of the proton driving force with subsequent decrease in intracellular pH. Apart from affecting yeast membrane composition, there are several other effects of ethanol upon yeast physiology during fermentation, including growth inhibition, and enzymatic inactivation which leads to a decreased cell viability. Cell re-use (yeast recycling) imposes a harsh condition towards industrial strains. Yeast cells must keep high viability at the end of each fermentation cycle in order to be able to cope with the following one. This is why a given strain can perform well in one fermentation cycle with a final ethanol content of 18 % (v/v), but cannot be recycled for subsequent fermentation cycles. Indeed, if yeast physiological condition, namely the vitality of the cells at the end of the fermentation, is not a matter of concern, higher ethanol contents could easily be achieved. This is observed in some corn and cereal-based fermentation processes, where 17 to 23 % (v/v) of ethanol titers are obtained when wheat and formulated mashes are used (Jones *et al* 1994; Bayrock and Ingledew, 2001). Despite these problems, high ethanol fermentations are desirable in order to reduce water consumption and energy costs during the distillation step. It is also expected that this fermentation condition will favour the energy balance of the produced ethanol and improve the sustainability of the industrial process. However, in most distilleries the final ethanol

content is limited by the ethanol tolerance of the fermenting strain. Additionally, ethanol stress can be intensified by high temperature and acidity, and all these stressing factors are imposed simultaneously or sequentially towards yeasts in industrial fermentations (Dorta *et al* 2006). A great research effort is now being conducted to search for ethanol tolerant yeast strains, in order to be used in high ethanol fermentations, specifically for the case of cell recycling. The genetic basis for ethanol tolerance is polygenic and complex. More than 250 genes seem to be involved with this trait (Alexandre *et al* 2001; Hu *et al* 2007). The majority of these genes are related to energy metabolism, lipid synthesis, ionic homeostasis, trehalose synthesis, etc. This means that yeast improvement for ethanol tolerance through a rational approach is a difficult task, and a more “blind” approach (such as genome shuffling, mutagenesis, directed evolution) could be helpful in the search for such strains (Stephanopoulos, 2002; Çakar *et al* 2005; Giudici *et al* 2005).

CHAPTER-III

MATERIAL AND METHODS

3.1 Procurement of Sweet Sorghum and Sugarcane juices

Mature plant varieties of CSV19SS of Sweet sorghum and CoJ-89 of sugarcane were used in the present studies and the juices of these varieties were procured from sugarcane section, Department of Plant Breeding and Genetics PAU, Ludhiana. The juices were extracted using cane crusher as shown in figure1 and were stored at 0°C and -20°C separately for further studies.



Fig 3.1: Cane crusher

3.2 Culture

Saccharomyces cerevisiae strain 35 and *Saccharomyces cerevisiae* strain NRRL Y-2034 used in the present study were obtained from the industrial microbiology laboratory, Department of Microbiology, PAU, Ludhiana. These yeast strains were maintained on glucose yeast extract medium slants by subculturing at regular intervals and storage at 4°C.

The composition of GYE (g/litre) is as under:

Components	g/l
Glucose	10
Yeast extract	5
Peptone	5
Agar powder	15
Distilled water	1000ml
pH	5.5

All ingredients except agar of above composition was weighed and dissolved in distilled water. After setting the required pH agar was added and mixed by heating. The medium is dispensed on flasks, cotton plugs and sterilized by autoclaving at 121°C for 20 min.



Fig 3.2 Culture of yeast *Saccharomyces cerevisiae* strain NRRL Y-2034



Fig 3.3 Culture of yeast *Saccharomyces cerevisiae* strain -35

3.3 Storage Stability of sweet sorghum juice

The sweet sorghum juice was filtered through muslin cloth and was stored at 0°C (freezer chest of refrigerator) and -20°C (deep freezer) in sampling bottles. Various parameters like TSS (°B) (section 3.6.1), pH (section 3.6.2), reducing sugars (section 3.6.3), total acidity (section 3.6.5), were measured to check the storage stability after 0, 15, 30, 45, 60 and 75 days.

3.4 Ethanol Fermentation of sweet sorghum juice

Ethanol production from fresh sweet sorghum juice was studied using two strains of *S. cerevisiae* namely strain-35 and NRRL Y-2034.

(a) Prepration of inoculum

The inoculum was prepared by inoculating 100ml of glucose yeast extract broth in 250 Erlenmeyer flasks with a loopful of 24 hour old actively growing culture of yeast (*S. cerevisiae*) strains. These flasks were incubated in BOD incubator and shaker at 28°C for 24 hours. The broth containing 10⁶ yeast cells/ml was used as starter culture to carry out the ethanol fermentation.

(b) Fermentation of the juice

The initial Brix of sweet sorghum juice was measured using a refractrometer. Flasks containing fifty ml of filtered juice was pasteurized at 63°C for 30 min and was inoculated with 5ml (v/v) of starter culture of *S.cerevisiae* strain 35. The fermentation was carried out at 28° C at 50 rpm. The observations were made till constant values of °Brix were obtained. The fermentation broth was analyzed for ethanol concentration (%v/v) as described in section 3.6.4. The experiment was similarly performed using *S. cerevisiae* strain NRRL Y-2034.

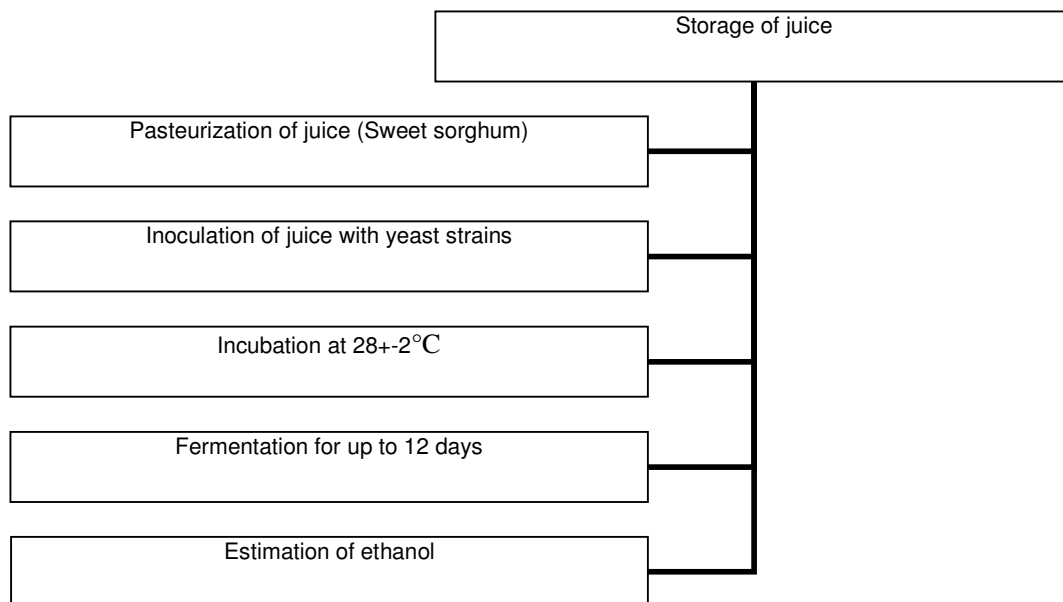


Fig 3.4 Flow chart of ethanol fermentation



Fig 3.5 Flasks showing fermentation of sweet sorghum juice for ethanol production

3.5 Optimization of process parameters for ethanol production using Response Surface Methodology

For optimization, the analysis of variance (ANOVA) for overall effect of three factor variables i.e. inoculum size, agitation rate, and temperature on three response variables pH, total acidity and ethanol content according to fitted model was done using software Statgraphics Centurion XVI.I and the least significant factor affecting the response variable was selected. The three dimensional plots and contour plots according to the fitted model were drawn using software. Response Surface Methodology was used for multiple regression analysis of the experiment and F-test was employed to evaluate the statistical significance of quadratic polynomial equation. Coefficient of correlation (r) and determination coefficient of correlation (R^2) were employed to evaluate the performance of regression equation. Following sets for optimizing the cultural parameters:

Sr. No.	Inoculum size (%)	Agitation rate (rpm)	Temperature (°C)
1	7.5	50	30
2	10	50	30
3	5	50	30
4	10	50	25
5	5	50	25
6	5	100	30

7	7.5	100	30
8	10	100	30
9	7.5	75	30
10	10	75	30
11	10	75	25
12	5	75	25
13	7.5	75	25
14	5	75	20
15	7.5	75	20
16	10	75	20
17	7.5	50	20
18	5	50	20
19	7.5	100	25
20	10	100	25
21	5	100	20
22	10	100	20

Activities of *S.cerevisiae* strain 35 and *S.cerevisiae* strain NRRL Y-2034 were analyzed and pH, Total acidity and ethanol content were determined using these sets of conditions.

3.5.1 Effect of optimizing parameters on sweet sorghum juice inoculated with *Saccharomyces cerevisiae* strain NRRL Y-2034

The initial Brix of sweet sorghum juice was measured using a refractometer. Triplicate flasks containing fifty ml of filtered juice was pasteurized at 63⁰C for 30 min and was inoculated with starter culture of *S.cerevisiae* strain NRRL Y-2034. The fermentation was carried out according to the sets obtained from response surface methodology for optimizing the cultural parameters (section 3.5). The fall in °Brix was noted at specific intervals with the help of hand refractometer. The observations were made till constant values of °Brix were obtained. The fermentation broth was analyzed for pH, ethanol concentration (% v/v) and total acidity (%) as described in section, 3.6.2, 3.6.4 and 3.6.5 respectively.

3.5.2 Effect of optimizing parameters on sweet sorghum juice inoculated with *Saccharomyces cerevisiae* strain-35

3.5.3 Effect of optimizing parameters on sugarcane juice inoculated with *Saccharomyces cerevisiae* strain NRRL Y-2034

3.5.4 Effect of optimizing parameters on sugarcane juice inoculated with *Saccharomyces cerevisiae* strain-35

For 3.5.2, 3.5.3, and 3.5.4 the experimental setup was same as 3.5.1.

3.5.5 Comparison of ethanol production from sweet sorghum and sugarcane juice

The experiment for ethanol production from sweet sorghum and sugarcane juice was performed under optimized conditions as per the RSM studies given in section 3.5.1, 3.5.2, 3.5.3 and 3.5.4. The ethanol content and fermentation efficiencies were calculated as per the procedure given in the section 3.6.4.

3.6 Analytical Procedures

3.6.1 Total Soluble Sugars (TSS)

The total soluble solids of any juice determine the amount of sugars in degree brix of the sweet sorghum and sugarcane juice. The TSS (°Brix) were determined using hand refractometer, model A-contrast 11-502-0.

3.6.2 pH

The pH was determined by pH meter. The pH meter was standardized to pH 4 with a standard buffer solution of pH 4.0 before determining the pH of sample.

3.6.3 Estimation of reducing sugars

Reducing sugars were estimated by the method of Miller *et al* (1959).

Reagents

- 1. Dinitro salicylic acid (DNS) reagent:** Ten gram 3, 5-dinitrosalicylic acid, 2g phenol and 0.5g sodium sulphite were dissolved in 500ml of 1% NaOH solution. The volume was made 1Litre with additional alkaline solution. It was filtered and stored in dark coloured bottle at room temperature.
- 2. Sodium-potassium tartarate (Rochelle salt) solution:** Forty gram sodium potassium tartarate was dissolved in distilled water and volume was made 100ml.

Procedure

Triplicate test tubes containing 3ml of sample each was taken and 3ml DNS reagent was added. The test tubes were kept in boiling water bath for 15 min. One ml Rochelle salt solution was added in each test tube and was cooled to room temperature. The absorbance was measured at 575nm using UV-VIS Spectrophotometer (Hitachi U-2800 model). A control containing 1ml of distilled water instead of sample was also run simultaneously. The optical density was used to determine reducing sugar concentration using glucose standard curve taking 0.1 to 1mg/ml of glucose as a stock solution.

3.6.4 Estimation of Ethanol

Ethanol was estimated by the chemical oxidation method of Caputi and Wright (1969).

Reagents

1. **Potassium dichromate ($K_2Cr_2O_7$) solution:** A volume of 325 ml concentrated sulphuric acid was mixed with 400 ml distilled water and the resulting solution was cooled slightly. Potassium dichromate (33.768 g) was added in it and dissolved properly. The final volume of this solution was made 1000 ml with distilled water.
2. **Dehydrated alcohol** – Absolute alcohol of 90% purity was used in the present studies.

Procedure

One ml fermentation broth was taken in a 500 ml distillation flask containing 30 ml distilled water. The distillate was collected in 50 ml flask containing 25 ml $K_2Cr_2O_7$ solution. The condenser tip was kept dipped in the solution throughout. About 20 ml distillate including rinsed water from the tip of the condenser was collected. The distillate flask was then kept in water bath maintained at 62.5°C for 20 min. The flask was then cooled to room temperature and the final volume was made 50 ml. Five ml of this solution was diluted with 5 ml distilled water and the absorbance was recorded at 600nm using UV-VIS Spectrophotometer (Hitachi U-2800 model). The percent ethanol was determined from the standard curve which was prepared by using different concentrations of ethanol ranging between 0-10% (v/v) absolute alcohol in distilled water.

Calculations

a) Sugar utilized = initial available sugars (%) - residual sugars after fermentation (%)

b) Fermentation efficiency (%) of the process is determined by the following formula

$$\frac{\text{Actual ethanol recovery (v/v)}}{\text{Theoretical ethanol recovery (v/v)}} \times 100$$

Where, Theoretical ethanol recovery (v/v) = Sugars utilized \times 0.64

Actual ethanol produced (% v/v) = Actual ethanol production (%v/v) was determined from the standard curve using absorbance values at 600nm

3.6.5 Determination of Titrable Acidity

Titrable acidity expressed as tartaric acid was estimated by the procedure of AOAC (1999).

Reagents

1. Sodium hydroxide solution (0.1 N)
2. Phenolphthalein indicator (1%) - One gram phenolphthalein was dissolved in 100 ml of dehydrated ethanol.

Procedure

Titration acidity was determined by titrating known quantity of wine sample (10 ml) against 0.1 N NaOH using a few drops of 1% phenolphthalein solution as indicator. The change of pink colour to colourless solution for atleast 15 seconds is considered as the end point. Results were expressed as grams of tartaric acid per 100ml as per following formula:

Calculations

Assuming 1ml 0.1 N.NaOH = 0.0075g tartaric acid.

$$\text{Titration Acidity (g/L tartaric acid)} = \frac{\text{Volume of alkali used} \times \text{Normality of alkali used} \times 75 \times 100}{\text{Volume of sample}}$$

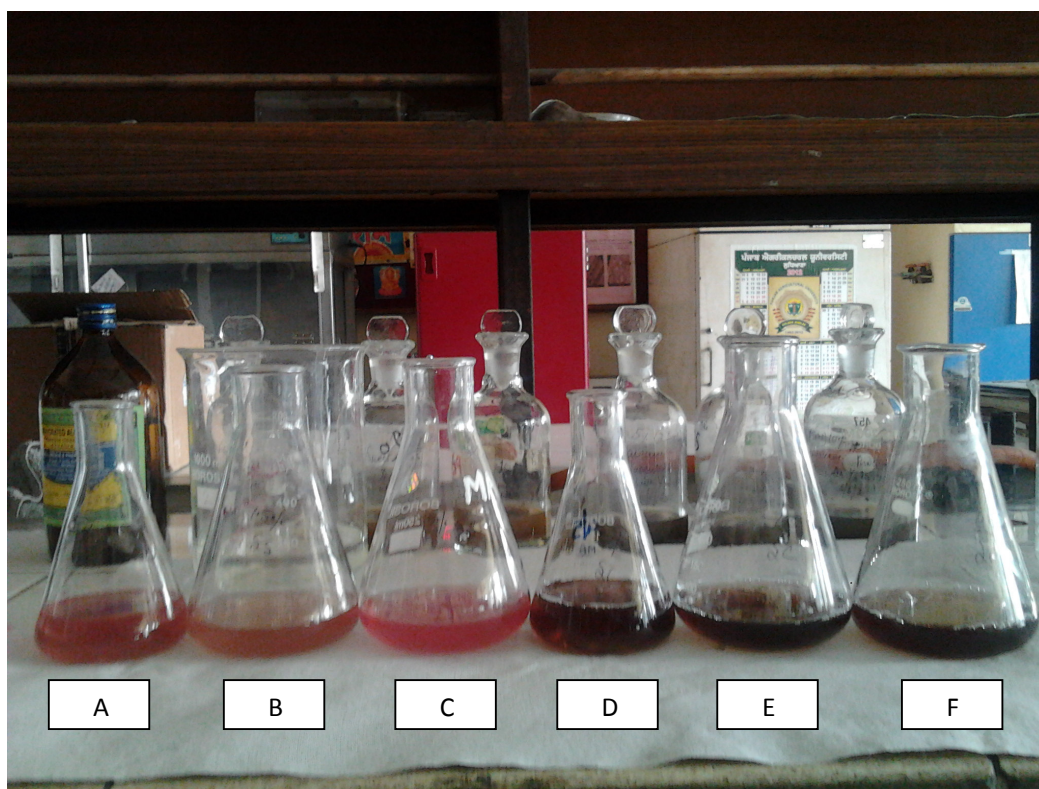


Fig 3.6 Flasks A, B, C showing titration acidity determination of fermented sweet sorghum juice inoculated with *Saccharomyces cerevisiae* Strain NRRL Y-2034 with inoculum concentration of 5%, 7.5% and 10% respectively at an agitation rate of 100 rpm under the temperature of 30°C and flasks D, E, F showing titration acidity determination of fermented sugarcane juice inoculated with *Saccharomyces cerevisiae*

Strain NRRL Y-2034 with inoculum concentration of 5%, 7,5% and 10% respectively at an agitation rate of 100 rpm and temperature of 30°C

CHAPTER – IV

RESULTS AND DISCUSSION

4.1 Shelf Life Study of Sweet Sorghum Juice

Mature plants of sweet sorghum and sugarcane of variety CSV19SS and COJ-89 respectively were collected from the field and juice was extracted from the stem of the sweet sorghum using cane crusher. The initial analysis of various parameters like reducing sugars, total acidity, pH and Total Soluble Sugars (°B) analyzed during the study is represented in table 4.1.

Table 4.1 Compositional analysis of sweet sorghum juice stored at 0°C and -20°C

Day(s) Storage temp.	Reducing Sugars (mg/ml)		Total Acidity (%)		pH		Total Soluble Sugars (°B)	
	0°C	-20°C	0°C	-20°C	0°C	-20°C	0°C	-20°C
0	1.75± 0.003	1.75±0.003	0.93±0.006	0.93±0.003	5.27±0.006	5.27±0.003	20±0.033	20±0.033
15	1.85±0.006	1.89±.0057	0.80±0.008	0.77±0.008	5.32±0.003	5.37±0.003	20±0.034	20±0.033
30	1.92±0.003	1.98±0.005	0.67±0.005	0.60±0.006	5.43±0.008	5.50±0.003	19.9±0.033	19.9±0.057
45	2.08±0.008	2.13±0.006	0.78±0.006	0.60±0.003	5.36±0.003	5.50±0.005	19.9±0.033	19.8±0.033
60	2.19±0.005	2.24±0.0057	0.91±0.005	0.60±0.005	5.25±0.003	5.50±0.006	19.9±0.034	19.8±0.033
75	2.27±0.005	2.32±0.008	0.91±0.003	0.60±0.005	5.25±0.003	5.50±0.006	19.9±0.034	19.8±0.033

#Average of triplicate data± values indicate standard error

The results indicate that there is 0.52% enhancement in the reducing sugar level of sweet sorghum juice, when stored at 0°C and 0.57% enhancement when juice was stored at -20°C for 75 days. Wu *et al* (2010) reported that when the juice was stored in refrigerator sugar losses were less than 1% and 3% after one week of storage respectively.

There was no significant change in total acidity at 0°C and whereas it decreased by 0.33% during storage at -20°C after 75 days. The pH of the juice stored at 0°C varied between 5.25 to 5.43 whereas at -20°C, there was slight increase in pH values from 5.27-5.50.

However, Wu *et al* (2010) reported that the juice stored at room temperature, showed an average decrease of pH from 4.7 to 3.8 in one week whereas the pH increased from 4.7-5.1 when stored in refrigerator. Ronghou and Shen (2008) reported that pH 5.0 is suitable for ethanol fermentation instead of 3.5.

The total soluble sugars of the fresh juice was 20 °B which remained almost constant i.e. 19.9 °B and 19.8 °B at 0°C and -20°C after 75 days of storage. Ratnavathi *et al* (2010) reported the TSS value of sweet sorghum juice in the range of 16-23 °B. This indicates that sweet sorghum juice cannot be stored at room temperature. There was no noticeable change in the sugar profile in refrigerated juices.

No significant difference in ethanol yields and sugar conversion efficiencies was observed for refrigerated juices during 2-week storage period (Wu *et al* 2010).

4.2 Ethanol production from sweet sorghum juice

Table 4.2; fig 4.1 represents the ethanol production profile of sweet sorghum juice using *Saccharomyces cerevisiae* Strain-35. With the increase of fermentation period, percent ethanol content was increased while percent total soluble sugars and reducing sugars decreased. There is 15.5 °B (from 20 to 4.5°B) decrease in the fermented sweet sorghum juice and 1.74% (from 1.75 to 0.01%) decrease in reducing sugars after 12 days of fermentation period. The maximum ethanol content of the juice was 8.6% which corresponds to fermentation efficiency of 86.69%.

Table 4.2 Ethanol production profile of Sweet Sorghum juice using *Saccharomyces cerevisiae* Strain-35

Fermentation day(s)	Brix(°B)	Reducing sugars (%)	% Ethanol	Fermentation Efficiency(%)
0	20.0±0.333	1.75±0.003	0.3±0.003	
2	16.4±0.057	1.41±0.033	5.2±0.033	
4	15±0.333	1.08±0.057	5.8±0.033	
6	13.8±0.003	0.77±0.003	6.1±0.057	
8	7.1±0.333	0.49±0.003	7.3±0.057	
10	5.0±0.333	0.17±0.057	8.0±0.057	
12	4.5±0.003	0.01±0.005	8.6±0.033	86.69%

#Average of triplicate data± values indicate standard error

Cultural conditions: Volume of sweet sorghum juice=50ml; Incubation temperature= 30°C; Agitation rate= 50 rpm; Inoculum concentration= 5%; pH=5.4

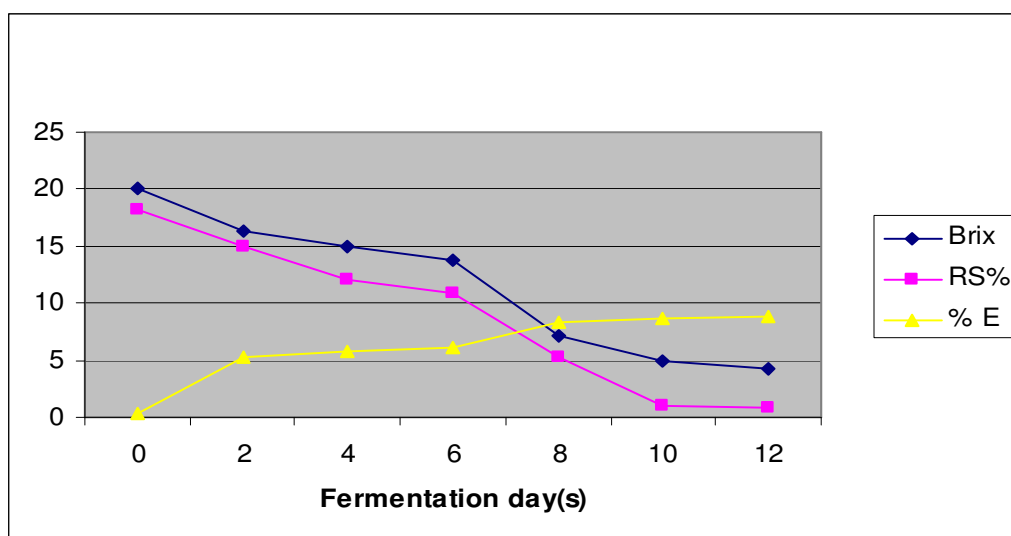


Fig 4.1 Ethanol production profile using *S. cerevisiae* strain-35

Table 4.3 and fig 4.2 indicates the ethanol production profile using *Saccharomyces cerevisiae* NRRL Y-2034. There is 15.2 °B (from 20 to 4.8°B) decrease in the fermented sweet sorghum juice and 1.73% (from 1.75 to 0.02%) decrease in reducing sugars and final % ethanol recovered is 8.4% after 12 days of fermentation period which corresponds to fermentation efficiency of 86.34%. Here also with the increase of fermentation period, percent ethanol content was increased while percent total soluble sugars and reducing sugars decreased.

Table 4.3 Ethanol production profile of Sweet Sorghum juice using *Saccharomyces cerevisiae* NRRL Y-2034

Fermentation day(s)	Brix (°B)	Reducing sugars(%)	% Ethanol	Fermentation Efficiency(%)
0	20.0±0.033	1.75±0.033	0.3±0.005	
2	17.7±0.057	1.48±0.057	2.7±0.033	
4	15.8±0.066	1.12±0.057	4.4±0.057	
6	13.2±0.033	0.89±0.033	5.5±0.033	
8	8.9±0.033	0.56±0.033	7.7±0.057	
10	6.4±0.033	0.12±0.057	7.9±0.033	
12	4.8±0.057	0.02±0.003	8.4±0.033	86.34%

#Average of triplicate data± values indicate standard error

Cultural conditions: Volume of sweet sorghum juice=50ml; Incubation temperature= 30°C; Agitation rate= 50 rpm; Inoculum concentration= 5%; pH=5.4

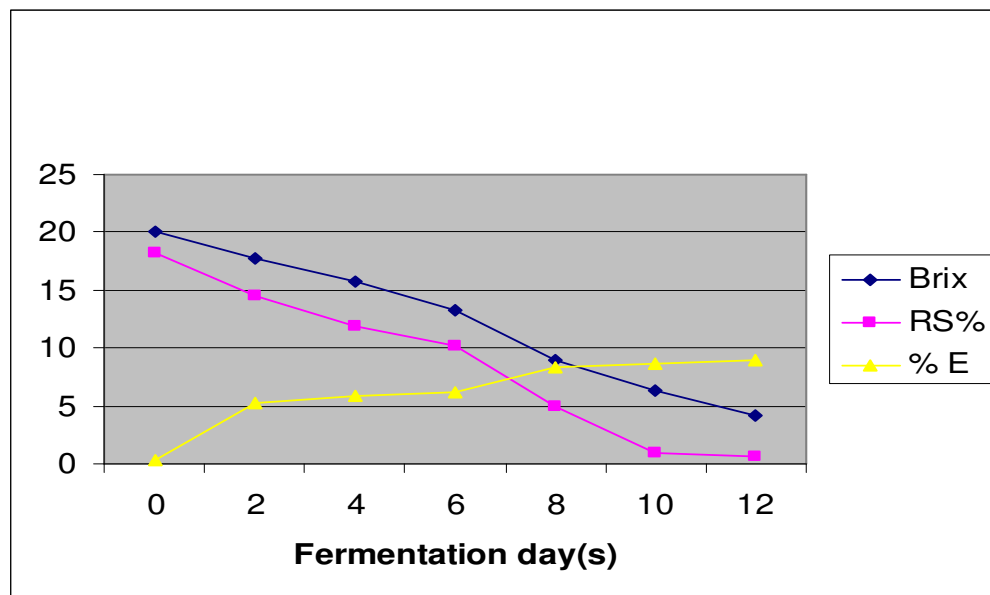


Fig 4.2 Ethanol production profile using *S. cerevisiae* strain NRRL Y-2034

Wu *et al* (2011) reported 90% of fermentation efficiency with a mixture of sweet sorghum juice and bagasse. Similarly, ethanol titers of 17-23% (v/v) were reported when

wheat and formulated mashes were used in fermentation process (Jones *et al* 1994; Bayrock and Ingledew 2001). Kundiyana *et al* (2010) reported maximum ethanol i.e. 79.6 g/l with sugar conversion efficiency of 95.6% at pH 4.3 with no added urea.

Patil *et al* (1998) reported that 7-8% (v/v) ethanol was produced by batch fermentation of cane molasses with 80-85% efficiency. Wu *et al* (2010) reported that most tested strains showed a sugar to ethanol conversion efficiency of more than 90%. Belloch *et al* (2008) reported that most yeast strains can ferment juices of 20°B with high efficiency batch fermentation showing 10-12% (v/v) ethanol content. Laopaiboon *et al* (2009) reported 1.8-1.85% w/v residual sugars in high gravity sweet sorghum juice fermentation. From the above studies *Saccharomyces cerevisiae* strain-35 was found to be better in terms of ethanol content and fermentation efficiency.

4.3 Optimization of cultural conditions by Response Surface Methodology

Statgraphics Centurion XVI.I software was used for the optimization of different cultural conditions for ethanol production. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. The effect of three factors viz. inoculum size, agitation rate and temperature at different levels was studied using 22 run Box Behnken design. The independent variables included inoculum level (5%, 7.5%, 10%), agitation rate (50 rpm, 75 rpm, 100 rpm) and temperature (20°C, 25°C, 30°C) and various response variables were total acidity, pH and % ethanol. Runs suggested by RSM were performed separately as given below:

4.3.1 Optimization of sweet sorghum juice using *Saccharomyces cerevisiae* strain NRRL Y-2034.

4.3.2 Optimization of sweet sorghum juice using *Saccharomyces cerevisiae* strain-35.

4.3.3 Optimization of sugarcane juice using *Saccharomyces cerevisiae* strain NRRL Y-2034.

4.3.4 Optimization of sugarcane juice using *Saccharomyces cerevisiae* strain-35.

Table 4.4 Effect of cultural parameters by Response Surface Methodology of sweet sorghum juice inoculated with *Saccharomyces cerevisiae* NRRL Y-2034

Sr. No.	Inoculum size (%)	Agitation rate (RPM)	Temperature (°C)	Total Acidity (%)	pH	%Ethanol		Fermentation Efficiency(%)
						v/v	w/v	
1	7.5	50	30	0.60	5.02	8.83	7.06	87.33
2	10	50	30	0.66	4.97	8.45	6.76	83.58
3	5	50	30	0.69	5.02	8.50	6.80	84.07
4	10	50	25	0.64	4.95	7.10	5.68	70.22
5	5	50	25	0.64	4.94	7.04	5.63	69.63
6	5	100	30	0.93	3.37	7.44	5.95	73.59
7	7.5	100	30	0.88	3.88	7.47	5.97	73.88
8	10	100	30	0.95	3.33	8.37	6.69	82.78
9	7.5	75	30	0.75	4.49	8.54	6.83	84.47
10	10	75	30	0.75	4.49	7.49	5.99	74.08
11	10	50	20	0.98	3.39	7.01	5.60	69.33
12	5	100	25	0.97	3.43	6.98	5.58	69.04
13	7.5	75	25	0.94	3.42	8.40	6.72	83.08
14	5	75	20	0.82	4.20	7.39	5.91	73.09
15	5	75	30	0.70	4.45	7.79	6.23	77.05
16	10	75	20	0.85	3.99	7.81	6.24	77.25
17	7.5	50	20	0.76	4.58	7.58	6.06	74.97
18	5	50	20	0.79	4.61	7.43	5.94	73.49
19	7.5	100	20	0.66	4.68	7.45	5.96	73.68
20	10	100	25	0.87	4.10	7.85	6.28	77.64
21	5	100	20	0.76	4.58	7.05	5.64	69.73
22	10	100	20	0.80	4.18	7.15	5.72	70.72

Cultural conditions: Volume of sweet sorghum juice=50ml; Fermentation period =12 days
Adjusted Brix= 20°B; pH= 5.5

Average of triplicate data

Table 4.5 Statistical analysis of table 4.4 for fitting of model

Model	Total Acids	Ethanol Content	pH
Transformation	none	none	none
Model d.f.	9	9	9
P-value	0.0491	0.0497	0.0223
Error d.f.	12	12	12
Std. error	0.0886617	0.436415	0.40769
R-squared	67.84	67.76	72.67
Adj. R-squared	43.72	43.58	52.18

4.3.1 Effect of cultural conditions on ethanol content

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (figure 4.3). Table 4.6 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 67.7583% of the variability in ethanol content. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 43.577%. The standard error of the estimate shows the standard deviation of the residuals to be 0.436415. The mean absolute error (MAE) of 0.259102 is the average value of the residuals.

The regression equation of the fitted model for ethanol is:

$$5.67858 + 0.693902*A + 0.0524152*B - 0.248634*C - 0.0643101*A^2 + 0.00308*A*B + 0.0032*A*C - 0.000303101*B^2 - 0.00142*B*C + 0.00800217*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE.

TABLE 4.6 Estimated effects for Ethanol Content (%)

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	7.9471	0.301809	
A:Inoculum Size	0.20125	0.218208	1.0
B:Agitation Rate	-0.2725	0.218208	1.0
C:Temperature+block	0.689746	0.213411	1.01359
AA+block	-0.803877	0.421944	1.01976
AB	0.385	0.251964	1.0
AC	0.08	0.251964	1.0
BB+block	-0.378877	0.421944	1.01976
BC+block	-0.355	0.251964	1.0
CC+block	0.400109	0.447347	1.0149

Standard errors are based on total error with 12 d.f.

Plot of Ethanol Content

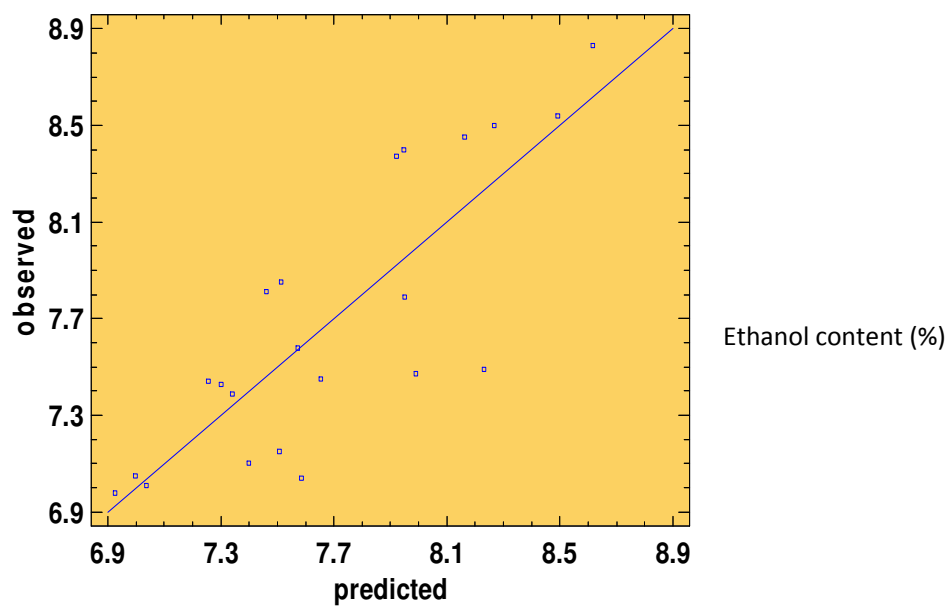


Fig 4.3 Experimental v/s predicted results of ethanol production

TABLE 4.7 Analysis of Variance for Ethanol Content

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.162006	1	0.162006	0.85	0.3746
B:Agitation Rate	0.297025	1	0.297025	1.56	0.2355
C:Temperature+block	1.9895	1	1.9895	10.45	0.0072
AA+block	0.691303	1	0.691303	3.63	0.0810
AB	0.444675	1	0.444675	2.33	0.1524
AC	0.0192	1	0.0192	0.10	0.7563
BB+block	0.153563	1	0.153563	0.81	0.3869
BC+block	0.378075	1	0.378075	1.99	0.1842
CC+block	0.152359	1	0.152359	0.80	0.3887
Total error	2.2855	12	0.190458		
Total (corr.)	7.08864	21			

Table 4.8 Regression coefficients for Ethanol Content

Coefficient	Estimate
constant	5.67858
A:Inoculum Size	0.693902
B:Agitation Rate	0.0524152
C:Temperature	-0.248634
AA	-0.0643101
AB	0.00308
AC	0.0032
BB	-0.000303101
BC	-0.00142
CC	0.00800217

4.3.2 Effect of cultural conditions on total acids

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.4). Table 4.9 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 67.8376% of the variability in Total Acids. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 43.7158%. The standard error of the estimate shows the standard deviation of the residuals to be 0.0886617.

The mean absolute error (MAE) of 0.0499308 is the average value of the residuals.

The regression equation of the fitted model for total acids is:

$$1.05694 - 0.014942*A - 0.00847754*B + 0.0037029*C + 0.00510725*A^2 - 0.000266667*A*B - 0.00146667*A*C - 0.0000369275*B^2 + 0.000746667*B*C - 0.00104203*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE.

Table 4.9 Estimated effects for Total Acids (%)

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	0.804928	0.0613153	
A:Inoculum Size	0.025	0.0443309	1.0
B:Agitation Rate	0.1325	0.0443309	1.0
C:Temperature+block	-0.0339855	0.0433564	1.01359
AA+block	0.0638406	0.0857218	1.01976
AB	-0.0333333	0.0511889	1.0
AC	-0.0366667	0.0511889	1.0
BB+block	-0.0461594	0.0857218	1.01976
BC+block	0.186667	0.0511889	1.0
CC+block	-0.0521014	0.0908826	1.0149

Standard errors are based on total error with 12 d.f.

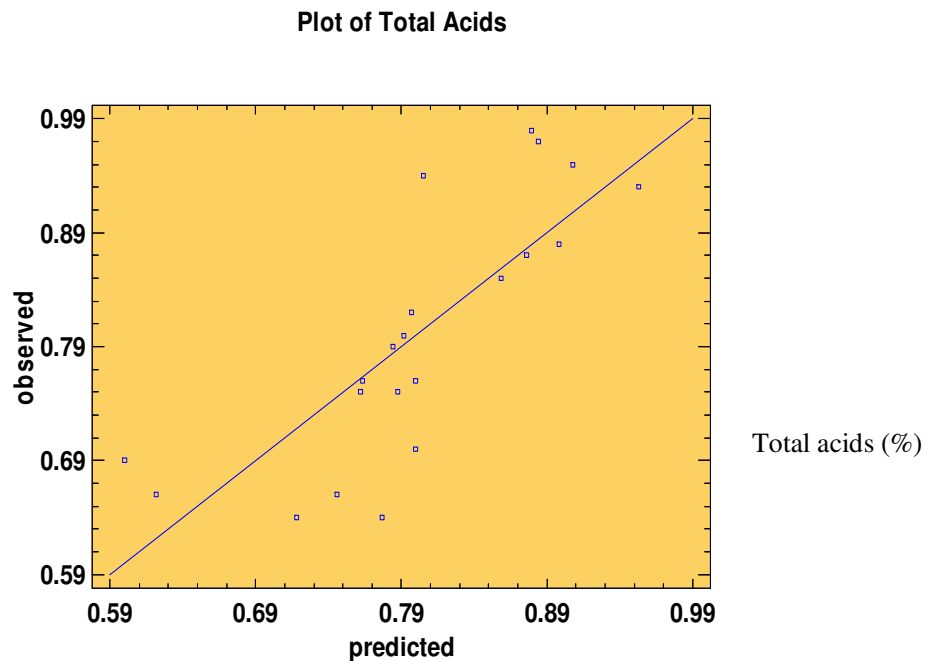


Fig 4.4 Experimental v/s predicted results of Total acids

Table 4.10 Analysis of Variance for Total Acids

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.0025	1	0.0025	0.32	0.5832
B:Agitation Rate	0.070225	1	0.070225	8.93	0.0113
C:Temperature+block	0.00483006	1	0.00483006	0.61	0.4483
AA+block	0.00435997	1	0.00435997	0.55	0.4708
AB	0.00333333	1	0.00333333	0.42	0.5272
AC	0.00403333	1	0.00403333	0.51	0.4875
BB+block	0.00227935	1	0.00227935	0.29	0.6001
BC+block	0.104533	1	0.104533	13.30	0.0033
CC+block	0.00258351	1	0.00258351	0.33	0.5770
Total error	0.0943308	12	0.0078609		
Total (corr.)	0.293295	21			

Table 4.11 Regression coefficients for Total Acids

Coefficient	Estimate
Constant	1.05694
A:Inoculum Size	-0.014942
B:Agitation Rate	-0.00847754
C:Temperature	0.0037029
AA	0.00510725
AB	-0.000266667
AC	-0.00146667
BB	-0.0000369275
BC	0.000746667
CC	-0.00104203

4.3.3 Effect of cultural conditions on pH:

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.5). Table 4.12 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a

perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 72.6744% of the variability in pH. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 52.1802%. The standard error of the estimate shows the standard deviation of the residuals to be 0.40769. The mean absolute error (MAE) of 0.250949 is the average value of the residuals.

The regression equation of the fitted model for pH is:

$$6.30394 - 0.256493*A + 0.0185591*B - 0.107475*C - 0.0146116*A^2 + 0.00198667*A *B + 0.0118667*B *C + 0.000265884*B^2 - 0.00352667*B *C + 0.00579058*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE.

Table 4.12 Estimated effects for pH

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	4.10812	0.281944	
A:Inoculum Size	-0.15	0.203845	1.0
B:Agitation Rate	-0.74125	0.203845	1.0
C:Temperature+block	0.0655435	0.199364	1.01359
AA+block	-0.182645	0.394172	1.01976
AB	0.248333	0.23538	1.0
AC	0.296667	0.23538	1.0
BB+block	0.332355	0.394172	1.01976
BC+block	-0.881667	0.23538	1.0
CC+block	0.289529	0.417902	1.0149

Standard errors are based on total error with 12 d.f.

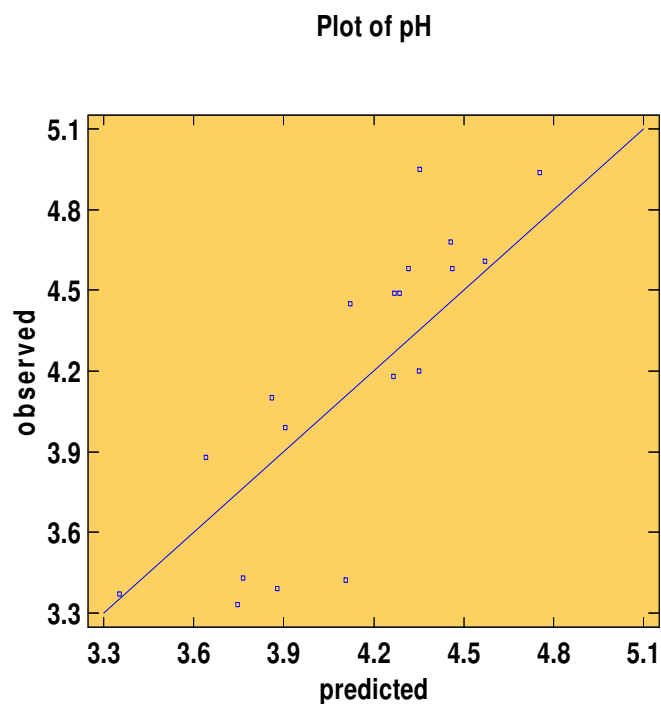


Fig 4.5 Experimental v/s predicted results of pH

Table 4.13 Analysis of Variance for pH

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.09	1	0.09	0.54	0.4760
B:Agitation Rate	2.19781	1	2.19781	13.22	0.0034
C:Temperature+block	0.0179649	1	0.0179649	0.11	0.7480
AA+block	0.0356866	1	0.0356866	0.21	0.6514
AB	0.185008	1	0.185008	1.11	0.3122
AC	0.264033	1	0.264033	1.59	0.2315
BB+block	0.118166	1	0.118166	0.71	0.4156
BC+block	2.33201	1	2.33201	14.03	0.0028
CC+block	0.0797802	1	0.0797802	0.48	0.5016
Total error	1.99453	12	0.166211		
Total (corr.)	7.29913	21			

Table 4.14 Regression coefficients for pH

Coefficient	Estimate
constant	6.30394
A:Inoculum Size	-0.256493
B:Agitation Rate	0.0185591
C:Temperature	-0.107475
AA	-0.0146116
AB	0.00198667
AC	0.0118667
BB	0.000265884
BC	-0.00352667
CC	0.00579058

4.4 RESPONSE SURFACE METHODOLOGY: TWO VARIABLE INTERACTION STUDIES

The surface plots for different interaction of any two independent variables, while holding the third variable constant, on enzyme production were generated using software, Statgraphics Centurion XVI.I. The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable in order to deduce the optimum conditions.

4.4.1 Effect of agitation rate and inoculum size on total acids, ethanol content and pH

A direct correlation was found between inoculum size and agitation rate and graphs were plotted to understand their effect on ethanol production. The response of experimental levels of each variable was examined and concluded that with the increase of agitation rate from 50, 75 and 100rpm and inoculums size from 5, 7.5 and 10%, the ethanol content, total acids and pH varied in the range of 0.62- 0.74% and ethanol content in range of 8.52- 9.0% and pH in range of 5.0-5.3 respectively as shown in Figure 4.6a, 4.6b, 4.6c, 4.7a, 4.7b and 4.7c.

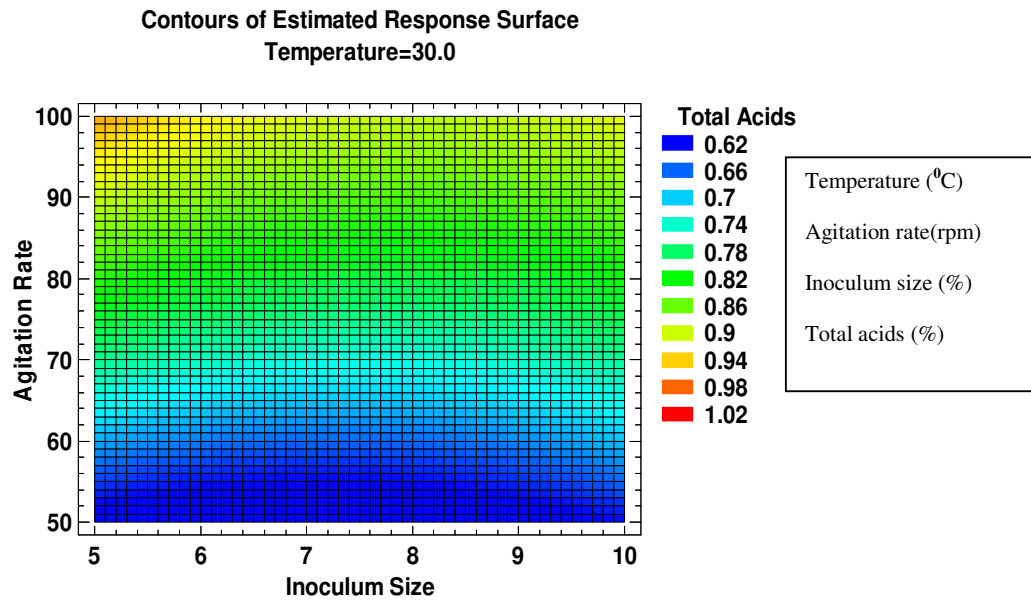


Fig 4.6a Contour plots for total acids (agitation rate vs. inoculum size)

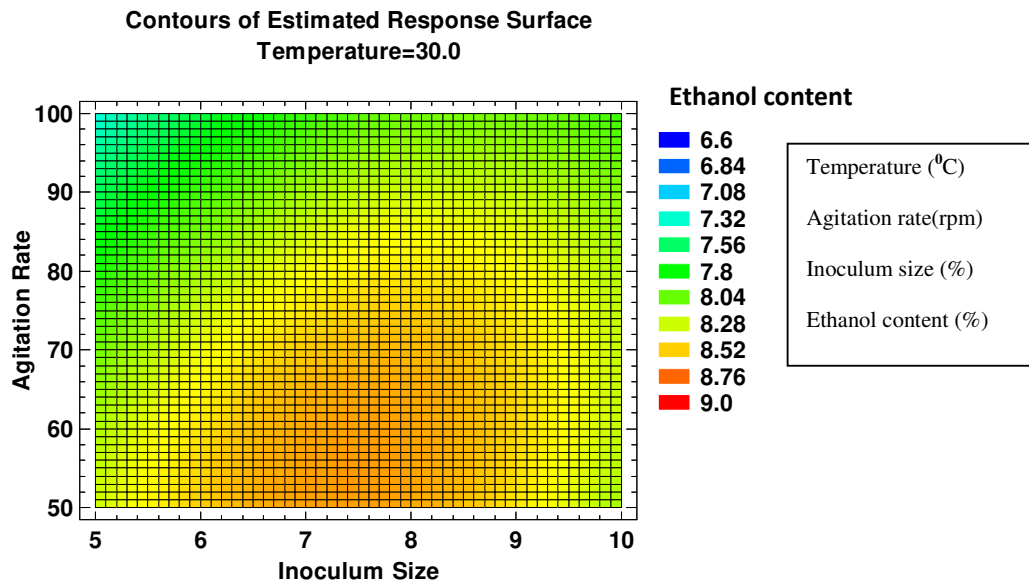


Fig 4.6b Contour plots for ethanol content (agitation rate vs. inoculum size)

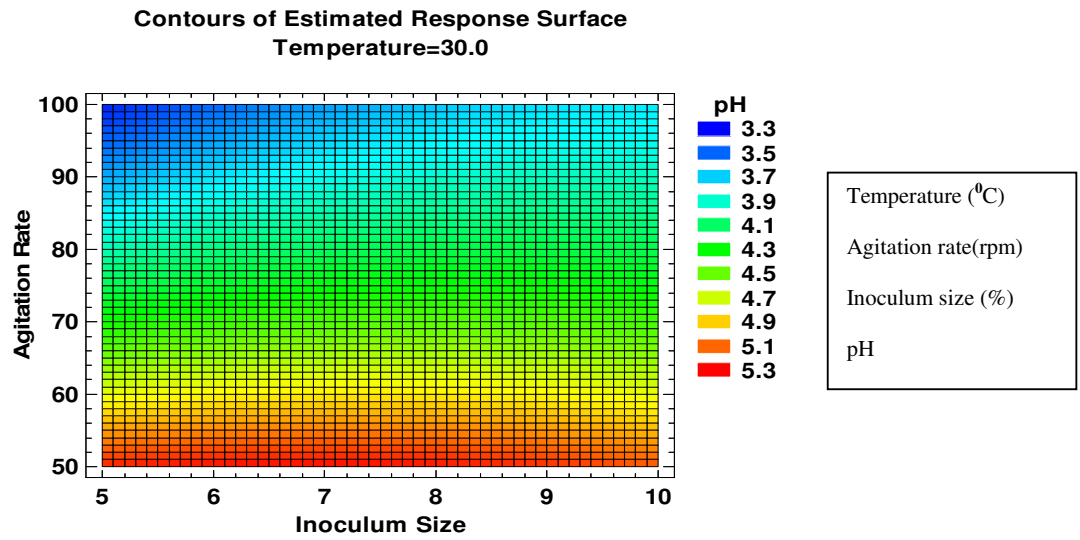


Fig 4.6c Contour plots for pH (agitation rate vs. inoculum size)

Fig 4.6 a, b and c Contour plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

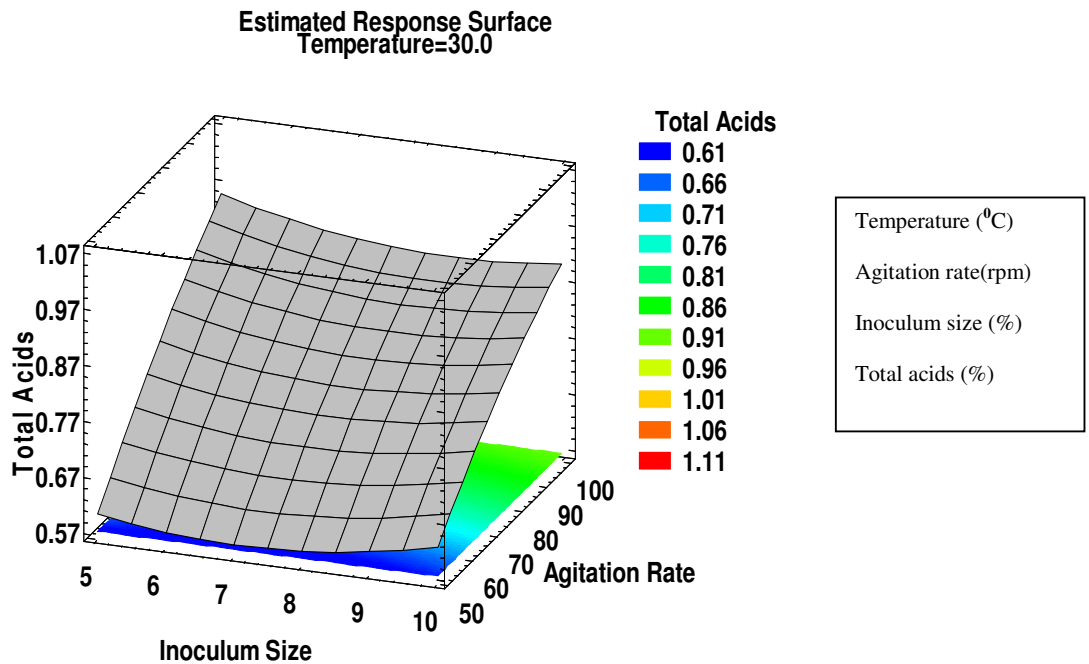


Figure 4.7a Surface plots for Total acids (Inoculum size v/s Agitation rate)

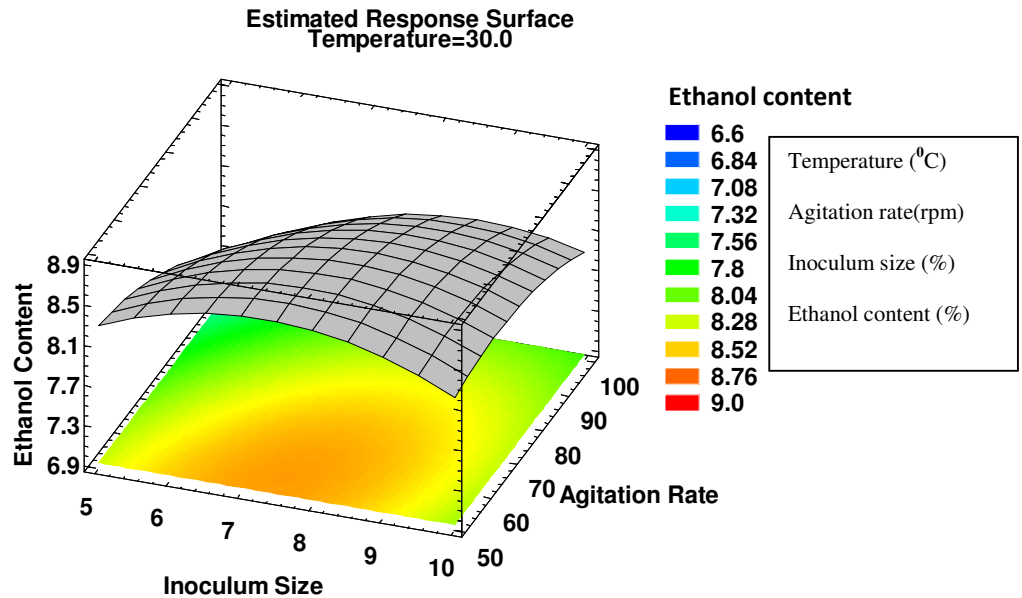


Figure 4.7b Surface plots for Ethanol content (Inoculum size v/s Agitation rate)

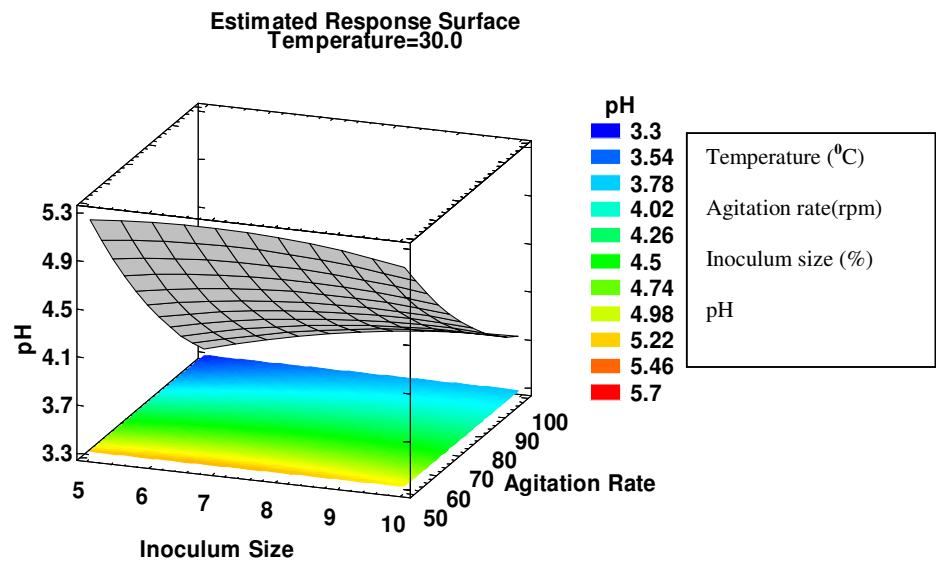


Figure 4.7c Surface plots for pH (Inoculum size v/s Agitation rate)

Fig 4.7 a, b and c Surface plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

4.4.2 Effect of inoculum size and temperature on total acids, ethanol content and pH

Figure 4.8a, 4.8b and 4.8c showed the contour plots 4.9a, 4.9b and 4.9c showed the surface plots of inoculum size v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25 30°C resulted in variation of total acids in the range of 0.62- 0.73% and ethanol content from 8.52- 9.0%. The pH was found to be in the range of 5.1-5.3.

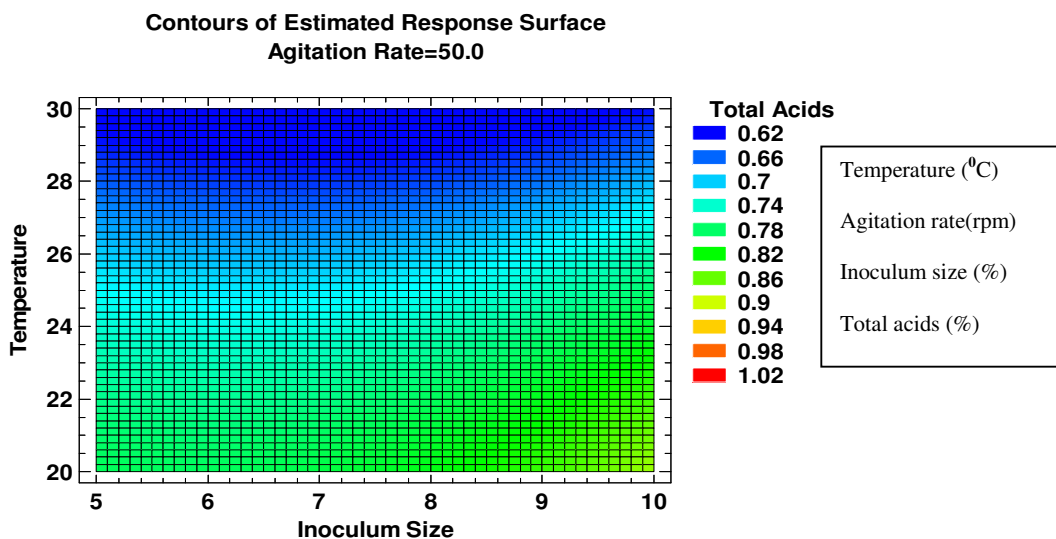


Fig 4.8a Contour plots for total acids (temperature vs. inoculum size)

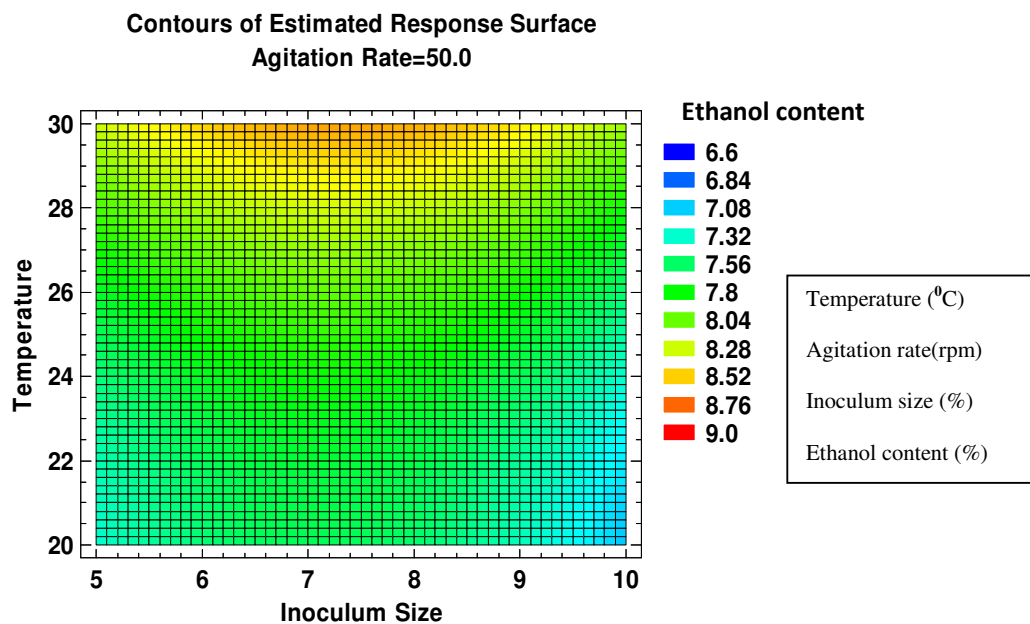


Fig 4.8b Contour plots for ethanol content (temperature vs. inoculum size)

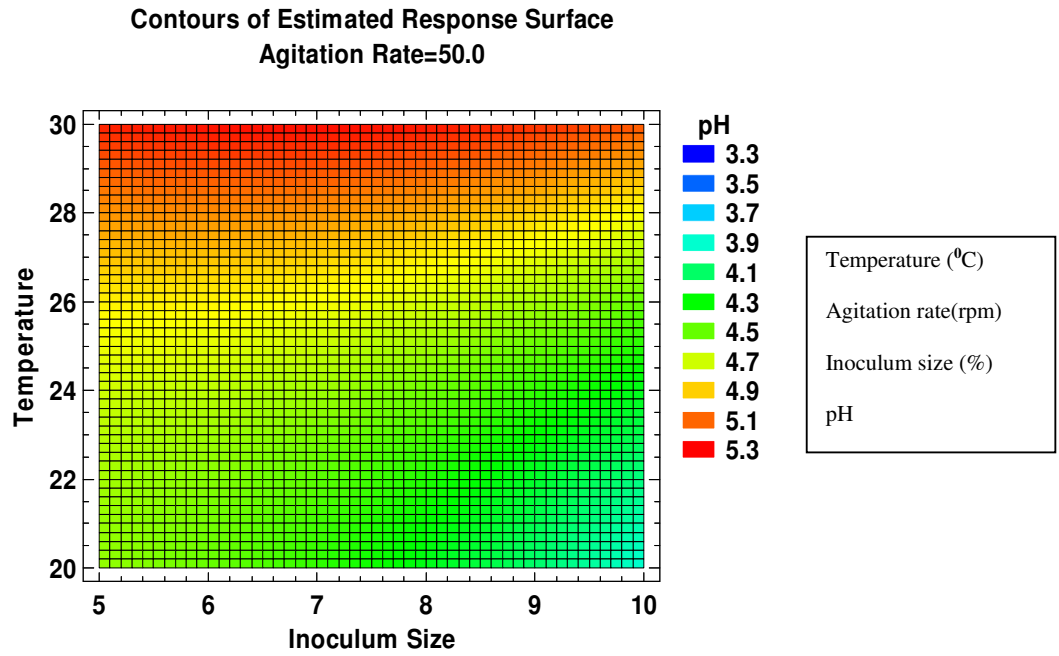


Fig 4.8c Contour plots for pH (temperature vs. inoculum size)

Fig 4.8 a, b and c Contour plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

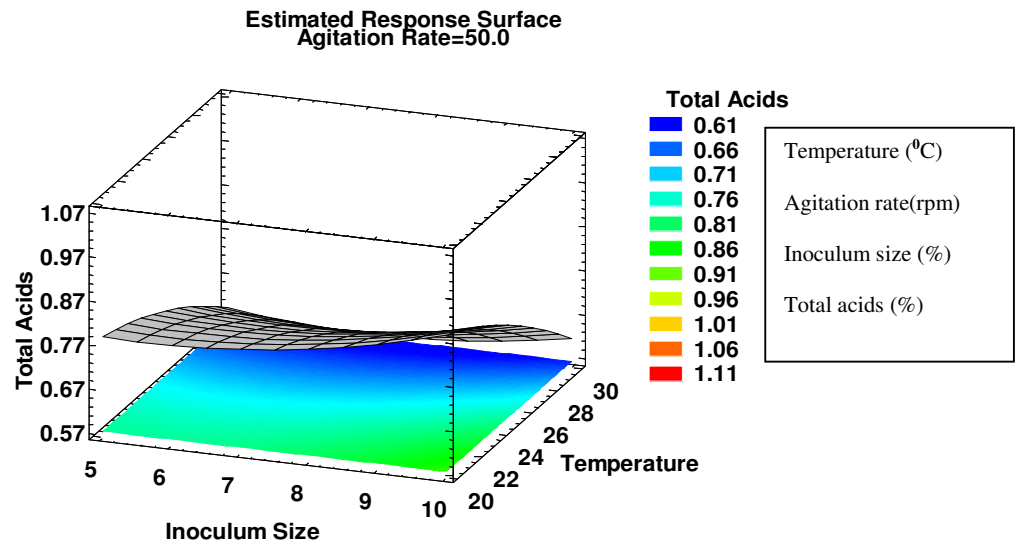


Figure 4.9a Surface plots for Total acids (Inoculum size v/s Temperature)

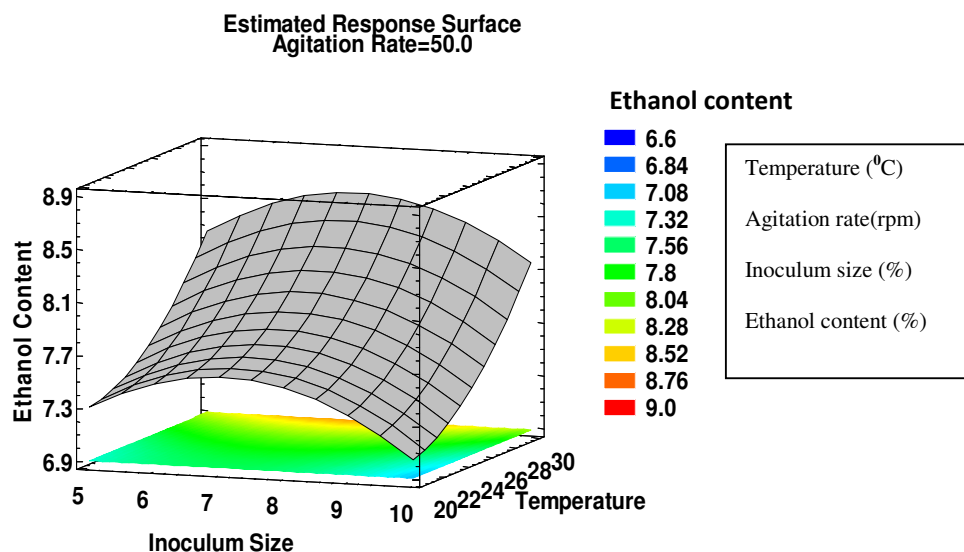


Figure 4.9b Surface plots for Ethanol content (Inoculum size v/s Temperature)

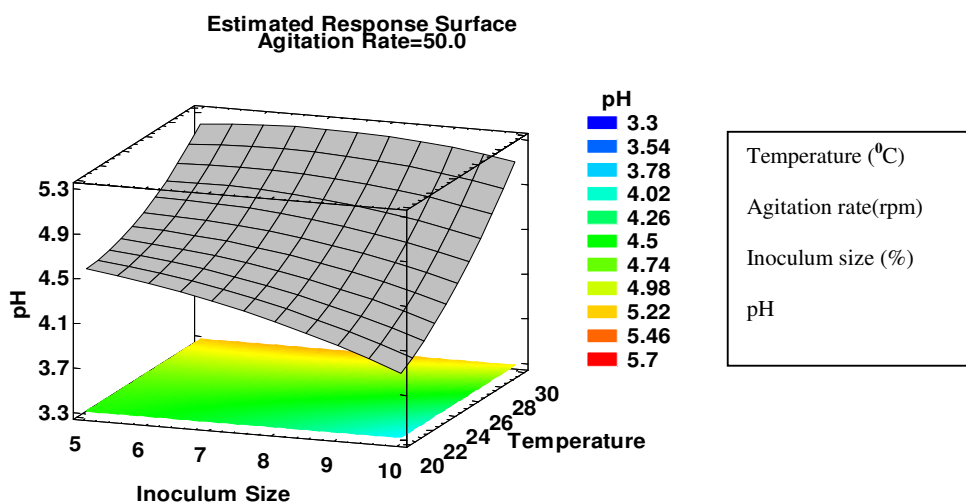


Figure 4.9c Surface plots for pH (Inoculum size v/s Temperature)

Fig 4.9 a, b and c Surface plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

4.4.3 Effect of Agitation rate and temperature on total acids, ethanol content and pH

Figure 4.10a, 4.10 b and 4.10 c showed contour plots, 4.11a, 4.11b and 4.11c showed the surface plots of agitation rate v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25 30°C resulted in variation of total acids in the range of 0.62- 0.73% and ethanol content from 8.52- 9.0%. The pH was found to be in the range of 5.1-5.3.

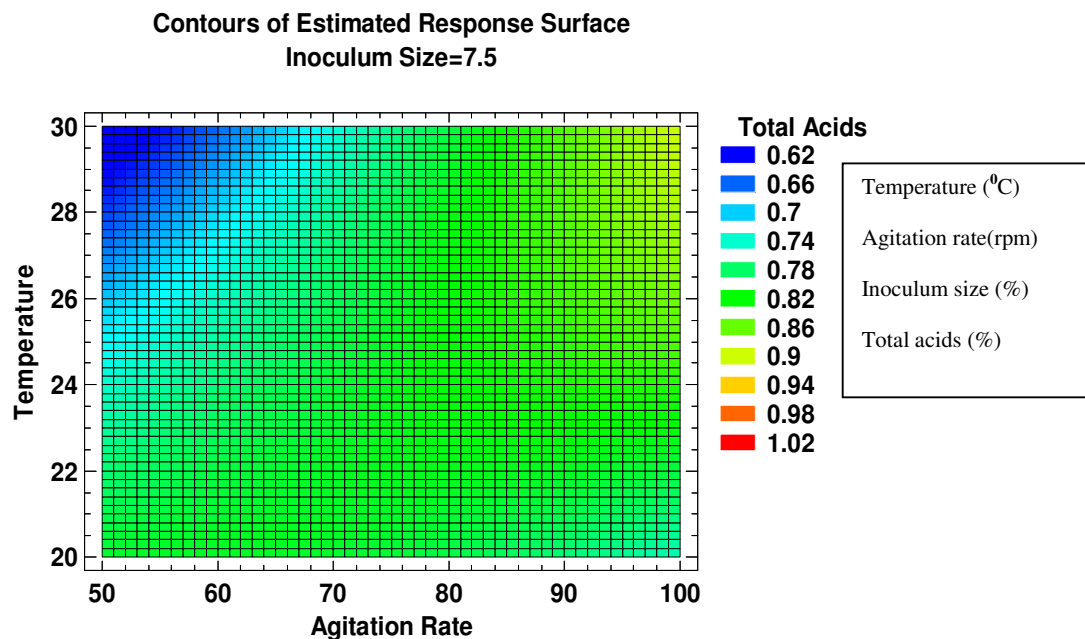


Figure 4.10a Contour plots for Total acids (Agitation rate v/s Temperature)

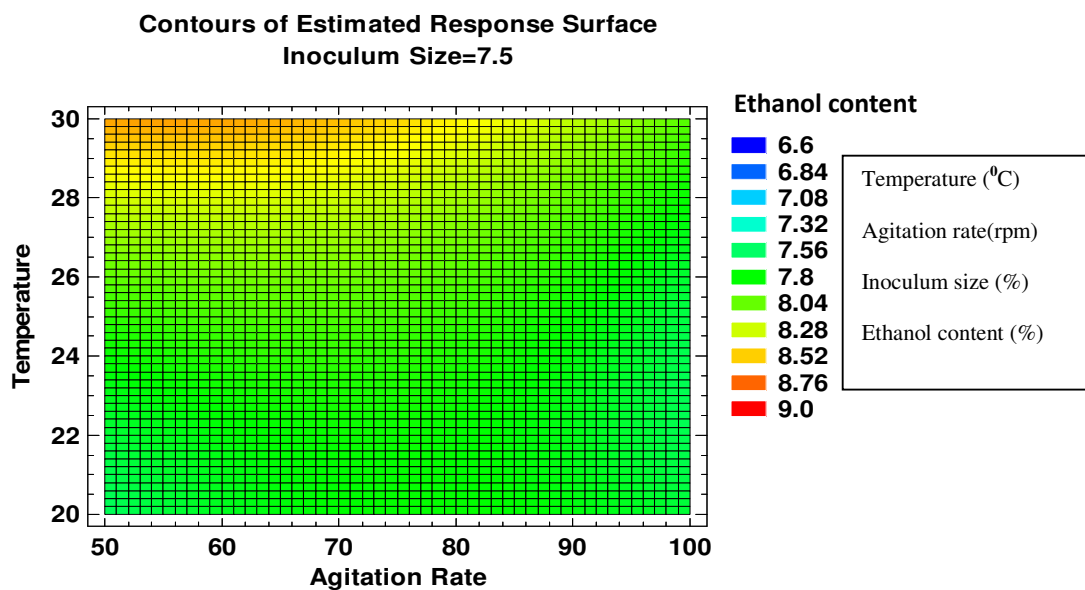


Figure 4.10b Contour plots for Ethanol content (Agitation rate v/s Temperature)

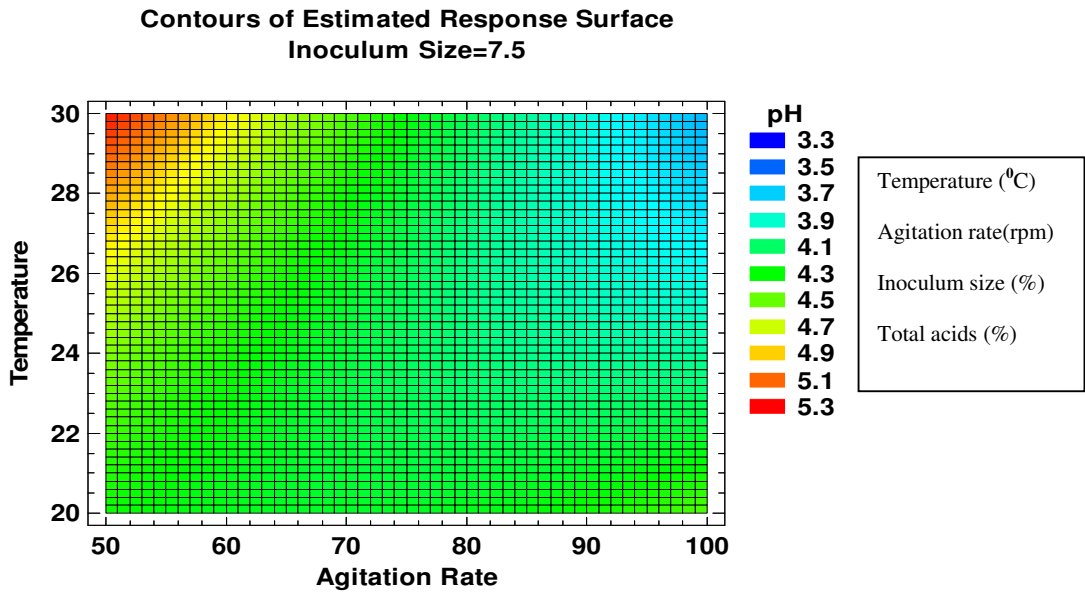


Figure 4.10c Contour plots for pH (Agitation rate v/s Temperature)

Fig4.10 a, b and c: Contour plots showing effect of temperature and agitation rate on total acids, ethanol content and pH

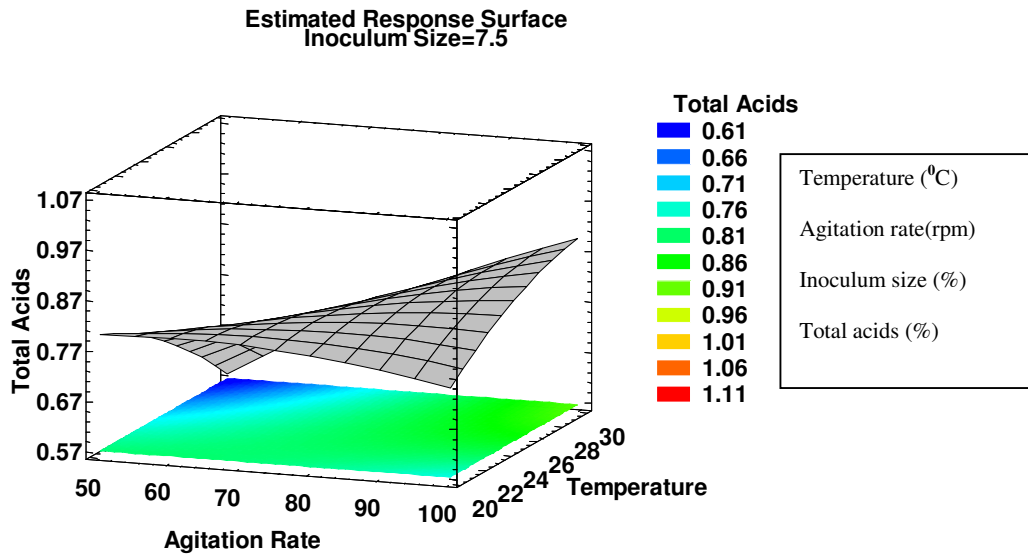


Figure 4.11a Surface plots for Total acids (Agitation rate v/s Temperature)

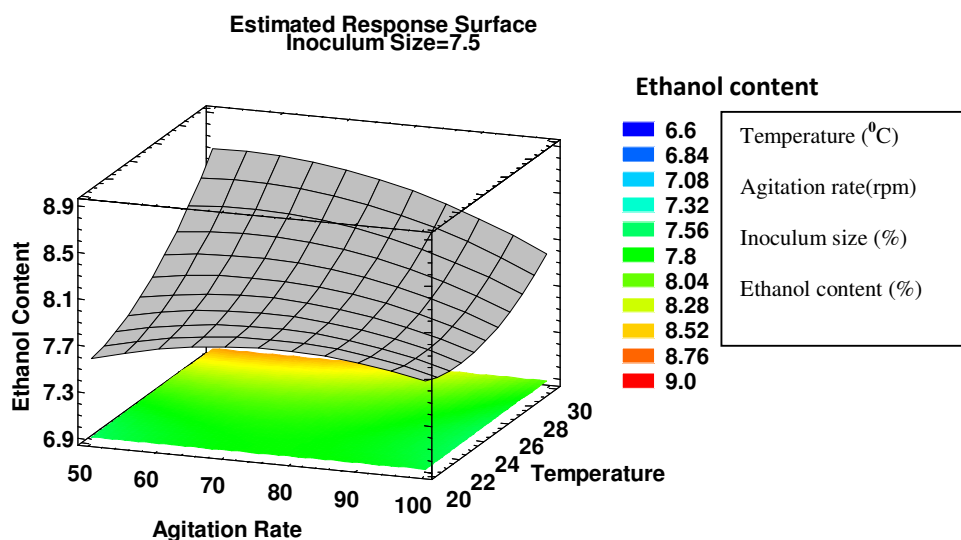


Figure 4.11b Surface plots for Ethanol content (Agitation rate v/s Temperature)

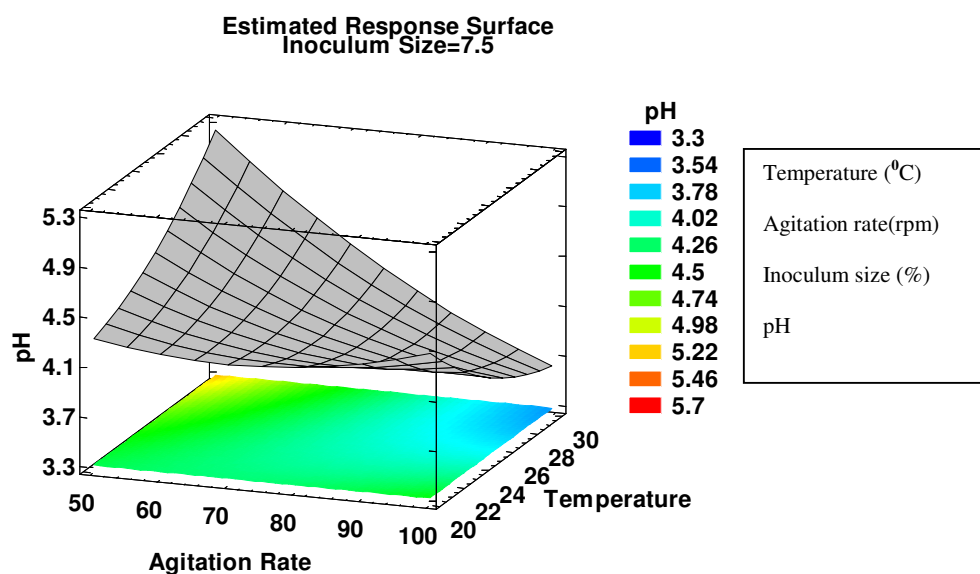


Figure 4.11c Surface plots for pH (Agitation rate v/s Temperature)

Fig 4.11 a, b and c Surface plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

4.5 Response Surface Methodology for Three Variable Interactions

Responses were optimized using software Statgraphics Centurion XVII.I. The optimum value for each dependent variable had different set of treatment conditions. The optimization was done with the target of maximum ethanol content and pH with minimum total acidity. To consider all the responses simultaneously for optimization, surface plots were generated for each response. The most desired combinations were selected for maximum

ethanol production from desirability surface plots (Fig 4.12, 4.13, 4.14). To get maximum ethanol production, surface plots were overlaid with the targets mentioned above. Overlay plots describing the optimum conditions has been shown in Fig. 4.15, 4.16 and 4.17. Hence inoculum size of 5%, agitation rate of 50 rpm and fermentation temperature of 30 °C were found to be the best parameters for optimum responses i.e. total acidity, ethanol content and pH values in sweet sorghum juice inoculated with strain *Saccharomyces cerevisiae* NRRL Y-2034.

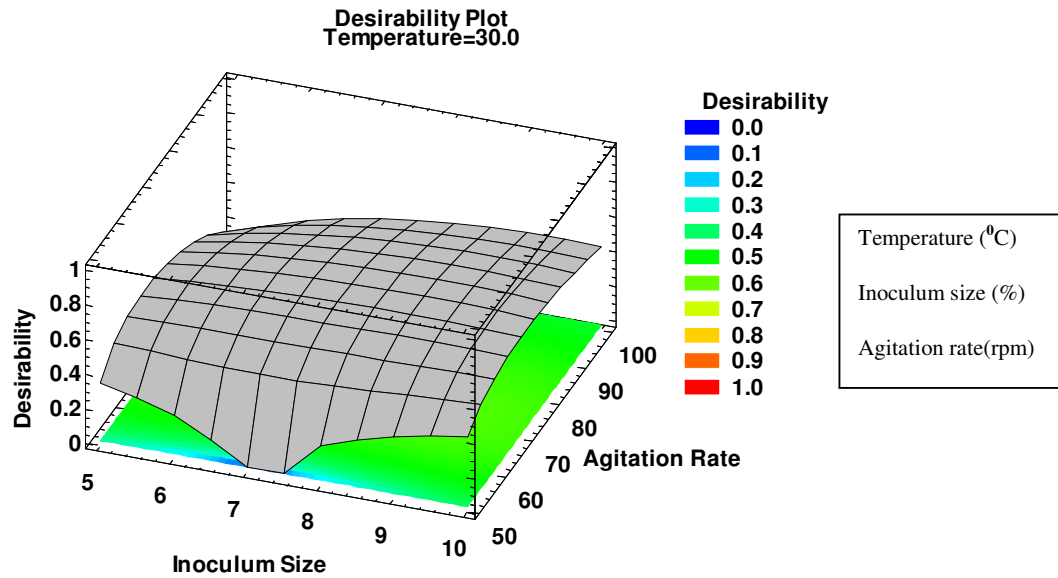


Fig 4.12 Surface plot for desirability: Inoculum size v/s Agitation rate

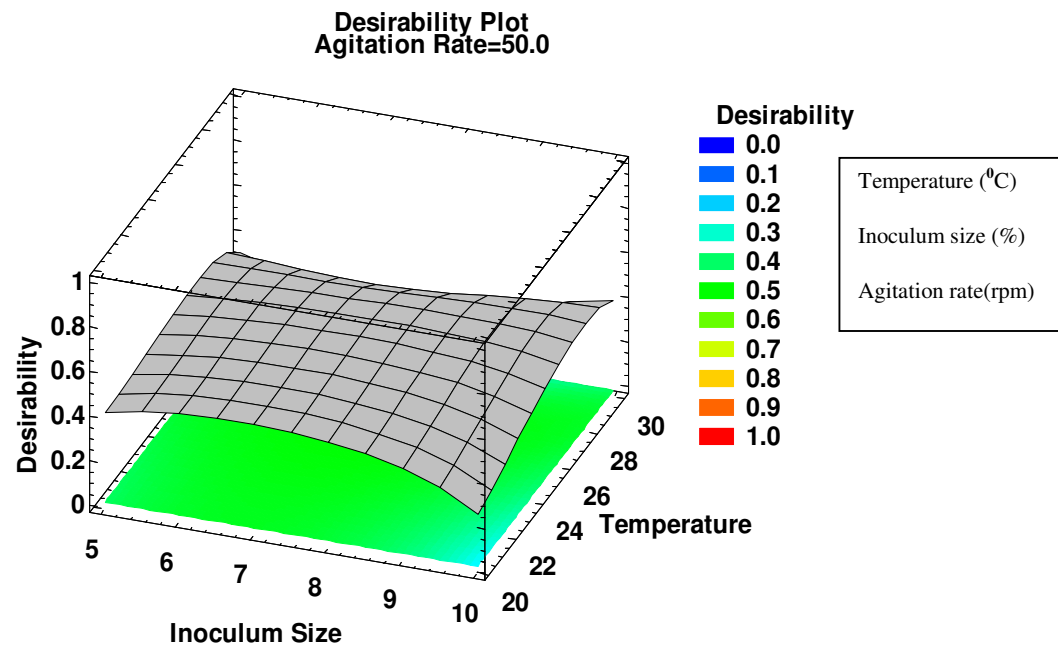


Fig 4.13 Surface plot for desirability: Inoculum size v/s Temperature

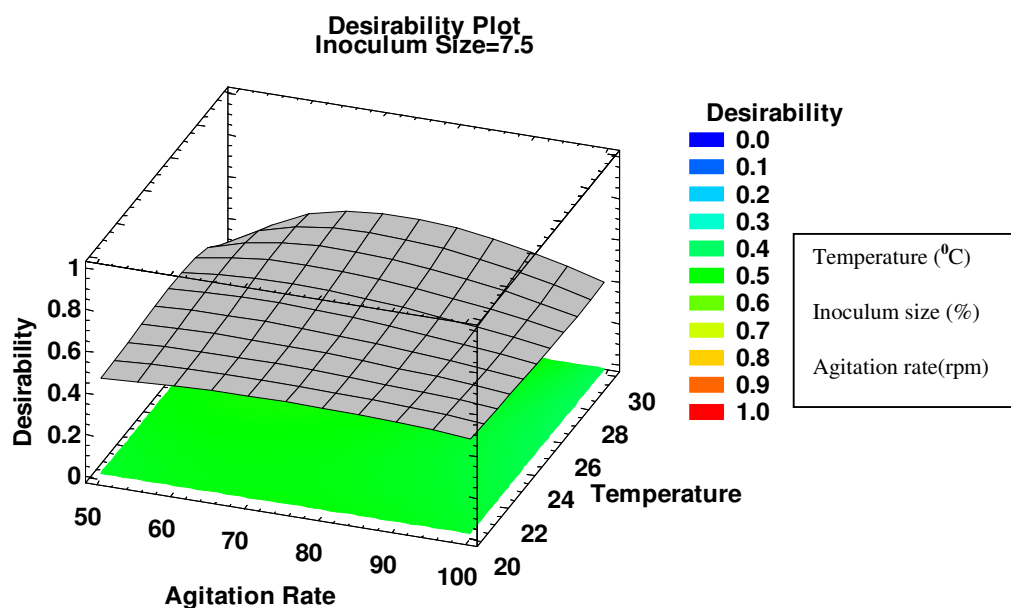


Fig 4.14 Surface plot for desirability: Agitation rate v/s Temperature

OVERLAY PLOTS:

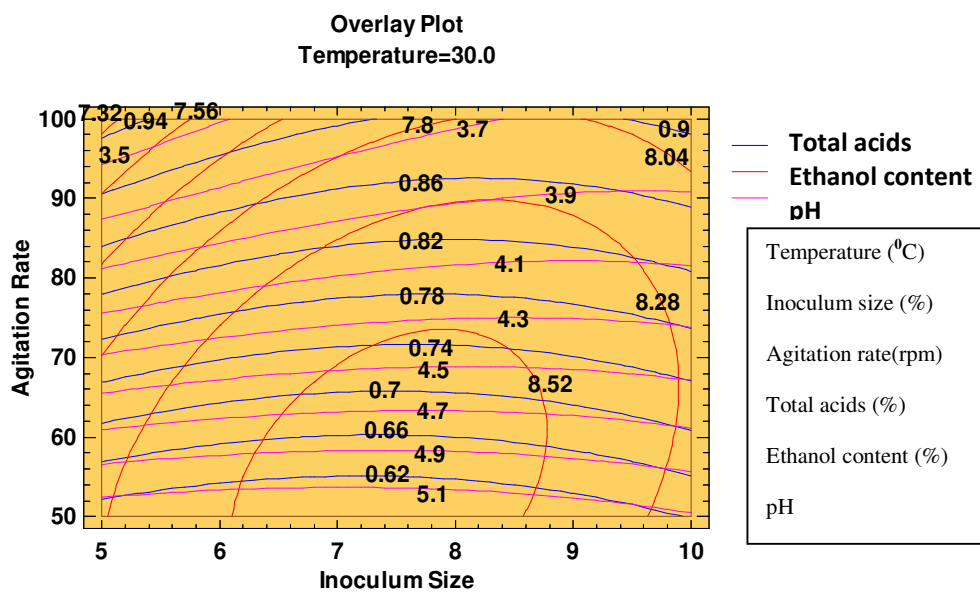


Fig 4.15 Agitation rate v/s inoculum size

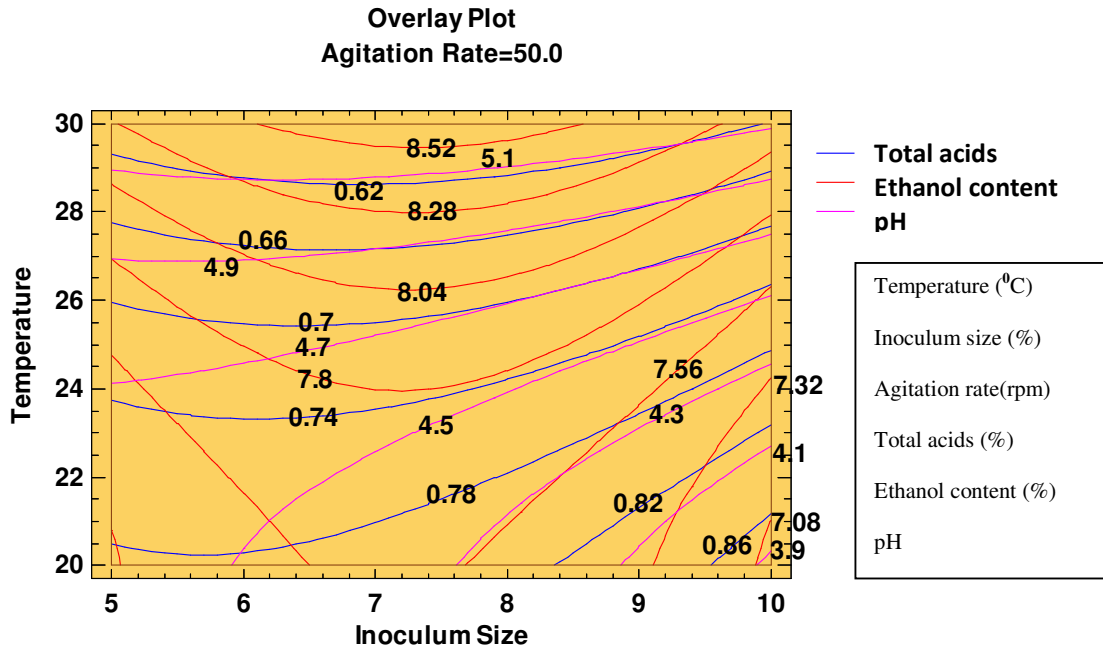


Fig 4.16 Temperature v/s Inoculum size

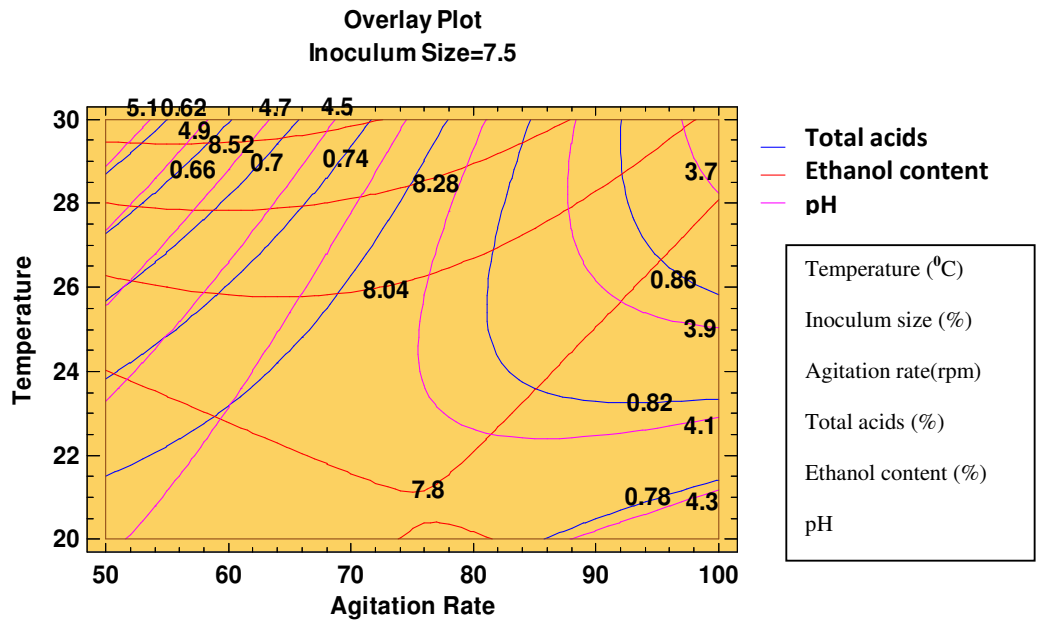


Fig 4.17 Temperature v/s Agitation rate

Table 4.15 Optimization of cultural parameters by Response Surface Methodology of sweet sorghum juice inoculated with strain- 35

Sr. No.	Inoculum size (mg/l substrate)	Agitation rate (RPM)	Temperature (°C)	Total acidity (%)	pH	% Ethanol		Fermentation Efficiency(%)
						v/v	w/v	
1	7.5	50	30	0.52	4.66	7.71	6.16	76.26
2	10	50	30	0.53	4.61	7.45	5.96	73.68
3	5	50	30	0.54	4.64	8.45	6.76	83.58
4	10	50	25	0.89	4.18	7.61	6.08	75.27
5	5	50	25	0.93	4.01	7.78	6.22	76.95
6	5	100	30	0.58	4.58	7.05	5.64	69.73
7	7.5	100	30	0.80	4.31	7.35	5.88	72.70
8	10	100	30	0.80	4.31	8.40	6.72	83.68
9	7.5	75	30	0.79	4.25	7.76	6.20	76.75
10	10	75	30	0.82	4.24	7.89	6.31	78.04
11	10	75	25	0.93	4.01	7.14	5.71	70.62
12	5	75	25	0.86	4.19	7.30	5.84	72.20
13	7.5	75	25	0.86	4.20	7.17	5.73	70.92
14	5	75	20	0.80	4.25	7.28	5.82	72.00
15	7.5	75	20	0.82	4.24	7.21	5.76	71.31
16	10	75	20	0.82	4.31	7.09	5.67	70.12
17	7.5	50	20	0.88	4.17	7.19	5.75	71.11
18	5	50	20	0.89	4.16	7.58	6.06	74.97
19	7.5	100	25	0.76	4.29	7.23	5.78	71.51
20	10	100	25	0.87	4.19	7.84	6.27	77.54
21	5	100	20	0.87	4.19	8.01	6.40	79.22
22	10	100	20	0.64	4.52	7.73	5.85	76.45

Cultural conditions: Volume of sweet sorghum juice=50ml; Fermentation period =12 days
Adjusted Brix= 20°B; pH= 5.5
Average of triplicate data

Table 4.16 Statistical analysis of table 4.15 for fitting of model

Model	Total Acids	Ethanol Content	pH
Transformation	none	none	none
Model d.f.	9	9	9
P-value	0.0213	0.0496	0.0492
Error d.f.	12	12	12
Std. error	0.0903613	0.302612	0.141405
R-squared	72.93	67.77	67.82
Adj. R-squared	52.62	43.60	43.69

4.6 Effect of cultural conditions on ethanol content

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.18). Table 4.17 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 67.7721% of the variability in Ethanol Content. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 43.6011%. The standard error of the estimate shows the standard deviation of the residuals to be 0.302612. The mean absolute error (MAE) of 0.17982 is the average value of the residuals.

The regression equation of the fitted model for ethanol content is:

$$14.1497 - 1.17139*A - 0.0554638*B - 0.0763152*C + 0.035887*A^2 + 0.00429333*A*B + 0.0129333*A*C + 0.00036287*B^2 - 0.00124667*B*C + 0.00211232*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.17 Estimated effects for Ethanol Content (%)

Effect	Estimate	Std. Error	V.I.F.
average+block	7.17913	0.209276	
A:Inoculum Size	0.06125	0.151306	1.0
B:Agitation Rate	0.0	0.151306	1.0
C:Temperature+block	0.328007	0.14798	1.01359
AA+block	0.448587	0.292578	1.01976
AB	0.536667	0.174713	1.0
AC	0.323333	0.174713	1.0
BB+block	0.453587	0.292578	1.01976
BC+block	-0.311667	0.174713	1.0
CC+block	0.105616	0.310192	1.0149

Standard errors are based on total error with 12 d.f.

Plot of Ethanol Content

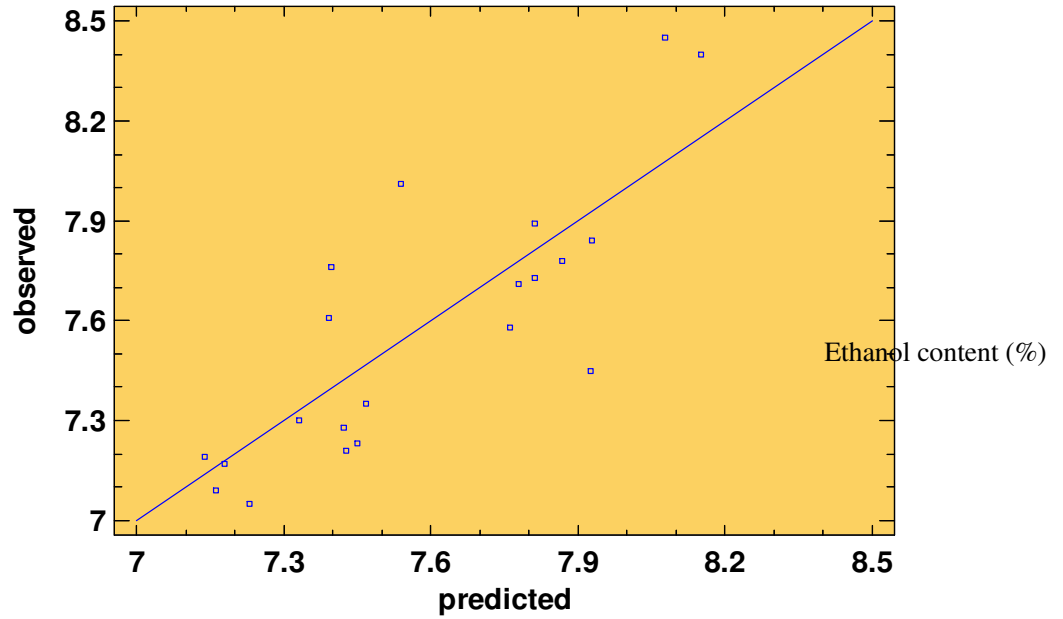


Fig 4.18 Experimental vs predicted results of ethanol production

Table 4.18 Analysis of Variance for Ethanol Content

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.0150063	1	0.0150063	0.16	0.6927
B:Agitation Rate	0.0	1	0.0	0.00	1.0000
C:Temperature+block	0.449917	1	0.449917	4.91	0.0467
AA+block	0.21527	1	0.21527	2.35	0.1512
AB	0.864033	1	0.864033	9.44	0.0097
AC	0.313633	1	0.313633	3.42	0.0890
BB+block	0.220095	1	0.220095	2.40	0.1470
BC+block	0.291408	1	0.291408	3.18	0.0997
CC+block	0.0106162	1	0.0106162	0.12	0.7394
Total error	1.09889	12	0.0915743		
Total (corr.)	3.40975	21			

Table 4.19 Regression coefficients for Ethanol Content

Coefficient	Estimate
constant	14.1497
A:Inoculum Size	-1.17139
B:Agitation Rate	-0.0554638
C:Temperature	-0.0763152
AA	0.035887
AB	0.00429333
AC	0.0129333
BB	0.00036287
BC	-0.00124667
CC	0.00211232

4.6.1 Effect of cultural conditions on total acids:

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.19). Table 4.20 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 72.9277% of the variability in Total Acids. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 52.6235%. The standard error of the estimate shows the standard deviation of the residuals to be 0.0903613. The mean absolute error (MAE) of 0.0542194 is the average value of the residuals.

The regression equation of the fitted model for total acids is:
 $-0.912862 - 0.0686051*A + 0.000622826*B + 0.180341*C + 0.000301449*A^2 + 0.0000133333*A*B + 0.00253333*A*C - 0.000116986*B^2 + 0.00068*B*C - 0.00528841*C^2$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.20 Estimated effects for Total Acids (%)

Effect	Estimate	Std. Error	V.I.F.
average+block	0.938986	0.0624907	
A:Inoculum Size	0.00125	0.0451807	1.0
B:Agitation Rate	0.00875	0.0451807	1.0
C:Temperature+block	-0.140797	0.0441876	1.01359
AA+block	0.00376812	0.0873651	1.01976
AB	0.00166667	0.0521701	1.0
AC	0.0633333	0.0521701	1.0
BB+block	-0.146232	0.0873651	1.01976
BC+block	0.17	0.0521701	1.0
CC+block	-0.26442	0.0926248	1.0149

Standard errors are based on total error with 12 d.f.

Plot of Total Acids

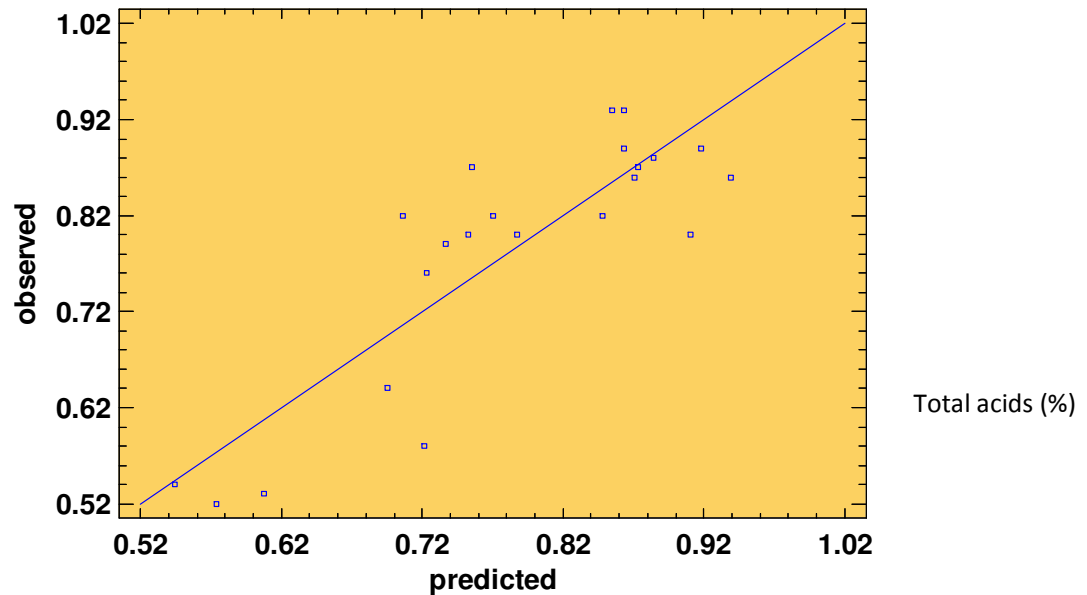


Fig 4.19 Experimental vs predicted results of total acids

Table 4.21 Analysis of Variance for Total Acids

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.00000625	1	0.00000625	0.00	0.9784
B:Agitation Rate	0.00030625	1	0.00030625	0.04	0.8497
C:Temperature+block	0.0828996	1	0.0828996	10.15	0.0078
AA+block	0.0000151893	1	0.0000151893	0.00	0.9663
AB	0.00000833333	1	0.00000833333	0.00	0.9750
AC	0.0120333	1	0.0120333	1.47	0.2481
BB+block	0.0228757	1	0.0228757	2.80	0.1200
BC+block	0.0867	1	0.0867	10.62	0.0068
CC+block	0.0665427	1	0.0665427	8.15	0.0145
Total error	0.0979821	12	0.00816517		
Total (corr.)	0.361927	21			

Table 4.22 Regression coefficients for Total Acids

Coefficient	Estimate
constant	-0.912862
A:Inoculum Size	-0.0686051
B:Agitation Rate	0.000622826
C:Temperature	0.180341
AA	0.000301449
AB	0.0000133333
AC	0.00253333
BB	-0.000116986
BC	0.00068
CC	-0.00528841

4.6.2 Effect of cultural conditions on pH:

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.20). Table 4.23 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted)

values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 67.8244% of the variability in pH. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 43.6926%. The standard error of the estimate shows the standard deviation of the residuals to be 0.141405. The mean absolute error (MAE) of 0.0887064 is the average value of the residuals.

The regression equation of the fitted model for total acids is:

$$6.7528 + 0.0858804*A - 0.000903623*B - 0.258793*C - 0.00000869565*A^2 + 0.0000933333*A*B - 0.0036*A*C + 0.000155913*B^2 - 0.000913333*B*C + 0.00748043*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.23 Estimated effects for pH

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	4.07609	0.0977905	
A:Inoculum Size	0.01375	0.0707024	1.0
B:Agitation Rate	0.0175	0.0707024	1.0
C:Temperature+block	0.197283	0.0691483	1.01359
AA+block	-0.000108696	0.136716	1.01976
AB	0.0116667	0.0816401	1.0
AC	-0.09	0.0816401	1.0
BB+block	0.194891	0.136716	1.01976
BC+block	-0.228333	0.0816401	1.0
CC+block	0.374022	0.144947	1.0149

Standard errors are based on total error with 12 d.f.

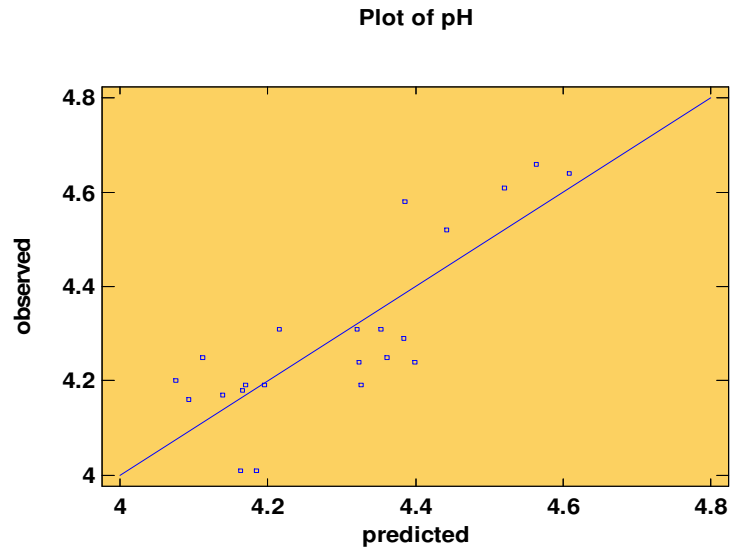


Fig 4.20 Experimental vs predicted results of pH

Table 4.24 Analysis of Variance for pH

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.00075625	1	0.00075625	0.04	0.8491
B:Agitation Rate	0.001225	1	0.001225	0.06	0.8087
C:Temperature+block	0.162758	1	0.162758	8.14	0.0145
AA+block	1.2639E-8	1	1.2639E-8	0.00	0.9994
AB	0.000408333	1	0.000408333	0.02	0.8887
AC	0.0243	1	0.0243	1.22	0.2919
BB+block	0.0406326	1	0.0406326	2.03	0.1795
BC+block	0.156408	1	0.156408	7.82	0.0161
CC+block	0.133139	1	0.133139	6.66	0.0241
Total error	0.239944	12	0.0199953		
Total (corr.)	0.745732	21			

Table 4.25 Regression coefficients for pH

Coefficient	Estimate
constant	6.7528
A:Inoculum Size	0.0858804
B:Agitation Rate	-0.000903623
C:Temperature	-0.258793
AA	-0.00000869565
AB	0.0000933333
AC	-0.0036
BB	0.000155913
BC	-0.000913333
CC	0.00748043

4.7 RESPONSE SURFACE METHODOLOGY: TWO VARIABLE INTERACTION STUDIES

The surface plots for different interaction of any two independent variables, while holding the third variable constant, on enzyme production were generated using software, Statgraphics Centurion XVI.I. The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable in order to deduce the optimum conditions.

4.7.1 Effect of agitation rate and inoculum size on total acids, ethanol content and pH

A direct correlation was found between inoculum size and agitation rate and graphs were plotted to understand their effect on ethanol production. The response of experimental levels of each variable was examined and concluded that with the variation in agitation rate from 50, 75 and 100rpm and inoculums size from 5, 7.5 and 10%, the ethanol content, total acids and pH varied in the range of 0.52-0.65 %, ethanol content in range of 8.7-10.3% and pH in range of 4.9-5.2 respectively as shown in Figure 4.21a, 4.21b, 4.21c, 4.22a, 4.22b and 4.22c.

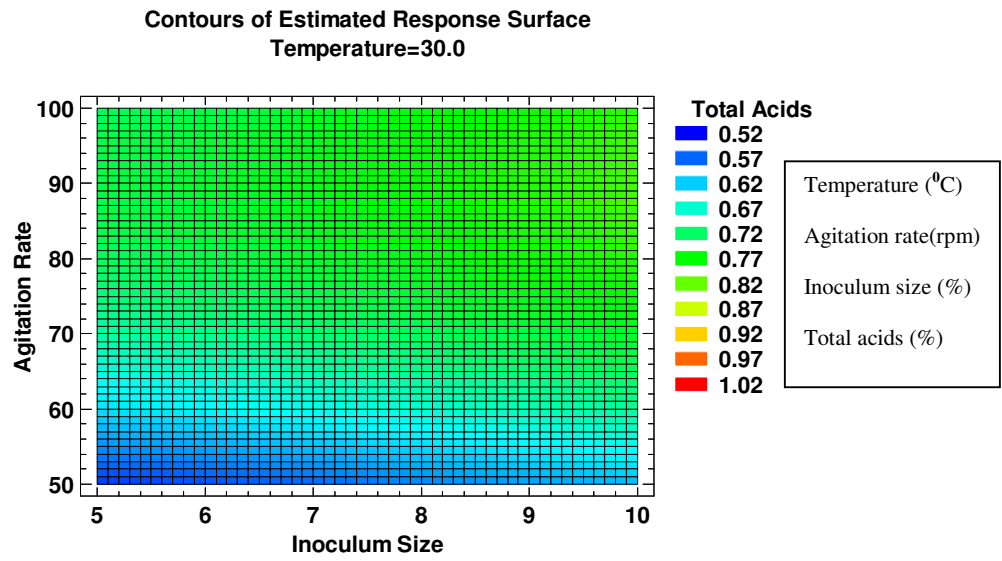


Fig 4.21a Contour plots for total acids (agitation rate vs. inoculum size)

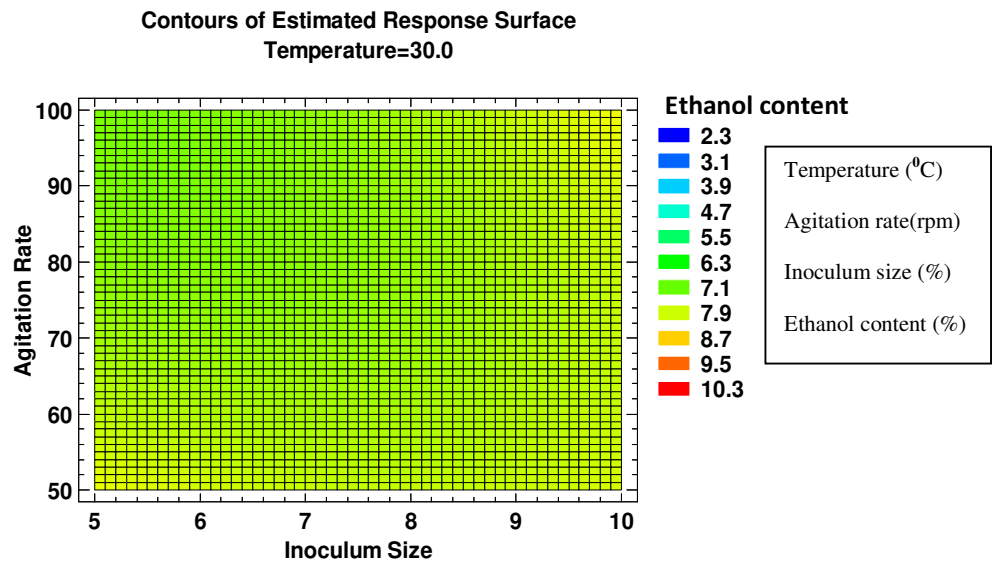


Fig4.21b Contour plots for ethanol content (agitation rate vs. inoculum size)

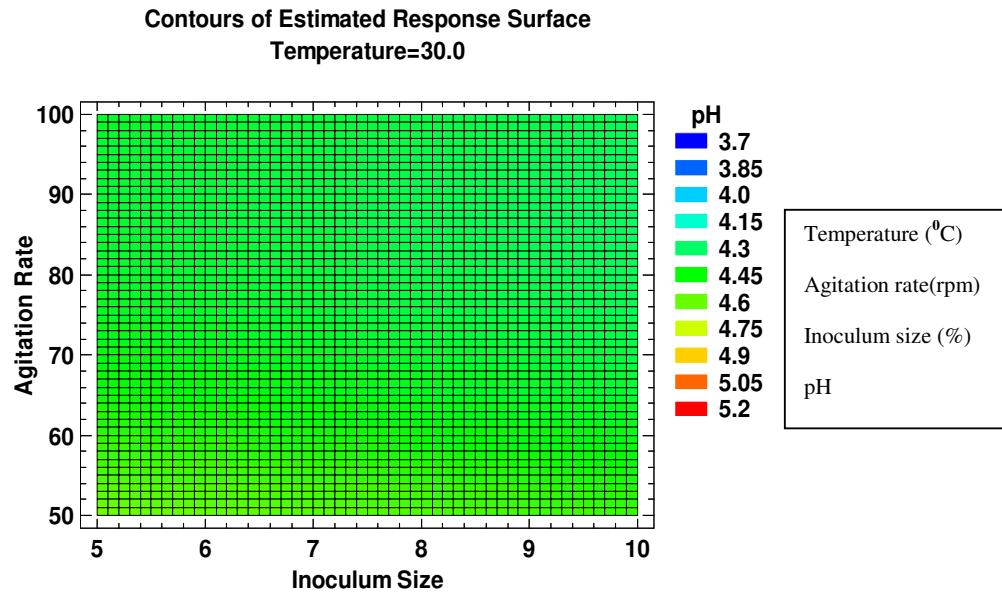


Fig4.21c Contour plots for pH (agitation rate vs. inoculum size)

Fig 4.21a, b and c Contour plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

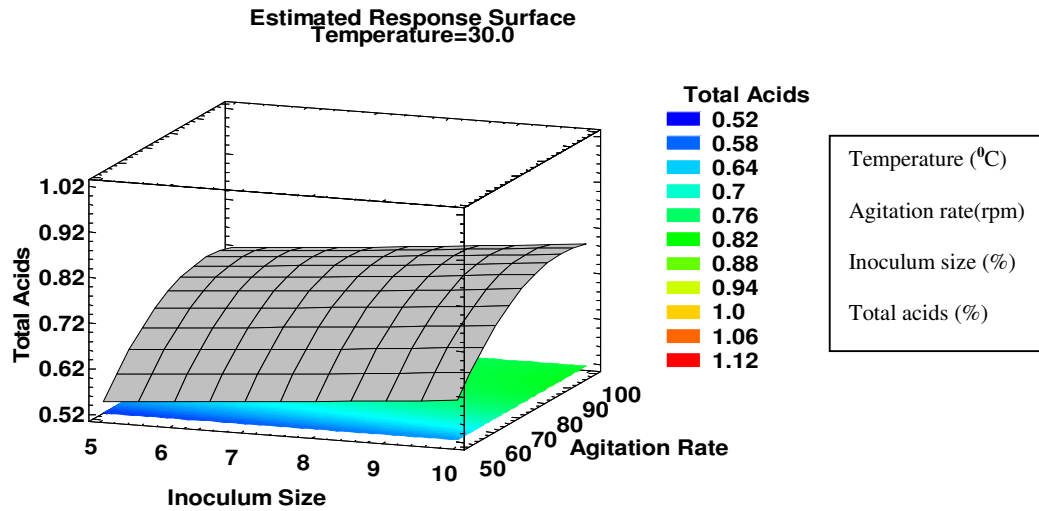


Fig 4.22a Surface plots for total acids (agitation rate vs. inoculum size)

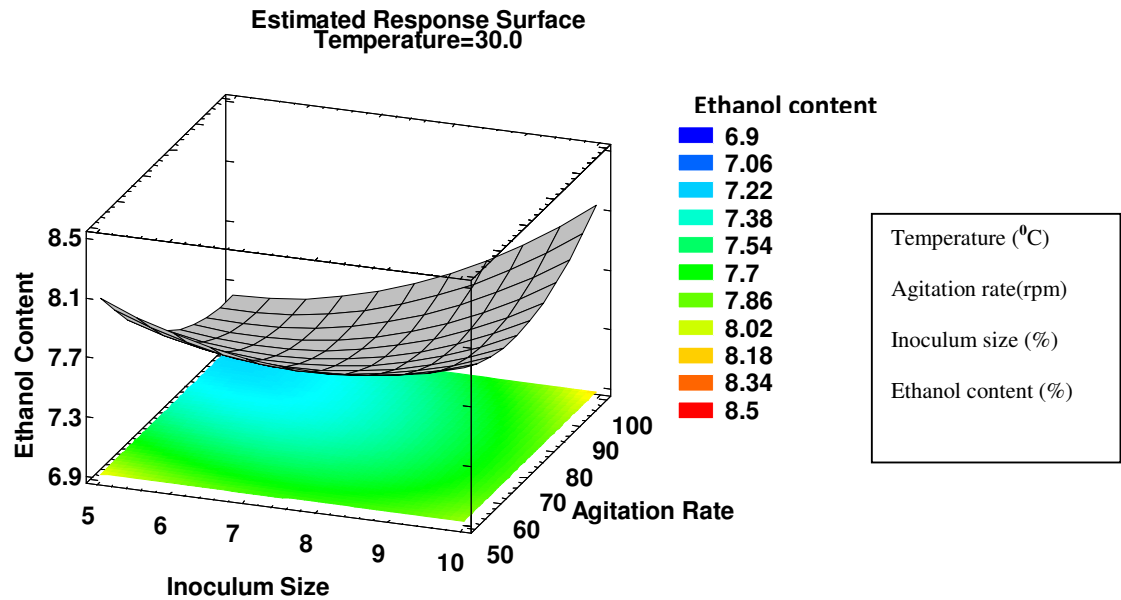


Fig 4.22b Surface plots for ethanol content (agitation rate vs. inoculum size)

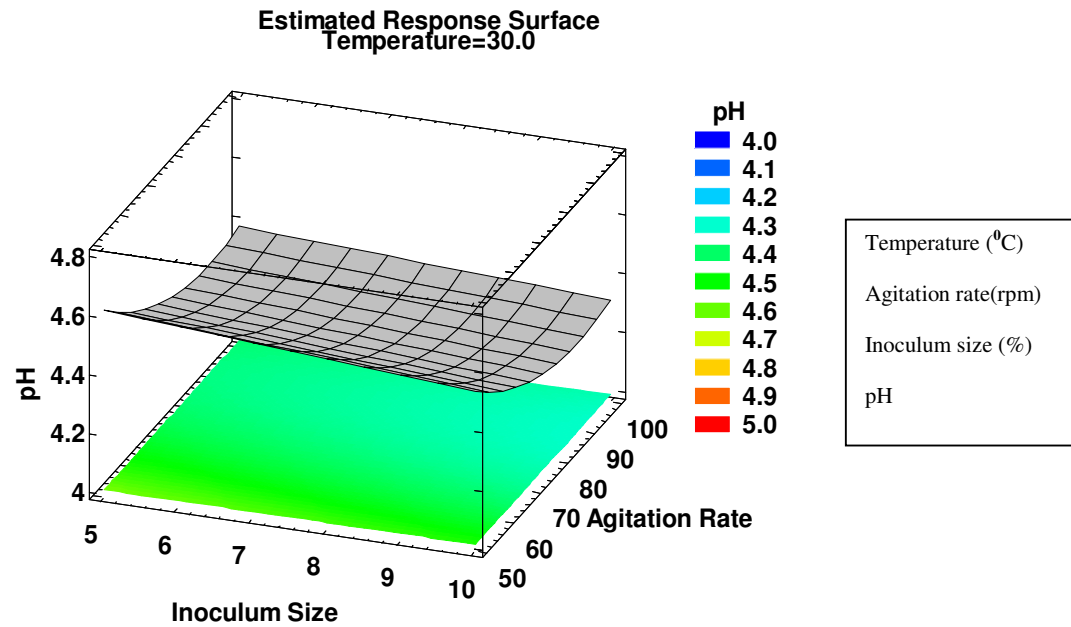


Fig 4.22c Surface plots for pH (agitation rate vs. inoculum size)

Fig 4.22 a, b and c Surface plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

4.7.2 Effect of inoculum size and temperature on total acids, ethanol content and pH

Figure 4.23a, 4.23b and 4.23c showed the contour plots, 4.24a ,4.24 b and 4.24c showed the surface plots of inoculum size v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25, 30°C resulted in variation of total acids in the range of 0.52-0.67 % and ethanol content from 8.7-10.3% . The pH was found to be in the range of 4.9-5.2.

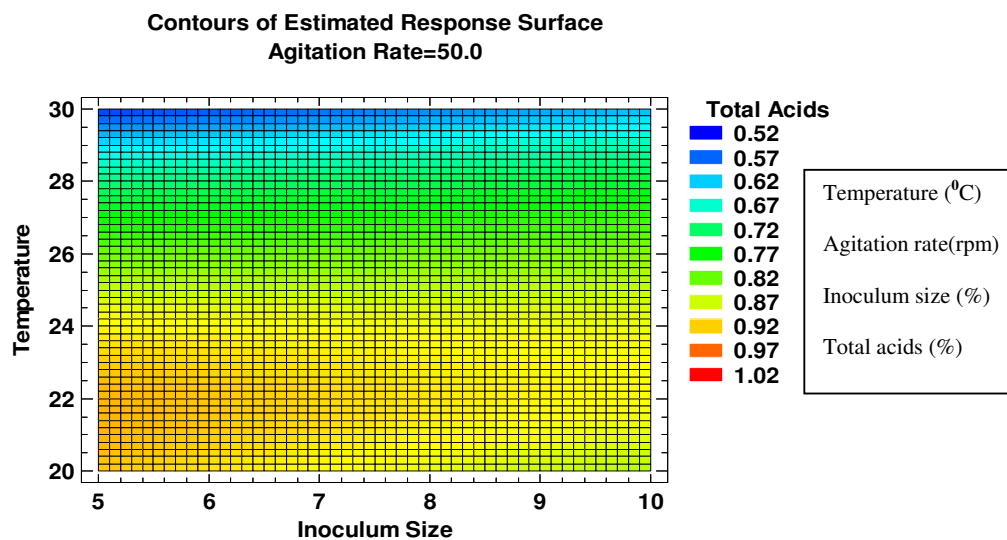


Fig4.23a Contour plots for total acids (temperature vs. inoculum size)

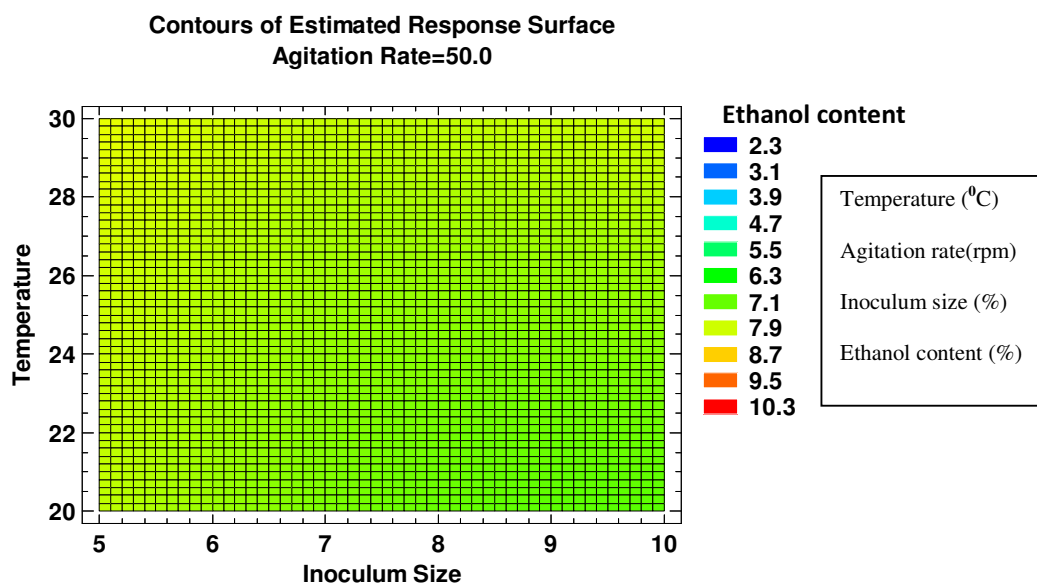


Fig4.23b Contour plots for ethanol content (temperature vs. inoculum size)

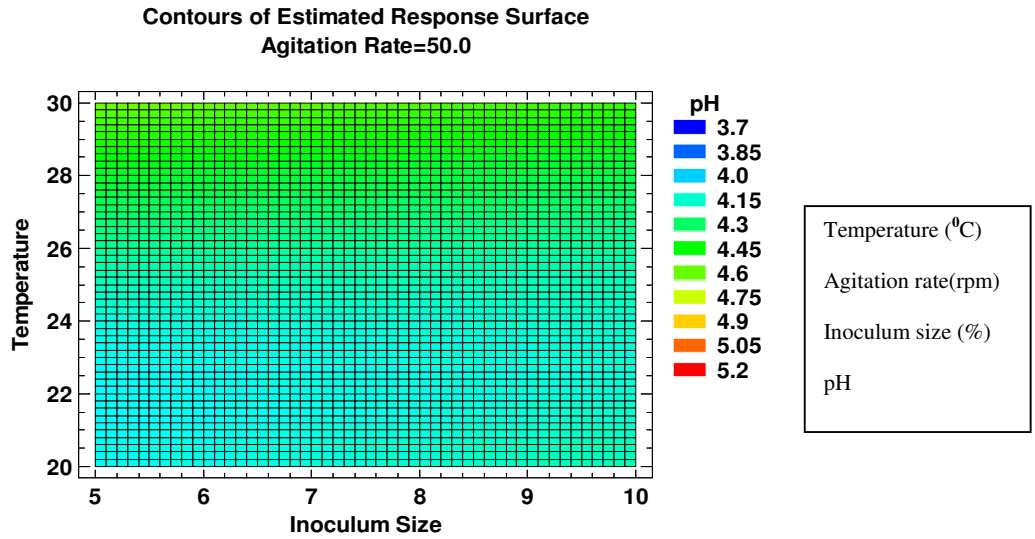


Fig4.23c Contour plots for pH (temperature vs. inoculum size)

Fig4.23 a, b and c: Contour plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

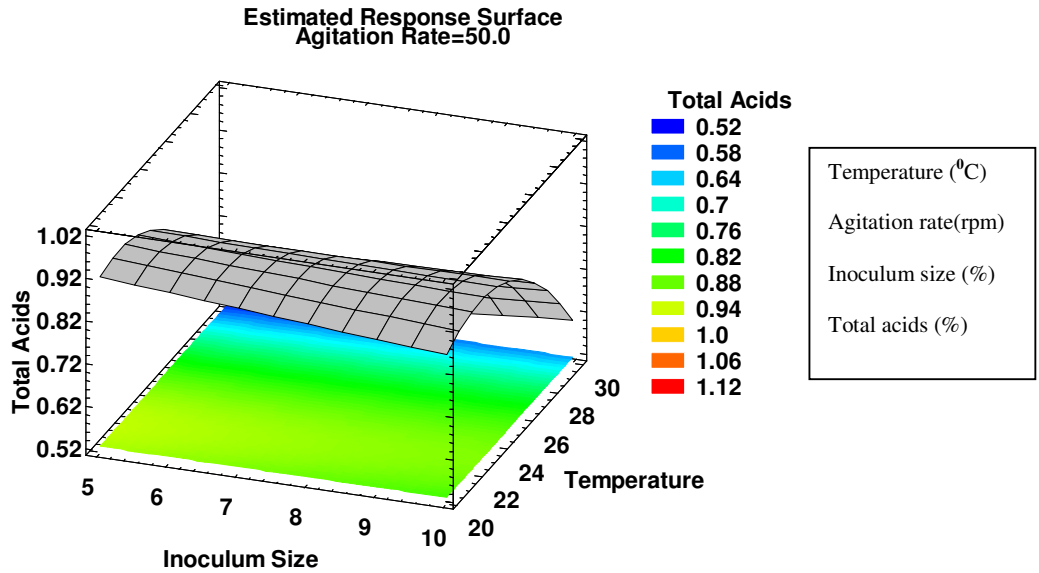


Fig 4.24a Surface plots for total acids (temperature vs. inoculum size)

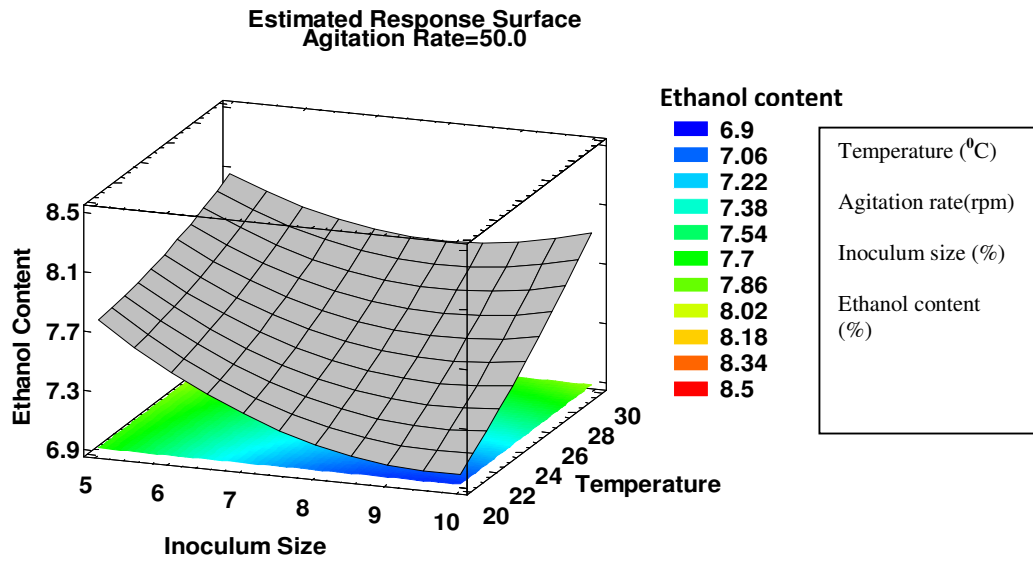


Fig 4.24b Surface plots for ethanol content (temperature vs. inoculum size)

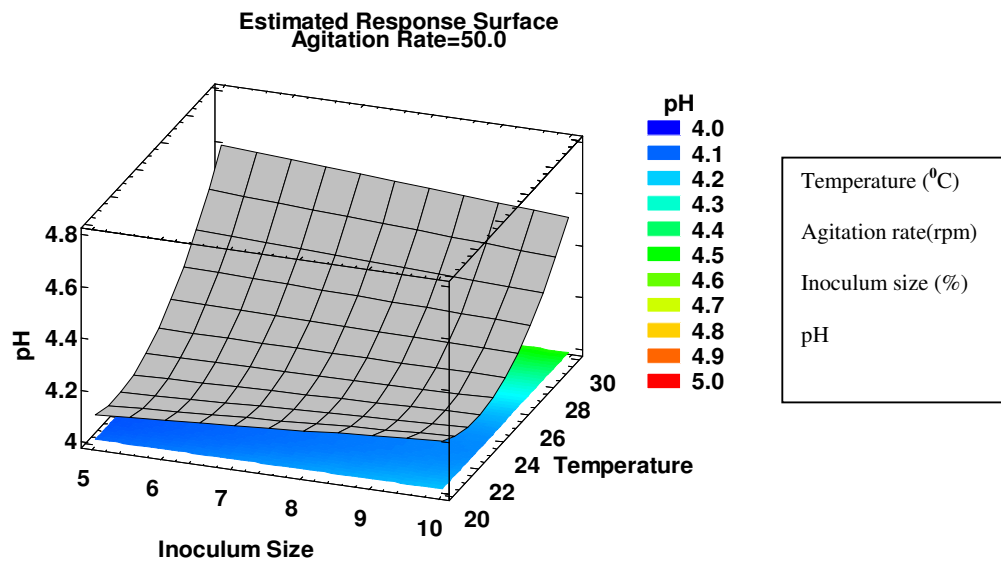


Fig 4.24c Surface plots for pH (temperature vs. inoculum size)

Fig 4.24 a, b and c Surface plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

4.7.3 Effect of Agitation rate and temperature on total acids, ethanol content and pH

Figure 4.25a, 4.25b and 4.25c showed contour plots, 4.26a, 4.26b and 4.26c showed the surface plots of agitation rate v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25, 30°C resulted in variation of total acids in the range of 0.52-0.67 % and ethanol content from 8.7-10.3%. The pH was found to be in the range of 4.9-5.2.

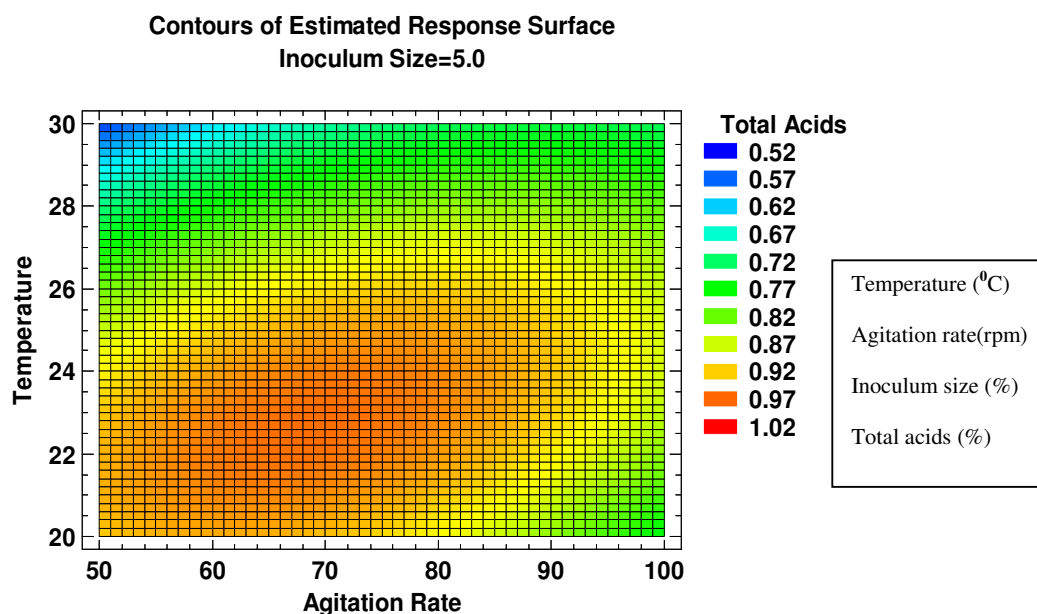


Fig 4.25a Contour plots for total acids (temperature vs. agitation rate)

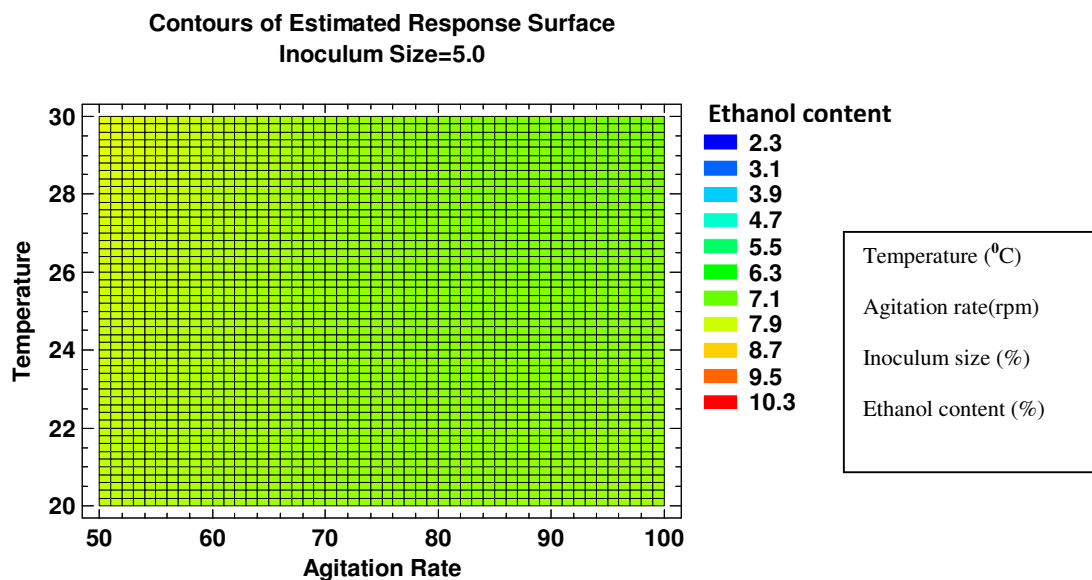


Fig 4.25b Contour plots for ethanol content (temperature vs. agitation rate)

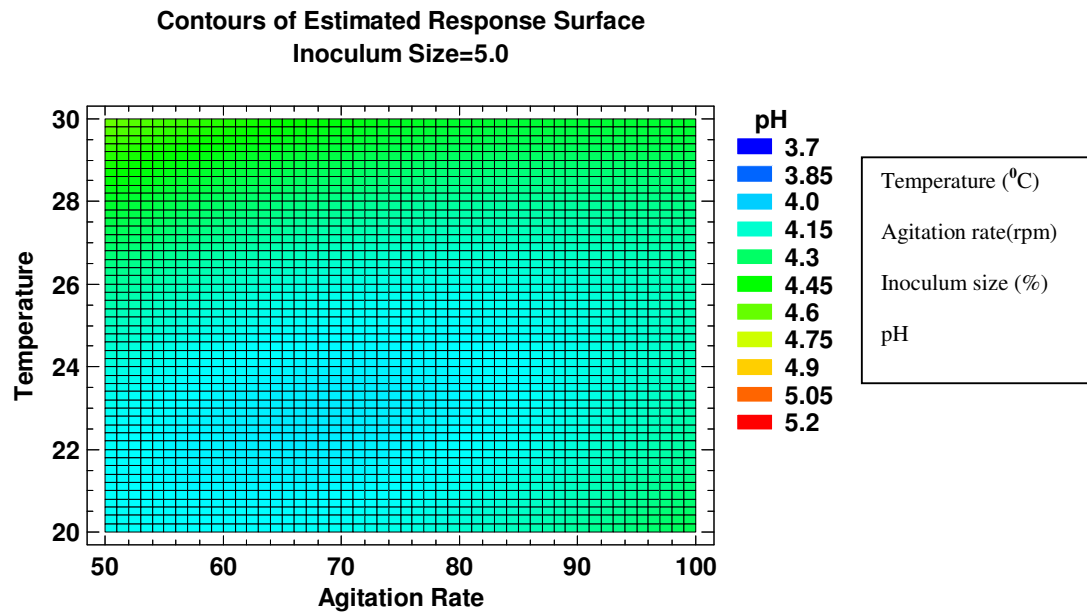


Fig4.25c Contour plots for pH(temperature vs. agitation rate)

Fig. 4.25 a, b and c Contour plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

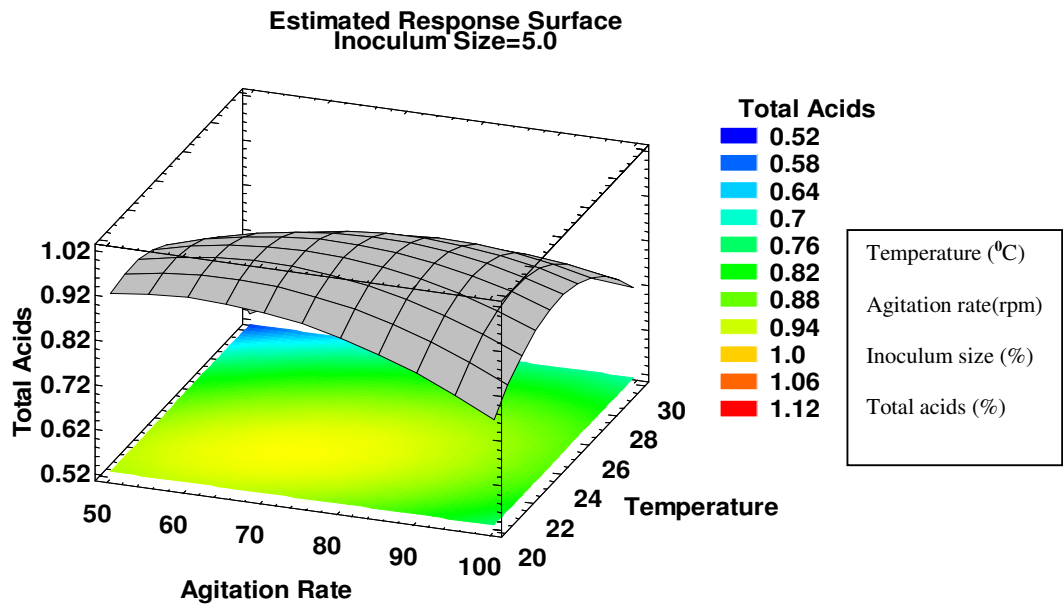


Fig 4.26a Surface plots for total acids (temperature vs. agitation rate)

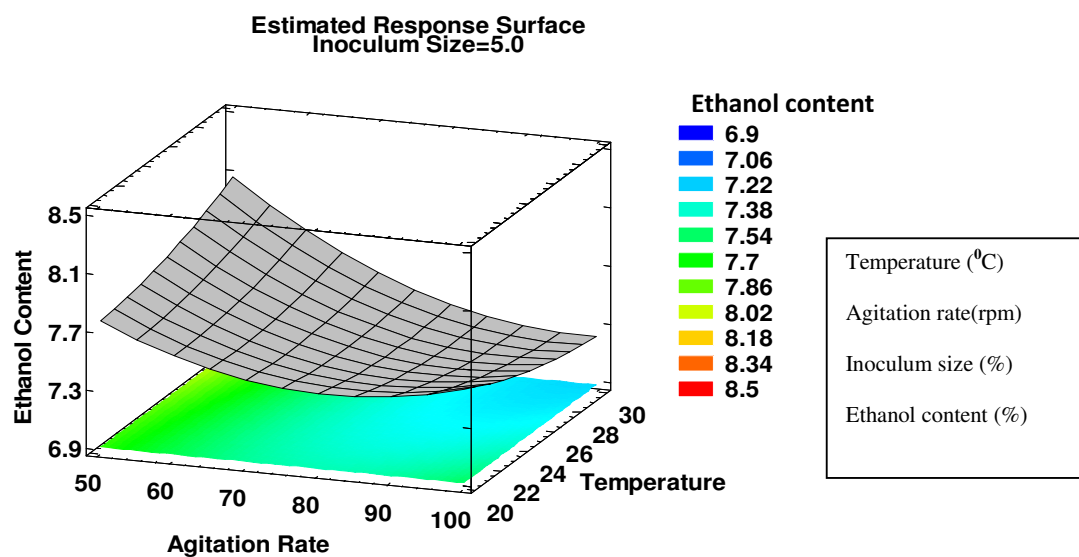


Fig 4.26b Surface plots for ethanol content (temperature vs. agitation rate)

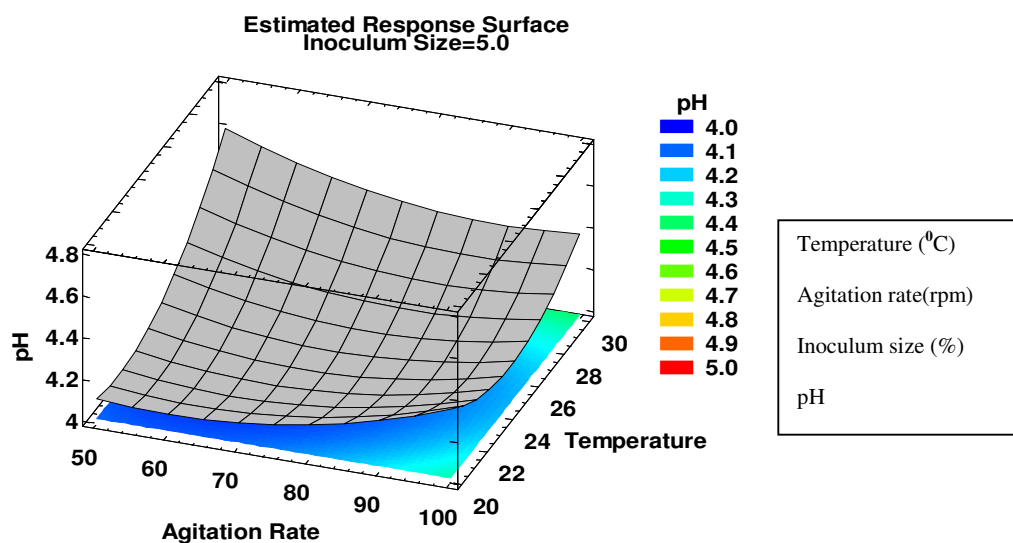


Fig 4.26c Surface plots for pH (temperature vs. agitation rate)

Fig 4.26 a, b and c Surface plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

4.8 Response Surface Methodology for Three Variable Interactions

Responses were optimized using software Statgraphics Centurion XVII.I. The optimum value for each dependent variable had different set of treatment conditions. The optimization was done with the target of maximum ethanol content and pH with minimum

total acidity. To consider all the responses simultaneously for optimization, surface plots were generated for each response. The most desired combinations were selected for maximum ethanol production from desirability surface plots (Fig4.27, 4.28, 4.29). To get maximum ethanol production, surface plots were overlaid with the targets mentioned above. Overlay plots describing the optimum conditions has shown in Fig 4.30, 4.31 and 4.32. Hence inoculum size of 5%, agitation rate of 50 rpm and fermentation temperature of 30 °C were found to be the best parameters for optimum responses i.e. total acidity, ethanol content and pH values in sweet sorghum juice inoculated with *Saccharomyces cerevisiae* strain-35.

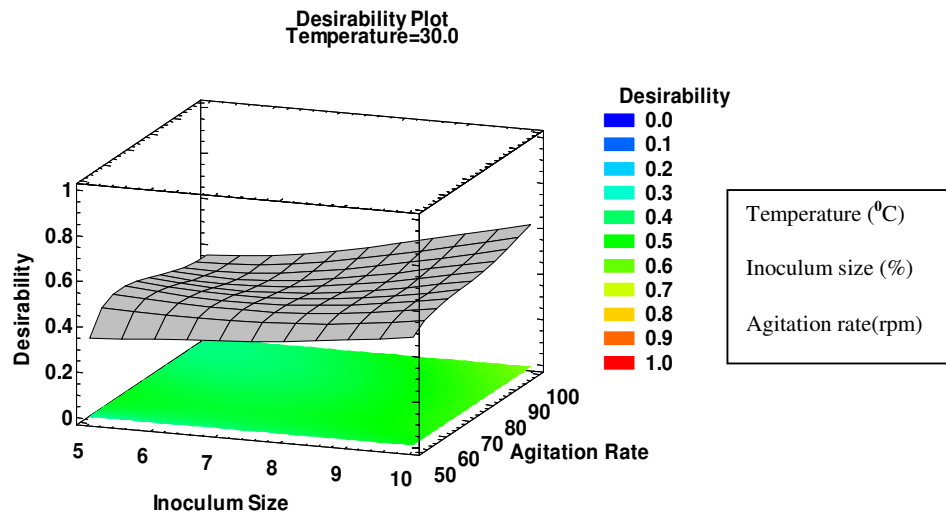


Fig4.27 Surface plot for desirability: Inoculum size vs. Agitation rate

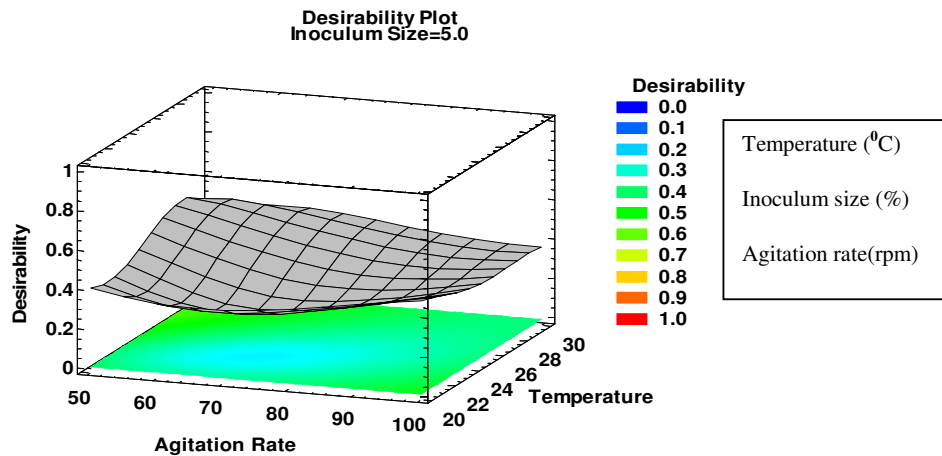


Fig 4.28 Surface plot for desirability: Temperature vs. Agitation rate

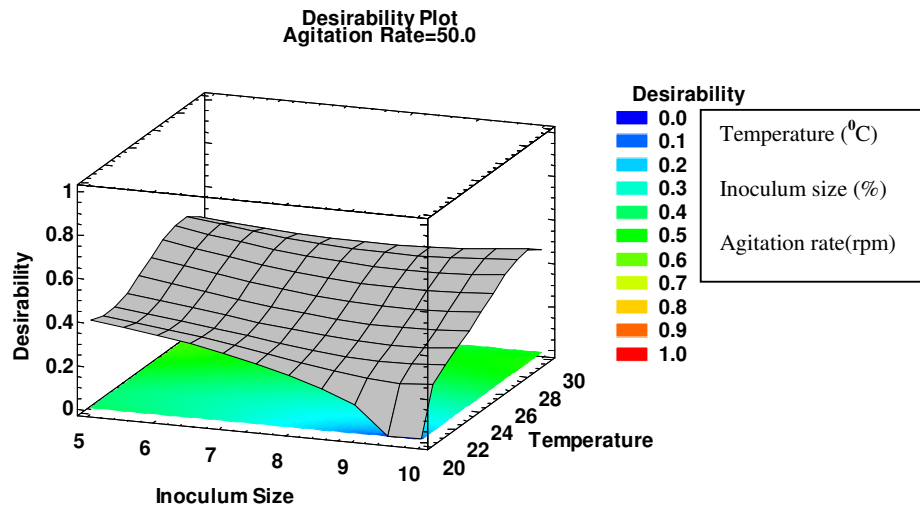


Fig 4.29 Surface plot for desirability: Inoculum size vs. Temperature

OVERLAY PLOTS:

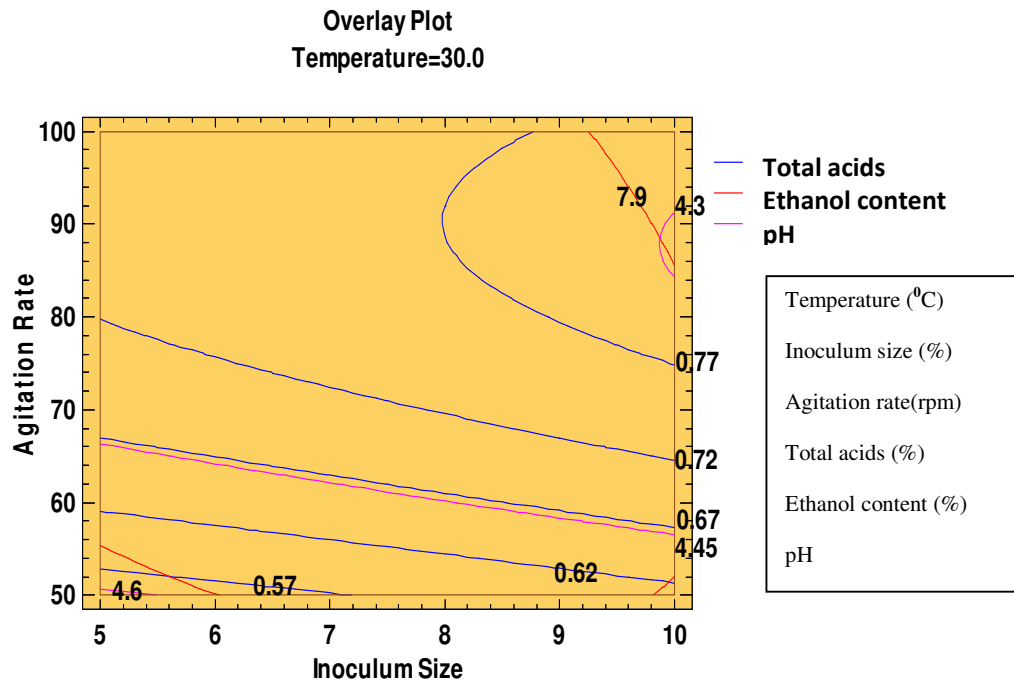


Fig 4.30 Agitation rate vs. inoculum size

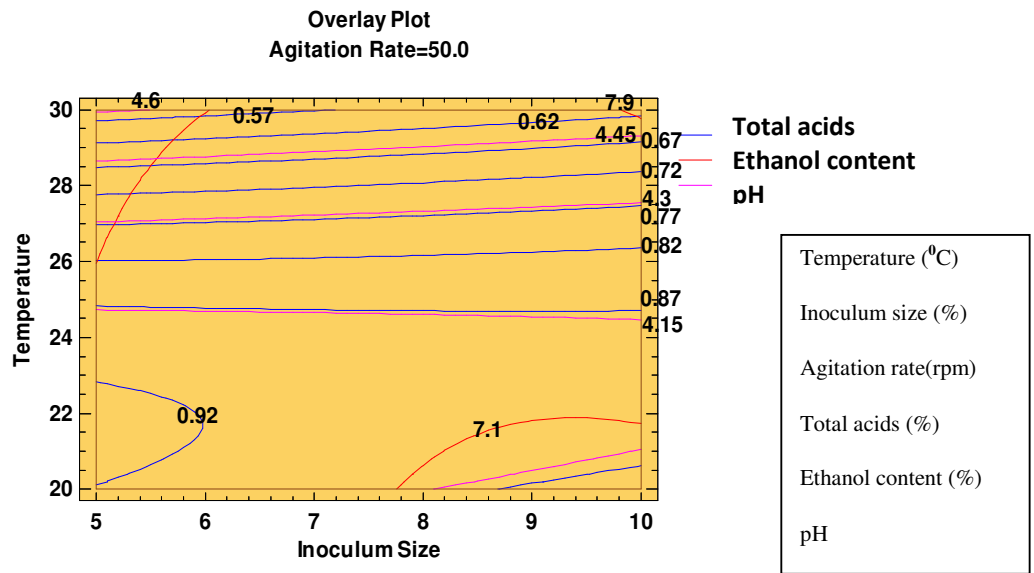


Fig 4.31 Temperature vs. Inoculum size

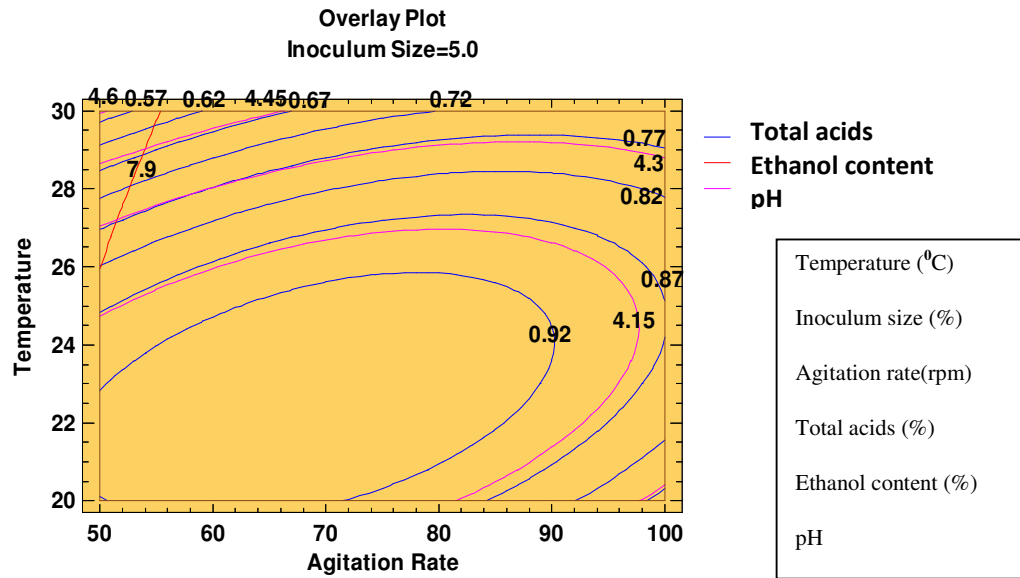


Fig 4.32 Temperature vs. Agitation rate

Table 4.26 Optimization of cultural parameters by Response Surface Methodology of sugarcane juice inoculated with NRRL Y-2034:

Sr. No.	Inoculum size (mg/l substrate)	Agitation rate (RPM)	Temperature (°C)	Total acidity %	pH	Ethanol (%)		Fermentation Efficiency(%)
						v/v	w/v	
1	7.5	50	30	0.36	4.96	8.00	6.4	80.64
2	10	50	30	0.34	5.01	8.38	6.70	84.47
3	5	50	30	0.37	4.97	9.39	7.15	94.65
4	10	50	25	0.34	5.01	8.45	6.76	85.18
5	5	50	25	0.36	4.96	8.48	6.78	85.48
6	5	100	30	0.52	4.62	10.12	8.09	97.82
7	7.5	100	30	0.43	4.70	9.84	7.87	96.09
8	10	100	30	0.38	4.98	9.55	7.64	96.27
9	7.5	75	30	0.89	4.08	8.40	6.72	84.67
10	10	75	30	0.86	4.20	8.35	6.68	84.17
11	10	75	25	0.72	4.20	8.78	7.02	88.50
12	5	75	25	0.81	4.25	8.94	7.15	90.12
13	7.5	75	25	0.56	4.58	8.54	6.83	86.08
14	5	75	20	0.79	4.13	8.04	6.43	81.04
15	7.5	75	20	0.79	4.13	8.28	6.62	83.46
16	10	75	20	0.83	4.23	8.19	6.55	82.56
17	7.5	50	20	0.34	5.01	9.25	7.40	93.24
18	5	50	20	0.33	5.02	9.05	7.24	91.22
19	7.5	100	25	0.58	4.59	8.98	7.18	89.91
20	10	100	25	0.49	4.64	8.85	7.08	89.21
21	5	100	20	0.53	4.60	9.01	7.20	90.82
22	10	100	20	0.37	4.97	8.87	7.09	89.41

Cultural conditions: Volume of sweet sorghum juice=50ml; Fermentation period =12 days

Adjusted Brix= 20°B; pH= 5.5

Average of triplicate data

Table 4.27 Statistical analysis of table 4.26 for fitting of model

Model	Total Acids	Ethanol Content	pH
Transformation	none	none	none
Model d.f.	9	9	9
P-value	0.0149	0.0038	0.0135
Error d.f.	12	12	12
Std. error	0.135002	0.331107	0.233329
R-squared	74.81	80.64	75.29
Adj. R-squared	55.91	66.11	56.75

4.9 Effect of cultural conditions on ethanol content

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.33). Table 4.28 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 80.6364% of the variability in Ethanol Content. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 66.1138%. The standard error of the estimate shows the standard deviation of the residuals to be 0.331107.

The regression equation of the fitted model for ethanol content is:

$$23.4298 + 0.259431*A - 0.237815*B - 0.629533*C - 0.00995652*A^2 + 0.00068*A*B - 0.00833333*A*C + 0.00118443*B^2 + 0.00264*B*C + 0.0102978*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.28 Estimated effects for Ethanol Content (%)

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	8.10957	0.228982	
A:Inoculum Size	-0.23625	0.165553	1.0
B:Agitation Rate	0.5475	0.165553	1.0
C:Temperature+block	0.208587	0.161914	1.01359
AA+block	-0.124457	0.320128	1.01976
AB	0.085	0.191165	1.0
AC	-0.208333	0.191165	1.0
BB+block	1.48054	0.320128	1.01976
BC+block	0.66	0.191165	1.0
CC+block	0.514891	0.339401	1.0149

Standard errors are based on total error with 12 d.f.

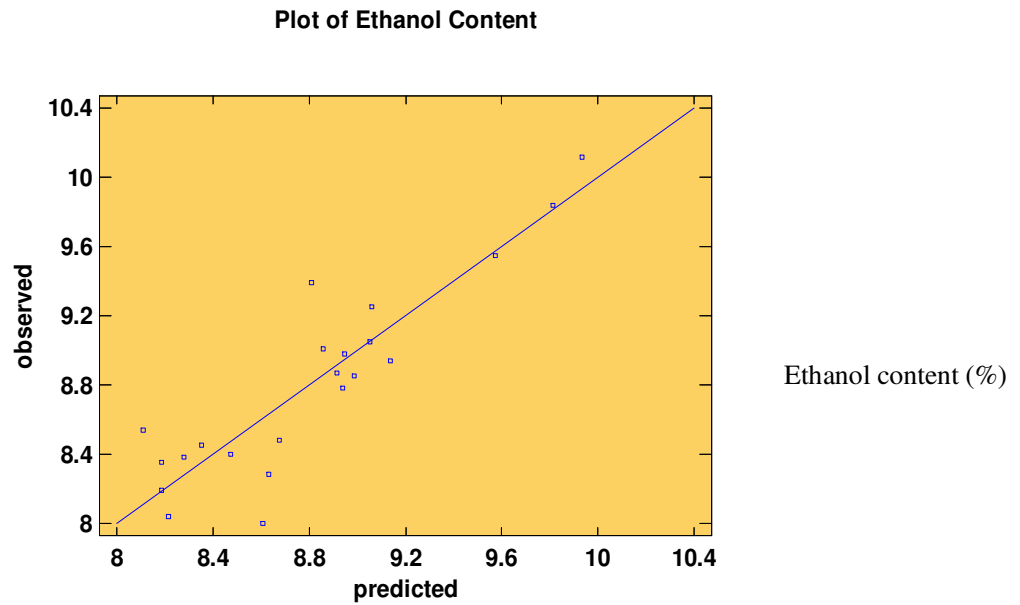


Fig 4.33 Experimental vs predicted results of ethanol production

Table 4.29 Analysis of Variance for Ethanol Content

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.223256	1	0.223256	2.04	0.1791
B:Agitation Rate	1.19902	1	1.19902	10.94	0.0063
C:Temperature+block	0.181945	1	0.181945	1.66	0.2219
AA+block	0.0165701	1	0.0165701	0.15	0.7043
AB	0.021675	1	0.021675	0.20	0.6645
AC	0.130208	1	0.130208	1.19	0.2972
BB+block	2.34494	1	2.34494	21.39	0.0006
BC+block	1.3068	1	1.3068	11.92	0.0048
CC+block	0.252314	1	0.252314	2.30	0.1551
Total error	1.31558	12	0.109632		
Total (corr.)	6.79411	21			

Table 4.30 Regression coefficients for Ethanol Content

Coefficient	Estimate
constant	23.4298
A:Inoculum Size	0.259431
B:Agitation Rate	-0.237815
C:Temperature	-0.629533
AA	-0.00995652
AB	0.00068
AC	-0.00833333
BB	0.00118443
BC	0.00264
CC	0.0102978

4.9.1 Effect of cultural conditions on total acids

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.34). Table 4.31 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a

perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 74.8079% of the variability in Total Acids. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 55.9139%. The standard error of the estimate shows the standard deviation of the residuals to be 0.135002. The mean absolute error (MAE) of 0.0817021 is the average value of the residuals.

The regression equation of the fitted model for total acids is:

$$-2.44226 + 0.0291558*A + 0.0903156*B - 0.0272464*C + 0.00828406*A^2 - 0.00128*B*B - 0.00246667*A *C - 0.000541159* B^2 + 0.000113333*B *C + 0.000672464*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.31 Estimated effects for Total Acids (%)

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	0.938986	0.0624907	
A:Inoculum Size	0.00125	0.0451807	1.0
B:Agitation Rate	0.00875	0.0451807	1.0
C:Temperature+block	-0.140797	0.0441876	1.01359
AA+block	0.00376812	0.0873651	1.01976
AB	0.00166667	0.0521701	1.0
AC	0.0633333	0.0521701	1.0
BB+block	-0.146232	0.0873651	1.01976
BC+block	0.17	0.0521701	1.0
CC+block	-0.26442	0.0926248	1.0149

Standard errors are based on total error with 12 d.f.

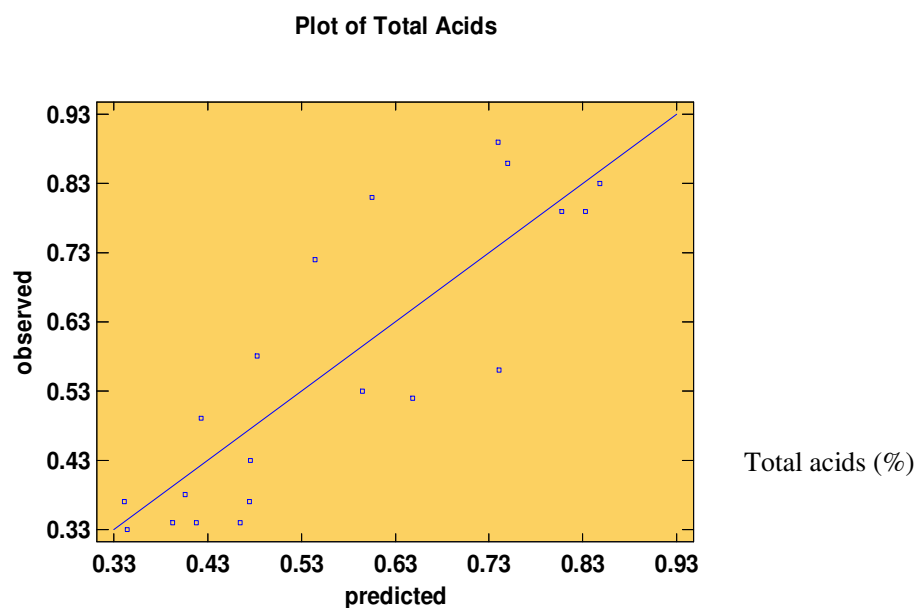


Fig 4.34 Experimental vs predicted results of total acids

Table 4.32 Analysis of Variance for Total Acids

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.00180625	1	0.00180625	0.10	0.7583
B:Agitation Rate	0.0564062	1	0.0564062	3.09	0.1040
C:Temperature+block	0.00548968	1	0.00548968	0.30	0.5932
AA+block	0.0114709	1	0.0114709	0.63	0.4430
AB	0.0768	1	0.0768	4.21	0.0626
AC	0.0114083	1	0.0114083	0.63	0.4442
BB+block	0.489508	1	0.489508	26.86	0.0002
BC+block	0.00240833	1	0.00240833	0.13	0.7225
CC+block	0.00107594	1	0.00107594	0.06	0.8121
Total error	0.218705	12	0.0182254		
Total (corr.)	0.86815	21			

Table 4.33 Regression coefficients for Total Acids

Coefficient	Estimate
constant	-2.44226
A:Inoculum Size	0.0291558
B:Agitation Rate	0.0903156
C:Temperature	-0.0272464
AA	0.00828406
AB	-0.00128
AC	-0.00246667
BB	-0.000541159
BC	0.000113333
CC	0.000672464

4.9.2 Effect of cultural conditions on pH:

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.35). Table 4.34 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 75.2871% of the variability in pH. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 56.7524%. The standard error of the estimate shows the standard deviation of the residuals to be 0.233329.

The regression equation of the fitted model for total acids is:

$$9.62733 - 0.123014*A - 0.14921*B + 0.0598659*C - 0.0123101*A^2 + 0.00246667*A *B + 0.00546667*A *C + 0.000904899*B^2 - 0.00038*B *C - 0.00129783*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.34 Estimated effects for pH

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	4.2971	0.161362	
A:Inoculum Size	0.07	0.116664	1.0
B:Agitation Rate	-0.22375	0.116664	1.0
C:Temperature+block	0.0747464	0.1141	1.01359
AA+block	-0.153877	0.225592	1.01976
AB	0.308333	0.134712	1.0
AC	0.136667	0.134712	1.0
BB+block	1.13112	0.225592	1.01976
BC+block	-0.095	0.134712	1.0
CC+block	-0.0648913	0.239173	1.0149

Standard errors are based on total error with 12 d.f.

Plot of pH

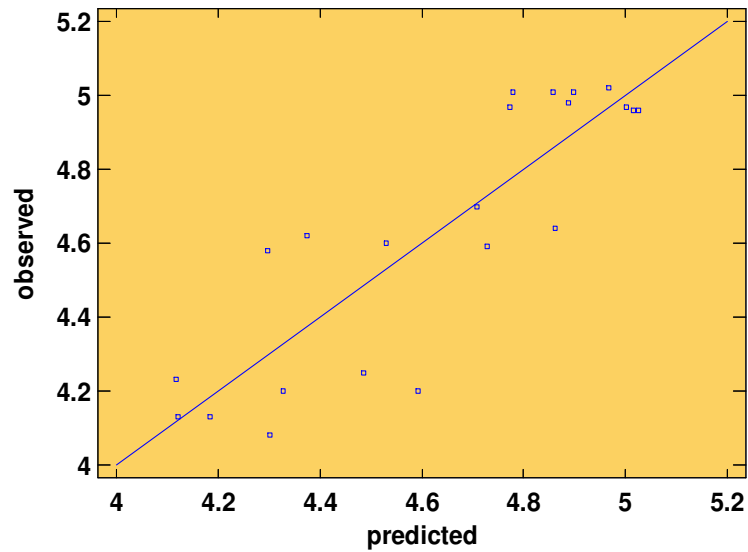


Fig 4.35 Experimental vs predicted results of pH

Table 4.35 Analysis of Variance for pH

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.0196	1	0.0196	0.36	0.5597
B:Agitation Rate	0.200256	1	0.200256	3.68	0.0792
C:Temperature+block	0.0233639	1	0.0233639	0.43	0.5248
AA+block	0.02533	1	0.02533	0.47	0.5081
AB	0.285208	1	0.285208	5.24	0.0410
AC	0.0560333	1	0.0560333	1.03	0.3304
BB+block	1.3687	1	1.3687	25.14	0.0003
BC+block	0.027075	1	0.027075	0.50	0.4941
CC+block	0.0040076	1	0.0040076	0.07	0.7908
Total error	0.653307	12	0.0544422		
Total (corr.)	2.64358	21			

Table 4.36 Regression coefficients for pH

Coefficient	Estimate
constant	9.62733
A:Inoculum Size	-0.123014
B:Agitation Rate	-0.14921
C:Temperature	0.0598659
AA	-0.0123101
AB	0.00246667
AC	0.00546667
BB	0.000904899
BC	-0.00038
CC	-0.00129783

4.10 RESPONSE SURFACE METHODOLOGY: TWO VARIABLE INTERACTION STUDIES

The surface plots for different interaction of any two independent variables, while holding the third variable constant, on enzyme production were generated using software, Statgraphics Centurion XVI.I. The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable in order to deduce the optimum conditions.

4.10.1 Effect of agitation rate and inoculum size on total acids, ethanol content and pH

A direct correlation was found between inoculum size and agitation rate and graphs were plotted to understand their effect on ethanol production. The response of experimental levels of each variable was examined and concluded that with the variation in agitation rate

from 50, 75 and 100 rpm and inoculums size from 5, 7.5 and 10%, the ethanol content, total acids and pH varied in the range of 0.32-0.5 %, ethanol content in range of 9.72-10.2 % and pH in range of 4.9-5.1 respectively as shown in Figure 4.36a, 4.36b, 4.36c, 4.37a, 4.37b and 4.37c.

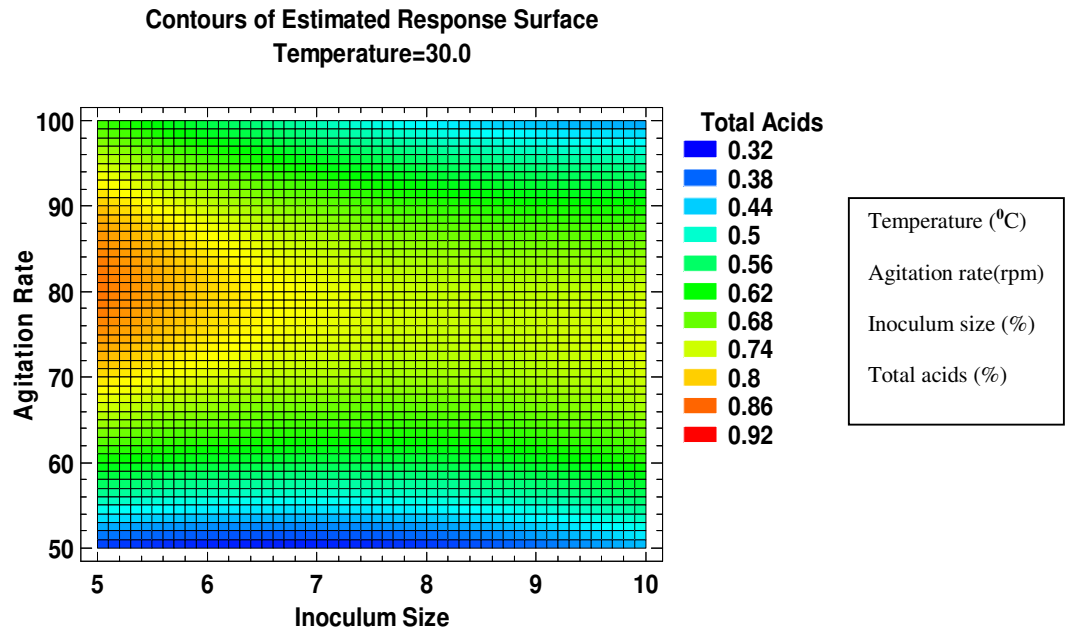


Fig 4.36a Contour plots for total acids (agitation rate vs. inoculum size)

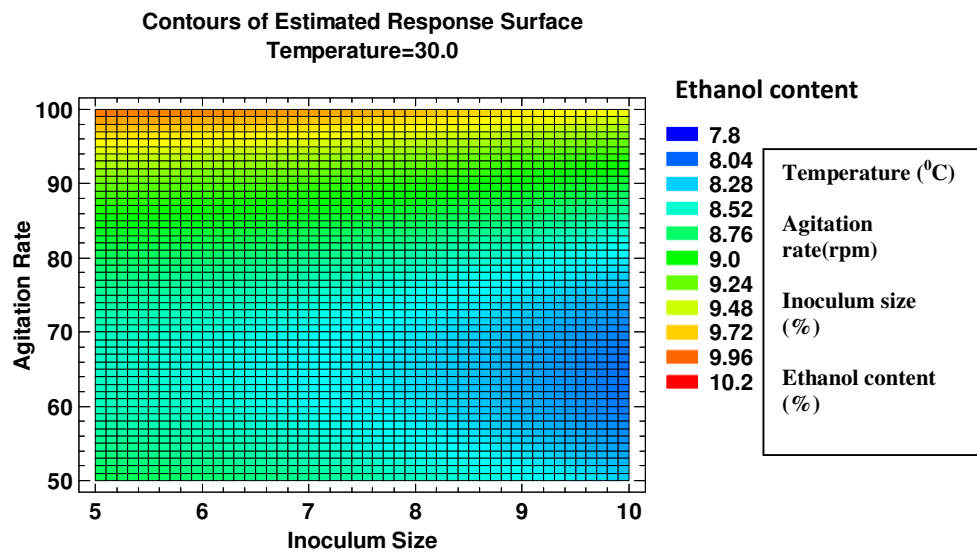


Fig4.36b Contour plots for ethanol content (agitation rate vs. inoculum size)

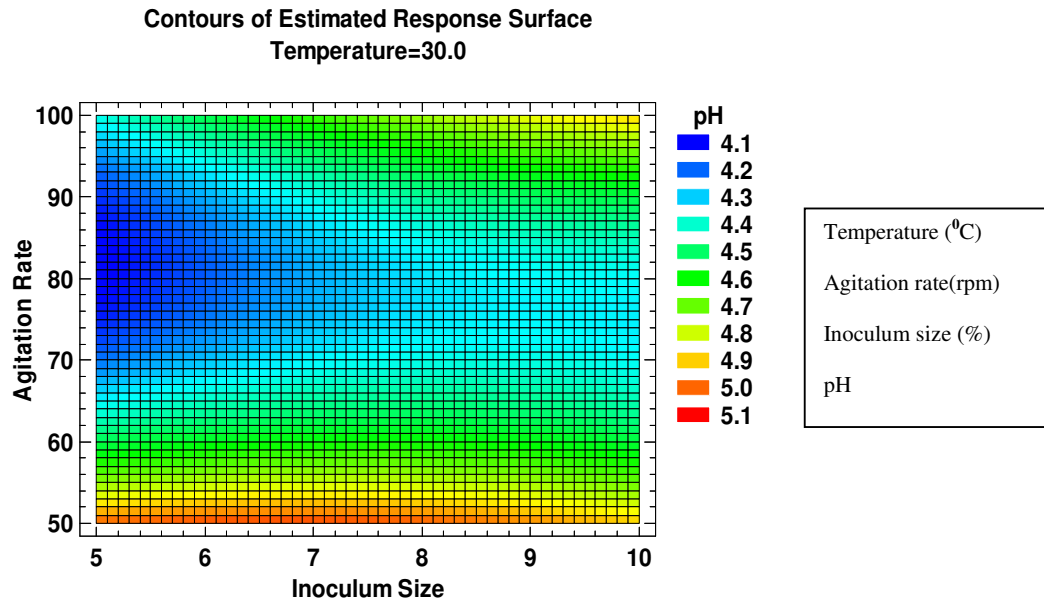


Fig4.36c Contour plots for pH (agitation rate vs. inoculum size)

Fig4.36 a, b and c Contour plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

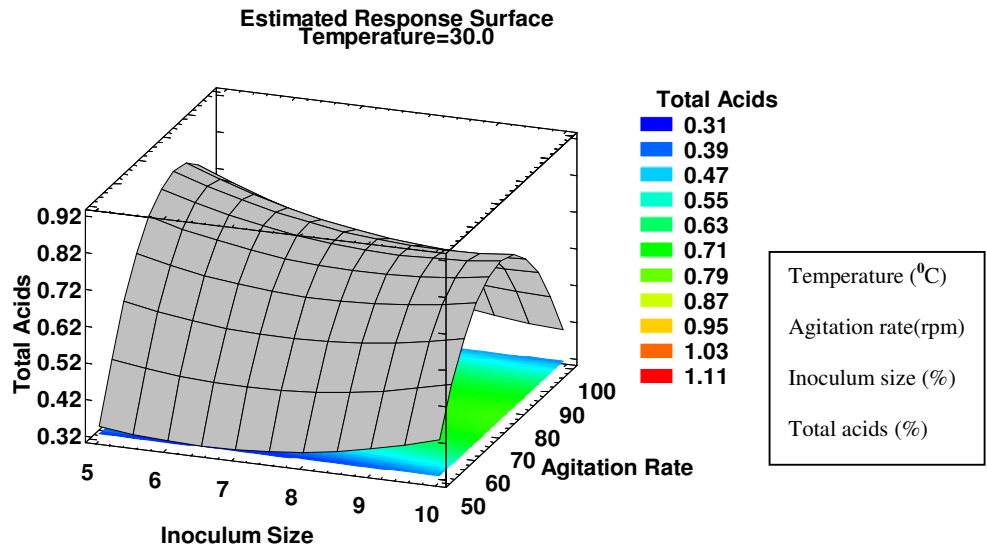


Fig4.37a Surface plots for total acids (agitation rate vs. inoculum size)

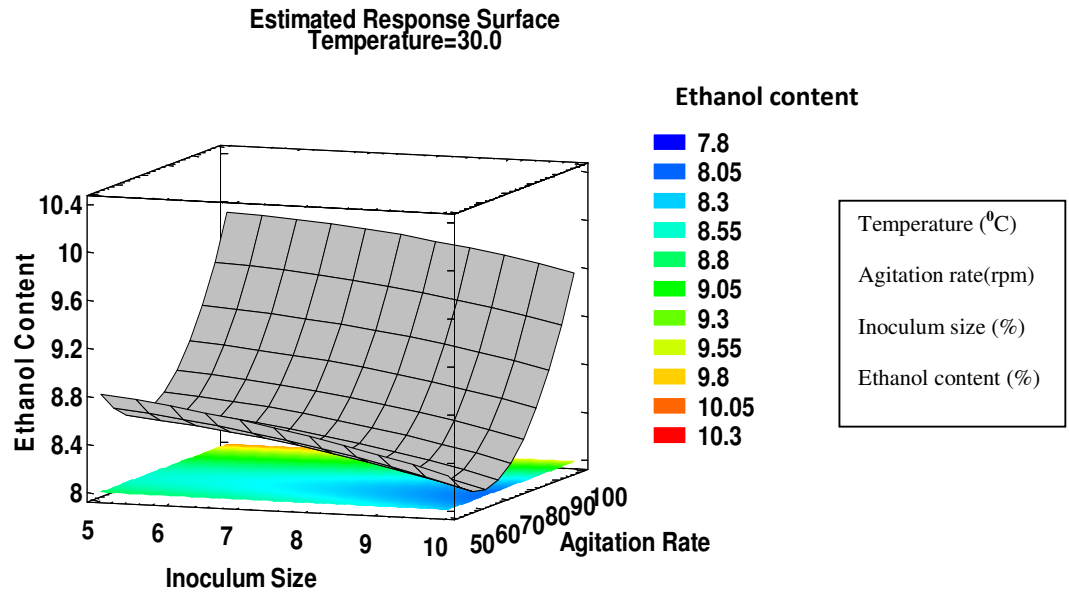


Fig 4.37b Surface plots for ethanol content (agitation rate vs. inoculum size)

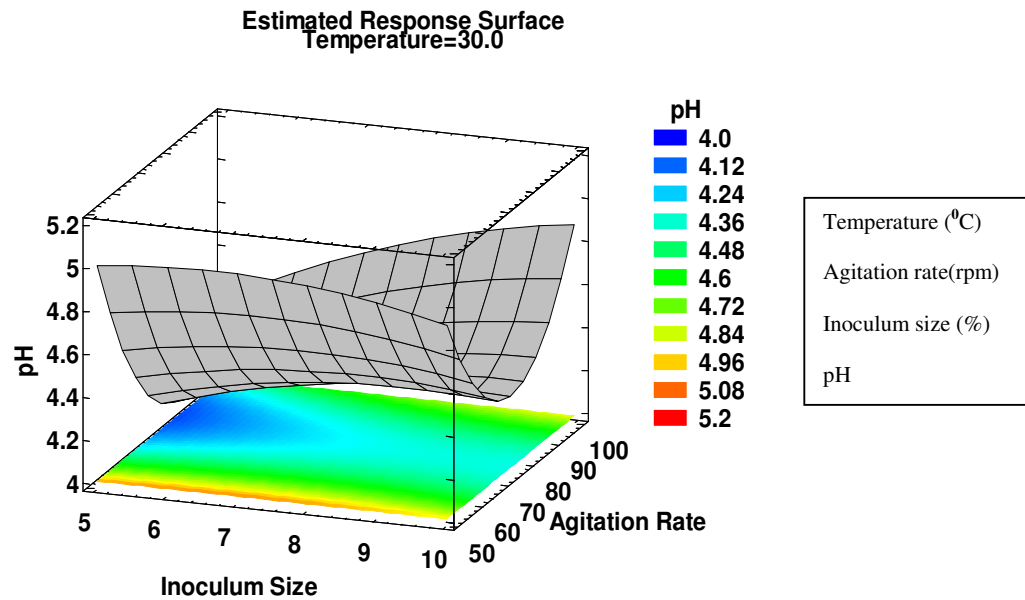


Fig 4.37c Surface plots for pH (agitation rate vs. inoculum size)

Fig4.37 a, b and c Surface plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

4.10.2 Effect of inoculum size and temperature on total acids, ethanol content and pH

Figure 4.38a, 4.38b and 4.38c showed the contour plots, 4.39a, 4.39b and 4.39c showed the surface plots of inoculum size v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25 30°C resulted in variation in total acids of the range of 0.32-0.5 % and variation in ethanol content from 9.72-10.2 %. The pH was found to be in the range of 4.9-5.1.

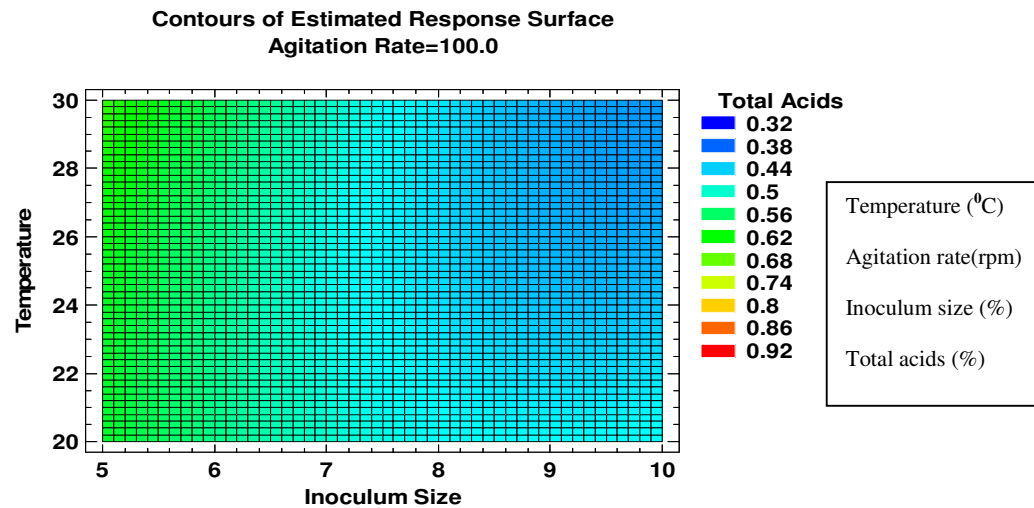


Fig 4.38a Contour plots for total acids (temperature vs. inoculum size)

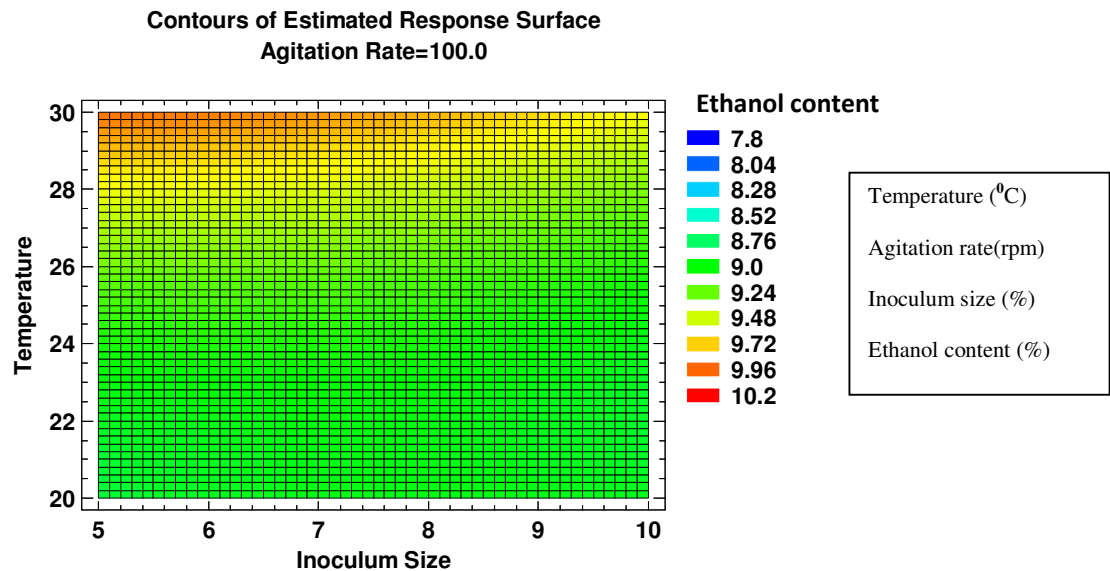


Fig 4.38b Contour plots for ethanol content (temperature vs. inoculum size)

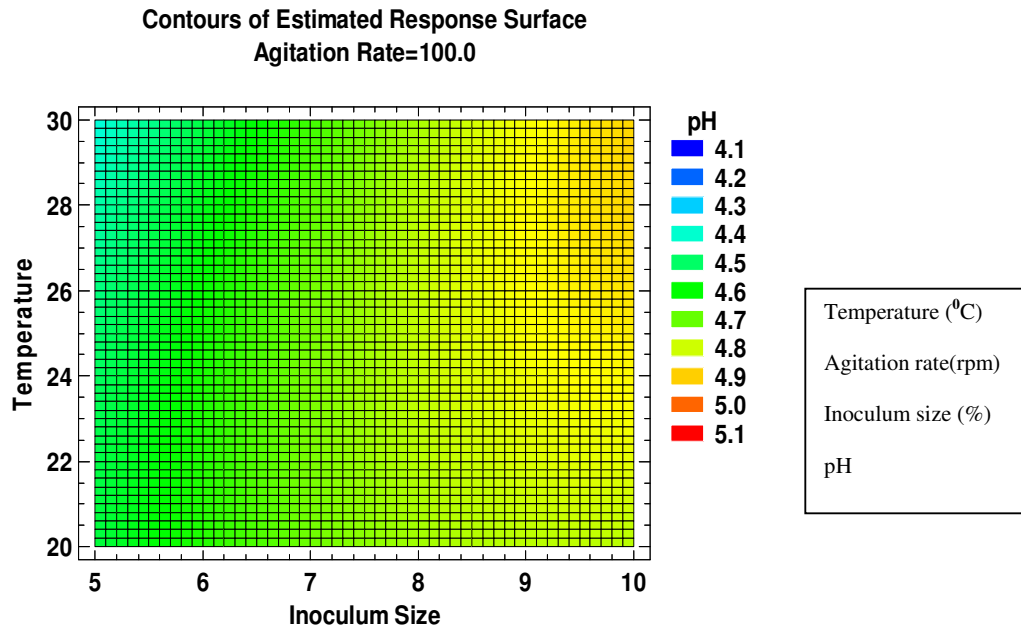


Fig 4.38c Contour plots for pH (temperature vs. inoculum size)

Fig4.38 a, b and c Contour plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

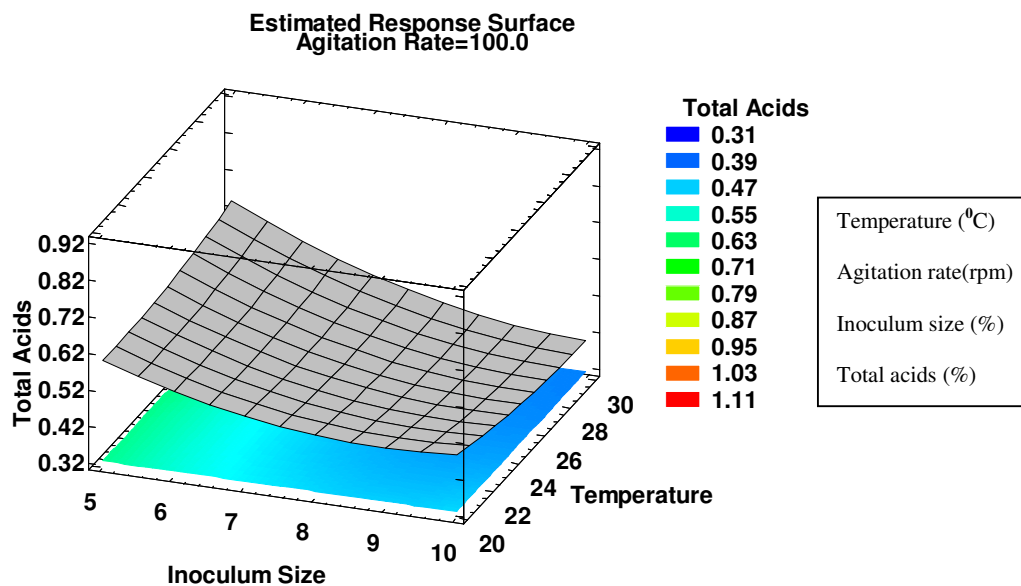


Fig 4.39a Surface plots for total acids (temperature vs. inoculum size)

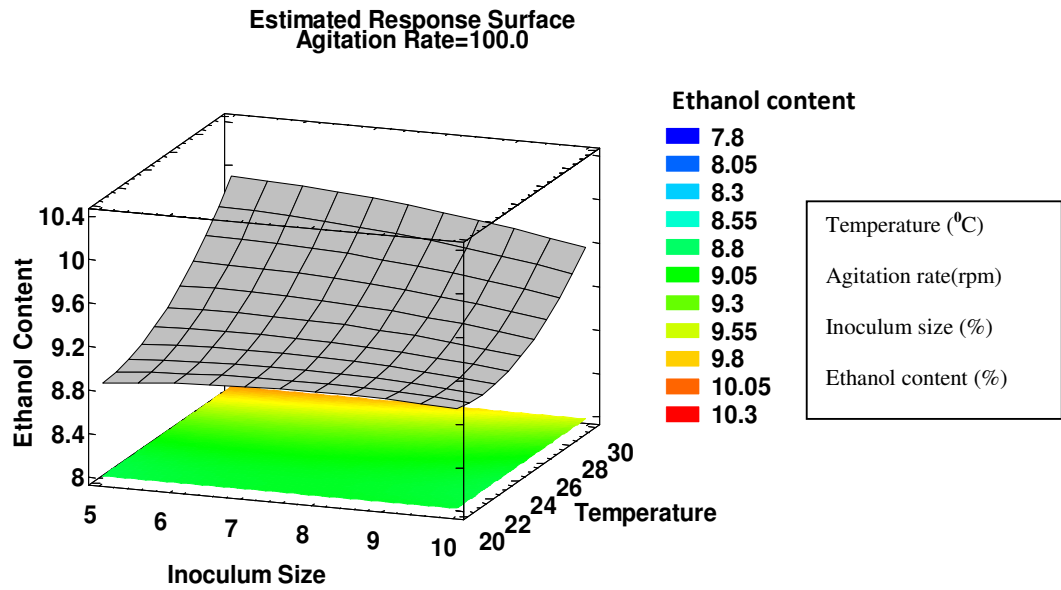


Fig4.39b Surface plots for ethanol content (temperature vs. inoculum size)

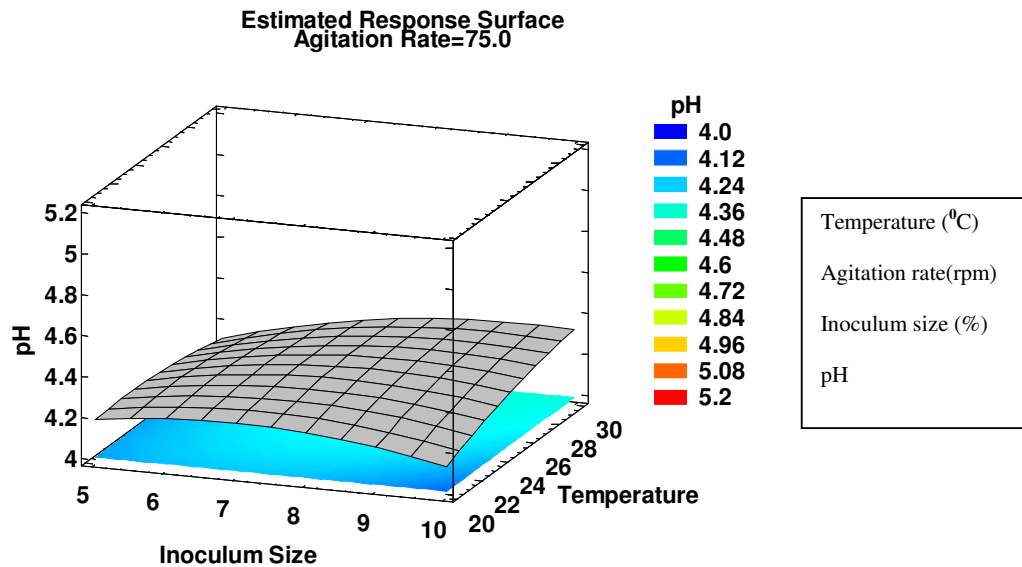


Fig4.39c Surface plots for pH (temperature vs. inoculum size)

Fig 4.39 a, b and c Surface plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

4.10.3 Effect of Agitation rate and temperature on total acids, ethanol content and pH

Figure 4.40a, 4.40b and 4.40c showed the contour plots, 4.41a, 4.41b and 4.41c showed the surface plots of agitation rate v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25 30°C resulted in variation of total acids in the range of 0.32-0.5 % and ethanol content from 9.72-10.2 %. The pH was found to be in the range of 4.9-5.1.

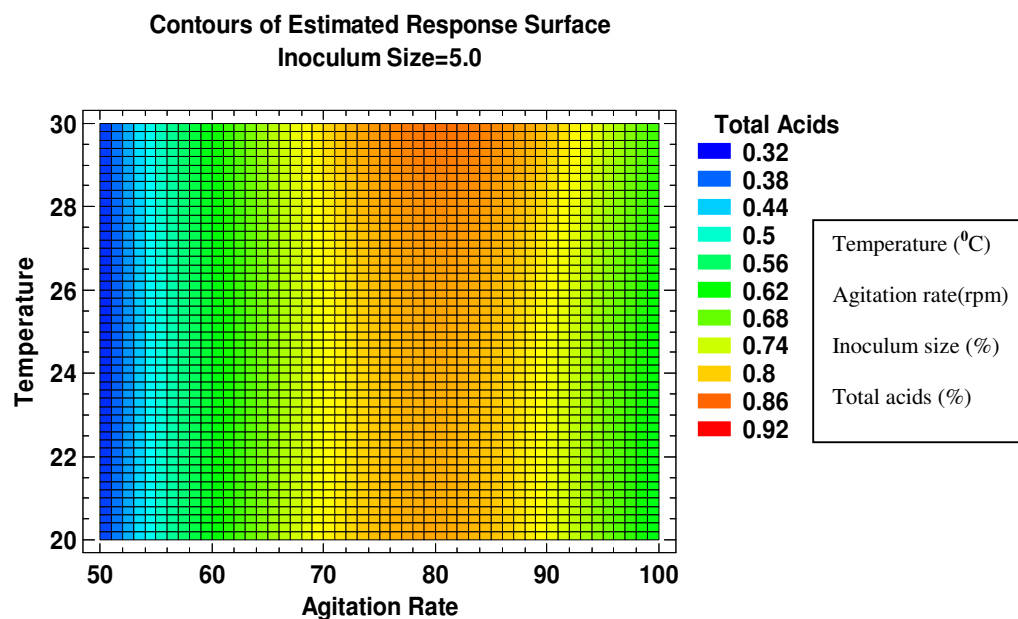


Fig 4.40a Contour plots for total acids (temperature vs. agitation rate)

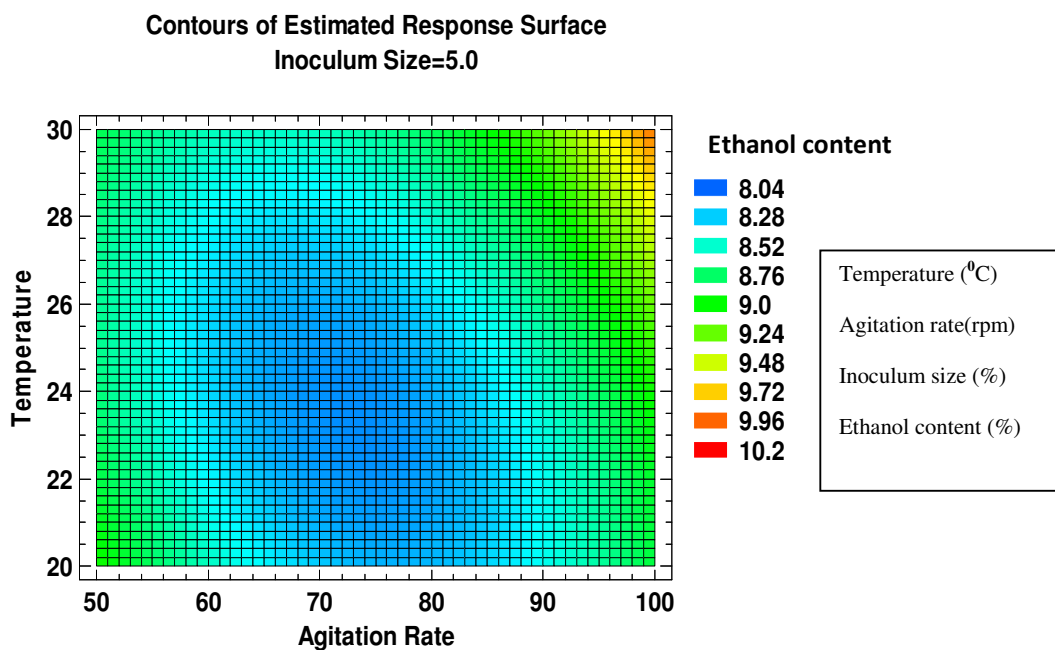


Fig4.40b Contour plots for ethanol content (temperature vs. agitation rate)

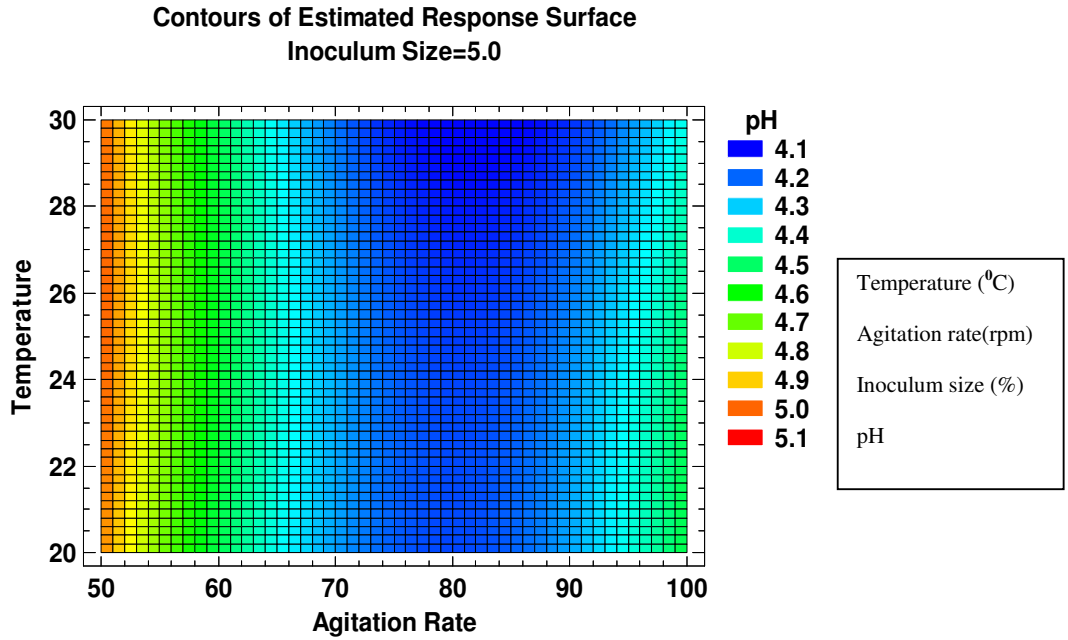


Fig4.40c Contour plots for pH(temperature vs. agitation rate)

Fig4.40a, b and c: Contour plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

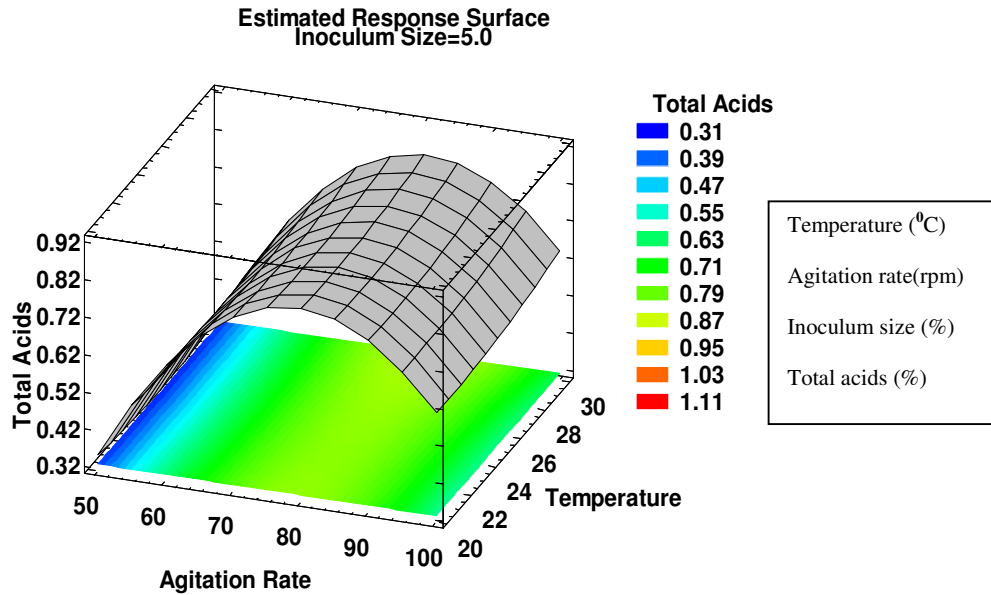


Fig 4.41a Surface plots for total acids (temperature vs. agitation rate)

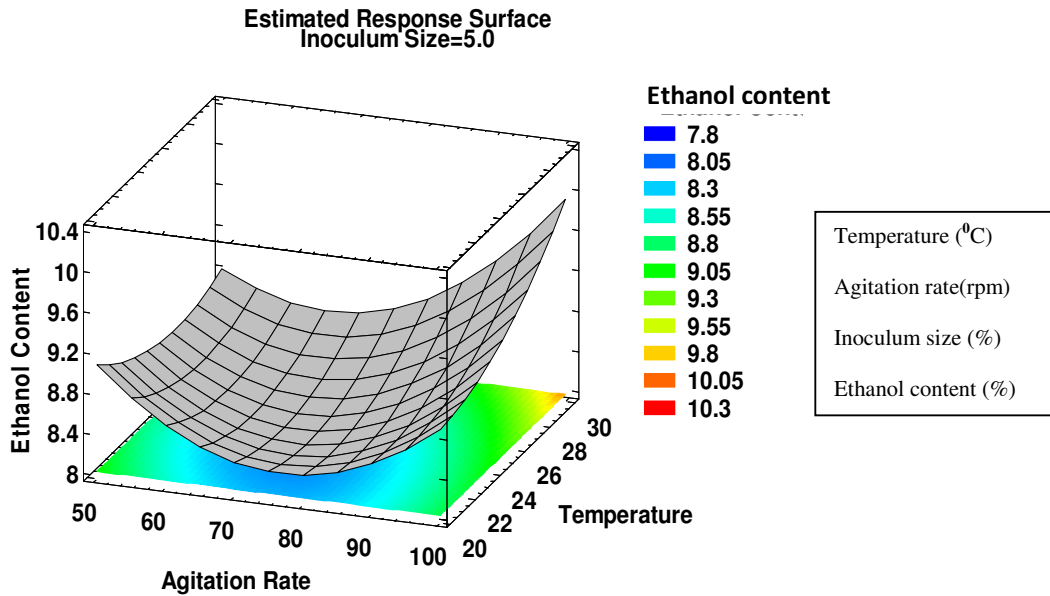


Fig4.41b Surface plots for ethanol content (temperature vs. agitation rate)

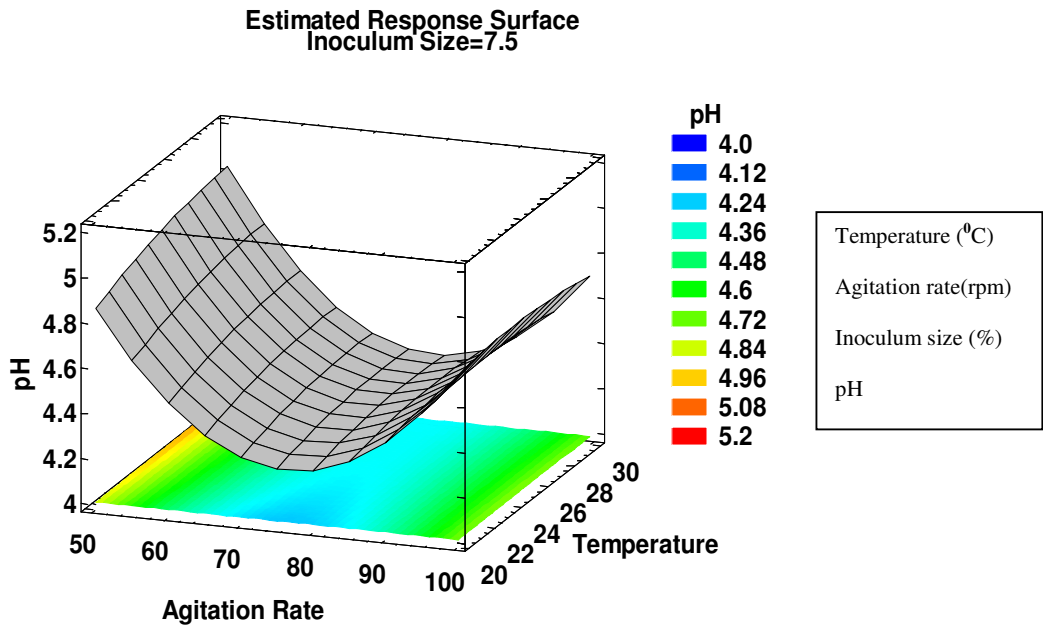


Fig4.41c Surface plots for pH (temperature vs. agitation rate)

Fig4.41 a, b and c Surface plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

4.11 Response Surface Methodology for Three Variable Interactions

Responses were optimized using software Statgraphics Centurion XVII.I. The optimum value for each dependent variable had different set of treatment conditions. The optimization was done with the target of maximum ethanol content and pH with minimum total acidity. To consider all the responses simultaneously for optimization, surface plots were generated for each response. The most desired combinations were selected for maximum ethanol production from desirability surface plots (Fig4.42, 4.43, 4.44). To get maximum ethanol production, surface plots were overlaid with the targets mentioned above. Overlay plots describing the optimum conditions has shown in Fig. 4.45, 4.46 and 4.47. Hence inoculum size of 5%, agitation rate of 50 rpm and fermentation temperature of 30 °C were found to be the best parameters for optimum responses i.e. total acidity, ethanol content and pH values in sugarcane juice inoculated with strain *Saccharomyces cerevisiae* NRRL Y-2034.

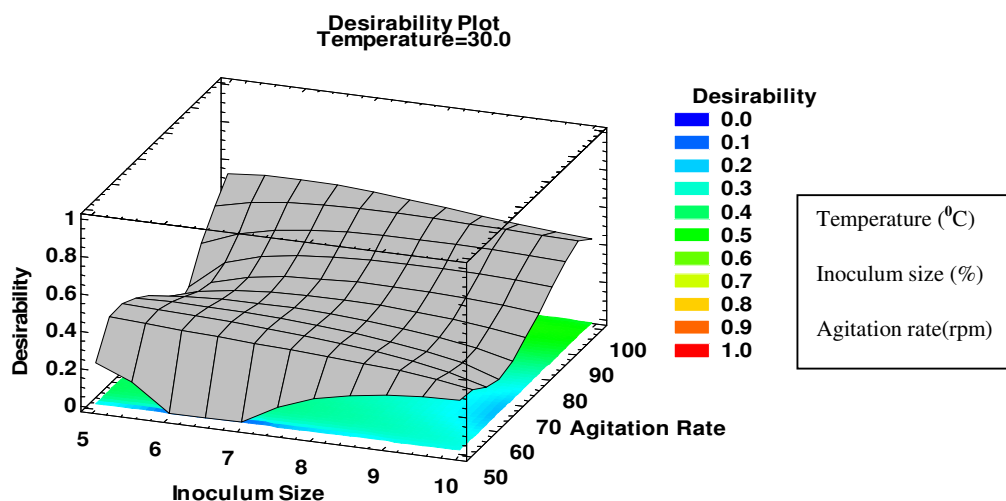


Fig 4.42 Surface plot for desirability: Inoculum size vs. Agitation rate

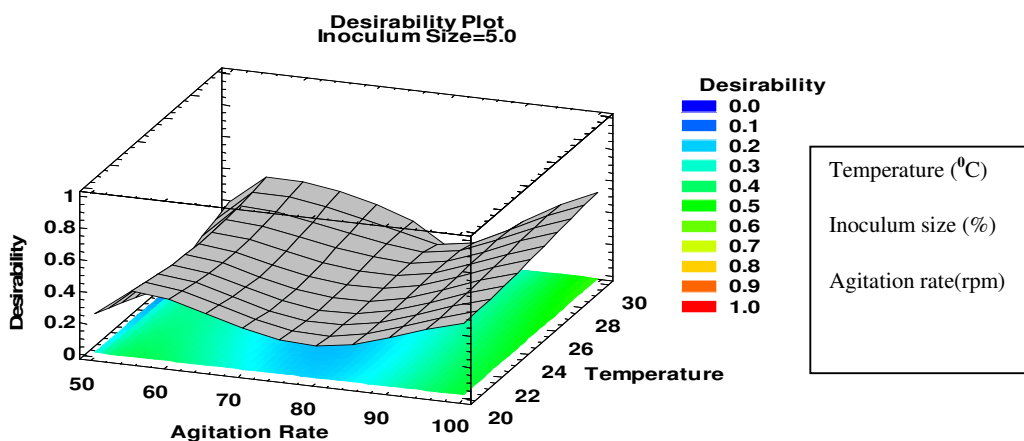


Fig4.43 Surface plot for desirability: Temperature vs. Agitation rate

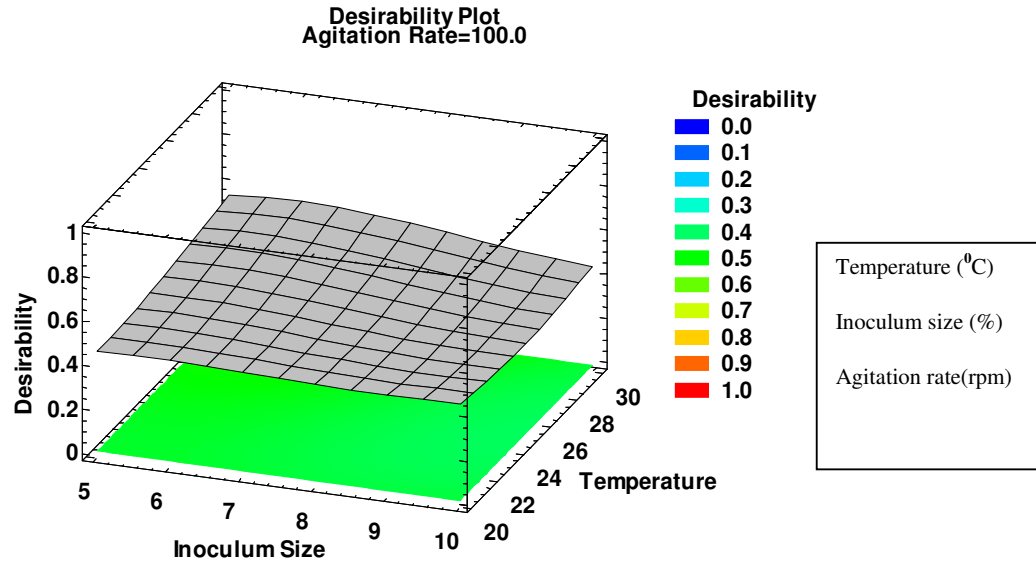


Fig4.44 Surface plot for desirability: Inoculum size vs. Temperature

OVERLAY PLOTS:

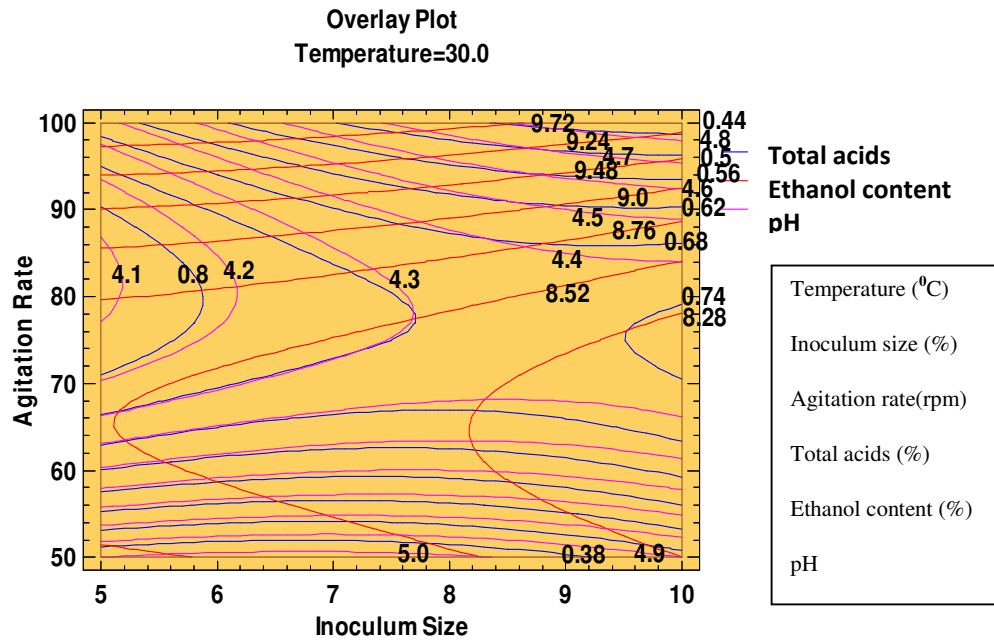


Fig4.45 Agitation rate vs. inoculum size

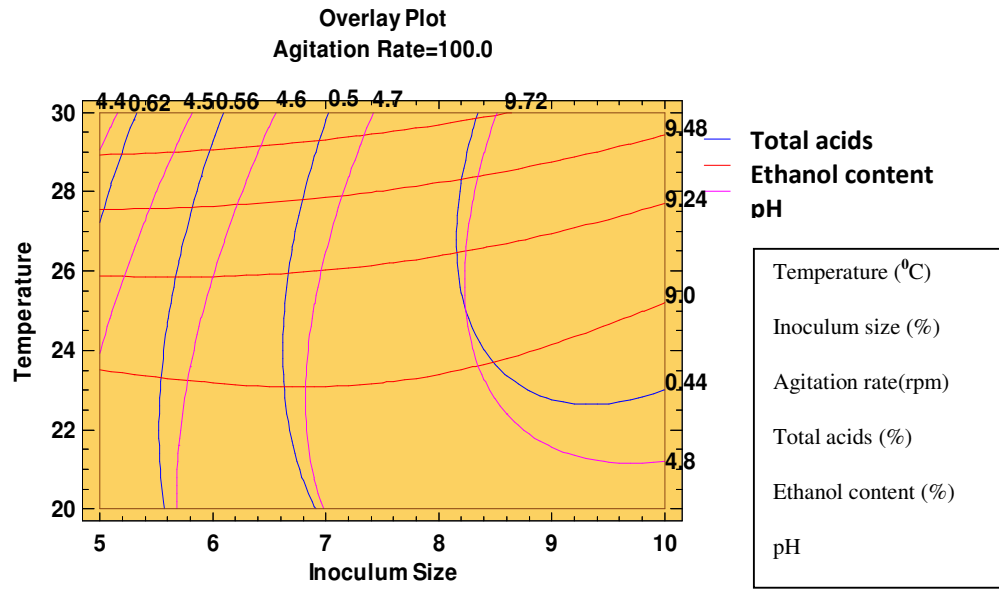


Fig4.46 Temperature vs. Inoculum size

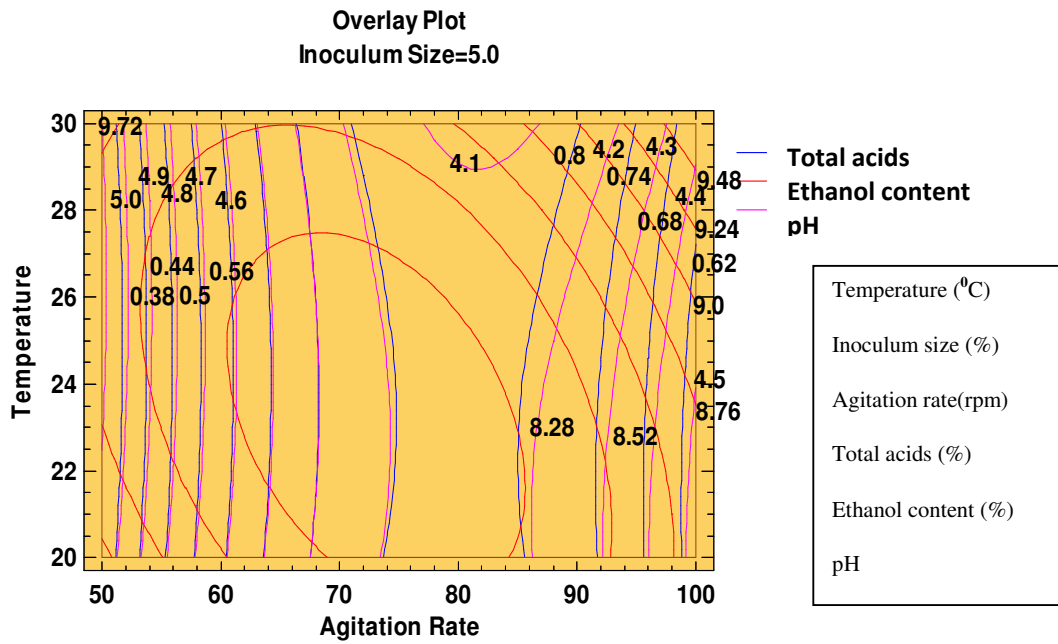


Fig4.47 Temperature vs. Agitation rate

Table 4.37 Optimization of cultural parameters by Response Surface Methodology of sugarcane juice inoculated with strain-35

Sr. No.	Inoculum size (mg/l substrate)	Agitation rate (RPM)	Temperature (°C)	Total acidity (%)	pH	%Ethanol		Fermentation Efficiency(%)
						v/v	w/v	
1	7.5	50	30	0.48	4.42	9.39	7.51	86.30
2	10	50	30	0.49	4.43	8.89	7.11	81.71
3	5	50	30	0.49	4.43	9.01	7.20	82.81
4	10	50	25	0.67	4.07	8.85	7.08	81.34
5	5	50	25	0.67	4.07	8.92	7.13	81.98
6	5	100	30	0.43	4.49	9.69	7.75	89.06
7	7.5	100	30	0.41	4.51	11.27	9.01	95.75
8	10	100	30	0.41	4.51	9.98	7.98	91.72
9	7.5	75	30	0.59	4.58	9.21	7.36	84.65
10	10	75	30	0.59	4.58	9.17	7.33	84.28
11	10	50	20	0.93	3.82	9.23	7.38	84.83
12	5	100	25	0.63	4.29	9.05	7.24	83.18
13	7.5	75	25	0.61	4.34	8.95	7.16	82.26
14	5	75	20	0.66	4.30	7.79	6.37	73.25
15	5	75	30	0.67	4.07	8.40	6.72	77.20
16	10	75	20	0.67	4.07	8.56	6.84	78.67
17	7.5	50	20	0.64	4.32	8.41	6.72	77.29
18	5	50	20	0.62	4.33	8.66	6.92	79.59
19	7.5	100	20	0.62	4.33	10.24	8.19	94.11
20	10	100	25	0.61	4.34	9.96	7.96	91.54
21	5	100	20	0.54	4.62	9.89	7.61	90.90
22	10	100	20	0.56	4.60	9.35	7.48	85.93

Cultural conditions: Volume of sweet sorghum juice=50ml; Fermentation period =12 days

Adjusted Brix= 20°B; pH= 5.5

Average of triplicate data

Table 4.38 Statistical analysis of table 4.37 for fitting of model

Model	Total Acids	Ethanol Content	pH
Transformation	none	none	none
Model d.f.	9	9	9
P-value	0.0149	0.0063	0.0327
Error d.f.	12	12	12
Std. error	0.0765206	0.455587	0.151337
R-squared	74.79	78.69	70.47
Adj. R-squared	55.88	62.72	48.32

4.12 Effect of cultural conditions on ethanol content

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.48). Table 4.39 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 78.6948% of the variability in Ethanol Content. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 62.7159%. The standard error of the estimate shows the standard deviation of the residuals to be 0.455587. The mean absolute error (MAE) of 0.273799 is the average value of the residuals.

The regression equation of the fitted model for ethanol content is:

$$12.4802 + 1.24399*A - 0.190776*B - 0.213692*C - 0.0820551*A^2 + 0.000373333*A*B + 0.000933333*A*C + 0.00133545*B^2 + 0.000313333*B*C + 0.00457609*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.39 Estimated effects for Ethanol Content (%)

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	8.88855	0.315068	
A:Inoculum Size	0.3225	0.227794	1.0
B:Agitation Rate	1.00875	0.227794	1.0
C:Temperature+block	0.456123	0.222787	1.01359
AA+block	-1.02569	0.440481	1.01976
AB	0.0466667	0.263033	1.0
AC	0.0233333	0.263033	1.0
BB+block	1.66931	0.440481	1.01976
BC+block	0.0783333	0.263033	1.0
CC+block	0.228804	0.466999	1.0149

Standard errors are based on total error with 12 d.f.

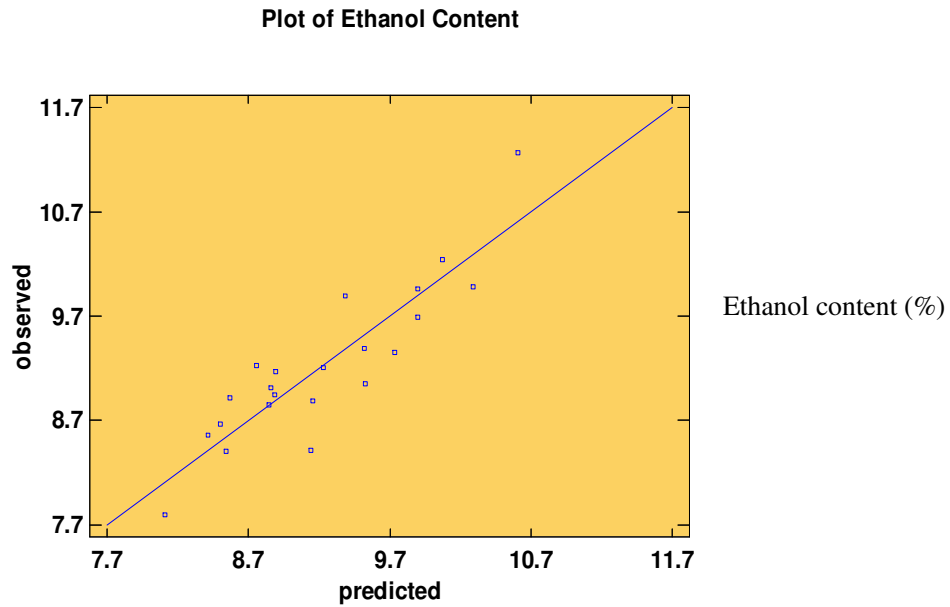


Fig4.48 Experimental vs predicted results of ethanol production

Table 4.40 Analysis of Variance for Ethanol Content

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.416025	1	0.416025	2.00	0.1823
B:Agitation Rate	4.07031	1	4.07031	19.61	0.0008
C:Temperature+block	0.87002	1	0.87002	4.19	0.0632
AA+block	1.12543	1	1.12543	5.42	0.0382
AB	0.00653333	1	0.00653333	0.03	0.8621
AC	0.00163333	1	0.00163333	0.01	0.9308
BB+block	2.98102	1	2.98102	14.36	0.0026
BC+block	0.0184083	1	0.0184083	0.09	0.7709
CC+block	0.0498241	1	0.0498241	0.24	0.6330
Total error	2.49072	12	0.20756		
Total (corr.)	11.6907	21			

Table 4.41 Regression coefficients for Ethanol Content

Coefficient	Estimate
constant	12.4802
A:Inoculum Size	1.24399
B:Agitation Rate	-0.190776
C:Temperature	-0.213692
AA	-0.0820551
AB	0.000373333
AC	0.000933333
BB	0.00133545
BC	0.000313333
CC	0.00457609

4.12.1 Effect of cultural conditions on total acids

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.49). Table 4.42 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted)

values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 74.7879% of the variability in Total Acids. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 55.8789%. The standard error of the estimate shows the standard deviation of the residuals to be 0.0765206. The mean absolute error (MAE) of 0.0459404 is the average value of the residuals.

The regression equation of the fitted model for total acids is:

$$-1.24388 + 0.0330507*A + 0.0155384*B + 0.116261*C + 0.00525217*A^2 - 0.00044*A *B - 0.00293333*A *C - 0.000123478*B^2 + 0.000173333*B *C - 0.00244928*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.42 Estimated effects for Total Acids (%)

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	0.673478	0.0529189	
A:Inoculum Size	0.0275	0.0382603	1.0
B:Agitation Rate	-0.0975	0.0382603	1.0
C:Temperature+block	-0.152029	0.0374193	1.01359
AA+block	0.0656522	0.0739833	1.01976
AB	-0.055	0.0441792	1.0
AC	-0.0733333	0.0441792	1.0
BB+block	-0.154348	0.0739833	1.01976
BC+block	0.0433333	0.0441792	1.0
CC+block	-0.122464	0.0784373	1.0149

Standard errors are based on total error with 12 d.f.

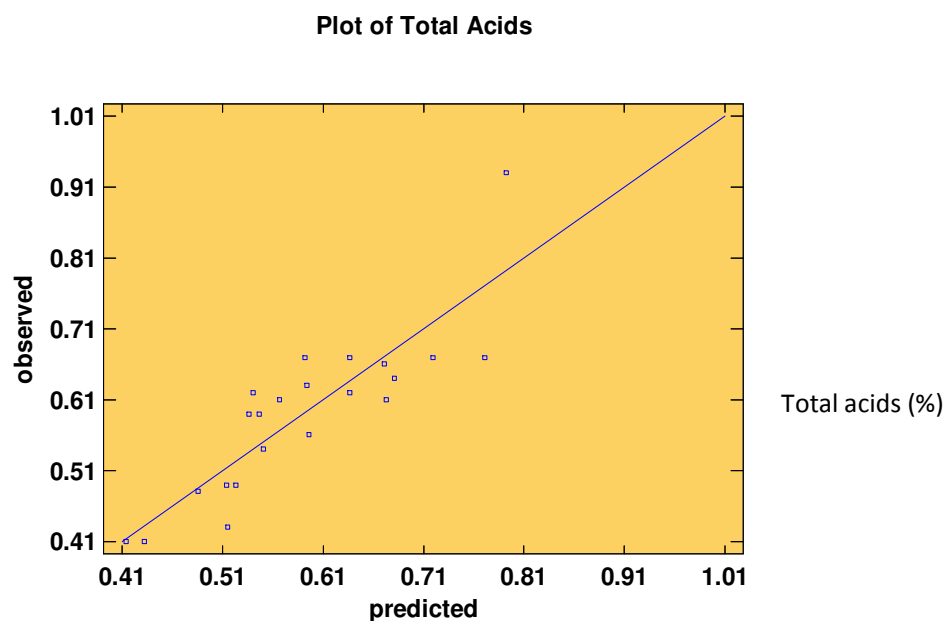


Fig4.49 Experimental vs predicted results of total acids

Table 4.43 Analysis of Variance for Total Acids

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.003025	1	0.003025	0.52	0.4860
B:Agitation Rate	0.038025	1	0.038025	6.49	0.0255
C:Temperature+block	0.0966536	1	0.0966536	16.51	0.0016
AA+block	0.00461092	1	0.00461092	0.79	0.3923
AB	0.009075	1	0.009075	1.55	0.2369
AC	0.0161333	1	0.0161333	2.76	0.1228
BB+block	0.0254853	1	0.0254853	4.35	0.0590
BC+block	0.00563333	1	0.00563333	0.96	0.3460
CC+block	0.0142734	1	0.0142734	2.44	0.1444
Total error	0.0702649	12	0.0058554		
Total (corr.)	0.278695	21			

Table 4.44 Regression coefficients for Total Acids

Coefficient	Estimate
constant	-1.24388
A:Inoculum Size	0.0330507
B:Agitation Rate	0.0155384
C:Temperature	0.116261
AA	0.00525217
AB	-0.00044
AC	-0.00293333
BB	-0.000123478
BC	0.000173333
CC	-0.00244928

4.12.2 Effect of cultural conditions on pH

For appropriate fitting of each model, the analysis of variance (ANOVA) was carried out for ethanol production. This gives good correlation between input factors and their responses. Experimental results were close to predicted ones for ethanol production (Figure 4.50). Table 4.45 shows each of the estimated effects and interactions. The straight line represents perfect agreement between experimental (observed) and calculated (predicted) values. The distance of each symbol from straight line corresponds to its deviation from related experimental values. Standard error of each of the effects is shown which measures their sampling error. Here the largest variance inflation factor (V.I.F.) equals 1.01976. For a perfectly orthogonal design, all of the factors would equal to 1. Factors of 10 or larger are usually interpreted as indicating serious confounding amongst the effects. Block effects are included in the model to reduce the magnitude of experimental error.

The ANOVA table partitions the variability in ethanol into separate pieces for each of the effects. It tests the statistical significance of each effect of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 70.471% of the variability in pH. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 48.3243%. The standard error of the estimate shows the standard deviation of the residuals to be 0.151337. The mean absolute error (MAE) of 0.0935063 is the average value of the residuals.

The regression equation of the fitted model for total acids is:

$$8.01567 - 0.0759348*A - 0.000576812*B - 0.304022*C - 0.0133043*A^2 + 0.000746667*A*B + 0.0086*A*C + 0.0000909565*B^2 - 0.000566667*B*C + 0.00593188*C^2$$

Where A= INOCULUM SIZE, B= AGITATION RATE, C= TEMPERATURE

Table 4.45 Estimated effects for pH

Effect	Estimate	Standard Error	Variance Inflation Factor
average+block	4.24304	0.104659	
A:Inoculum Size	-0.0225	0.0756685	1.0
B:Agitation Rate	0.225	0.0756685	1.0
C:Temperature+block	0.145725	0.0740052	1.01359
AA+block	-0.166304	0.146319	1.01976
AB	0.0933333	0.0873744	1.0
AC	0.215	0.0873744	1.0
BB+block	0.113696	0.146319	1.01976
BC+block	-0.141667	0.0873744	1.0
CC+block	0.296594	0.155128	1.0149

Standard errors are based on total error with 12 d.f.

Plot of pH

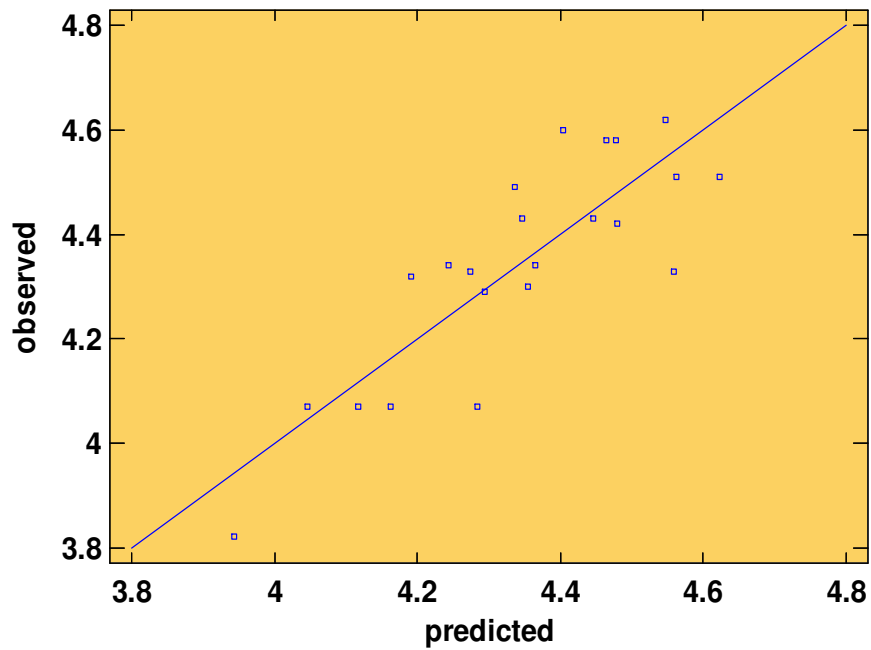


Fig 4.50 Experimental vs predicted results of pH

Table 4.46 Analysis of Variance for pH

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Size	0.002025	1	0.002025	0.09	0.7713
B:Agitation Rate	0.2025	1	0.2025	8.84	0.0116
C:Temperature+block	0.0888037	1	0.0888037	3.88	0.0725
AA+block	0.0295867	1	0.0295867	1.29	0.2779
AB	0.0261333	1	0.0261333	1.14	0.3064
AC	0.138675	1	0.138675	6.05	0.0300
BB+block	0.0138286	1	0.0138286	0.60	0.4522
BC+block	0.0602083	1	0.0602083	2.63	0.1309
CC+block	0.0837214	1	0.0837214	3.66	0.0801
Total error	0.274834	12	0.0229029		
Total (corr.)	0.930727	21			

Table 4.47 Regression coefficients for pH

Coefficient	Estimate
constant	8.01567
A:Inoculum Size	-0.0759348
B:Agitation Rate	-0.000576812
C:Temperature	-0.304022
AA	-0.0133043
AB	0.000746667
AC	0.0086
BB	0.0000909565
BC	-0.000566667
CC	0.00593188

4.13 RESPONSE SURFACE METHODOLOGY: TWO VARIABLE INTERACTION STUDIES

The surface plots for different interaction of any two independent variables, while holding the third variable constant, on enzyme production were generated using software, Statgraphics Centurion XVI.I. The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable in order to deduce the optimum conditions.

4.13.1 Effect of agitation rate and inoculum size on total acids, ethanol content and pH

A direct correlation was found between inoculum size and agitation rate and graphs were plotted to understand their effect on ethanol production. The response of experimental levels of each variable was examined and concluded that with the variation in agitation rate

from 50, 75 and 100rpm and inoculums size from 5, 7.5 and 10%, the ethanol content, total acids and pH varied in range of 0.41-0.59 %, ethanol content in range of 10.9-11.7% pH in range of 4.66-4.9 respectively as shown in Figure 4.51a, 4.51b, 4.51c, 4.52a, 4.52b and 4.52c.

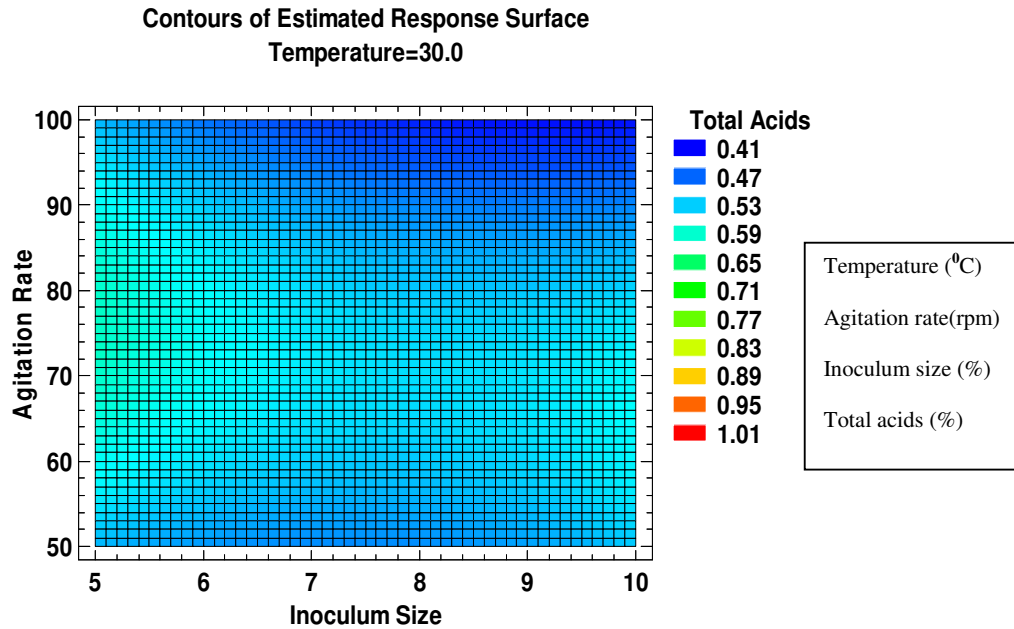


Fig 4.51a Contour plots for total acids (agitation rate vs. inoculum size)

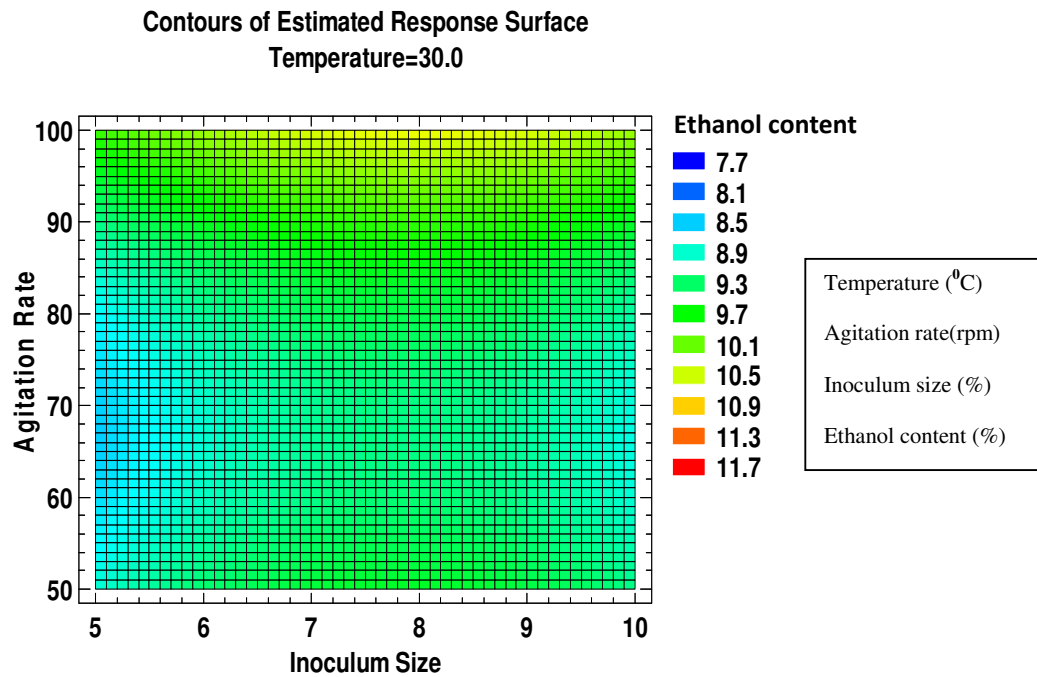


Fig 4.51b Contour plots for ethanol content (agitation rate vs. inoculum size)

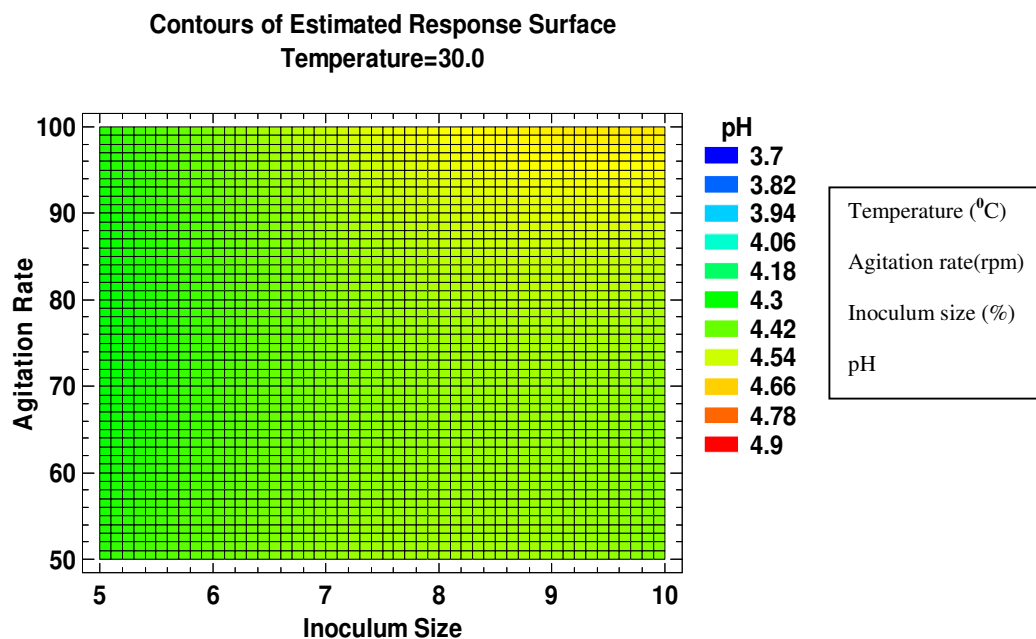


Fig 4.51c Contour plots for pH (agitation rate vs. inoculum size)

Fig4.51a, b and c Contour plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

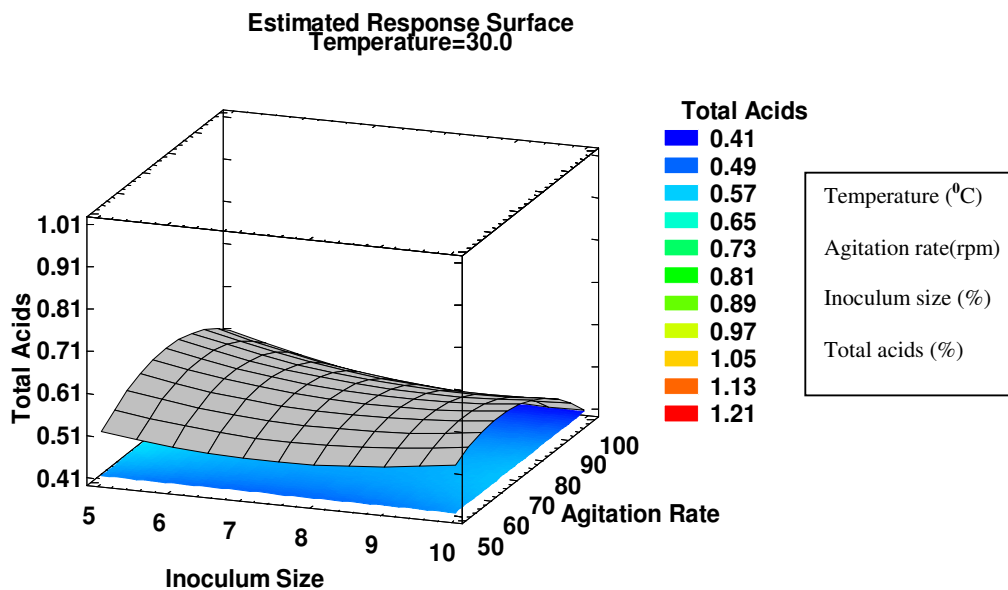


Fig4.52a Surface plots for total acids (agitation rate vs. inoculum size)

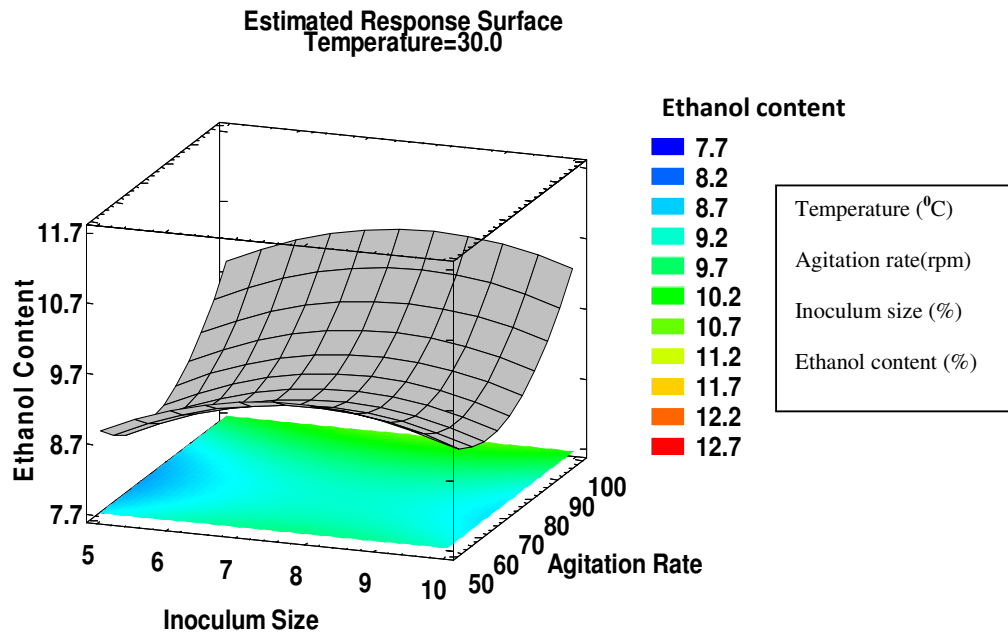


Fig 4.52b Surface plots for ethanol content (agitation rate vs. inoculum size)

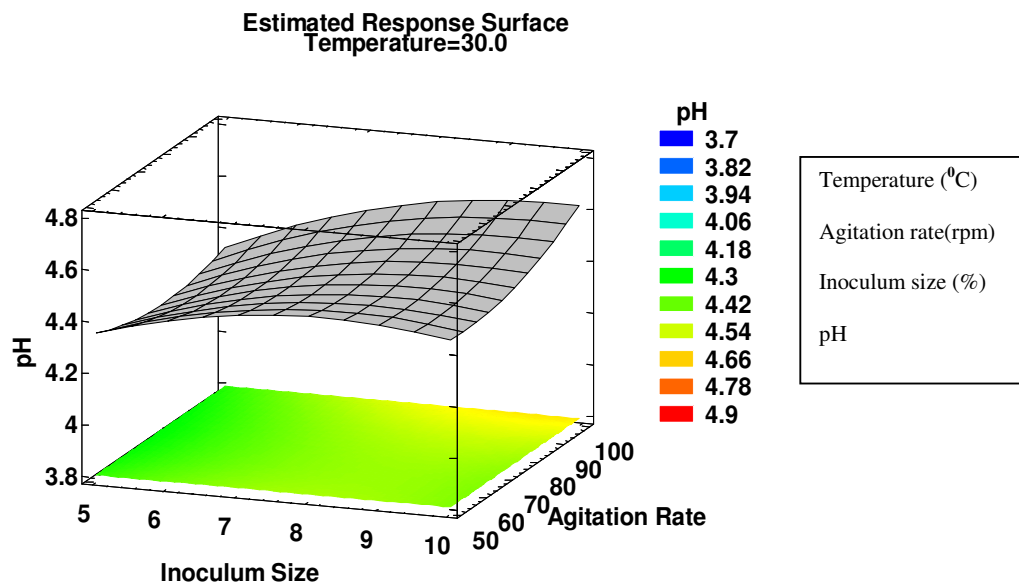


Fig 4.52c Surface plots for pH (agitation rate vs. inoculum size)

Fig 4.52 a, b and c Surface plots showing effect of agitation rate and inoculum size on total acids, ethanol content and pH

4.13.2 Effect of inoculum size and temperature on total acids, ethanol content and pH

Figure 4.53a, 4.53b and 4.53c showed the contour plots, 4.54a, 4.54b and 4.54c showed the surface plots of inoculum size v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25 30°C resulted in variation in total acids of the range of 0.41- 0.59% and ethanol content from 10.9-11.7 % The pH was found to be in the range of 4.66-4.9.

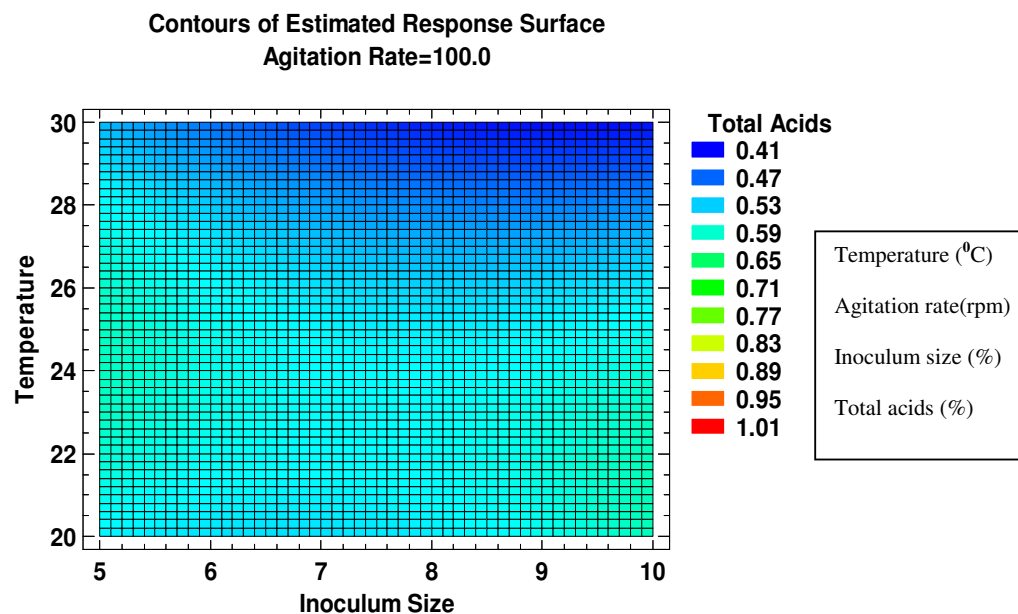


Fig 4.53a Contour plots for total acids (temperature vs. inoculum size)

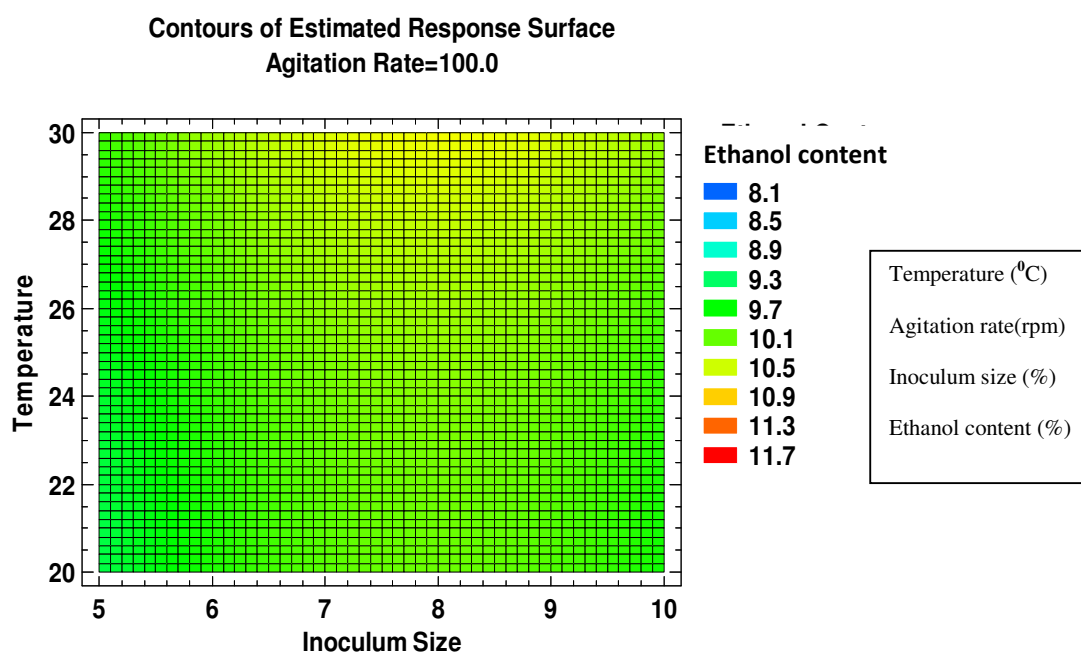


Fig 4.53b Contour plots for ethanol content (temperature vs. inoculum size)

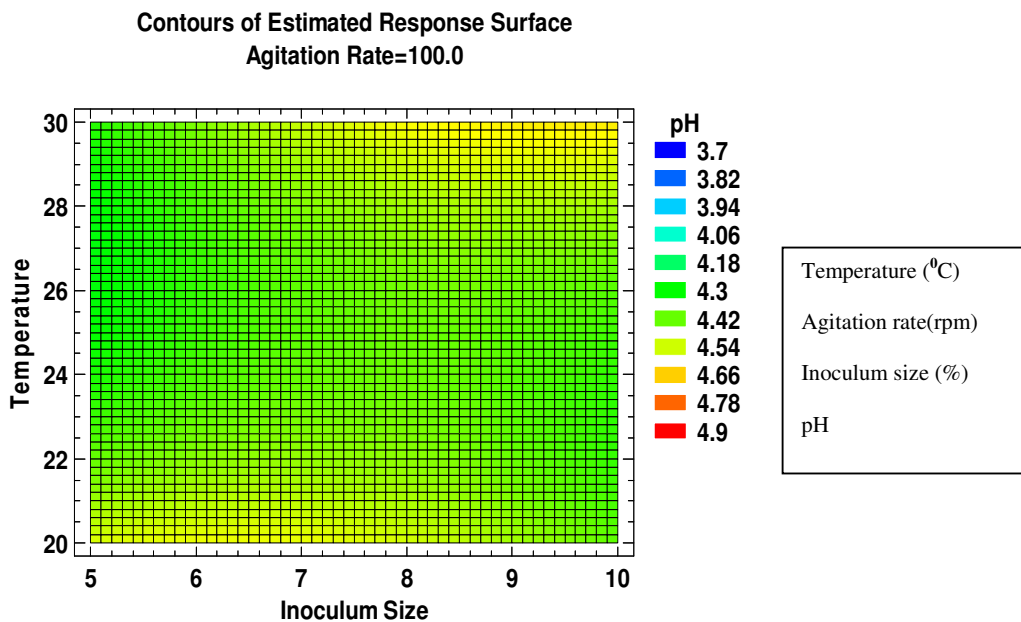


Fig 4.53c Contour plots for pH (temperature vs. inoculum size)

Fig 4.53 a, b and c: Contour plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

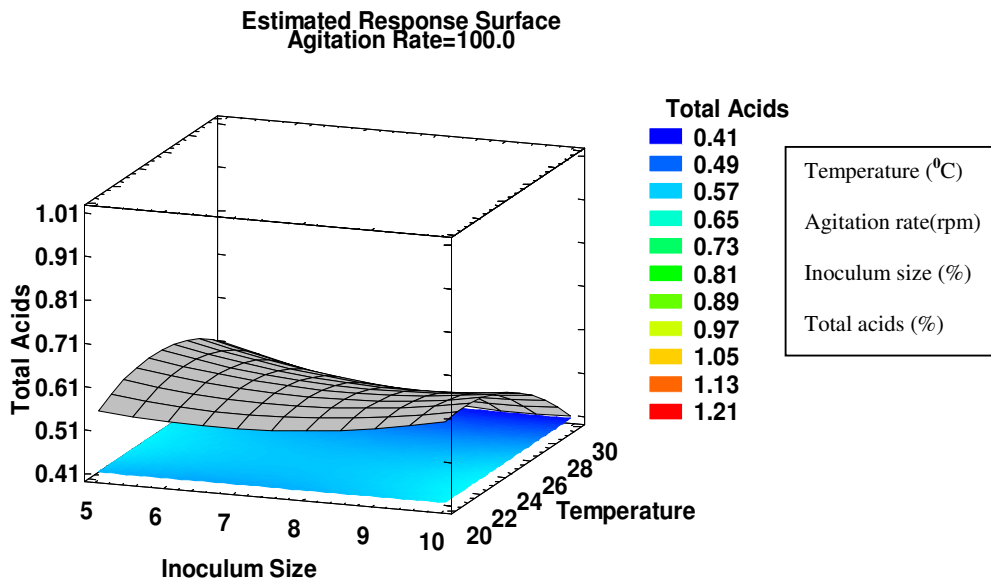


Fig4.54a Surface plots for total acids (temperature vs. inoculum size)

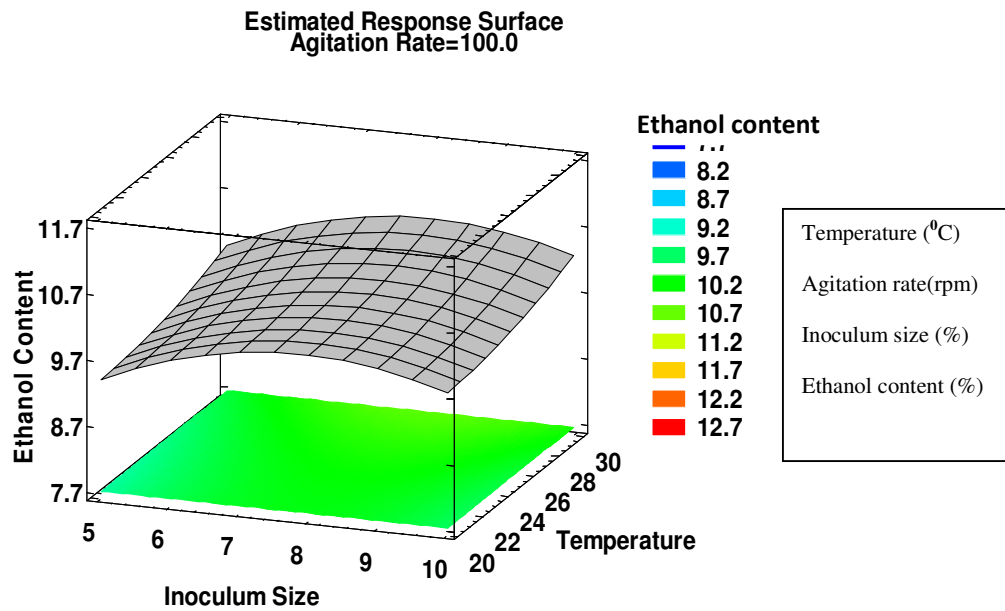


Fig4.54b Surface plots for ethanol content (temperature vs. inoculum size)

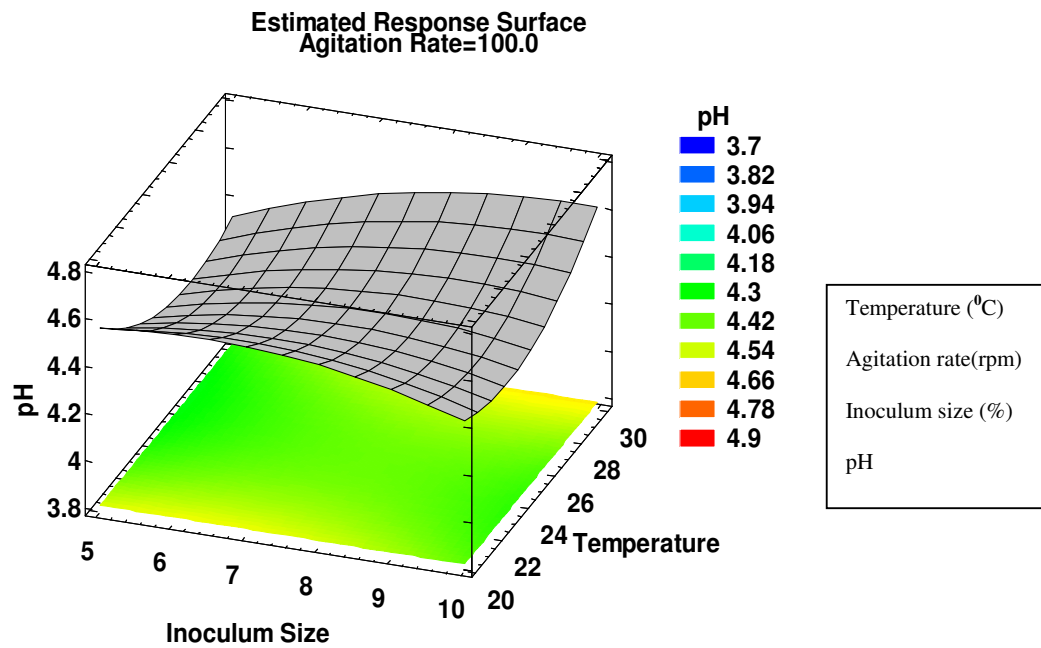


Fig4.54c Surface plots for pH (temperature vs. inoculum size)

Fig 4.54 a, b and c Surface plots showing effect of temperature and inoculum size on total acids, ethanol content and pH

4.13.3 Effect of Agitation rate and temperature on total acids, ethanol content and pH

Figure 4.55a, 4.55b and 4.55c showed the contour plots, 4.56a, 4.56b and 4.56c showed the surface plots of agitation rate v/s temperature for total acids, ethanol content and pH. Increase in inoculum size from 5, 7.5 and 10% and temperature from 20, 25 30°C resulted in variation of total acids in the range of 0.41- 0.59% and ethanol content from 10.9-11.7 % The pH was found to be in the range of 4.66-4.9.

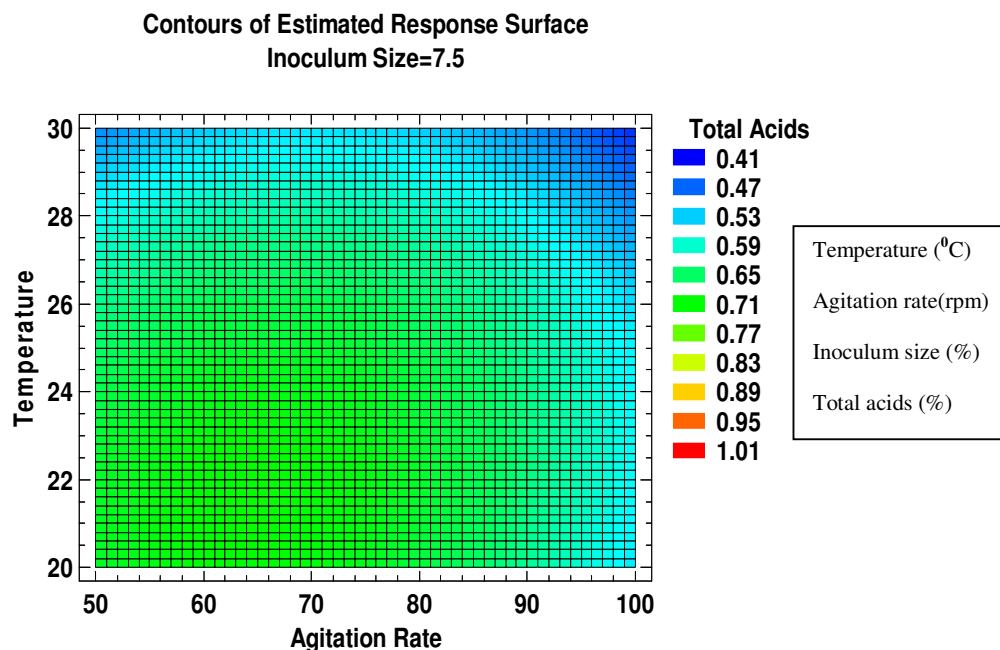


Fig 4.55a Contour plots for total acids (temperature vs. agitation rate)

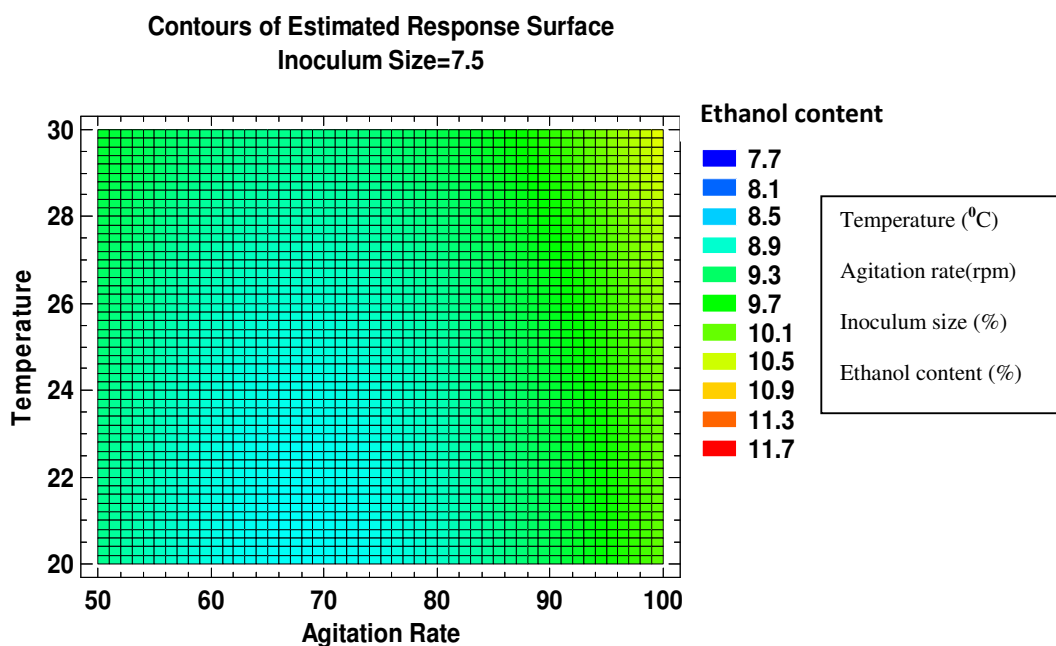


Fig 4.55 b Contour plots for ethanol content (temperature vs. agitation rate)

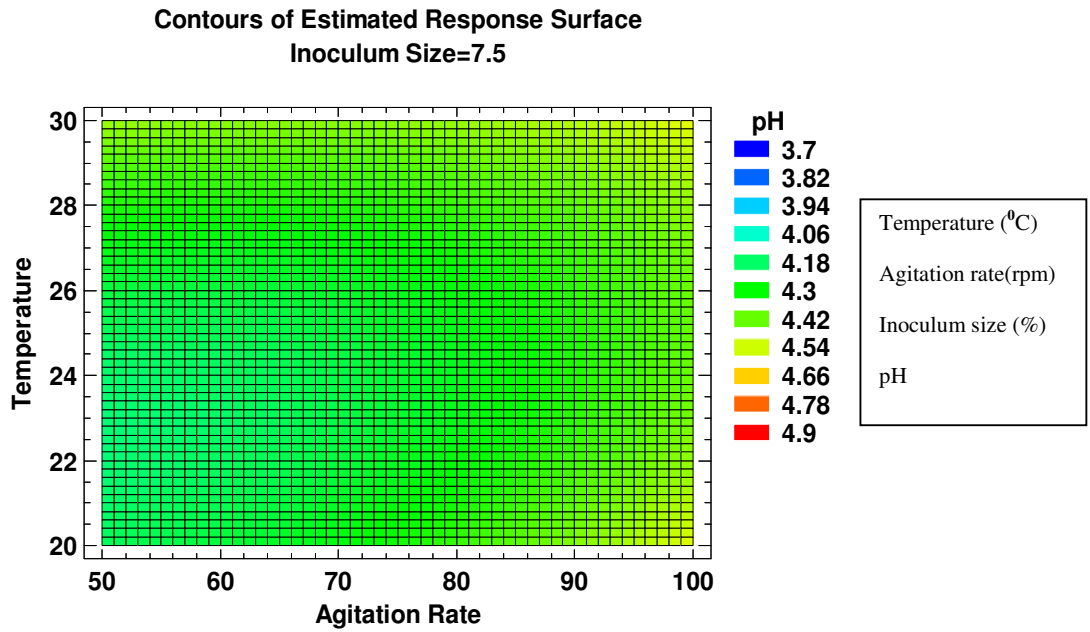


Fig 4.55c Contour plots for pH(temperature vs. agitation rate)

Fig 4.55 a, b and c Contour plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

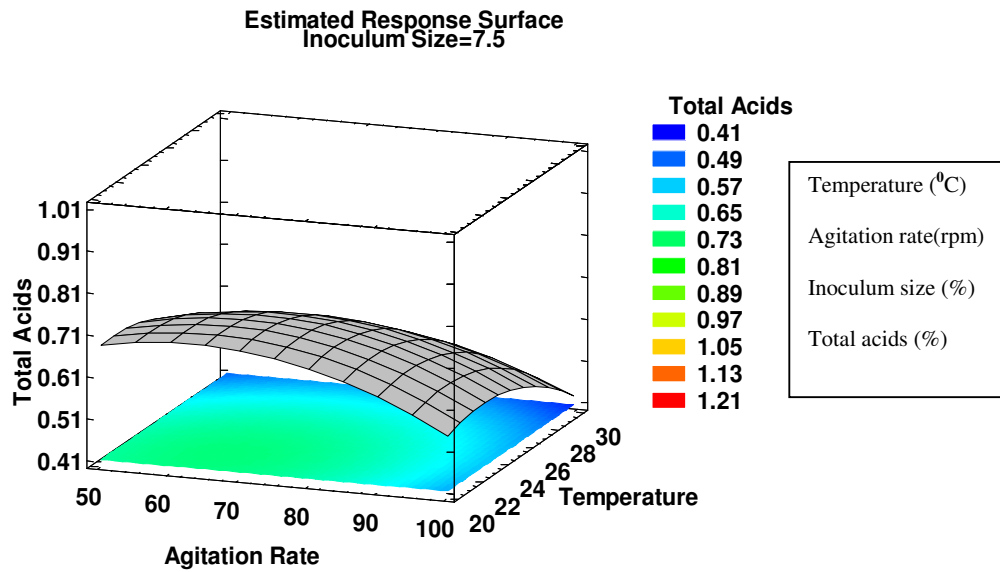


Fig 4.56a Surface plots for total acids (temperature vs. agitation rate)

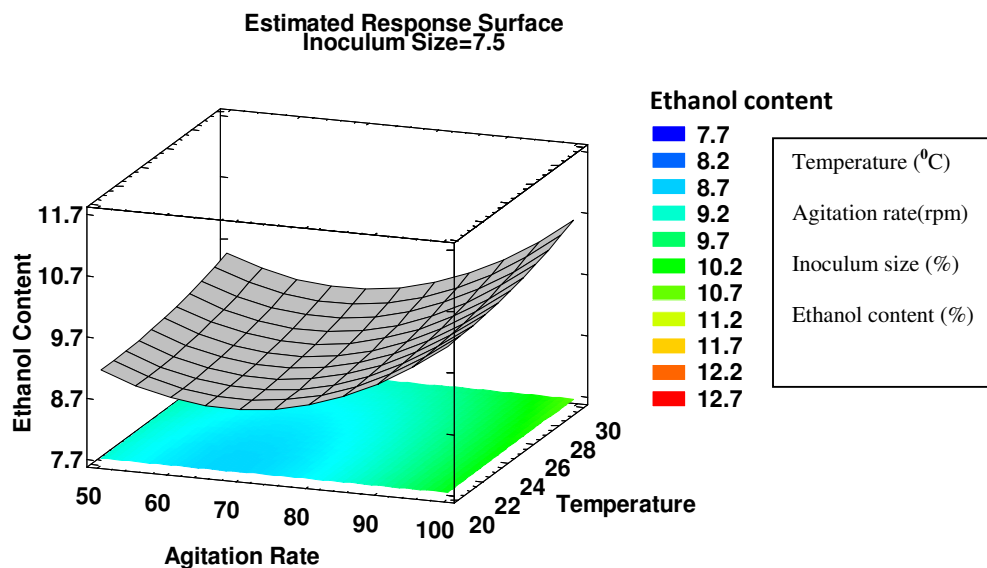


Fig 4.56b Surface plots for ethanol content (temperature vs. agitation rate)

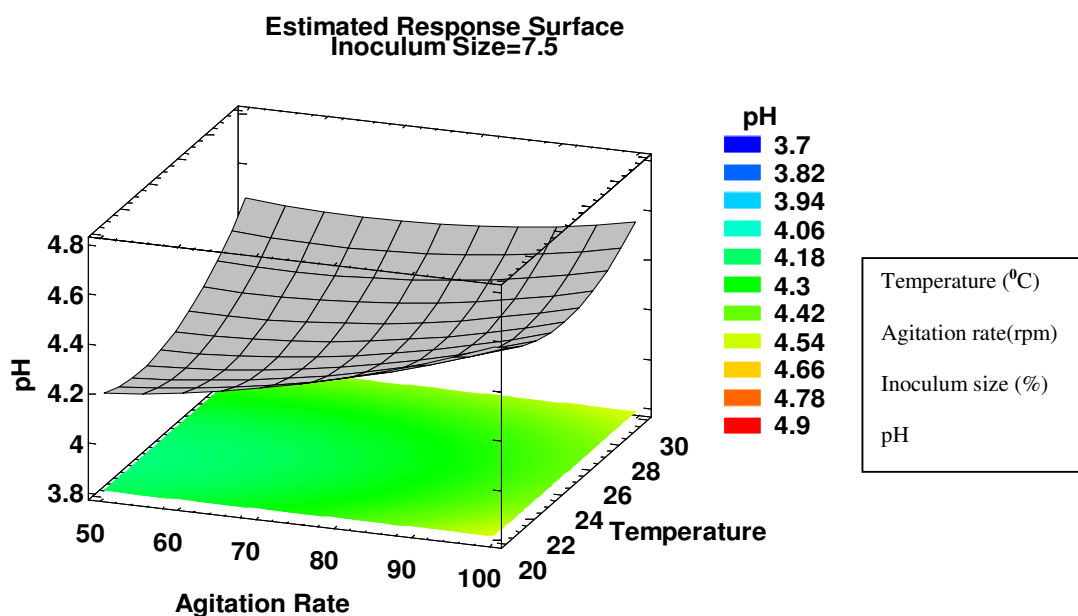


Fig 4.56c Surface plots for pH (temperature vs. agitation rate)

Fig 4.56a, b and c Surface plots showing effect of agitation rate and temperature on total acids, ethanol content and pH

4.14 Response Surface Methodology for Three Variable Interactions

Responses were optimized using software Statgraphics Centurion XVII.I. The optimum value for each dependent variable had different set of treatment conditions. The optimization was done with the target of maximum ethanol content and pH with minimum total acidity. To consider all the responses simultaneously for optimization, surface plots were generated for each response. The most desired combinations were selected for maximum

ethanol production from desirability surface plots (Fig4.57, 4.58, 4.59). To get maximum ethanol production, surface plots were overlaid with the targets mentioned above. Overlay plots describing the optimum conditions has shown in Fig. 4.60, 4.61 and 4.62. Hence inoculum size of 5%, agitation rate of 50 rpm and fermentation temperature of 30 °C were found to be the best parameters for optimum responses i.e. total acidity, ethanol content and pH values in sweet sorghum juice inoculated with *Saccharomyces cerevisiae* strain-35.

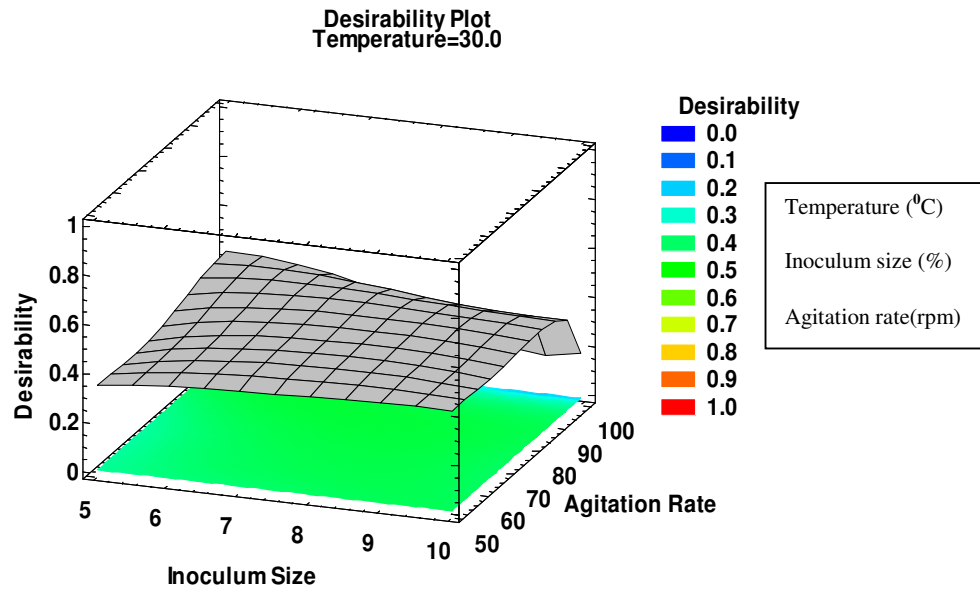


Fig 4.57 Surface plot for desirability: Inoculum size vs. Agitation rate

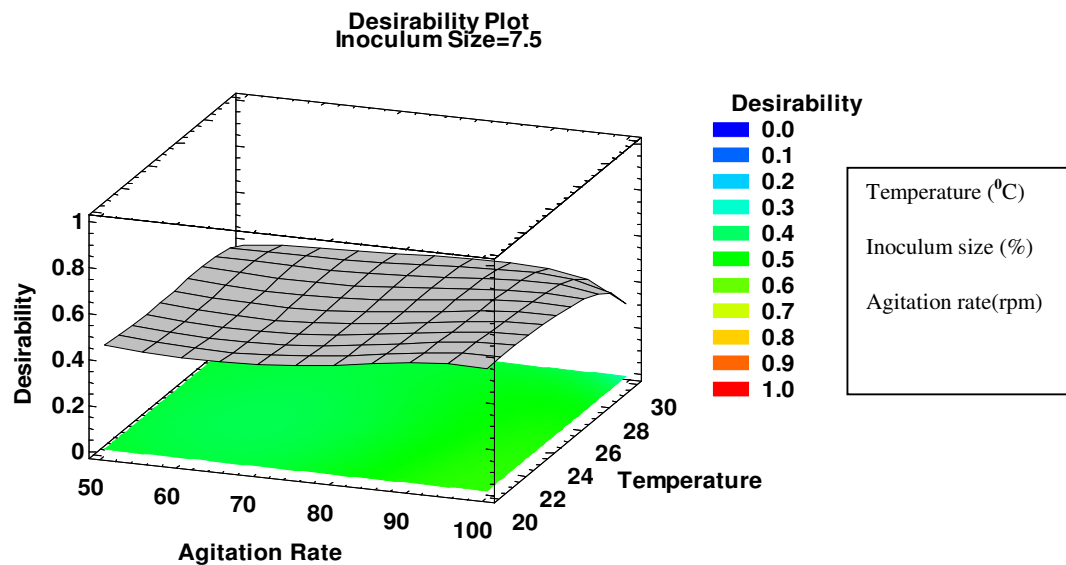


Fig4.58 Surface plot for desirability: Temperature vs. Agitation rate

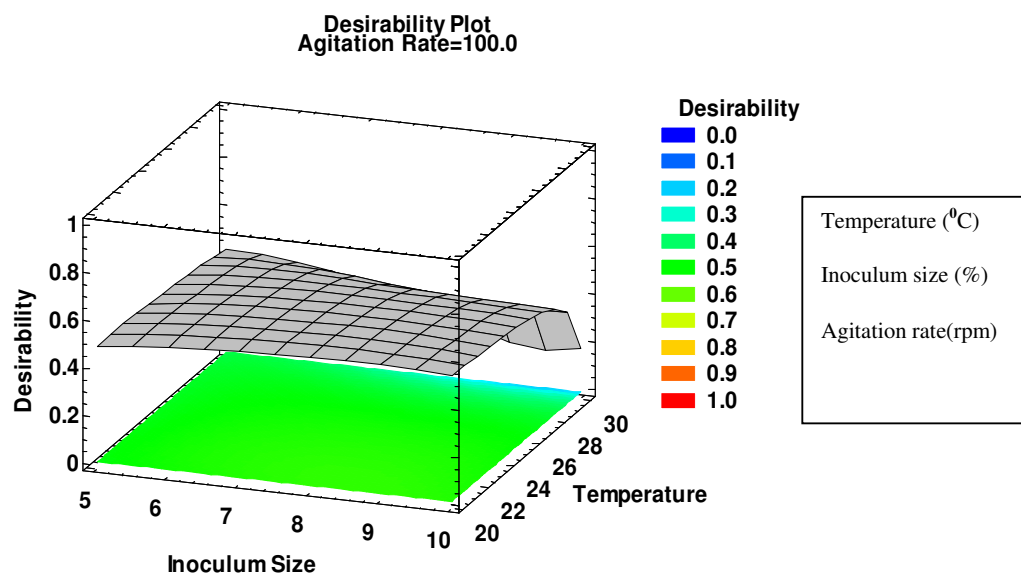


Fig4.59 Surface plot for desirability: Inoculum size vs. Temperature

OVERLAY PLOTS:

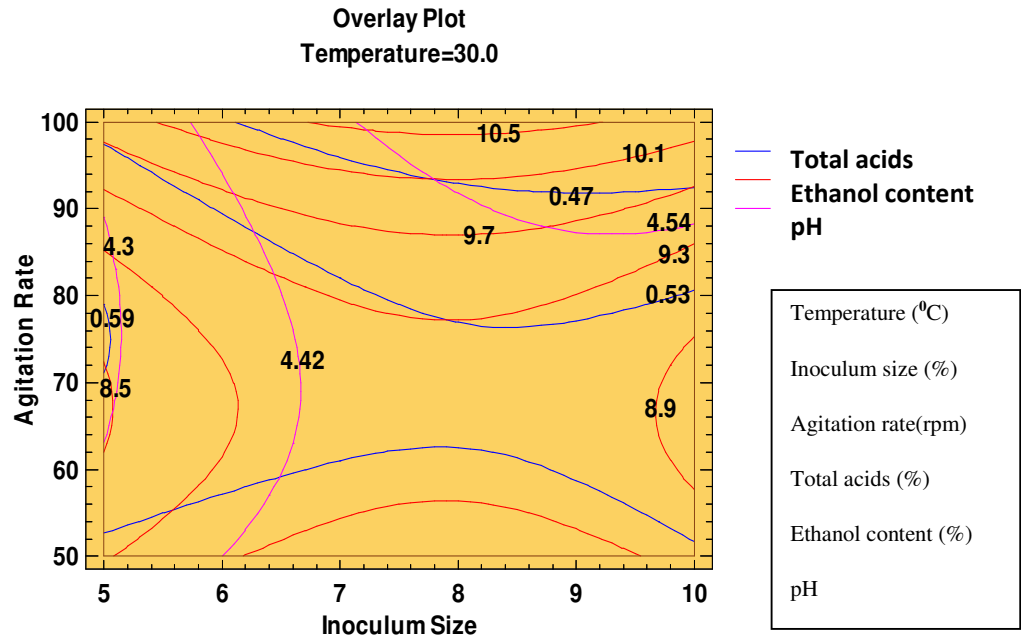


Fig4.60 Agitation rate vs. inoculum size

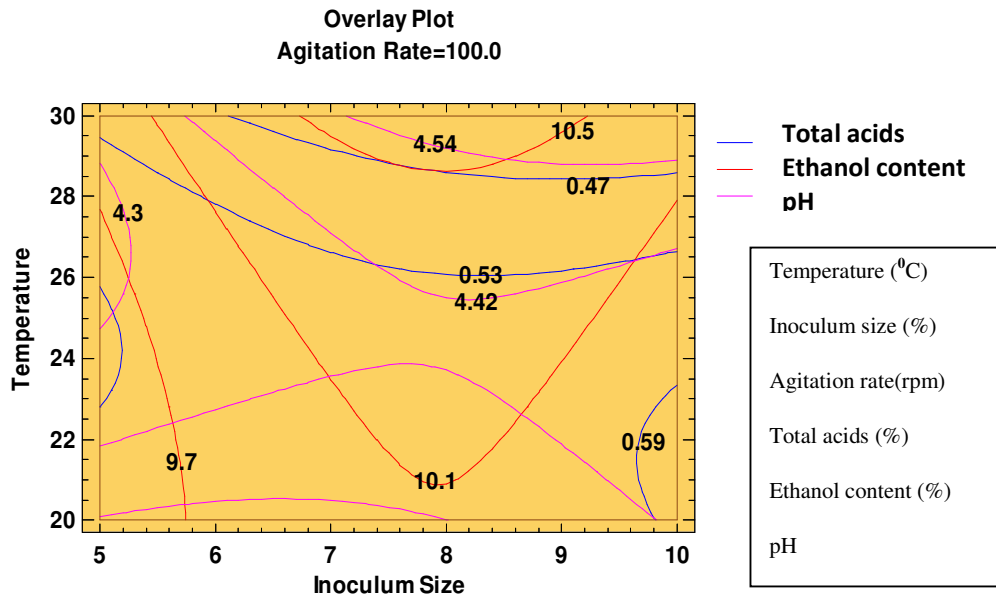


Fig4.61 Temperature vs. Inoculum size

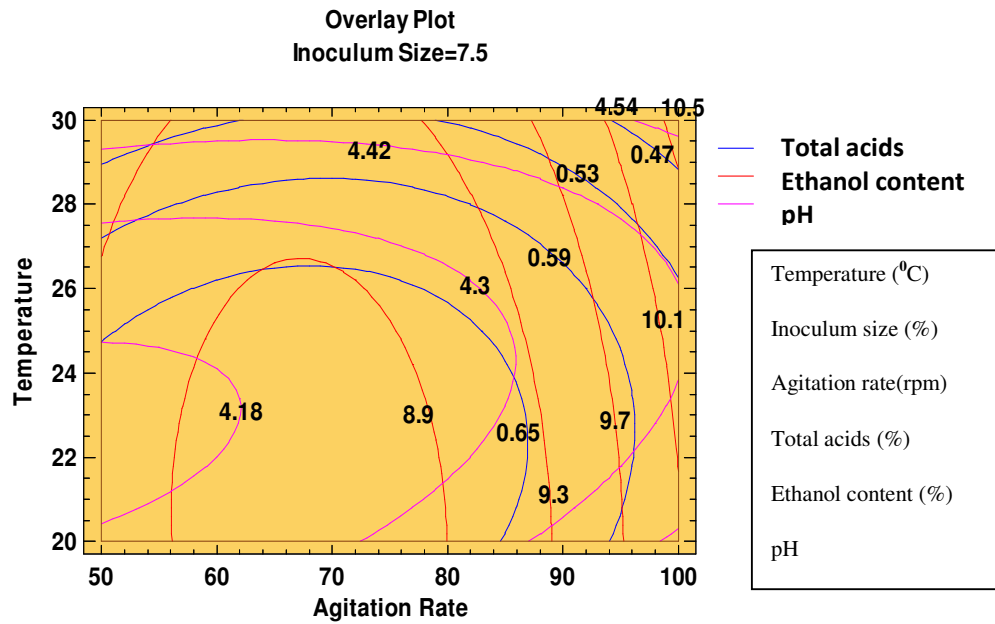


Fig4.62 Temperature vs. agitation rate

Jin *et al* (2011) reported that Hinshelwood model could give an adequate fit to the dynamics of ethanol fermentation with immobilized *Saccharomyces cerevisiae* growing on sweet sorghum stalk juice of initial reducing sugar concentrations in the range from 85-156g/l. Optimized bioethanol production from intermediates of sugarbeet processing by response surface methodology and reported that the ethanol production from sugarbeet processing intermediates is technically possible (Jovana *et al*, 2011).

4.15 Comparison of ethanol production from sweet sorghum and sugarcane juice under optimized conditions

Results from table 4.48 indicates that sweet sorghum juice inoculated with *Saccharomyces cerevisiae* strain NRRL Y-2034 produced 8.85% (v/v) ethanol under optimized conditions of 30°C temperature, 50 rpm and 7.5% (v/v) inoculum size whereas in sugarcane juice a maximum of 10.14% (v/v) ethanol content was obtained under optimized conditions of 30°C temperature, 100 rpm and 5% (v/v) inoculum size. Sweet sorghum juice with *Saccharomyces cerevisiae* strain-35 showed 8.47% (v/v) ethanol under optimized conditions of 30°C temperature, 50 rpm and 5% (v/v) inoculum size whereas from sugarcane juice 11.23% (v/v) ethanol under optimized conditions of 30°C temperature, 100 rpm and 7.5% (v/v) inoculum size.

Table 4.48 Ethanol production from sweet sorghum and sugarcane juice under optimized conditions

Sweet sorghum juice								
<i>Saccharomyces cerevisiae</i>	Optimization parameters			Responses				
	Inoculum size (%)	Agitation rate (rpm)	Temperature (°C)	Total Acidity (%)	pH	Percent Ethanol		Percent FE
						v/v	w/v	
Strain-NRRL Y-2034	7.5	50	30	0.62	5.01	8.85	7.04	87.35
Strain-35	5	50	30	0.55	4.62	8.47	6.73	83.56
Sugarcane juice								
Strain-NRRL Y-2034	5	100	30	0.54	4.65	10.14	8.11	97.67
Strain-35	7.5	100	30	0.44	4.55	11.23	8.99	95.62

Rathnavathi *et al* (2010) has recovered maximum ethanol production of 9.0% (w/v) with fermentation efficiency of 94.7% when *S. cerevisiae* was used under optimized conditions. However, Ratnavathi *et al* (2011) reported 7.9% (w/v) ethanol from sweet sorghum juice in 120 hr under ambient temperature. Under same cultural conditions, unsterile sweet sorghum juice has recovered 6.47% (w/v) ethanol content. Liu *et al* (2008) reported 91.61% fermentation efficiency of sweet sorghum juice using *Saccharomyces cerevisiae*. Similarly Mamma *et al* (1995) reported that sweet sorghum juice supplemented with minerals recovered

9.2% (w/v) ethanol inoculated with *S. cerevisiae* at pH 5.5, inoculum concentration of 10% (v/v) at 33°C, after 48 hrs of incubation.

Liang *et al* (2008) reported that *Saccharomyces cerevisiae* strain AS2.1190 under repeated batch fermentation yielded ethanol concentrations of about 89.73–77.13 g/l showing conversion efficiency of 90.11–94.28 % using sugarcane juice. The thermotolerant yeast strain isolated from sugarcane juice through enrichment technique was identified as *Pichia kudriavzevii* (Issatchenkiaorientalis) through molecular characterization. The *P. kudriavzevii* cells adapted to galactose medium produced about 30% more ethanol from sugarcane juice than the non-adapted cells. The recycled cells could be used for four successive cycles without a significant drop in ethanol production (Dhaliwal *et al* 2011). Rolz and Deleon (2011) reported that as the yeast is capable of fermenting not only sucrose but also hexoses into ethanol, thus the proper development stage to harvest sugarcane directed only for ethanol production was different than one for sugar production. Wu *et al* (2011) reported that fermentable sugars in the mixture of sugarcane juice and bagasse hydrolysate were readily converted to ethanol by *S. cerevisiae* within 12 hours showing fermentation efficiency of 88%.

It was reported that under appropriate environmental and nutritional conditions, *S. cerevisiae* can produce and tolerate high ethanol concentration (Thomas *et al* 1996; Bafrencova *et al* 1999). In view of the high titers of ethanol towards the end of each fermentation cycle [8-12 % (v/v)], the ethanol is one of the major stress factors that act upon yeast. The inhibitory role of ethanol upon *S. cerevisiae* is not fully understood until now. Yeast cells must keep high viability at the end of each fermentation cycle in order to be able to cope with the next fermentation cycle. A given strain can perform well in one fermentation cycle, but cannot be recycled for subsequent fermentation cycles. This was observed in some corn and cereal-based fermentation processes, where 17 to 23 % (v/v) of ethanol titers were obtained when wheat and formulated mashes were used (Jones *et al* 1994; Bayrock and Ingledew, 2001). Research is being conducted to search for ethanol tolerant yeast strains, in order to be used in high ethanol fermentations. Under the present study the fermentation efficiency of sweet sorghum juice was found to be 87.35% and 83.56% when inoculated with *Saccharomyces cerevisiae* strain NRRL Y-2034 and *Saccharomyces cerevisiae* strain-35 respectively. However, fermentation efficiency of sugarcane juice was found to be 98.67% and 95.62% inoculated with *Saccharomyces cerevisiae* strain NRRL Y-2034 and *Saccharomyces cerevisiae* strain-35 respectively.

CHAPTER - V

SUMMARY

Petroleum is a non-renewable source of energy once depleted will not be replenished back. Hike in the prices of fuel and increasing environmental pollution is a major concern now days. To overcome this problem, an alternate fuel has to be introduced at the place of petroleum. Ethanol is the most common biofuel used all over the world. Ethanol is a solvent miscible in water and other organic solvents. For the production of ethanol, crop plants are one of the best sources to renew the lost energy. Corn, cassava, sugarcane molasses, wheat, and sweet sorghum are the crops, which are being used for ethanol production. Brazil and USA are the major producers of ethanol all over the world. Ethanol is mixed in the petroleum engine at the place of MTBE (Methyl –tert- butyl ether) as a fuel oxygenate to raise the octane number (standard measure of the performance of a motor). Therefore, ethanol (an oxygenous biomass fuel) is considered as a predominant alternative to MTBE for its biodegradable nature, low toxicity, persistence and regenerative characteristic.

Sweet sorghum has many advantages over other crops for ethanol production. Sweet sorghum, a C₄ Gramineous crop is sugar rich and can yield higher amount of ethanol at lower costs. It is the only crop that provides grain and sugar rich stem and can be used for sugar, alcohol, syrup, jaggery, fodder, fuel, bedding, roofing, fencing, paper and chewing purposes. It is easier and less expensive to cultivate than corn, need fewer pesticides and fertilizers. Due to remarkably short growth cycle, two crops can be grown per year in warm climates, has higher tolerance to salt and drought comparing to sugarcane and corn that are currently being used for biofuel production. Sweet sorghum juices usually contain approximately 16-18% fermentable sugar, which can be directly fermented into ethanol by yeast at room temperature. These desirable agricultural characteristics make sweet sorghum a promising alternative feedstock for fuel ethanol production.

For the fermentation, use of *Saccharomyces cerevisiae* (baker's yeast) is the easiest way to produce ethanol from ancient times. Yeast can also tolerate high ethanol concentrations under appropriate conditions. Temperature can affect sensitivity of yeast to ethanol concentration, growth rate and rate of fermentation. Many projects on sweet sorghum as renewable energy source have been started all over the world such as Agrifuels (Australia), NARI (India), TATA Group (India), Renegie (USA), Akuo Energy (France), Tectane (Canada), Anantha Energy (India) and Ceres (Brazil) etc.

Under the present study, sweet sorghum juice of variety CSV19SS and sugarcane juice of variety COJ-89 were procured from the Sugarcane section of Plant Breeding and Genetics Department, PAU, Ludhiana. Procured juice was then stored at 0°C and -20°C for 75 days to study its storage stability. Juice was fermented with the yeast *Saccharomyces*

cerevisiae strain NRRL Y-2034 and *Saccharomyces cerevisiae* strain-35 maintained on GYE slants. Fermentation with both the strains was carried out at a temperature of $28\pm 2^{\circ}\text{C}$, agitation rate of 50 rpm and inoculum concentration of 5%, for 10-12 days with sweet sorghum juice. Variation in the reducing sugars, total soluble sugars ($^{\circ}\text{B}$), total acidity and ethanol content produced was observed at regular intervals. Optimization of cultural parameters for maximum ethanol production and fermentation efficiency was done with the help of statistical software Statgraphics Centurion XVI.I using Response Surface Methodology. Various optimizing parameters were inoculum concentration, agitation rate and temperature and the resultant responses were ethanol content, total acidity and pH, using sugarcane and sweet sorghum juice inoculated separately by *Saccharomyces cerevisiae* strain NRRL Y-2034 and *Saccharomyces cerevisiae* strain-35. Production of ethanol from sweet sorghum juice and sugarcane juice was also compared under optimized conditions.

Storability studies of sweet sorghum juice pertains to measurement of TSS, reducing sugars, total acids and pH after juice was stored at 0°C and -20°C for 75 days. There is enhancement of 0.52% and 0.57% of reducing sugar at 0°C and -20°C respectively. This might be due to the presence of high invertase activity in sweet sorghum juice which converts sucrose into simple sugars such as glucose. Total acidity decreased from 0.93% to 0.67% in 30 days at 0°C but after that increase in total acidity was observed. Whereas at -20°C , it decreased from 0.93 to 0.60 in 30 days and remained constant thereafter. The pH was found to be almost constant at 0°C but increased from 5.27 to 5.5 at -20°C . There was no significant change in TSS ($^{\circ}\text{B}$) both at 0°C and -20°C .

Ethanol production profile from sweet sorghum juice using *Saccharomyces cerevisiae* Strain-35 and *Saccharomyces cerevisiae* NRRL Y-2034 at the temperature of $28\pm 2^{\circ}\text{C}$, agitation rate of 50 rpm and inoculum concentration of 5% was studied. A 15.5 $^{\circ}\text{B}$ decrease in the TSS and 1.74% decrease in reducing sugars were observed. The maximum ethanol content of 8.6% was obtained corresponding to fermentation efficiency of 86.69% using *Saccharomyces cerevisiae* Strain-35 after 12 days of fermentation. Whereas there was 15.2 $^{\circ}\text{B}$ decrease in TSS, 1.73% decrease in reducing sugars and maximum ethanol content of 8.4% was recovered with fermentation efficiency of 86.34% using *Saccharomyces cerevisiae* NRRL Y-2034 after 12 days of fermentation.

The effect of three factors viz. inoculum size, agitation rate and temperature at different levels was studied using 22 run Box Behnken design. The independent variables included inoculum level (5%, 7.5%, and 10%), agitation rate (50 rpm, 75 rpm, and 100 rpm) and temperature (20°C , 25°C , and 30°C) and various response variables were total acidity, pH and percent ethanol content.

Runs suggested by RSM were performed using sweet sorghum juice inoculated with *S. cerevisiae* strain NRRL Y-2034, sweet sorghum juice inoculated with *S. cerevisiae*

strain-35, sugarcane juice inoculated with *S. cerevisiae* strain NRRL Y-2034 and using sugarcane juice inoculated with *S. cerevisiae* strain-35. Multiple regression analysis, analysis of variance (ANOVA), estimated effects and interactions and standard error was tested for appropriate fitting of each model. The surface plots and contour plots for different interaction of any two independent variables, while holding the third variable constant, on ethanol production were generated using the software. The most desired combinations were selected for maximum ethanol production from desirability surface plots and also the overlay plots for describing the optimum conditions.

Ethanol fermentation of Sweet Sorghum and Sugarcane juice was compared using Response Surface Methodology (Statgraphics Centurion XVI.I software) with optimized parameters such as temperature, agitation rate and inoculum size, with the initial brix level of 20°B, pH 5.5. Sweet sorghum juice inoculated with *S. cerevisiae* NRRL Y-2034 yielded 8.85% (v/v) ethanol with fermentation efficiency of 87.35% under optimized conditions of temperature (30°C), agitation rate (50 rpm) and inoculum size (7.5%) (v/v). The sugarcane juice inoculated with *S. cerevisiae* NRRL Y-2034 yielded 10.14% (v/v) ethanol under optimized conditions of temperature (30°C), agitation rate (100 rpm) and inoculum size (5%) (v/v) with fermentation efficiency of 97.67%. When sweet sorghum juice inoculated with *S. cerevisiae* Strain-35 8.47% (v/v) ethanol was produced under optimized conditions of temperature (30°C), agitation rate (50 rpm) and inoculum size (5%) (v/v) with fermentation efficiency of 83.56%. However in sugarcane the ethanol production was observed (11.23%) (v/v) with fermentation efficiency of 95.62% under optimized conditions of temperature (30°C), agitation rate (100 rpm) and inoculum size (7.5%) (v/v).

Conclusions

1. From the above studies it is concluded that sweet sorghum juice can be stored under refrigeration conditions (0°C and -20°C) for 75 days without any significant changes in sugar content, acidity and pH.
2. The results of optimization studies for ethanol production from sweet sorghum and sugarcane juice using *Saccharomyces cerevisiae* strain NRRL Y-2034 and *Saccharomyces cerevisiae* strain-35 indicates variation in the optimized parameters as follows:
 - Sweet sorghum juice inoculated with *Saccharomyces cerevisiae* strain NRRL Y-2034 showed maximum ethanol concentration of 8.85 % (v/v) with fermentation efficiency of 87.35% under optimized conditions of temperature (30°C), agitation rate (50 rpm) and inoculum size (7.5%) (v/v).
 - Sugarcane juice with *Saccharomyces cerevisiae* strain NRRL Y-2034 yielded 10.14% (v/v) ethanol under optimized conditions of temperature (30°C), agitation rate (100 rpm) and inoculum size (5%) (v/v) with fermentation efficiency of 97.67%.

- Sweet sorghum juice with *Saccharomyces cerevisiae* strain-35 yielded 8.47% (v/v) ethanol content under optimized conditions of temperature (30°C), agitation rate (50 rpm) and inoculum size (5%) (v/v) with fermentation efficiency of 83.56%.
 - Sugarcane juice with *Saccharomyces cerevisiae* strain-35 yielded 11.23% (v/v) ethanol content with fermentation efficiency of 95.62% under optimized conditions of temperature (30°C), agitation rate (100 rpm) and inoculum size (7.5%) (v/v).
3. Sweet sorghum juice and sugarcane juices are good substrates for ethanol production. However, lifecycle assessment of both the crops has an edge towards the utilization of sweet sorghum juice for ethanol production.

Future prospective

1. Development of improved strains is required to enhance the overall fermentation efficiency and also to reduce the time of fermentation.
2. Development of ethanol tolerant, hexose and pentose fermenting yeast strains.
3. Upgradation of technology from laboratory scale level to fermenter level for its commercialization.
4. The experiments were conducted with the juices without any nutrient supplementation to make the process economical. However, the nutrient supplementation may lead to further increase in fermentation efficiency and decrease in fermentation period.

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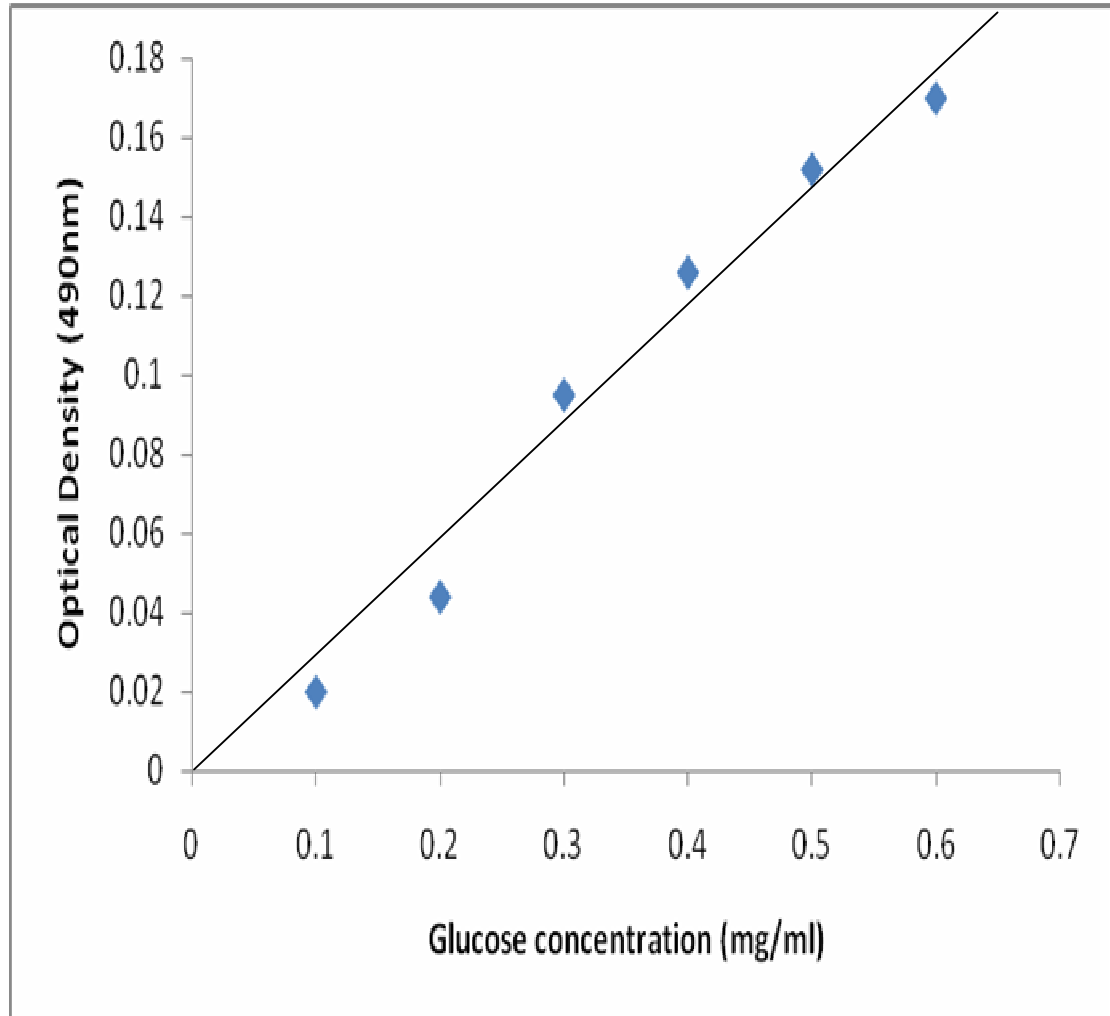
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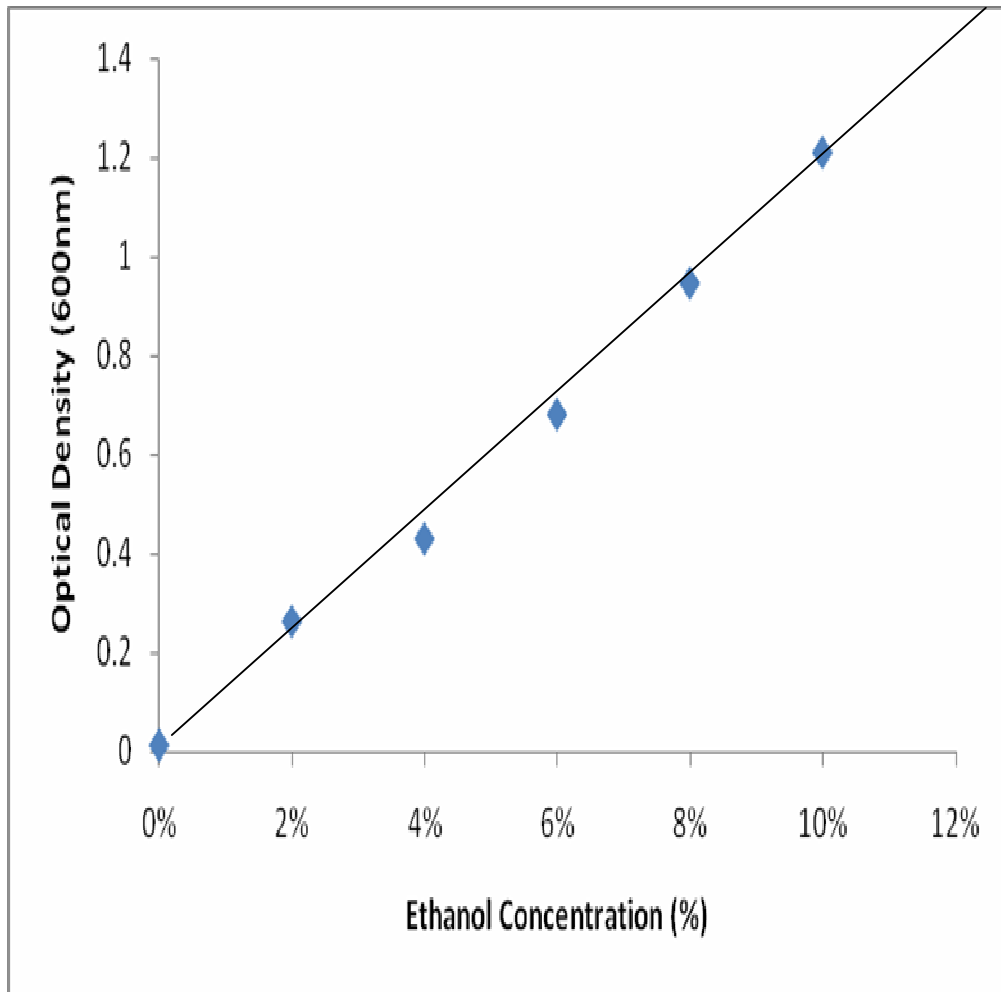
ANNEXURE I

STANDARD CURVE OF GLUCOSE



ANNEXURE II

STANDARD CURVE OF ETHANOL



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