Optimization of Fermentation Parameters For The Bioconversion of Corn To Ethanol Using Response Surface Methodology

DISSERTATION

SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE DEGREE OF

MASTER OF SCIENCE

(BIOTECHNOLOGY)

By

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This is to certify that **Ms Navpreet Kaur Walia** student of M.Sc. Biotechnology has successfully completed her dissertation entitled, **"Optimization of Fermentation Parameters for Bioconversion of Corn to Ethanol Using Response Surface Methodology",** submitted to the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala in partial fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology**. This is a record of **bona fide** research work carried out by her under my guidance and supervision and that no part of the thesis has been submitted anywhere for the publication or for any other degree or diploma.

The assistance and help received by her during the course of this investigation has been duly acknowledged.

11/7/13

(Dharam Paul Chaudhary)

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CERTIFICATE

This is to certify that the Dissertation entitled — **"Optimization of Fermentation Parameters for Bioconversion of Corn to Ethanol Using Response Surface Methodology"**, submitted by Navpreet Kaur Walia in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her at Directorate of Maize Research-ICAR, New Delhi. The report has not been submitted for the award of any other degree to any university or institute by her.

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Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled "Optimization of Fermentation Parameters for The Bioconversion of Corn To Ethanol Using Response Surface Methodology" in partial fulfillment for the requirements of award of degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University Patiala is my own laboratory work done at Directorate of Maize Research, PUSA, New Delhi during the period of january2013 to june 2013, under the supervision of Dr. Dharam Paul Chaudhary, Sr. Scientist, DMR, IARI Campus, PUSA , New Delhi. I have not submitted the matter embodied in this thesis for the award of any other degree.

Patiala Navpreet Kaur Walia

Date…….

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It is the grace and the will of almighty that finally I have reached at this point where the thesis is in hand and I feel myself at the zenith of 6 months project where I am now is from when the wind is against my sail, but this is the same which support in my voyage .

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ABBREVIATIONS

RSM	Response surface methodology
CCD	Central composite design
Conc.	Concentration
Temp.	Temperature
Fig.	Figure
µL	Micro liter
g/L	Gram per liter
$	Dollar
MGY	Million gallons per year
RFA	Renewable Fuel Association
EPA	Environment Protection Agency
NAD	Nicotinamide adenine dinucleotide
ATP	Adenosine triphosphate
MTBE	Methyl tert-butyl ether
DDGS	Dried distiller's grain with solids
GDP	Gross domestic product
NPPD	Nebraska Public Power District
BTU	British thermal unit
LCA	Life cycle assessment

O. D.	Optical density
ANOVA	Analysis of variance
%	Percent
R^2	Coefficient of determination
Adeq precision	Adequate precision
Adj. R- squared	Adjusted R- squared
Pred R- squared	Predicted R- squared
C.V.	Coefficient of variation

Abstract

The present study was conducted to optimize the ethanol production potential of maize (*Zea mays)*. In order to achieve maximum ethanol production two experiments were conducted by optimizing three fermentation variables i.e. pH, temperature and substrate concentration which were optimized at different conditions using response surface methodology by design- expert software (version 8. 0.7.1 Stat-Ease Inc; USA). Parameters were optimized by two ways: the first experiment is conducted manually by changing one variable at a time and keeping the other two invariable without RSM and second experiment was conducted by central composite design observing the effect of combination of two variables and keeping the one constant on ethanol production. During first experiment, maximum ethanol i.e 78.4 g/L was observed at conditions: pH 5.5, temperature 350C and substrate concentration of 160g. During the second experiment, the maximum ethanol production was 74.6 g/L at conditions: pH 5.8, temperature 310C and substrate concentration 160 g. The RSM is a better method for optimization of parameters as it is less labor intensive. It reduces the number of fermentation batches. The adequacy of all the models was satisfactory as coefficients of determination were found to be (0.9923) (0.9735) (0.9662).

1. INTRODUCTION

The perception of sustainable development has been greatly developed as a means of integrating the environmental, social and economic objectives of the society in order to maximize human well-being in the present system without compromising the ability of future generations. Development that is not sustainable will inevitably lead to negative environmental, social and economic repercussions (OECD 2001). Energy is the vital need of mankind and is the priceless gift offered by the nature. Energy is needed in various forms in our day-to-day life. At present, most of our energy requirements are fulfilled by non-renewable sources.

The ever increasing use of fossil fuel and the fear of extinguishing the petroleum stocks has led us to rethink about the use of renewable energy sources that reduce carbon dioxide (CO_2) emissions. Among the most promising renewable energy sources is the use of bio-fuels as an economical substitute for petroleum-based fuels. Bio-fuels, such as ethanol, are generally considered renewable since the CO_2 emitted into the atmosphere is recaptured by the growing crop in the next growth cycle.

The most important issues relevant to the conversion of carbohydrates to ethanol are the cost and availability of substrate. It is as a consequence worthy to develop an economical process which allows the use of cheap substrates such as starch and cellulose into fermentable sugars and their successive conversion to ethanol. For the long term planning various cellulosic substances appear to be striking as raw materials but currently are not competitive as ethanol sources. Starchy materials, however, have been proposed and have proven viable as a substrate for ethanol production (Lee *et al*., 1985). The key to success of conversion of starch into sugars is the availability of highly active enzymes, suitable strain and the optimization conditions of substrate concentration, temperature and pH.

Maize is the top ranking crop of the world. India is at sixth position in maize production and fifteenth position in its productivity worldwide. It is the third major crop of India after rice and wheat that provides food, feed and fodder to the livestock and serves as a source of basic raw materials for a number of industrial products mainly

starch, corn oil, corn syrup, alcoholic beverages, cosmetics, biofuel and many more. As per the latest figures available around 11 per cent of the maize produced is used as human food, 53 as animal feed, 14 per cent as industrial raw material and around 22 per cent is being export. As a result of single cross hybrid technology, maize achieved the highest growth rate (6.7%) amongst cereals as against the required growth rate of 4.7 % set by the XI planning commission of India. Consequently, India became importer to exporter of maize. Father of green revolution, renowned Nobel laureate Dr. Norman E. Borlaug mentioned that maize is the crop of future. Maize is one of the nature's greatest multiplier of starch. Exactly 3 months after sowing, a single corn kernel produces more than 600 kernels. Maize kernel possesses around 70 per cent of starch which can be one of the best natural sources to be converted to ethanol. Due to increase in area production and productivity, India is producing surplus maize and the trend is likely to go on and the excess from the food basket can be used to secure us at the platform of energy. Today, maize has set the stage to roll the dice and definitely ethanol will hit the right note. Keeping in view the increasing production of maize, the present study was designed to optimize the conditions required for conversion of maize for efficient ethanol production.

2. HISTORY AND REVIEW OF THE LITERATURE

2.1 History

2.1.1 Ethanol

The fermentation of sugar into ethanol is one of the most primitive organic reactions that man learned to carry out and the history of man-made ethanol is very long. Ethanol is a powerful psychoactive substance and ethanol history is filled with accounts detailing its use as a recreational drug. Dried ethanol residue has been found on 9000 year old pottery in China which indicates that Neolithic people in this part of the world may have consumed alcoholic beverages.

Ethanol, both liquor and a fuel, has been around in the form of Moonshine Whiskey since 15th Century Scotland. The year 1796 is significant for ethanol history because this is when Johann Tobias Lowitz obtained pure ethanol by filtering distilled ethanol through activated charcoal. Ethanol is not a new-fangled 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.

Antoine Lavoisier was able to ascertain that ethanol consists of hydrogen, oxygen and carbon, but it wasn't until the early 19th century that the chemical formula was determined by Nicolas-Théodore de Saussure. During the mid 1800s, ethanol became one of the first structural formulas to be determined. The scientist behind the description was Scottish chemist Archibald Scott Couper. Ethanol (CH_3CH_2OH) is a clear, colorless liquid with a characteristic agreeable odour. It is an alcohol, a group of chemical compounds whose molecules contain a hydroxyl group –OH, bonded to a carbon atom. The word alcohol derives from Arabic word- ‘al-kuhul’, which denotes a fine powder of antimony produced by distilling antimony and used as an eye make-up. Alcohol originally referred to as any fine powder, but medieval alchemists later applied the term

to the refined products of distillation, and this led to its present usage. Ethanol melts at -114.1°C, boils at 78°C and has a density of 0.789 g/ml at 20°C. Its low freezing point has made it useful as fluid for thermometers for temperature below -40°C, the freezing point of mercury, and for the other low temperature purposes, such as for antifreeze in automobile radiators. Ethanol has lower energy content than gasoline. That means that about one-third more ethanol is required to travel the same distance as on gasoline. But other ethanol fuel characteristics, including a high octane rating, result in increased engine efficiency and performance.

2.1.2 Raw material for ethanol production

Today, ethanol industry utilizes raw materials rich in saccharides, such as sugar cane or sugar beets, and raw materials rich in starch, such as corn and wheat (Rudolf *et al.,* 2009). Various raw materials like sugarcane juice and molasses (Morimura *et al*., 1997 and Aggarwal *et al.,* 1998), sugar beet, beet molasses (EI-Diwany *et al*., 1992 and Agrawal *et al,*. 1998), Sweet sorghum (Bulawayo *et al.,* 1996) and starchy materials like sweet potato (Sree *et al*., 1999), Corn cobs and hulls (Beall *et al.,* 1992 and Arni *et al*., 1999), cellulosic materials like cocoa, pineapples and sugarcane waste (Othman *et al.,* 1992) and milk/cheese/whey using lactose hydrolyzing fermenting strains (Silva *et al.,* 1995, Ghaly and Ben-Hassan 1995) have been reported. Dabas *et al* studied ethanol production from wheat starch. Hydrolyzed wheat starch was used as a substrate for ethanol production using 2 strains of *S.cerevisiae*. Wheat flour slurry (25%w/v) was gelatinized and conditions were standardized for saccharification and fermentation of wheat starch for ethanol production.

2.1.3 Corn as a source of ethanol

Maize, the American Indian word for corn, literally means "that which sustains life". It is the most important cereal grain in the world, providing nutrients for humans, poultry and livestock and serve as a basic raw material for the production of starch, oil, protein, alcoholic beverages, food sweeteners and, more recently, bio fuel. The silage made out of the green part provides a nutritious and palatable feed for livestock. After harvest of the

grain, the stover is used to provide good forage for ruminant animals owned by many small farmers particularly in the developing countries.

Botanically, maize (*Zea mays*) belongs to the grass family (*Gramineae*) and is a tall annual plant. It is a cross pollinating species, with the female (ear) and male (tassel) flowers in separate places on the plant. The grain develops in the ears, or cobs, often one on each stalk; each ear has about 200 to 1000 kernels. The kernels are often white or yellow in colour, although black, red and a mixture of colours is also found. There are a number of grain types, distinguished by differences in the chemical compounds deposited or stored in the kernel. In India about 52 per cent of maize produced is used as poultry feed, about 24 per cent as food, 11 per cent as livestock feed, 11 per cent in wet milling industry and rest for breweries and seed with 1 per cent each.

2.1.4 Structure of Maize Kernel

Maize kernels develop through accumulation of the products of photosynthesis, root absorption and metabolism of the maize plant on the female inflorescence called the ear. This structure may hold from 200 to 1000 single kernels depending on the number of rows, diameter and length of the cob. During harvest the ears of maize are removed from the maize plant either by hand or mechanically. The husks covering the ear are first stripped off, and then the kernels are separated by hand or, more often, by machine.

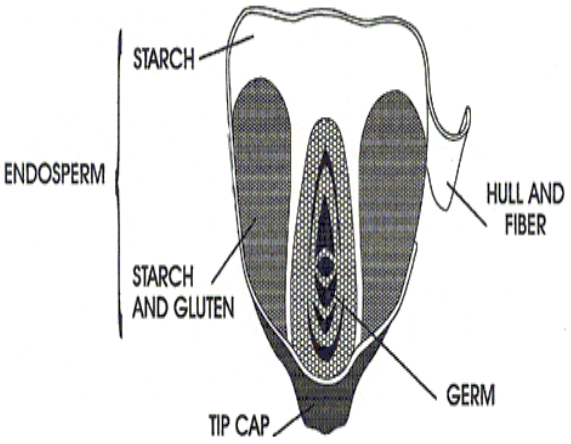


Figure 1: Internal structure of a Maize kernel

The maize kernel is known botanically as a caryopsis; a single grain contains the seed coat and the seed, as shown in **Figure 1**. The figure also shows four major physical structures of the kernel: the pericarp, hull or bran; the germ or embryo; the endosperm; and the tip cap (dead tissue found where the kernel joins the cob).

2.1.5 Fermentation

Fermentation is the chemical transformation of organic substances into simpler compounds by the action of enzymes, complex organic catalysts, which are produced by microorganisms such as molds, yeasts, or bacteria. Enzymes act by hydrolysis, a process of breaking down or predigesting complex organic molecules to form smaller (and in the case of foods, more easily digestible) compounds and nutrients. Fermentation is one of the oldest methods of food preparation and for the production of second generation fuels. The first fermented product prepared and consumed by humans was the fermented juice or the wine. Fermentation is the core of biotechnology where current methodologies span across technologies based on the use of either solid or liquid substrates.

During the course of human history, when a system of trial, error, and careful observation is used, cultures began producing fermented beverages. Mead, or honey wine, was produced in Asia during the Vedic times (around 1700–1100 BC), and the Greeks, Celts, Saxons, and Vikings also produced this beverage. In Egypt, Babylon, Rome, and China, people produced wine from grapes and beer from malted barley. In South America, people produced chicha from grains or fruits, mainly maize; while in North America, people made octli (now known as "pulque") from agave, a type of cactus.

At that time, people knew that leaving fruits and grains in covered containers for a long time produced wine and beer; however, no one fully understood the reason behind it. The process was named fermentation, from the Latin word *fervere*, which means "to boil." The name came from the observation that mixtures of crushed grapes kept in large vessels produced bubbles, as though they were boiling. To produce fermented beverages was tri cky. If the mixture did not stand long enough, the product contained no alcohol; but if left for too long, the mixture rotted and was undrinkable. Through experimental observation, people learned that temperature and air exposure are key to the fermentation process.

Traditionally, wine producers used their feet to soften and grind the grapes before leaving the mixture to stand in buckets. During the process, they transferred

microorganisms from their feet into the mixture. At that time, no one knew that the alcohol produced during fermentation was produced because of one of these microorganisms — a tiny, one-celled eukaryotic fungus that is too small to see through the naked eye: yeast. It took several hundred years before quality lenses and microscopes revolutionized science and allowed researchers to detect these microorganisms.

In the seventeenth century, a Dutch tradesman named Anton van Leeuwenhoek, developed high-quality lenses and he was able to view yeast for the first time. Leeuwenhoek discovered that yeast consists of globules floating in a fluid, but he thought they were simply the starchy particles of the grain from which the wort (liquid obtained from the brewing of whiskey and beer) was made (Huxley 1894). Later, in 1755, yeast was defined in the Dictionary of the English Language by Samuel Johnson as "the ferment put into drink to make it work; and into bread to lighten and swell it." At that era nobody believed that yeast were alive; they were seen as just organic chemical agents required for fermentation.

In the eighteenth and nineteenth centuries, chemists worked hard to decode the nature of alcoholic fermentation through analytical chemistry and chemical nomenclature. In 1789, a French chemist Antoine Lavoisier, has worked on the transformations of substances. In his quest, he decided to use sugars for his experiments, and he gained new knowledge about the structures and chemical reactions of sugars. Using quantitative studies, he learned that sugars are composed of a mixture of hydrogen, charcoal (carbon), and oxygen. Although, Lavoisier was also interested in analyzing the mechanism by which sugarcane is transformed into alcohol and carbon dioxide during fermentation. He estimated the proportions of sugars and water at the beginning of the chemical reaction and compared them with the alcohol and carbon dioxide proportions obtained at the end. He also added yeast paste (or "ferment," as it was called) to proceed for the alcoholic reaction. He concluded that sugars were broken down through two chemical pathways: Two-thirds of the sugars were reduced to form alcohol, and the other third were oxidized to form carbon dioxide (the source of the bubbles observed during fermentation). Lavoisier predicted (according to his famous conservation-of-mass principle) that if it was possible to combine alcohol and carbon dioxide in the right

proportions, the resulting product would be sugar. The experiment provided a clear insight into the basic chemical reactions needed to produce alcohol. Later, the chemists hypothesized that the yeast initiated alcoholic fermentation but did not take part in the reaction. They assumed that the yeast remained unchanged throughout the chemical reactions

In 1815 the French chemist Joseph-Louis Gay-Lussac made some interesting observations about yeast. Gay-Lussac has experimented for preventing perishable food from rotting with a method developed by Nicolas Appert, a confectioner and cooker. He was interested in using the method to maintain grape juice wort in an unfermented state for an indefinite time. The method consisted of boiling the wort in a vessel, and then tightly closing the vessel containing the boiling fluid to avoid exposure to air. With this method, the grape juice remained unfermented for long periods as long as the vessel was kept closed. However, if yeast (ferment) was introduced into the wort after the liquid cooled, the wort would begin to ferment. There was now no doubt that yeast was indispensable for alcoholic fermentation. But their role in the process was still unidentified

When more powerful microscopes were developed, the nature of yeast came to be better understood. In 1835, a French inventor, Charles Cagniard de la Tour, observed that during alcoholic fermentation yeast multiply by gemmation (budding). His observation confirmed that yeasts are one-celled organisms and suggested that they were closely related to the fermentation process. Around the same time, Theodor Schwann, Friedrich Kützing, and Christian Erxleben independently concluded that "the globular, or oval, corpuscles which float so thickly in the yeast [ferment] as to make it muddy" were living organisms (Barnett 1998). The recognition that yeasts are living entities and not merely organic residues changed the prevailing idea that fermentation was only a chemical process. This discovery paved the way to understand the role of yeast in fermentation.

Pasteur was the first who demonstrated experimentally that fermented beverages resulted from the action of living yeast that was able to transform glucose into ethanol. Moreover, Pasteur demonstrated that only these microorganisms are capable of converting sugars into alcohol from grape juice, and that the process occurs in the

absence of oxygen. He concluded that fermentation is a vital process, and he defined it as respiration without air (Barnett 2000; Pasteur 1876).

Pasteur performed careful experiments and demonstrated that the end products of alcoholic fermentation are more numerous and complex than those initially reported by Lavoisier. Along with alcohol and carbon dioxide, there were also significant amounts of glycerin, succinic acid, and amylic alcohol (some of these molecules were optical isomers). These observations suggested that fermentation was an organic process. Pasteur reproduced fermentation under experimental conditions so as to confirm his hypothesis, and his results showed that fermentation and yeast multiplication occur in parallel. He realized that fermentation is a consequence of the yeast multiplication, and the yeast has to be alive for alcohol to be produced. Pasteur published his seminal results in a preliminary paper in 1857 and in a final version in 1860, which was titled "Mémoire sur la fermentation alcoolique" (Pasteur 1857).

In 1856, a man named Bigo sought Pasteur's help because he was having problems at his distillery, which produced alcohol from sugar beetroot fermentation. The contents of his fermentation containers were embittered, and instead of alcohol he was obtaining a substance similar to sour milk. Pasteur analyzed the chemical contents of the sour substance and found that it contained a substantial amount of lactic acid instead of alcohol. When he compared the sediments from different containers under the microscope, he noticed that large amounts of yeast were visible in samples from the containers in which alcoholic fermentation had occurred. In contrast, in the polluted containers, the ones containing lactic acid, he observed "much smaller cells than the yeast." Pasteur's finding showed that there are two types of fermentation: alcoholic and lactic acid. Alcoholic fermentation occurs by the action of yeast; lactic acid fermentation, by the action of bacteria.

By the end of the nineteenth century, Eduard Buchner had shown that fermentation could occur in yeast extracts of free cells, which made it possible to study fermentation biochemistry in vitro. He prepared cell-free extracts by carefully grinding yeast cells with a pestle and mortar. The resulting moist mixture was put through a press

to obtain a juice to which sugar was added. Using a microscope, Buchner confirmed that there were no living yeast cells in the extract.

Upon studying the cell-free extracts, Buchner detected zymase, the active constituent of the extracts that carries out fermentation. He realized that the chemical reactions responsible for fermentation were occurring inside the yeast. Today researchers know that zymase is a collection of enzymes (proteins that promote chemical reactions). Enzymes are part of the cellular machinery, and all of the chemical reactions that occur inside cells are catalyzed and modulated by enzymes. For his discoveries, Buchner was awarded the Nobel Prize in Chemistry in 1907 (Barnett 2000; Barnett & Lichtenthaler 2001; Encyclopaedia Britannica 2010).

2.1.6 Starch: the actual raw material

Starch is the basic precursor or substrate required for ethanol production. Corn is the most significant and economical source of starch. Corn kernel comprises 68-75% starch by weight. Starch is easily converted into glucose and fermented into ethanol.

2.1.6.1 Chemistry of Starch

Starch, is the energy-reserving compound. Starch is produced by green plants and can be found in different parts of the plant, including stems, leaves, fruits, tubers and roots. Starch granules for temporary storage are synthesized in chloroplasts, while for long-time storage, starch granules are produced and stored in amyloplasts (Robyt, 1998).

Starch is a high molecular weight compound, which on hydrolysis yield alpha-D-glucose. It consists of two polysaccharides: amylose and amylopectin, on complete hydrolysis both of these polysaccharides yield alpha-D-glucose. Amylose is a straight chain polymer composed of D-glucopyranose residues linked by α- 1, 4 glycosidic bonds. Amylopectin is a branched molecule having short linear chains, so amylopectine in addition to α- 1, 4 linkages also contains α-1, 6 glycosidic bonds linkages. (Banks *et al*., 1975 and Robyt, 1998). The amylose content in waxy starch is small (0-8% amylose), while high-amylose starch contains 50% or more amylose (Li *et al.,* 2008 and Perez *et al.,* 2010). High-amylose starch also consists of another polysaccharide known as the

intermediate component (IC), which has a similar molecular weight to that of amylose but contains relatively more branched structure (Baba and Arai, 1984). Amylose, amylopectin, and IC molecules have different characteristics of molecular weight, molecular structure, physical and chemical properties (e.g.crystallinity, complex formation). The composition, molecular structures and organization of these molecules in starch granules have significant effects on structures and properties of the starch.

2.1.6.2 Organization of starch granule

In a starch granule, amylopectin and amylose molecules are methodically oriented side by side from the hilum to the periphery. Amylopectin branch-chains form double helices and are clustered to form a crystalline lamella with a thickness of 9-10nm (Jenkins *et al.,* 1993). It is proposed that branch-chains of amylopectin are organized in parallel to form clusters and crystalline regions (Gallant *et al.,* 1997). The cluster structure is stabilized by hydrophobic interaction, hydrogen bonds and vander Waals forces between double helices of branch-chains (Imberty *et al*., 1988). Branch points of the branch-chains consist of the amorphous regions. The alternating crystalline and amorphous regions contribute to the semi-crystallinity of the starch granule. Amylose is in amorphous form and interdispersed among amylopectin molecules. Amylose molecules are more concentrated at the periphery of starch granule, and contributeto the integrity of the granular structure by intertlinking with amylopectin (Jane *et al.,* 1986).

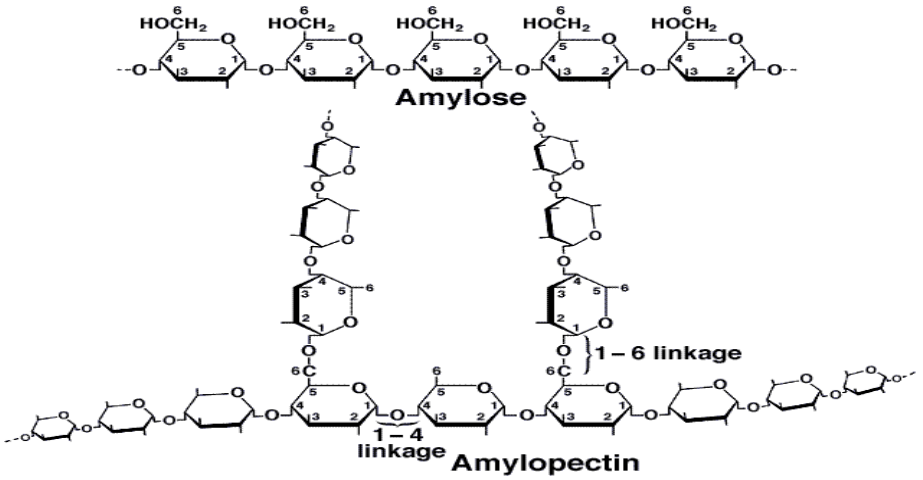


Figure 2: Structure of starch molecule

2.1.6.3 Starch hydrolysis

Hydrolysis of starch to glucose is important for the maximum utilization of starch to provide energy for animals and plants, as well as to provide the substrate for ethanol fermentation by yeast. The starch digestibility is significantly affected by amylose/ amylopectin ratio of starch. Digestibility of starch is impacted by a lot of factors, including the processing method, hydrolyzing enzymes, amylose/ amylopectin ratio, protein and lipid content, granular size and surface area, and starch granular structures (Rooney *et al*., 1986, Svihus *et al*., 2005 and Tester *et al*., 2004). Normal starch is usually less digestible than its waxy counterpart, and high-amylose starch has even poorer digestibility in both uncooked and cooked forms (Gallant *et al.,* 1972, Perera *et al*., 2001, Rooney *et al*., 1986). Amylose is known to be concentrated at the periphery of starch granule and intertwines with amylopectin, making the starch granule more resistant to enzyme hydrolysis (Jane, 2007). Studies on high amylose corn starch showed that the starch granules retained partially crystalline structures after cooking, and the resistant starch content positively correlated with the amylose content (Knutson *et al.,* 1982 and Li *et al.,* 2008).

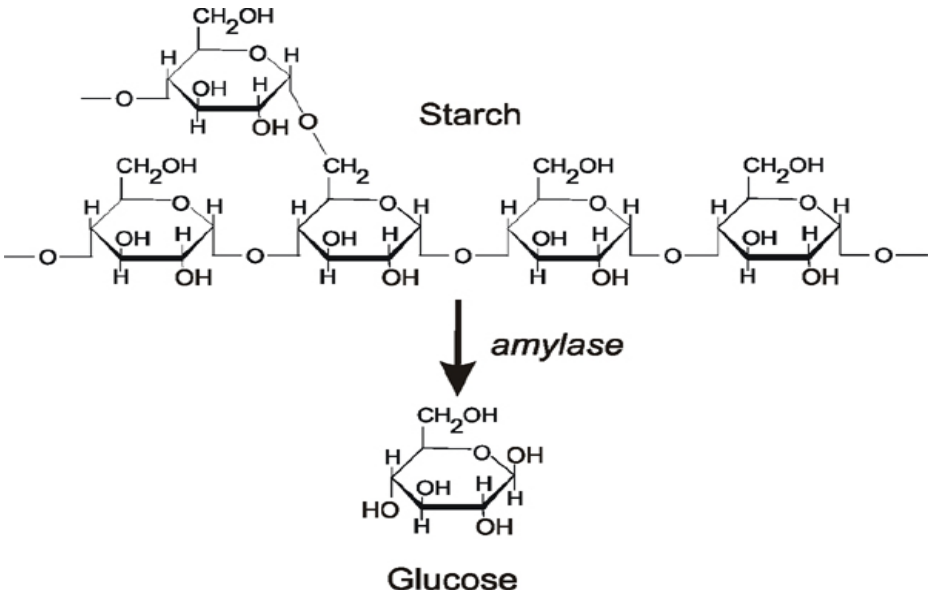


Figure 3: Hydrolysis of starch to glucose

2.1.7 Sugar Decomposition

The metabolic pathway which facilitates the conversion of glucose (a type of sugar) into pyruvate is known as glycolysis. It is the first major step of fermentation or respiration in cells. It is an ancient metabolic pathway that probably developed about 3.5 billion years ago, when no oxygen was available in the environment. Glycolysis occurs not only in microorganisms, but in every living cell (Nelson and Cox 2008).

As of its importance, glycolysis was the first metabolic pathway resolved by biochemists. However, the scientists that were studying glycolysis faced an enormous challenge as they figured out that many chemical reactions were involved, and the order in which these reactions took place. In glycolysis, a single molecule of glucose (with six carbon atoms) is transformed into two molecules of pyruvic acid (each with three carbon atoms). Around 1929, Karl Lohmann, Yellapragada Subbarao, and Cirus Friske independently discovered an essential molecule called adenosine triphosphate (ATP) in animal tissues. ATP is a versatile molecule used by enzymes and other proteins in many cellular processes. It is required for many chemical reactions, such as sugar degradation and fermentation (Voet and Voet 2004). The complete glycolytic pathway, which involves a sequence of ten chemical reactions, was elucidated around 1940. In 1941, Fritz Albert Lipmann proposed that ATP was the main energy transfer molecule in the cell.

Scientists began to understand glycolysis, by analyzing and purifying the labile component of cell-free extracts, which Buchner called zymase. They also detected a low-molecular-weight, heat-stable molecule, later called co-zymase. Using chemical analyses, they learned that zymase is a complex of several enzymes; and cozymase is a mixture of ATP, ADP (adenosine diphosphate, a hydrolyzed form of ATP), metals, and coenzymes (substances that combine with proteins to make them functional), such as NAD^+ (nicotinamide adenine dinucleotide). Both the components were required for the fermentation process.

In glycolysis, two molecules of ATP are produced for each broken molecule of glucose. During glycolysis, two reduction-oxidation (redox) reactions occur. In a redox reaction, one molecule is oxidized by losing electrons, while the other molecule is reduced by gaining those electrons. A molecule called NADH acts as the electron

carrier in glycolysis, and this molecule must be reconstituted to ensure continuity of the glycolysis pathway.

2.1.7.1 The Chemical Process of Fermentation

The glucose is converted into pyruvic acid during glycolysis. When oxygen is available, pyruvic acid enters a series of chemical reactions (known as the tricarboxylic acid cycle) and proceeds to the respiratory chain. As a result of respiration, cells produce 36–38 molecules of ATP for each molecule of glucose oxidized.

In the absence of oxygen, depending on the type of cell, pyruvic acid can follow two different routes. It can be converted into ethanol (alcohol) and carbon dioxide through the alcoholic fermentation pathway, or it can be converted into lactate through the lactic acid fermentation pathway.

The alcoholic fermentation is carried out by yeast of the genus *Saccharomyces*. The most common species involved is *S cerevisiae*. *Saccharomyces* converts the glucose or fructose into ethanol via a process of fermentation. During the process of fermentation, an organic compound serves as terminal electron acceptor. This leads to the production of ethanol.

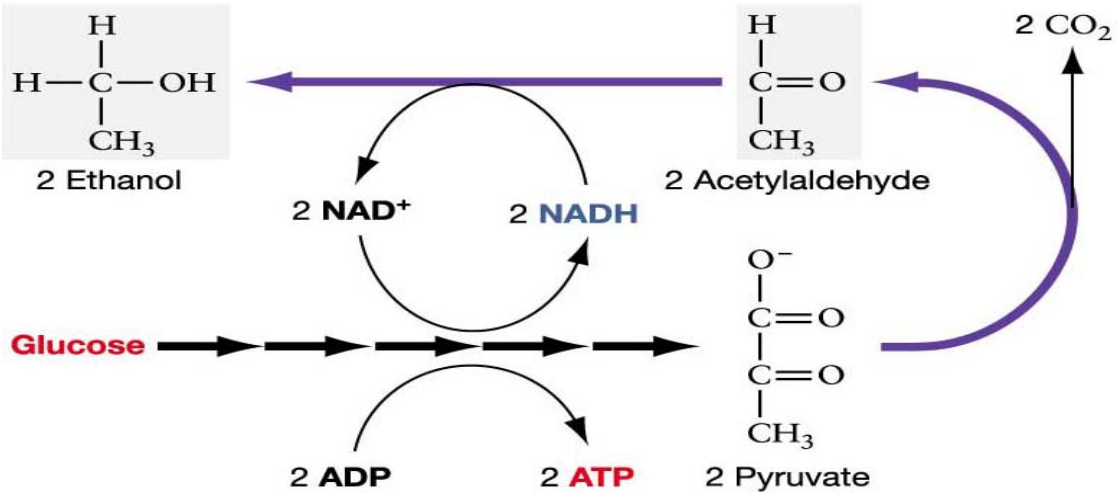


Figure 4: Pathway of ethanol production from glucose

2.1.8 Use of corn as ethanol: historical perspectives

More than decade ago the United States began a hard line search to find a practical source of renewable fuel to meet its voracious energy demands. Alternative fuels such as starch-based ethanol, cellulosic ethanol, and biodiesel are all considered to be potential solutions in a national effort to reduce gasoline usage by 20 percent over the next ten years (Bush, 2007). Corn-based ethanol emerged as an early leader due to the abundance of corn and the popularity of ethanol-gasoline mixtures.

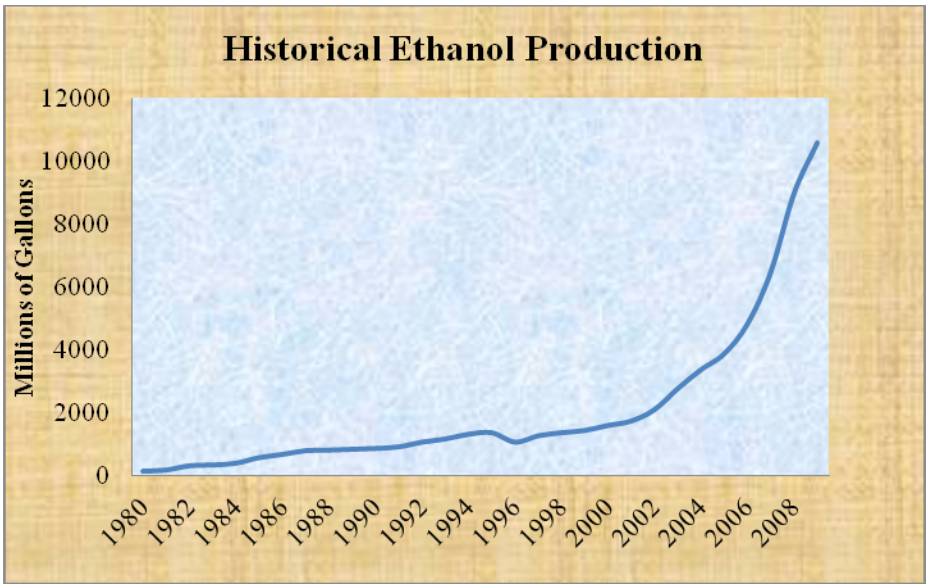
The United States is the major producer of corn world-wide (roughly 40% of the world's harvest in 2009). Since 2005, it is also the world's leading producer of ethanol fuel. Most of the ethanol fuel produced in the U.S. is derived from corn (maize). In the US, ethanol from corn history is the history of subsidies, since the sector didn't really take off until the government decided to subsidize it. The Energy Tax Act of 1978 created ethanol tax credits in an effort to decrease the America's susceptibility to oil.

Between 1979 and 1986, domestic production of ethanol rose considerably in the U.S., from a mere 20 million U.S. liquid gallons (over 75 million liters) to 750 million gallons (around 2.84 billion liters). In 1990, small-scale producers received an additional tax credit of 10 cents per gallon. By 2004, the US ethanol production had grown even more.

The Energy Policy Act of 2005 was another important step in corn ethanol history. It mandated an annual consumption of 7.5 billion gallons of ethanol by 2012. Two years later, the mandate was increased to 15 billion gallons of corn ethanol by 2015.

In 2009, the U.S. produced 10.6 billion gallons of ethanol fuel and a vast majority of this fuel was corn based. Together, the U.S. and Brazil stood for nearly 90% of all the ethanol fuel produced that year. However, Brazil does not rely on corn for ethanol production; their crop of choice is sugarcane.

The US government has supported the use of ethanol as a policy to reduce dependence on foreign oil since the 1970's. In the 1990's it became popular to blend ethanol as an oxygenate in conventional gasoline to reduce smog. Ethanol production



Renewable fuel association

standards were set in place by the Energy Policy Act of 2005, and then updated as part of the Energy Independence and Security Act of 2007. According to the 2010, ethanol Industry Outlook, the 2009 production of 10.6 billion gallons of ethanol reduced the demand for oil by 364 million barrels. Ethanol production is scheduled to reach 36 billion gallons by 2022.

The majority of American-made ethanol is produced from corn, while a small amount is produced from cheese whey, wood waste, or other grains. Corn-based ethanol plants represent 92 percent of all plants, but almost 99 percent of all U.S. ethanol production. Depending on plant technology, an average of 2.8 gallons of ethanol can be produced from one bushel of corn (RFA). As technology improves, plants are continually striving to produce more ethanol with fewer inputs to improve efficiency and reduce costs.

A traditional dry mill ethanol plant produces a number of co-products such as dried distillers' grains with solubles (DDGS), and carbon dioxide (CO_2). On average a plant can produce 18 pounds of DDGS per bushel of corn used in ethanol production and most plants are able to sell it to the livestock industry as a high value feed source. If there is a market opportunity, some plants are able to sell the CO_2 to the food processing and

bottling industries. Also, ethanol wet mills can produce corn gluten meal, corn gluten feed, sweeteners and corn oil which can also be sold to their respective industries

2.1.9 Brief overview of ethanol: from beverages to biofuel

- In 1826, Samuel Morey developed an engine that ran on ethanol and turpentine.
- In 1850's, During the Civil War, a liquor tax was placed on ethanol whisky, also called Moonshine, to raise money for the war.
- In 1876, Otto Cycle was the first combustion engine designed to use alcohol and gasoline.
- In 1896, Henry Ford built his first automobile, the quadricycle, to run on pure ethanol.
- In 1908, the first Ford Motor Company automobile, Henry Ford's Model T, was designed to use corn alcohol, called ethanol. The Model T ran on (ethanol) alcohol, fuel or a combination of the two fuels.
- In 1920's, Standard Oil began adding ethanol to gasoline to increase octane and reduce engine knocking.
- In 1940's, First U.S. fuel ethanol plant built. The U.S. Army built and operated an ethanol plant in Omaha, Nebraska, to produce fuel for the army and to provide ethanol for regional fuel blending.
- 1940's to late 1970's virtually no commercial fuel ethanol was sold to the general public in the U.S. - due to the low price of gasoline fuel.
- In 1975, U.S. begins to phase out lead in gasoline. MTBE eventually replaced lead.
- Later, in 2004 to 2006, MTBE banned in almost all states, due to groundwater contamination and health risks.

- In 1980's, Oxygenates added to gasoline included MTBE (Methyl Tertiary Butyl Ether - made from natural gas and petroleum) and ETBE (Ethyl Tertiary Butyl Ether - made from ethanol and petroleum).
- In 1988, Denver, Colorado, was the first state to mandate ethanol oxygenates fuels for winter use to control carbon monoxide emissions. Other cities soon followed.
- In 1990, Clean Air Act Amendments - Mandated the winter use of oxygenated fuels in 39 major carbon monoxide non-attainment areas (based on EPA emissions standards for carbon dioxide not being met) and required year-round use of oxygenates in 9 severe ozone non-attainment areas in 1995.
- In 1992, The Energy Policy Act of 1992 (EPAct) was passed by Congress to reduce nation's dependence on imported petroleum by requiring certain fleets to acquire alternative fuel vehicles, which are capable of operating on nonpetroleum fuels.
- The Clean Air Act (1990) and Alternative Motor Fuels Act (1998 & 1992) contain provisions for mandating oxygenated fuel (RFG =Ethanol and MTBE). Requirements set for 2 types of clean-burning gasoline, RFG Federal Reformulated Gasoline and Wintertime Oxygenated Fuel.
- In 1995, The EPA began requiring the use of reformulated gasoline year round in metropolitan areas with the most smog

2.1.10 Ethanol as a fuel

An important measure of the performance of a fuel is octane rating. High octane rating is needed, because it means that more compression the fuel can withstand before self-ignition, which allows enhancing the engine's compression ratio for better thermal efficiency (Freudenberger, 2009b). Ethanol fuel has higher octane rating (106) than conventional gasoline fuel (usually 87-93). The heating value of ethanol, however, is only about 63% of the gasoline, which consequence of the presence of oxygen in the

molecular structure of ethanol (Freudenberger 2009a). Ethanol blend fuels have been blamed for several issues about the engine performance. Because ethanol has higher flash point and latent heat of vaporization, it is less volatile than gasoline, which raises concerns for starting difficulties of engine using blend gasoline in the cold weather. In effect, this is not a major issue as engine can be started on gasoline in the blends and generates enough heat to vaporize ethanol sufficiently (Freudenberger 2009b). Another issue challenging the use of ethanol blend is that ethanol could cause corrosion and degradation to metal parts, fuel lines, seal, in the engine. But the damage is related to the water content in the ethanol. In the U.S., the ethanol blended into gasoline is anhydrous, and when the water content is below 5%, the corrosive effects are not significant (Freudenberger 2009b).

In India, the first phase of the project, ethanol- blended petrol is supplied through retail outlets in nine States and four Union Territories. These states are Andhra Pradesh, Goa, Gujarat, Haryana, Karnataka, Maharashtra, Punjab, Tamil Nadu and Uttar Pradesh. The four Union Territories include Chandigarh, Dadra and Nagar Haveli, Daman and Diu and Pondicherry. Petrol blended with 5 per cent ethanol is supplied by petrol pumps all over the country under the second phase towards the end of the year. The content of ethanol blending would be increased to 10 per cent in the third phase of the programme.

Ethanol is used as an automotive fuel by itself and can be mixed with gasoline called "gasohol" Fuel Ethanol- the most common blends contain 10% ethanol and 85% ethanol mixed with gasoline. In the United States, over 1 billion gallons of ethanol are blended with gasoline every year. Because the ethanol molecule contains oxygen, it allows the engine to more completely combust the fuel, resulting in fewer emissions. Since ethanol is produced from plants that harness the power of the sun, ethanol is also considered a renewable fuel. Therefore, ethanol has many advantages as an automotive fuel.

Many common ethanol fuel mixtures are in use around the world. The use of pure hydrous or anhydrous ethanol in internal combustion engines (ICEs) is only possible if the engines are designed or modified for that purpose. Anhydrous ethanol can be blended

with gasoline (petrol) in various ratios for use in unmodified gasoline engines, and with minor modifications, can also be used with a higher content of ethanol.

Ethanol fuel mixtures have "E" numbers which describe the percentage of ethanol fuel in the mixture by volume, for example, E85 is 85% anhydrous ethanol and 15% gasoline. Low-ethanol blends, from E5 to E25, are also known as gasohol, though internationally the most common use of the term refers to the E10 blend.

The push for ethanol as an alternative to imported oil spurred the construction of 172 plants in 25 states by the end of 2008. But during 2009 falling oil prices has made ethanol less cost effective. More than 20 plants have recently closed.

Despite 10% being the universally accepted legal limit for ethanol in conventional gas-powered engines, in March 2009 ACE, Growth Energy and 54 ethanol producers submitted a waiver application to increase E10 to E15 which was accepted and now E15 blend is used.

2.1.11 Use of ethanol as biofuel in India

India is one of the fastest growing economies of the world with its gross domestic product (GDP) growing at an average annual rate of over seven percent since 2005. To maintain this rate of growth, energy inputs are critical. However concerns, that the conventional source of energy will be exhausted have prompted the nation to view bio-fuels as a potential alternative to conventional liquid fossil fuels. In this regard ethanol has emerged as an important renewable fuel for transportation purposes.

Energy is the critical input required to achieve our socio-economic goals and sustain growth at the same rate. Of the total primary energy consumption of the country, a large share is accounted for by fossil fuels, of which oil constitutes a 32 percent share. Like all other fuel-importing economies in the world, India too, is facing a shortage of conventional fossil fuels. Biofuels have emerged as a substitute for fuel oil for countries like India. Hence many countries including India have both mandated biofuel use as well as offered fiscal incentives for the promotion of biofuel. To promote biofuels, ethanol in particular, the Indian Government initiated an ethanol blending policy in 2003.

India is initiating the use of ethanol as an automotive fuel. A move has been made by distilleries in India to use surplus alcohol either as a blending agent or as an oxygenate in gasoline.

2.2 Review of Literature

Over the decades, energy consumption has increased immensely by 16-fold as the world population quadrupled in the twentieth century, and more countries have become industrialized (Sun and Cheng 2002; Sanchez and Cordona 2008; Zhang 2008). If the current drift persists, the total energy consumption is expected to rise to 27–42 Terawatt (TW) from the current 13 TW by the year 2050 (Whitesides and Crabtree 2007).

Use of ethanol as fuel is not a novel concept; Henry Ford designed his 1908 Model T to run on ethanol (Kuvarik *et al.,* 1998). Although many process are employed for ethanol production from corn- quick fibre (Singh *et al.,* 1999), quick germ (Singh and Eckhoff 1996), COPE process (Cheryan 2002), enzymatic milling (Johnston *et al.,* 2003), dry grind and wet milling) .Dry grind process was found to be better due to less capital and energy intensive (Butzen and Haefele 2008). Fermentation is one of the oldest processes known to man (Bothast and Schilcher 2005)

Bialas *et al.,* (2010) conducted the experiment to examine the effect of stillage recirculationon of fuel ethanol production by using *saccharomyces cerevisiae* and a granular starch using hydrolyzing enzyme in a SSF by using a native starch from corn flour. They concluded that the ethanol yield was not influenced by the recycling of stillage. The results of their experiment showed that, repeated batch of SSF process conducted on native starch by using GSH novel enzymes have standed as an excellent alternative for traditional technology in the production.

Singh *et al.,* (1999) concluded that corn fibre samples can be easily recovered in dry-grind ethanol production by floatation. Dien *et al.,* (2002) studied the fate of recombinant Bt protein (CRY Ab from *Bacillus thuringiensis*) in different corn hybrids by both dry-grind and wet- milling processes. They concluded that ethanol yield is not completely depended on starch content.

Nikolic *et al.,* (2010) conducted the ethanol fermentation from ezymatically obtained corn meal hydrolyzates by free and immobilized *S.cerevisiae* and optimized the parameters such as the initial glucose concentration, inoculum concentration and fermentation time for efficient ethanol production. They found that optimal inoculum

concentration of 2% (v/v), fermentation time of 38 hrs for both free and immobilized yeast. However, the initial glucose concentration of 150 g/L in free yeast cells and 176 g/L in an immobilized yeast cells were found to be optimum. So, they concluded that immobilized cell system was superior to free cells.

Kunamneni and Singh (2005) studied the effects of doses of pre-cooking α- amylase, post- cooking α- amylase, glucoamylase and saccharification temperature to attain the maximum conversion efficiency of maize starch to glucose by using crude enzyme. They used CCD and RSM to design the experiments. The optimum values for maximum conversion efficiency they found were: pre-cooking α- amylase (2.243 U/mg), post-cooking α- amylase (3.383 U/mg), glucoamylase (0.073 U/mg) and saccharification temperature of 55.1^{o}C. The maximum conversion efficiency was of 96.25 %.

Torija *et al.,* (2001) observed a mixed response to fermentation temperature (15-35°C) on mixed strain population of S.cerevisiae. Some strains performed better at higher temperature, while others did so at lower temperature. Alcohol yield was higher at lower temperature while at higher temperature secondary metabolites increased. Yadav *et al*., (1997) found an increase in alcohol concentration, productivity as well as efficiency with an increase in pH from 4.0-5.0 and found that the optimum pH range for S.cerevisiae strain HAU-1 to be between pH 4.5-5.0.

Manikandan and Viruthagiri (2010) conducted the experiment to study the effect of substrate, enzyme concentration, pH and temperature on ethanol production. The optimum values of substrate concentration, the pH, temperature and the enzyme were found to be 160g/l, 5.5, 30ºC and 50 IU.

Rausch *et al.,* (2009) optimized parameters like mill type, dry solids, yeast, glucoamylase dose and found, yeast addition of 1.2g/100g of corn, glucoamylase dose of 80 or 160µl/100g of corn.

Kwiatkowski *et al.*, in 2005 have developed a new model for dry- grind process namely “super pro designer” which produces more ethanol than normal dry- grind process. Ethanol (55.3 g/l) was produced from sago starch using a starch concentration of 150 g/l. The optimum conditions they found to be a temperature of 32.4 ◦C, pH of 4.93 and time of fermentation of 17.24 h. (Bandaru *et al*., 2005)

Kademi *et al.,* (1996) studied the effect of susbstrate concentration on ethanol fermentation. They concluded that at high hydrolysate concentration growth parameters during fermentation were inhibited; however, catabolic parameters did not affected. This is due to increase in medium osmolality.

Stenberg *et al.,* (2000) investigated the effect of substrate concentration on ethanol production and concluded that the ethanol production is reduced at high substrate concentration. This is due to the fact that high substrate concentration cause inefficient fermentation while a lower substrate concentration resulted in increased formation of lactic acid, which lowered the yield. Compared with separate hydrolysis and fermentation, SSF gave a higher yield and doubled the productivity.

2.2.1 Ethanol economics and market

Record high fuel prices and public policy initiatives continue to rouse interest in renewable fuels including ethanol. Communities seeking economic development opportunities, job creation, tax base diversification and new capital investment are quick to recognize the economic benefits of a local ethanol processing plant. These benefits are obvious from the point of initial construction and continue to expand throughout the operating life of the plant. Several economic analyses have examined these impacts and quantified the benefits. Each ethanol plant generates similar but different benefits depending on local and state tax rates, employment requirements and a host of other factors.

The ethanol industry is an important provider to the employment, income of the rural families, and development of rural economies. At the end of 2011, the ethanol industry is comprised of about 209 plants in 29 states in U.S., with a gross production capacity of 14.7 billion gallons. The ethanol industry supported 90,200 direct jobs and 31,400 indirect jobs across the United States in the year 2011 and contributed 42.4 billion to the National gross domestic product (GDP) (Urbanchuk, 2012). The ethanol industry has increased 29.9 billion incomes to American families in 2011, mostly to corn growers who benefited from the demand of feedstocks. The increased use of biofuels contributes to the decline in foreign oil dependence, and also the extension of ethanol industry will enable the country to break its dependence on fossil fuels. The production of 13.9 billion

gallons of ethanol compensated for 485 million barrels of oil for refinery gasoline, which is equivalent of 13% total U.S. crude oil imports. (Urbanchuk, 2012)

The expansion of corn- based ethanol industry, however, has been blamed to be the motivating force behind higher agriculture prices in recent years. Those concerns, however, were based on unreliable evidences. Two independent studies by World Bank and OCED (Organization for Economic- Co-operation and Development) claimed that influences of biofuels on food/feed are much smaller than originally reported (Baffes and Haniotis 2010, OECD, 2008). Actually, no single factor is the driver of the food prices, but rather, food prices are influenced by the set of interrelated factors, such as increase in petroleum price, inflation pressures and supply, demand balances. Ethanol demand is not the only factor that influences corn prices. Based on the analyses of historical price data (RFA 2011) corn prices did not show strong effects on the prices of livestock, poultry, egg and milk. So, there is a very little statistic evidence that supports a conclusion that the growth of ethanol industry is the main driving force of the steep rise in the food prices.

The extensive list of quantifiable economic benefits is one reason many states and communities have identified ethanol plants as a primary economic development target. Nearly 100 communities across America host ethanol plants. Corn is the predominant feedstock which is converted into ethanol. The local economic impact of the plants varies but many plants consistently demonstrate the significant economic benefits illustrated in a report by the Nebraska Public Power District (NPPD). During the past decade NPPD has made a concerted effort to assist communities and companies in their efforts to develop ethanol plants in Nebraska. This spirit of cooperation and hard work is motivated by a clear understanding of the economic benefits generated by ethanol plants.

NPPD reports that a typical 40 million gallon ethanol plant will generate the following economic activity:

• The plant will provide a one-time boost of $71 million to the local economy during construction.

• The plant will expand the local economic base of the community by $70.2 million each year through the direct spending of $58 million.

• The plant will create at least 33 full-time jobs at the plant and a total of at least 120 jobs throughout the local economy.

• The plant will increase household income for the community by $6.7 million annually. (Source: Nebraska Public Power District, Employment and Other Economic Impacts Associated with the Construction of an Ethanol Production Facility (January 2005), and Estimated Economic Effects for the Prospective Ethanol Production Facility in Boone County, Nebraska (June 2004))

Scientific study has proven ethanol's energy balance to be positive. The most recent USDA figures show that ethanol made from the dry mill process provides at least 77% more energy as a fuel than the process it takes to make it. The bottom line is that it takes about 35,000 BTUs (British Thermal Units) of energy to create a gallon of ethanol, and that gallon of ethanol contains at least 77,000 BTUs of energy.

2.2.1.1 Ethanol market in India

The government of India has made it compulsory to sell ethanol doped petrol. With the current rates, oil firms such as Indian Oil, Bharat Petroleum and Hindustan Petroleum will save almost Rs 3 per liter on petrol if they sell it in blended form. Companies have finalized purchase of ethanol from domestic producers but the quantity is not sufficient. They have also opened price quotes from overseas to meet the demand. Imports of ethanol will also take place simultaneously. The Cabinet had made it mandatory to blend 5% ethanol with petrol across the country.

Ethanol production supports farmers and creates domestic jobs. And because ethanol is produced domestically, from domestically grown crops, it reduces nation's dependence on foreign oil and increases the nation's energy independence.

The banning of MTBE by many states created an almost instant demand for approximately 7 bgy of ethanol. MTBE is methyl tertiary butyl ether, a gasoline additive. Like ethanol, it is a fuel oxygenate, it adds oxygen to the gasoline to help it burn more cleanly. Unlike ethanol, MTBE is a toxic substance and even small spills or leaks of the

product have been found to contaminate ground water supplies. The use of MTBE has been banned in more than 25 states in U.S.

MTBE and ethanol were the two oxygenates of choice under the former Clean Air Act of 1990, but the use of MTBE went by the wayside in 2006 when its manufacturers did not receive the product liability protection they had hoped for in the Energy Policy Act of 2005. Ethanol, just as effective at adding octane and oxygen, has moved in to many former MTBE markets due to its beneficial properties and increasing availability. Initially, much of the investment in ethanol facilities focuses on dry mills that were funded by corn producers. Wet-mill facilities are more flexible than dry mills because they can produce products such as high-fructose corn syrup in addition to ethanol. However, dry-mill plants are less expensive to construct and can typically be built in less than two years.

2.3 Applications of ethanol

- **Ethanol is Better than gasoline**

- Ethanol is a much cleaner burning fuel than gasoline,
- Ethanol offers a significant reduction in carbon monoxide and hydrocarbon tailpipe emissions.
- Research shows that every city and state that has switched to ethanol-blended fuel has experienced improved air quality.
- According to the U.S. Department of Energy, in 2005 alone ethanol-blended fuels reduce CO2-equivalent greenhouse gas emissions by 7.8 million tons - this has the effect of removing the annual GHG emissions of more than 1 million automobiles from the road.

- **Ethanol is good for the environment**

  E85, a blend of 85 percent ethanol and 15 percent gasoline, also has fewer volatile components than gasoline, which means fewer emissions from evaporation. Adding ethanol to gasoline in lower percentages, such as 10 percent

ethanol and 90 percent gasoline (E10), reduces carbon monoxide emissions from the gasoline and improves fuel octane.

- **Ethanol is widely available and easy to use**

  Flexible fuel vehicles that can use E85 are widely available and come in many different styles from most major auto manufacturers. E85 is also widely available at a growing number of stations throughout the United States. Flexible fuel vehicles have the advantage of being able to use E85, gasoline, or a combination of the two, giving drivers the flexibility to choose the fuel that is most readily available and is best suited to their needs.

- **Ethanol is good for the economy**

  Ethanol production supports farmers and creates domestic jobs. As ethanol is produced domestically, from domestically grown crops, it reduces nation's dependence on foreign oil and increases the nation's energy independence.

2.3.1 Disadvantages of cellulosic ethanol over starch

- The resistance nature of biomass to breakdown
- The variety of sugar which are releases when the hemicellulose and cellulose polymers are broken
- The need to find or genetically engineer organisms to efficiently ferment these sugars
- Cost of selection and storage of low density biomass feedstock

2.4 Ethanol production processes

Ethanol can be produced in two different ways: through fermentation and through ethylene hydration. Fermentation is a biological process, while ethylene hydration is a petrochemical one.

- **Production of ethanol through fermentation**

Fermentation is the oldest way for humans to produce ethanol, and this is the conventional way of making alcoholic beverages. It is also the process used for the vast majority of ethanol fuels on the market.

When definite species of yeast metabolize sugar, the end result is ethanol and carbon dioxide. One example of such a species is *Saccharomyces cerevisiae*,.

This is the chemical formula for turning sugar into ethanol and carbon dioxide:

$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$

$C_6H_{12}O6$ is simple sugar, also known as dextrose or D-glucose.

CH_3CH_2OH is ethanol.

CO_2 is carbon dioxide.

One sugar molecule is turned into two ethanol molecules and two carbon dioxide molecules.

Most types of yeast will stop reproducing when the alcohol content reaches 15% ethanol by volume, or even earlier, putting a natural limit on the alcohol concentration achieved through fermentation.

- **Production of ethanol through ethylene hydration**

Ethanol produced through ethylene hydration is commonly referred to as synthetic ethanol, since it isn't the result of a biological process. Synthetic ethanol is chiefly used as a solvent and as an industrial feedstock. Acid is normally used as a catalyst for the process.

$C_2H_4(g) + H_2O(g) \rightarrow CH_3CH_2OH(l)$

C_2H_4 is ethylene.

H_2O is water.

CH_3CH_2OH is ethanol.

Earlier, a two step process was utilized. First, ethylene was allowed to react with concentrated sulfuric acid to form ethyl sulfate. The ethyl sulfate was then hydrolyzed to produce ethanol and sulfuric acid.

Step one: $C_2H_4 + H_2SO_4 \rightarrow CH_3CH_2SO_4H$

Step two: $CH_3CH_2SO_4H + H_2O \rightarrow CH_3CH_2OH + H_2SO_4$

H_2SO_4 is sulfuric acid.

$CH_3CH_2SO_4H$ is ethyl sulfate

2.5 Brief introduction of the conversion of corn to ethanol process

In the present study, ethanol is made through dry milling process. However, ethanol can be made both by dry and wet milling of corn. In dry milling the entire corn kernel is processed without separating out the various component parts of the grain such as the germ. Water is added to form the slurry to which the enzymes are added to convert the starch to dextrose, a simple sugar. Then it is cooked at high temperatures to reduce bacterial levels then cooled and transferred to fermentors where yeast is added to convert the sugars to ethanol. Cooking and fermentation converts the starch in the grain to sugar and ethanol. Left behind as the “stillage” this comprises of protein as well as fibre. This stillage is sent through the centrifuge that separates the solid matter and solubles which are concentrated to syrup of 30 % solids by evaporation. The solid matter and syrup are combined and dried together to produce a co-product called Distiller’s dry grain with solubles (DDGS). DDGS is a high quality, medium protein, nutritious feed ingredient widely used in beef and dairy cattle, poultry and swine feed

2.5.1 Enzymes involved in the fermentation process

α-Amylase is an enzyme having EC 3.2.1.1.. It is also present in seeds containing starch as a food reserve, and is secreted by many fungi. Amylase helps in the breakdown of starch to maltose. Alpha-amylase hydrolyzes bonds between glucose repeats. Carbohydrates and sugars are the major energy storage molecules used by living organisms. Plants store energy of the sun in sugars using photosynthesis. Alpha Amylase's official name is 1, 4-α-D-Glucan glucanohydrolase. Alpha amylase breaks down starch by hydrolysis to release maltose. Starch is a complex molecule mostly made in plants and bacteria. It consists of two types of polysaccharides amylose and amylopectin. A variety of amylase exists. There function is to "cut" the branches of starch molecules, each at a particular point. Depending on the type of amylase, the resulting compounds may be simple sugars such as glucose or fructose, compound sugars such as maltose, malt sugar, or special forms of starch such as dextrine. The alpha-amylase is a bacterial thermostable endo-amylase. It hydrolyzes α- 1, 4 bonds of starch molecules at random positions to rapidly reduce the viscosity of gelatinized starch solutions. This enzyme is a metal ion containing protein and requires a small amount of calcium ion during use for maximum activity and stability. The enzyme cannot hydrolyze α- 1, 6 bonds but can by-pass these branch points in amylopectin. The product of the reaction is dextrins- short glucose chains, and small amounts of glucose and maltose.

Glucoamylase (EC 3.2.1.3) is an important industrial enzyme. Glucoamylase, produced by fungi, is an exo-amylase. It hydrolyzes the maltose and dextrins from the non-reducing end of the molecule. Glucoamylase hydrolyzes α- 1, 6 bonds to completely degrade the dextrins to glucose. The enzyme is optimally active at pH 4-5 so pH adjustments after saccharification is not needed for the yeast fermentation. The yeast fermentation takes place at pH 3-5.

2.6 Dry grind ethanol process

There are two major traditional industrial processes for producing fuel ethanol- Wet milling and Dry milling. In the Wet milling process, corn kernels are soaked and softened before fractionation into germ, endosperm, fibre and gluten to produce a variety of

products separately. In the Dry grind process, the whole grain is processed to produce ethanol and co-products (e.g.DDGS) (Bothast and Schlicher, 2005). Dry- grind process produces about 2.8 gallons of ethanol and 7.7 kg of DDGS per bushel of corn (Mosier and Illeley 2006).

The large scale and capital- intensive wet milling process, results in higher costs of construction and operation. Therefore, with targeting ethanol as the main product, a dry- grind process is preferred for producing ethanol at lower cost (Tiffany *et al*., 2005, Dale *et al*., 2006). The various procedures involved in this process are discussed below:

There are five major steps in the dry-grind method of ethanol

1. Milling
2. Liquefaction
3. Saccharification
4. Fermentation
5. Distillation and recovery

Milling

Milling is the process which involves processing of corn through a hammer mill (with screens between 3.2 to 4.0 mm) to generate a corn flour (Rausch et al., 2005). This whole corn flour is slurried with water, and heat-stable enzyme (a-amylase) is added.

Liquefaction

This slurry is cooked, also known as “liquefaction.” Liquefaction is accomplished using jet-cookers that inject steam into the corn flour slurry to cook it at temperatures above 100°C (212°F). The heat and mechanical shear of the cooking process break apart the starch granules present in the kernel endosperm, and the enzymes break down the starch polymer into small fragments. The cooked corn mash is then allowed to cool to 80-90°C (175-195°F), additional enzyme (α-amylase) is added, and the slurry is allowed to continue liquefying for at least 30 minutes.

Simultaneous Saccharification and Fermentation

Simultaneous saccharification and fermentation (SSF) is the process which is used in the present study for the production of ethanol from corn. The prime benefits of performing the enzymatic hydrolysis together with the fermentation, instead of in a separate step after the hydrolysis, are the reduced end-product inhibition of the enzymatic hydrolysis, and the reduced investment costs. SSF is today important in the dry-milling process in the corn-based ethanol industry in the U.S and worldwide.

The initiative of performing the enzymatic hydrolysis and fermentation simultaneously was put forward by Gauss et al. in a patent from 1976. The glucose yield in a traditional separate enzymatic hydrolysis was less, most likely due to end-product inhibition of the hydrolysis by glucose. The combination of hydrolysis and fermentation decreases the number of vessels needed and thereby investment costs. The decrease in capital investment has been estimated to be larger than 20%. This is quite important, since the capital costs can be expected to be comparable to the raw material costs in ethanol production from corn.

Saccharification

After liquefaction, the slurry, is called "mash," is cooled to around 30°C, and a second enzyme (glucoamylase) is added. Glucoamylase completes the breakdown of the starch into simple sugar (glucose). This step, called "saccharification," often occurs while the mash is filling the fermentor in preparation for the next step (fermentation) and continues throughout the next step.

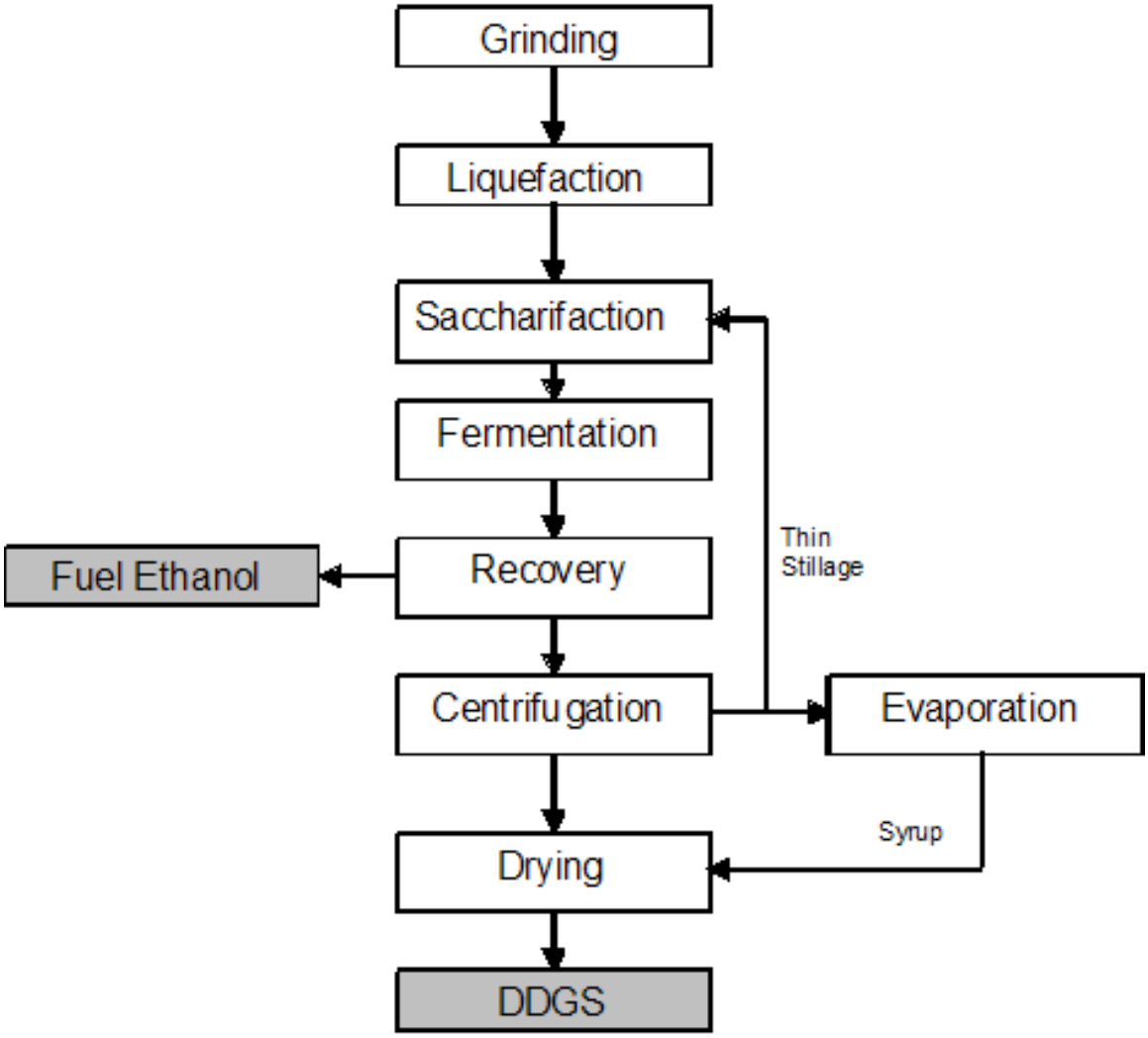
Fermentation

In the fermentation step, yeast grown in the flasks, are added to the corn mash to begin the process of converting the simple sugars to ethanol. The other components of the corn kernel (protein, oil, etc.) remain largely unchanged during the fermentation process. In most dry-grind ethanol plants, the fermentation process occurs in batches. A fermentation vessel is filled, and the batch ferments completely before the vessel is drained and refilled with a new batch.The up-stream processes (grinding, liquefaction, and saccharification)

and downstream processes (distillation and recovery) occur continuously (grain is continuously processed through the equipment). Thus, dry-grind facilities of this design usually have three fermentors (tanks for fermentation) where, at any given time, one is filling, one is fermenting (usually for 48 hours), and one is emptying and resetting for the next batch. Carbon dioxide is also produced during fermentation. Usually, the carbon dioxide is not recovered and is released from the fermentors to the atmosphere. If recovered, this carbon dioxide can be compressed and sold for carbonation of soft drinks or frozen into dry ice for cold product storage and transportation.After the fermentation is complete, the fermented corn mash (now called "beer") is emptied from the fermentor into a beer well. The beer well stores the fermented beer between batches and supplies a continuous stream of material to the ethanol recovery steps, including distillation.

Distillation and Recovery

After the process of fermentation, the liquid portion of the slurry has 8-12% ethanol by weight. Because ethanol boils at a lower temperature than water does, the ethanol can be separated by a process called "distillation." Conventional distillation/rectification systems can produce ethanol at 92-95% purity. The residual water is then removed using molecular sieves that selectively adsorb the water from an ethanol/water vapor mixture, resulting in nearly pure ethanol (>99%).The residual water and corn solids that remain after the distillation process are called "stillage." This whole stillage is then centrifuged to separate the liquid (thin stillage) from the solid fragments of the kernel (wet cake or distillers' grains). Some of the thin stillage (backset) is recycled to the beginning of the dry-grind process to conserve the water used by the facility.The remaining thin stillage passes through evaporators to remove a significant portion of the water to produce thickened syrup. Usually, the syrup is blended with the distillers' grains and dried to produce an animal feed called "distillers' dried grains with solubles" (DDGS). When markets for the feed product are close to the plant, the byproduct may be sold without drying as distillers' grains or wet distillers' grains.



Adapted from McAloon, A.; Tayor, F.; Yee, W.; Ibsen, K. and Wooley, R. (2000). Determining the Cost of Producing Ethanol from Corn Starch and Lignocellulosic Feedstocks. NREL/TP-580-28893.

.**Fig 5: Flowchart of dry- grind ethanol production process**

2.7 Objectives of the Current Study

- **To identify best corn hybrid having highest ethanol production potential.** Seven hybrids were screened for nutritional quality parameters. On the basis of starch concentration, three hybrids were chosen for fementation batches.

  DHM 117- Bold seeded high yielding (10 ton/ ha) single cross hybrid orange-yellow in colour

  PMH 3- Bold seeded high yielding (8-10 ton/ha) released for commercial cultivation all over India

  Bio 9681: High yielding private sector hybrid.

- **To optimize the fermentation parameters- pH, Temperature and substrate concentration for efficient ethanol production.**

  The fermentation parameters are to be optimized by response surface methodology. The parameters are optimized first by changing one variable at a time and than by combination of variables by CCD to see the effect of variables on ethanol concentration.

- **To statistically analysis the observed data.**

  Statistical analysis is to be done to know the significant level of the experimental model and to know that which parameter has more influence on ethanol concentration.

- **Optimization of variables by Response surface methodology.**

3. MATERIALS AND METHODS

3.1 Materials

Maize genotypes: Genetically pure seeds of seven single cross hybrids of maize were procured from Directorate of Maize Research, IARI campus, PUSA, New Delhi.

Fermenting Yeast: The fermenting yeast which was used in the present study was procured from IMTECH, Chandigarh. The name of the yeast was *Saccharomyces cerevisiae*, MTCC (4043)

Commercial Enzymes: Alpha amylase: An enzyme alpha enzyme which was used in the experiment was procured from HI-MEDIA chemicals having activity1:2000 I.P. Units. Glucoamylase: An enzyme glucoamylase used in the present study was procured from SRL, New Delhi. The enzyme having activity 64 units/ mg

Fermentor: An applikon fermentor having capacity 3 L was used in the experiment.

Distillation unit: A fractional distillation apparatus was used in the present study.

Equipments: The equipments used in the laboratory for the experiment:

- Autoclave
- Laminar air flow
- Distillation unit
- Spectrophotometer
- pH meter
- centrifuge
- Sample grinder
- Inoculation loops, conical flasks, etc.

Reagents:

DNS reagent, sodium potassium tartarate, sodium hydroxide, peptone, dextrose, yeast extract, starch, potassium dihydrogen, phosphor tungstic acid, hydro chloric acid.

3.2 METHODS

1.) Maintenance of culture:

The Yeast culture were maintained by subculturing them after every 15-20 days on YEPD agar plates in a BOD incubator at 30^{o} C, pH 5.4 for 24 hours.

MEDIUM COMPOSITION (g/L):

COMPONENTS	QUANTITY (g/L)
Yeast extract	1.5
Peptone	5
Starch	1
Dextrose	5
Agar	15
Potassium dihydrogen phosphate	0.5
dH_2O	1

The medium was sterilized in an autoclave at 121^{o}C, 15 psi for 20 min.

2.) Inoculum Preparation and Inoculation:

The inoculum for yeast was prepared in YEPD liquid medium. A loopfull of strain MTCC 4043 was inoculated in the liquid medium. The culture was incubated in a BOD incubator at 30^{o}C, 150 rpm for 24 hrs. This inoculum was used 10 percent in the sterilized corn mash.

3.) Grinding

The entire corn kernel of the three genotypes was grinded and very fine flour was made.:

4.) Fermentation

The substrate was dissolved in 1000 ml distilled water in the fermentation vessel. The vessel was autoclaved along with slurry. After autoclaving, when the temperature of fermentation vessel reaches to 60-70^{o}C, α- amylase was added in the fermentation vessel. The pH of the vessel was set to 5.4, temperature 35^{o}C, after 5 hrs. glucoamylase along with the 24 hrs old culture of *S.cerevisiae* (MTCC 4043) was added in the vessel. Run the fermentation cycle for 72 hrs. Fermentation was stopped after 72 hours. The sample was filtered and distilled after 72 hrs. Three cycles were run following the same procedure. Out of the three genotypes, the genotype which produced the maximum ethanol was taken further for the optimization studies. For the optimization studies the effect of pH, temperature, and substrate conc. were studied.

3.2.1 Optimization studies for fermentation

Response Surface Methodology is a major process optimization tool that is used to determine the optimum values of various factors critical for the process. Response surface methodology (RSM) is a compilation of statistical techniques to design experiments, build models, evaluate the effects of variables and thereby, seeking for the optimum conditions. It mostly is used in optimization of different types of fermentations and bioprocesses (Kristo *et al*., 2003; Wejse *et al*., 2003; Dey *et al*., 2001). The major advantage of RSM is reduced number of experimental runs required to provide sufficient information for statistically acceptable results, its suitability for multiple factor experiments and examination of common relationship between various factors towards finding the most suitable production conditions for the bioprocess and forecast response (Chang *et al*., 2006). This is a group of techniques that are used to study the reaction between one or more measured dependent factors (responses) and a number of input (independent) factors. It is a three factorial design where contour plots are generated by linear or quadratic effects of key factors and a model equation is derived that fits the experimental data to calculate optimal responses of the system. To calculate optimum values of three factors (pH, temp and substrate concentration) selected for Central Composite Design. . An optimization study seeks a solution to an objective (minimization and maximization of an analysis feature parameters) while being constrained by a set of

model dimensions and other analysis feature parameters. In order to maximize the ethanol production effective factors and their levels were selected based on literature review. The selected variables include pH, temperature and substrate concentration. High (+) and low (-) values of these three variables were examined. A Central Composite Design was constructed which gave different values in the form of a matrix, for the selected variables.

Experiment 1:

In the first set of experiments, no RSM was used, the variables were optimized manually. The experiment was conducted by changing one variable at a time while keeping the other two variables remains constant.

Experiment 2:

The second set of experiments was conducted by making combinations of the variables using two factorial composite design of RSM. Initially, the pH and temperature effects were observed on ethanol concentration. Thirteen experiments were conducted using CCD (design expert software) with 5 central points. Out of these thirteen cycles nine cycles were selected for fermentation batches. Further, the effect of temperature and substrate concentration was observed on ethanol concentration using the same method. Thirteen experiments were conducted by CCD (design expert software). Out of these thirteen cycles nine cycles were selected for fermentation batches. Finally, the effect of pH and substrate concentration on ethanol production was observed. Thirteen experiments conducted using CCD (design expert software). Out of these thirteen cycles, nine batches were performed for fermentation.

3.2.2 Optimization Studies for Experiment 1

1.) **Genotype selection**

Seven genotypes of *Zea mays* were taken in order to conduct the experiment. Nutrition quality parameters were analyzed of all the seven genotypes (i.e. protein, starch, moisture and sugar were analyzed). Out of the seven genotypes, the three genotypes (DHM 117, PMH 3, BIO 9681) having the high starch percentage were taken for the fermentation

batches. Among these three genotypes, the one which resulted in the maximum ethanol production was further taken for parameter optimization for efficient ethanol production.

2.) Effect of pH

The pH has profound effect on fermentation. It has also a deep effect on the growth of *S. cerevisiae*. The pH range of 4-5.6 has been reported as optimum for the fermentation and for the active functioning of *S. cerevisiae* (Arifa *et al.,* 2010). The pH ranges- 4.8, 5.4, 6.0, 6.8 were selected for optimization studies. The increase in pH inhibits the growth of *S. cerevisiae* as the yeast grows well on acidic pH. Also, at low pH there is no bacterial contamination. At high pH yeast produces acids rather than alcohol (Mathewson 1980, Arifa *et al*., 2010).

3.) Effect of temperature

Temperature also affects fermentation and yeast growth to a great extent. The optimum temperature reported for production of ethanol and for the growth of *S. cerevisiae* ranges from 30^{o}-35^{o}C. The lower temperature than the optimum temperature will result in extended lag phase and slower down the process of fermentation. The high temperatures resulted in the long exponential phase and fasten the fermentation process. But increasing temperature to a great extent may disrupt enzyme and membrane functions (Sener *et al*., 2007) .Yeast growth is also inhibited at higher temperature. The temperature ranges 22, 28, 35, 40 were taken for the optimization studies.

4.) Effect of substrate concentration

The fermentation process is greatly influenced by the amount of substrate added. The higher substrate concentration inhibits the growth of the yeast and thus slower down the process of fermentation because of lower heat and mass transfer rate in high substrate concentration. The substrate (corn flour) at three different concentrations were studied- 140, 160, 180 g. At high substrate conc. the growth parameters are inhibited due to high medium osmolality (Kademi *et al*., 1996). High substrate concentration also causes inefficient fermentation (Stenberg *et al*., 2000).

3.2.3 Analytical Methods

Moisture content analysis

Moisture can be determined by drying the sample in an oven. The oven drying method is preferred because of accuracy. 5 g of flour is taken in an aluminium box having a proper fitting lid. The uncovered aluminium box with its lid was placed in a well ventilated oven maintained at 100^{o}C. After five hours, cooled the box with lid replaced to room temperature in a desiccators and weighed. The box was again placed in the oven at 100^{o}C for an hour. Then cooled the box by putting it in desiccators and weighed. Repeated the process till a constant weight is obtained. The moisture % was calculated by determining the loss in weight sample during drying.

Calculations: $\frac{\text{Original wt. of the sample (g) - Dry wt. of the sample (g) * 100}}{\text{Original wt. of the sample (g)}}$

Starch estimation

Reagents:-.3N HCl, 4% Phosphotungstic Acid

50 g of sample was grinded to 20 meshes or finer. Moisture content of the ground samples was determined. About 2g of sample was weighed and transferred to a test tube. A 25ml of 0.3N HCl was added and shake vigoursly 2-3 times. The content was transferred to 100 ml volumetric flasks. Rinsed the tube with 25ml of 0.3N HCl and transferred to the volumetric flasks so that the total volume in volume flask is 50 ml. The flasks were kept in water bath at 100^{o}C for 15min. It was cooled under running water. 10 ml of 4% Phosphotungestic acid was added and made the volume up to 100ml. The content was filtered with Whatman no.1 filter paper and read in a polarimeter.

Sugar estimation

Reagents: - 1. Substrate solution: Standard solution of 1000 µ g/ml concentration was made by dissolving 100 mg of glucose in 100 ml of distilled water.

2. 3, 5 dinitrosalicylic acid (DNS) solution: It was prepared by dissolving 10.0g of 3, 5-DNS, and 0.5 g of sodium sulphitein 500 ml of 2% NaOH solution and then diluting it to 1 liter with distilled water. The reagent was filled in dark colored bottle.

3. Potassium sodium tartarate (Rochelle salt): 40 g of potassium sodium tartarate was dissolved in distilled water and the volume was made to 100 ml.

The reducing sugar concentration in the samples was analyzed by DNS method (Miller, 1959). The 100 mg/ml glucose was used as a stock. Six test tubes were taken with varying stock concentrations. Observed the O.D. at 575 nm.

Protein estimation

Estimation of proteins (AOAC, 1970)

Estimation of proteins is done by micro kjeldahl method. The sample is digested in concentrated sulphuric acid, which acts as an oxidising and dehydrating agent. So the carbon in the sample is oxidized to carbon dioxide which leaves as a gas. Nitrogen in the sample is held back in the form of ammonium sulphate. It is converted to ammonia by the addition of sodium hydroxide, which is distilled off, absorbed in boric acid solution and titrated with standard hydrochloric acid.

Reagents

Sulphuric acid (sp. gr. 1.84), catalyst mixture, sodium hydroxide solution(40%), boric acid solution(4%), hydrochloric acid solution(0.02N)

Preparation of reagents

1. Catalyst mixture: 99.0 g of K_2SO_4, 4.1 g of HgO and 0.8 g of $CuSO_4$ were grind in a mortar
2. Sodium hydroxide solution: 400 g NaOH in 1000 ml water.
3. Boric acid solution: 40 g boric acid in 1000 ml water.
4. Hydrochloric acid solution: 0.02 N

Method

- 100 mg sample was transferred into a digestion tube. To this 1 g of catalyst mixture and 5 ml of conc.sulphuric acid were added.
- The mixture was digested until the solution became colorless.

After cooling the protein content was distilled through auto analyzer.

Ethanol estimation

The fractional distillation apparatus was used for ethanol estimation. The boiling point of ethanol is 78^{o} C. The temperature of fractional distillation was set at 78^{o} C because at this temperature only the ethanol is separated out into the collecting flask. The concentration of ethanol in the fermentation broth was calculated as following:

Total broth = X ml

Distilled sample from the broth = Y ml

Ethanol produced per liter = Y* 1000/ X -------- (1)

Calculation in w/v

D = M/V

Where,

D= density of ethanol (0.789g/ ml)

V= Y*1000/X------- (from 1.)

Therefore, M= D* V; M= 0.789* Y*1000/X

4. Results and Discussion

As per the study plan we have optimized the variables such as pH, temperature, substrate concentration to maximize ethanol production from maize. For this purpose seven high yielding single cross maize hybrids were taken and analyzed for nutrition quality parameters i.e. moisture, starch, protein, sugar contents. Results showed that the genotypes DHM 117, PMH3 and BIO 9681 were found to be having maximum starch content and, therefore, were selected for the fermentation batches (**Table 1**). Starch is hydrolyzed to sugars which are further fermented to ethanol. Starch content, therefore, is considered as the criteria for selecting the maize hybrids for ethanol fermentation. The fermentation was carried out at the standard conditions of pH (5.5), temperature (35) and substrate concentration (160g/1000ml) as described by Viruthagiri *et al* (2005). However, different workers have reported different conditions for highest ethanol productions. .Out of these three hybrids, BIO 9681 has shown the maximum ethanol production (78 g/L) followed by DHM 117 (66 g/L) and PMH 3 (59.3 g) (**Table 2**.) at standard conditions (Viruthagiri *et al* 2005). As BIO 9681 has shown the maximum ethanol concentration, therefore, it was selected for optimization studies to evaluate effect of pH, temperature and substrate concentration for the efficient production of ethanol.

Hybrids	Oil %	Protein %	Starch %
BIO 9681	3.36	9.95	72.16
DHM 117	3.92	11.79	70.83
PMH 3	3.03	11.47	70.57
PMH 1	3.51	10.50	69.24
VIVEK QPM-9	4.09	8.24	69.21
MADHURI	10.00	15.67	57.29
JH 3459	4.44	9.78	69.75

Table 1: Biochemical components in corn hybrids

Hybrids	Ethanol conc. (g/L)
BIO 9681	**78**
DHM 117	**66**
PMH 3	**59.3**

Table 2: Ethanol concentration in selected corn hybrids

Optimization of pH

The effect of pH on the ethanol production was observed, while keeping substrate concentration (160 g/L) and temperature (35°C) constant (**Table 3**). It is clearly observed that pH has profound effect on ethanol concentration. The maximum ethanol (78.3 g/L) was produced at pH 5.5 followed by pH 4.8 (69.6g/L), 6.0 (51.43g/L) and 6.8 (43.6 g/L) (**Figure 1**). This implies that the microbial culture is highly effective at pH 5.5 .At higher pH yeast produce acids rather than alcohol (Mathewson 1980, Arifa, *et al*., (2010). Also at acidic pH there are no chances of bacterial contamination. Yadav *et al.,* (1997) found an increase in alcohol concentration, productivity as well as efficiency with an increase in pH from 4.0-5.0 and found that the optimum pH range for *S.cerevisiae* strain HAU-1 to be between pH 4.5-5.0.

Temp(^{o}C)	**Substrate conc. (g)**	**pH**	**Ethanol conc.(g/L)**			**Mean**
			R1	**R2**	**R3**	
35	160	4.8	69.4	69.6	70.0	69.66
35	160	5.5	78.5	78.2	78.4	78.36
35	160	6.0	51.5	51.1	51.7	51.43
35	160	6.8	43.9	43.3	43.6	43.6

Table No. 3: Effect of pH on ethanol concentration at constant temperature and substrate concentration

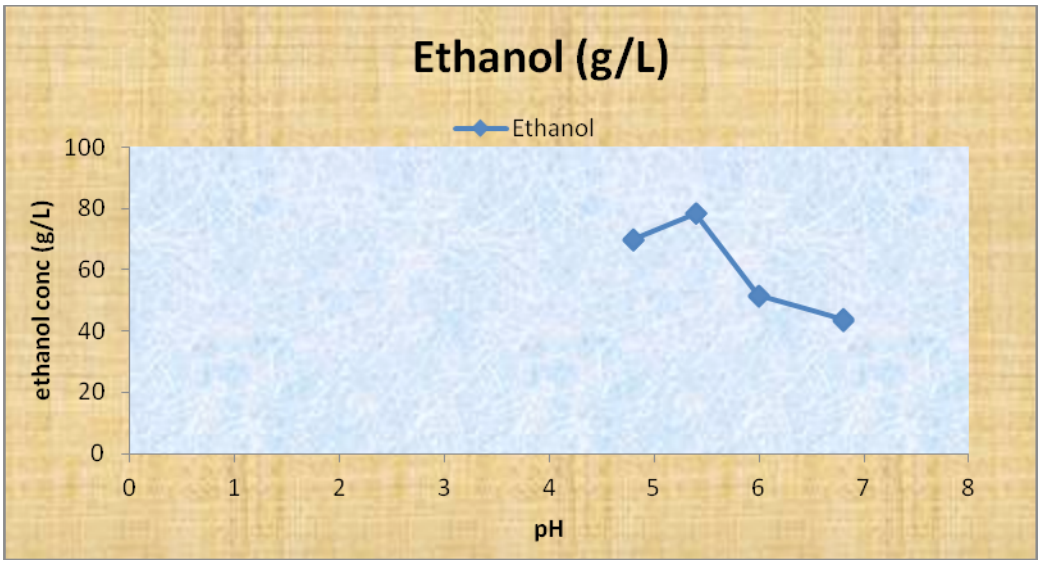


Figure 1: Ethanol concentration with change in pH

Optimization of temperature

Since maximum ethanol has been obtained at pH 5.5, therefore, the effect of temperature on the ethanol production is evaluated at constant pH (5.5) and substrate concentration (160g/L) (**Table 4.)** The temperature was found to be having profound effect on ethanol concentration as ethanol concentration varied from 44.1 g/L (40^{o}C) to 78.5 g/L (35^{o}C) (**Figure 2**) i.e. the highest ethanol was observed at a temperature of 35^{o}C. At high temperature there might be the thermal deactivation of enzymes as well as yeast which might be responsible for lesser production of ethanol. Torija *et al.,* (2001) observed different responses to fermentation temperatures (15-35^{o}C) on mixed strain population of *S.cerevisiae*. Some strains performed better at higher temperature, while others did so at lower temperature. Alcohol yield was higher at lower temperature while at higher temperature secondary metabolites increased. (Sener *et al.,* 2007).

pH	Substrate conc. (g)	Temp(^{o}C)	Ethanol conc.(g/L)			Mean
			R1	R2	R3	
5.5	160	22	49.6	50.2	49.8	49.86
5.5	160	28	58.9	59.1	58.7	58.9
5.5	160	35	78.5	78.2	78.4	78.36
5.5	160	40	44.1	44.3	44	44.13

Table 4: Effect of Temperature on ethanol concentration at constant pH and substrate concentration

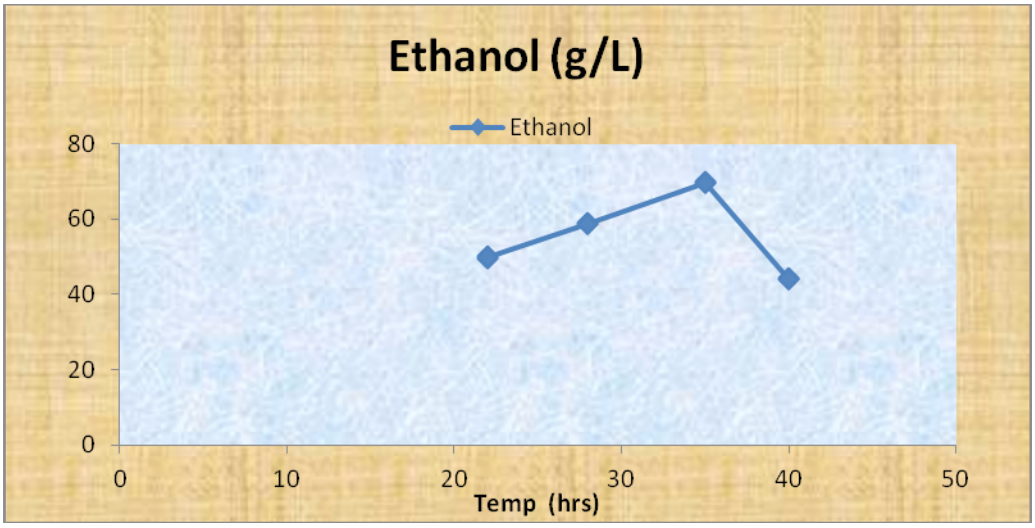


Figure 2: Ethanol concentration with change in temperature

Optimization of substrate concentration

Since maximum ethanol has been obtained at pH 5.5 and temperature 35^{o}C the effect of varying substrate concentration from 140 to 180 g/L on ethanol production ,therefore, is observed at constant temperature (35^{o}C) and pH (5.5) (**Table 5**). It is clearly depicted from the table that substrate concentration also plays a vital role in ethanol production during fermentation process. At high substrate concentration there is lower heat and mass transfer rate during fermentation process which will inhibit the growth of yeast. At high substrate conc. the growth parameters are inhibited due to high medium osmolality (Kademi *et al*., 1996). High substrate concentration also causes inefficient fermentation (Stenberg et al., 2000) In the present study, the maximum ethanol production ,(78.5 g/L) was observed at substrate concentration 160 g/L, followed by 140 g/L (59.8 g/L) and 180 g/L (51.8 g/L) (**Figure 3**)

pH	Temp (^{o}C)	Substrate conc. (g)	Ethanol conc.(g/L)			Mean
			R1	R2	R3	
5.5	35	140	59.8	60.1	59.5	59.8
5.5	35	160	78.5	78.2	78.4	78.36
5.5	35	180	51.8	52	51.6	51.8

Table 5: Effect of substrate concentration on ethanol production at constant temperature and pH

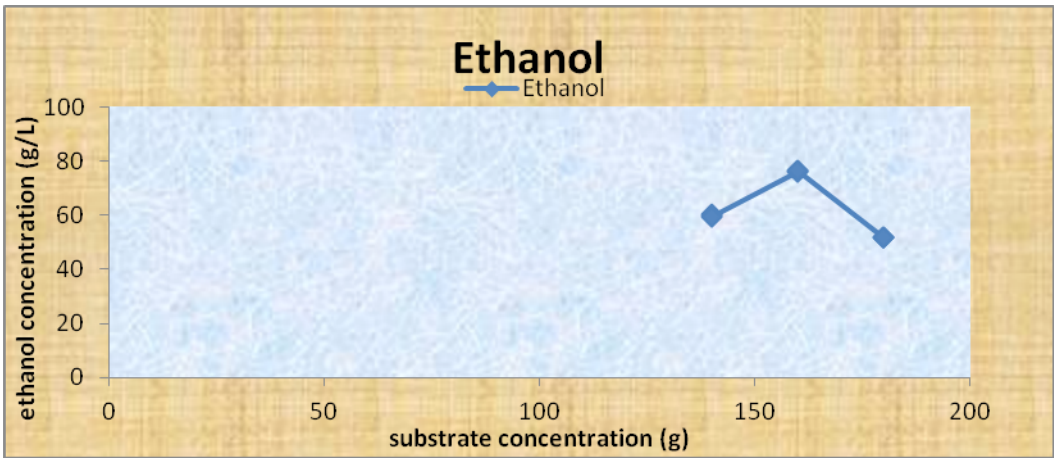


Figure 3: Effect of substrate concentration on ethanol production

Statistical analysis of results

Analysis of Variance is a method which is used to analyze the level of significance of the present study. The level of significance of the effect of pH, temperature and substrate concentration on ethanol concentration was analyzed from ANOVA. **(Table 6,7**and **8)**. Values of "Prob>F" less than 0.0500 indicate model terms are significant. In this study P-value 000*** indicates that the model is highly significant .Values greater than 0.0500 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. In this study, no insignificant value was observed.

Dependent Variable: ETH

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
REP	.302	2	.151	3.034	.123
pH	2312.327	3	770.776	15501.631	.000***
Error	.298	6	.050		
Corrected Total	2312.927	11			

(Adjusted R Squared = 1.000)

Table 6: Analysis of variance of pH to ethanol

Dependent Variable: ETH

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
REP	.245	2	.122	4.955	.054
TEMP	1135.729	3	378.576	15313.202	.000***
Error	.148	6	.025		
Corrected Total	1136.123	11			

(Adjusted R Squared = 1.000)

Table 7: Analysis of variance of temperature to ethanol

Dependent Variable: ETH

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
REP	.276	2	138	4.679	.090
SUB	937.069	2	468.534	15912.491	.000***
Error	.118	4	.029		
Corrected Total	937.462	8			

(Adjusted R Squared = 1.000)

Table 8: **Analysis of variance of substrate to ethanol**

Correlation analysis of three variables to ethanol concentration

Correlation analysis was also carried out to know as which factor more effect on ethanol production. All the three factors were found to have profound has more effect on ethanol concentration, however, it was observed that out of the three variables, ethanol

concentration is effected more with change in pH (**Table 9**) followed by substrate concentration (**Table 10**) and temperature (**Table 11**). All the three variables have shown the negative correlation to ethanol concentration.

		ETH	pH
ETH	Pearson Correlation	1	-.851(**)
			.000

** Correlation **is significant at the 0.01 level (2-tailed).**

Table 9: Correlation of pH with ethanol conc.

		ETH	TEM
ETH	Pearson Correlation	1	-.019
			.954
			12

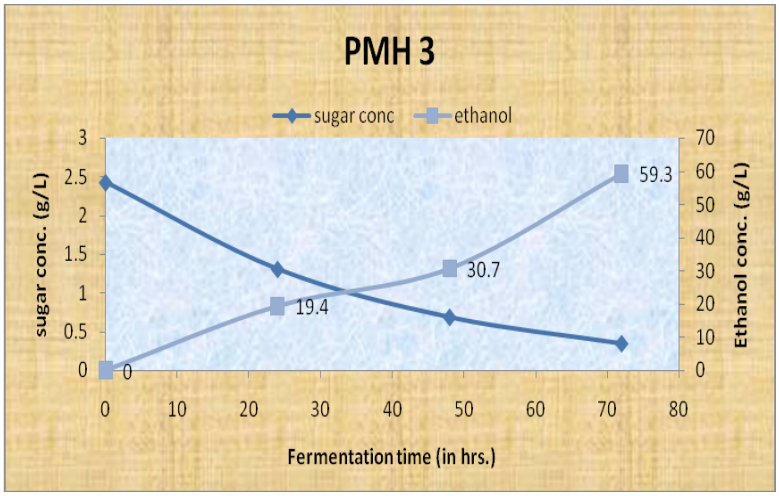
Table 10 : Correlation of temp with ethanol conc.

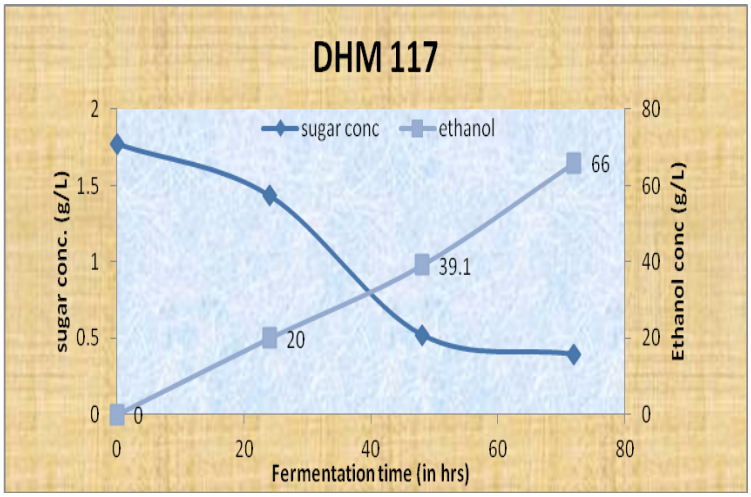
		ETH	SUB
ETH	Pearson Correlation	1	-.319
			.403

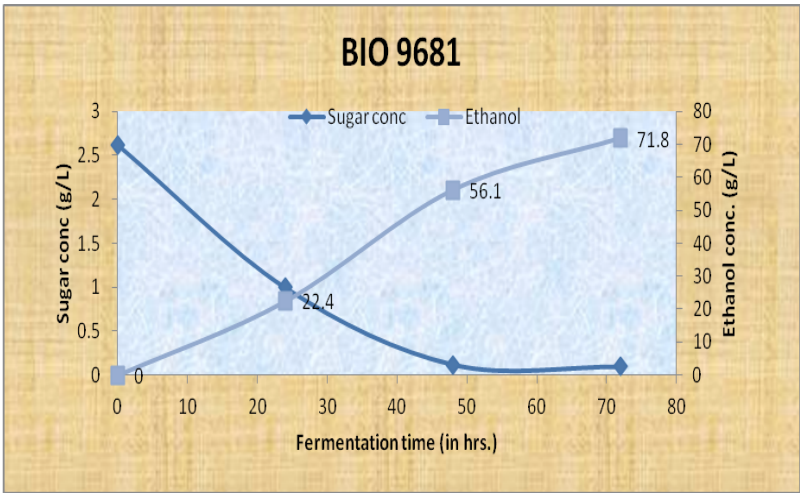
Table 11 : Correlation of substrate conc .with ethanol conc

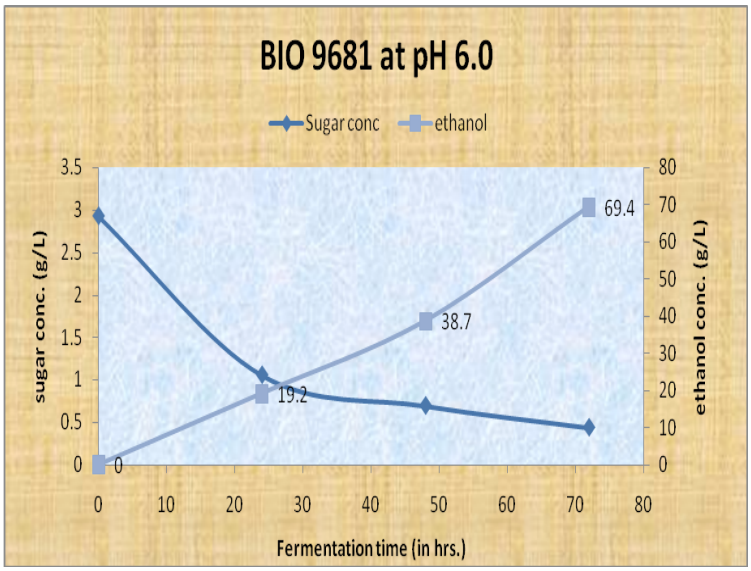
Estimation of ethanol concentration at different time intervals in different batches

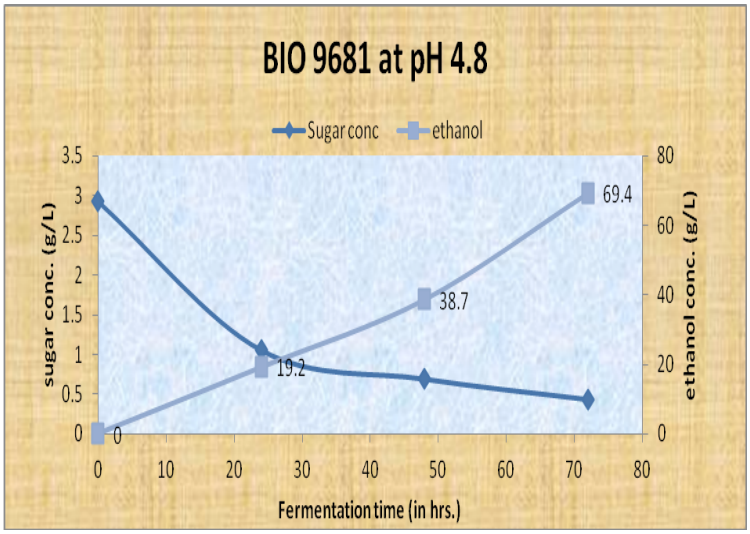
The ethanol concentration was also analyzed at different time intervals during the fermentation cycles. In the fermentation process the starch is first broken down to glucose (starch hydrolysis) with the help of amylases, the glucose is then broken down to ethanol by *S.cerevisiae.* For ethanol production it is necessary that maximum starch is converted to sugar to obtain high fermentation efficiency. (Figure **4-17**) shows that as the sugar concentration decreased, the ethanol concentration increased in all batches

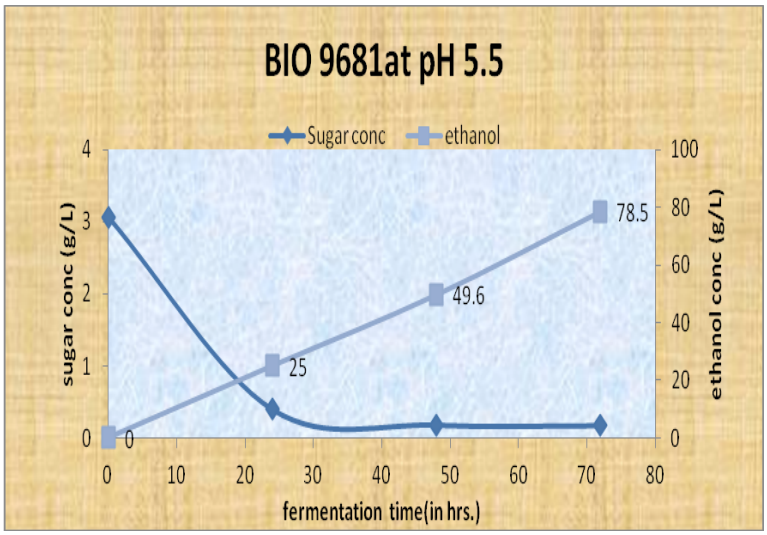

PMH 3
sugar conc
ethanol
sugar conc. (g/L)
Ethanol conc. (g/L)
Fermentation time (in hrs.)
59.3
30.7
19.4
0

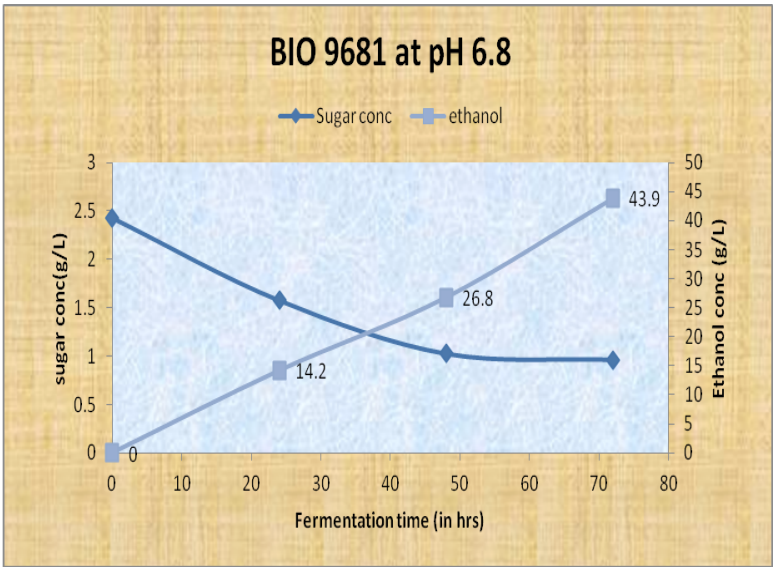

DHM 117
sugar conc
ethanol
sugar conc. (g/L)
Ethanol conc (g/L)
Fermentation time (in hrs)
66
39.1
20
0

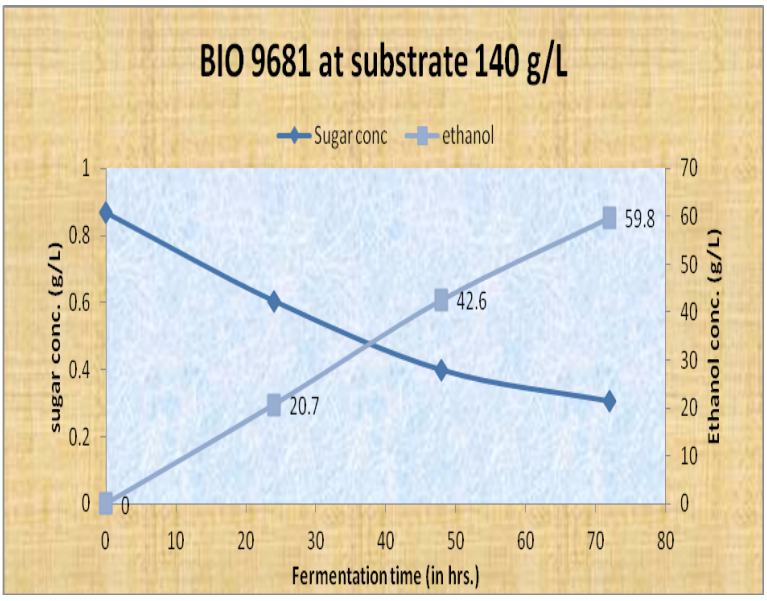

BIO 9681
Sugar conc
Ethanol
Sugar conc (g/L)
Ethanol conc. (g/L)
Fermentation time (in hrs.)
71.8
56.1
22.4
0

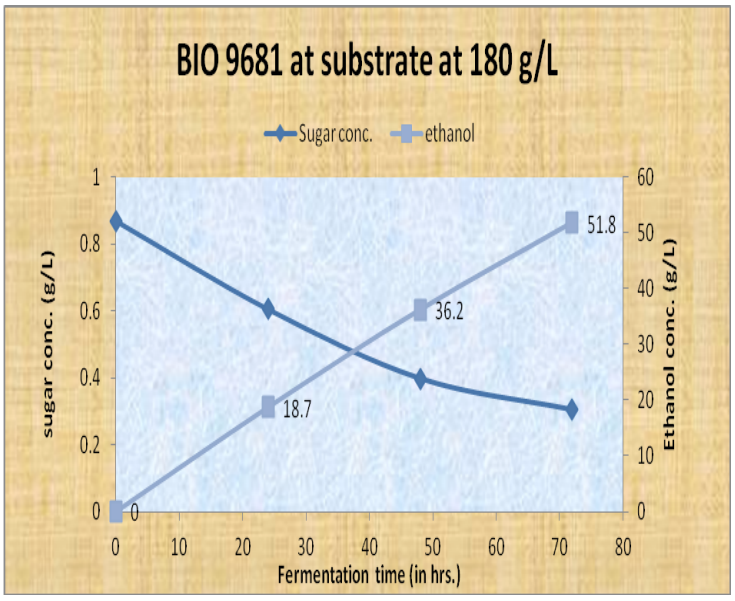

BIO 9681 at pH 6.0
Sugar conc
ethanol
sugar conc. (g/L)
ethanol conc. (g/L)
Fermentation time (in hrs.)
0
19.2
38.7
69.4

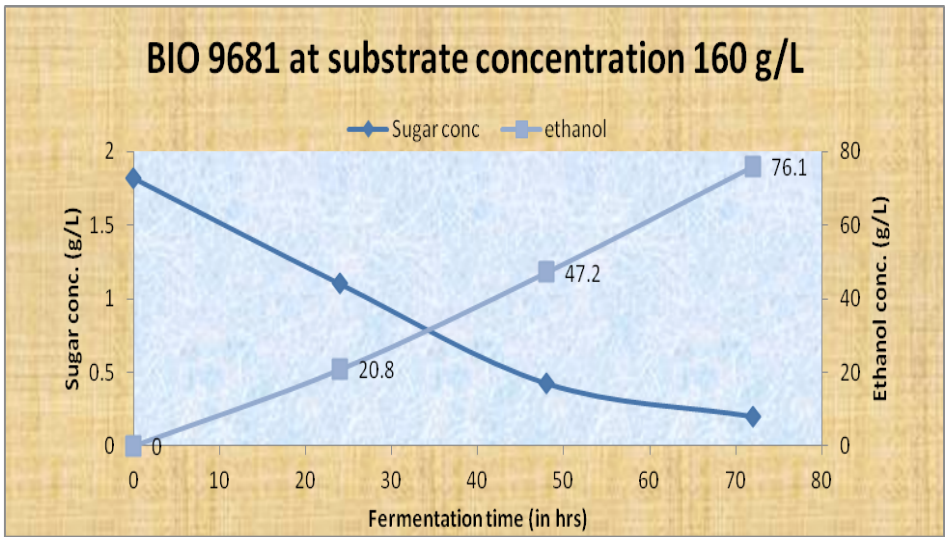

BIO 9681 at pH 4.8
Sugar conc
ethanol
sugar conc. (g/L)
ethanol conc. (g/L)
Fermentation time (in hrs.)
0
19.2
38.7
69.4

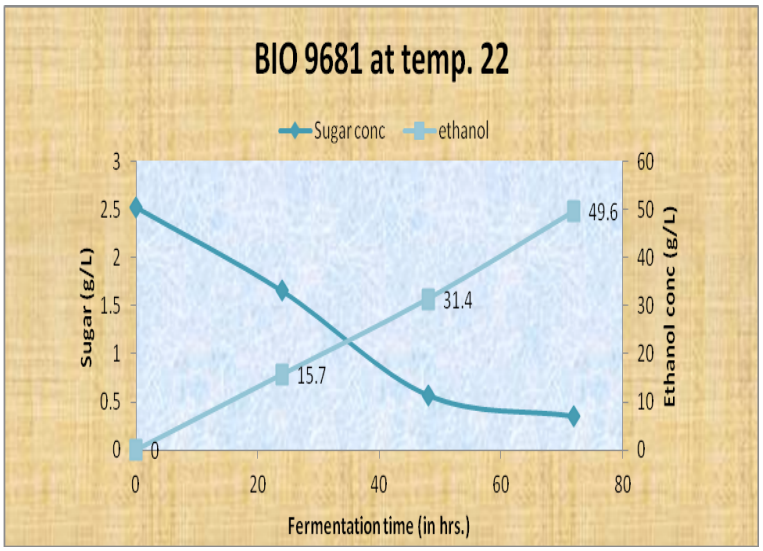

BIO 9681at pH 5.5
Sugar conc
ethanol
sugar conc (g/L)
ethanol conc (g/L)
fermentation time(in hrs.)
0
25
49.6
78.5

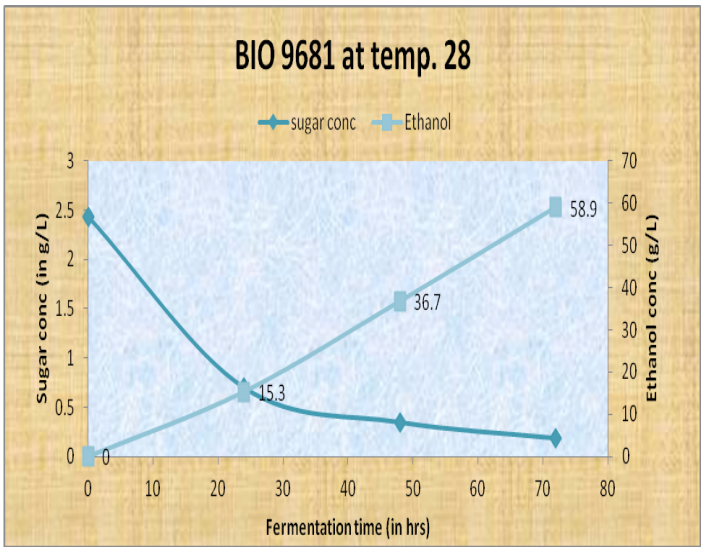

BIO 9681 at pH 6.8
Sugar conc
ethanol
sugar conc(g/L)
Ethanol conc (g/L)
Fermentation time (in hrs)
0
14.2
26.8
43.9

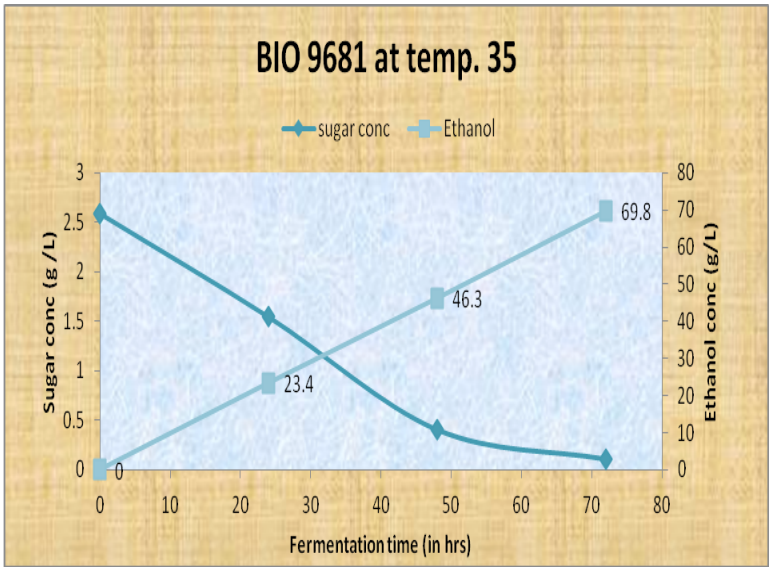

BIO 9681 at substrate 140 g/L
Sugar conc
ethanol
sugar conc. (g/L)
Ethanol conc. (g/L)
Fermentation time (in hrs.)
0
20.7
42.6
59.8

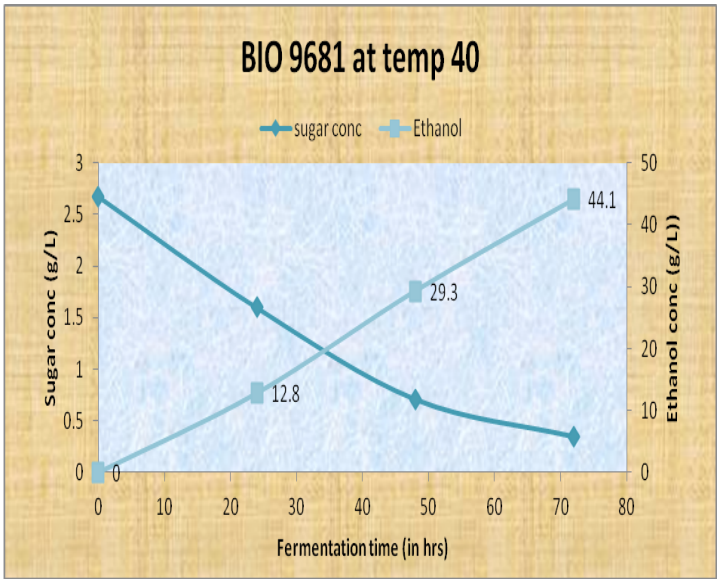

BIO 9681 at substrate at 180 g/L
Sugar conc.
ethanol
sugar conc. (g/L)
Ethanol conc. (g/L)
Fermentation time (in hrs.)
0
18.7
36.2
51.8


BIO 9681 at substrate concentration 160 g/L
Sugar conc
ethanol
Sugar conc. (g/L)
Ethanol conc. (g/L)
Fermentation time (in hrs)
0
20.8
47.2
76.1


BIO 9681 at temp. 22
Sugar conc
ethanol
Sugar (g/L)
Ethanol conc (g/L)
Fermentation time (in hrs.)
0
15.7
31.4
49.6


BIO 9681 at temp. 28
sugar conc
Ethanol
Sugar conc (in g/L)
Ethanol conc (g/L)
Fermentation time (in hrs)
0
15.3
36.7
58.9


BIO 9681 at temp. 35
sugar conc
Ethanol
Sugar conc (g /L)
Ethanol conc (g/L)
Fermentation time (in hrs)
0
23.4
46.3
69.8


BIO 9681 at temp 40
sugar conc
Ethanol
Sugar conc (g/L)
Ethanol conc (g/L))
Fermentation time (in hrs)
0
12.8
29.3
44.1

Experiment 2:

Interactive effects of variables on ethanol production

For the optimization of process parameters, statistical experimental design advance approach was used to provide information on the interactive effect of variables; finally, verification of experiments is used to validate the results under specific experimental conditions (Chen *et al*., 2002). The influence of temperature ,pH and substrate concentration on ethanol production was determined using RSM .The results of a, two level factorial experiment designs with five replications of the central point and six axial points are summarized in **Table 12,13,14** with alpha value of 1.414 and 13 runs . The effect of each factor and their interactions were analyzed using the analysis of variance (ANOVA)

Optimization by Central composite rotable design

Nowadays, RSM (Response surface methodology) is being widely used in optimizing different types of fermentations and bioprocess. It is an empirical statistical modelling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously. Response surface methodology is applied to evaluate the effect of pH, temperature and substrate concentration on ethanol production by making combination of variables.

Central composite design (CCD) is the most common experimental design used in RSM, and the design exhibits equal certainty in all directions from the center. The F-test analysis of variance (ANOVA) was used to check the statistical significance of model equation. Experimental data was analyzed via response surface methodology in order to fit the following second order polynomial equation generated by Design Expert software (Trial VERSION 8.0.7.1 Stat-Ease Inc; USA). Second order coefficients were generated via regression. The response was initially fit to the factors via multiple regressions. The quality of the fit of the three models was evaluated using the coefficients of determination and analysis of variance. The three quadratic response surface models conducted by Central composite design were fit to the following equations:

□□ **$Y = +76.40 - 7.85A - 1.25B - 0.57AB - 13.78A^2 - 12.95B^2$**

Where,

A= temperature

B= pH

Y= Ethanol concentration

□□□ **$Y = +74.60 - 1.95A - 1.92B - 0.37AB - 13.33A^2 - 5.83B^2$**

Where,

A= temperature

B= substrate concentration

Y= Ethanol concentration

□□□□ **$Y = +74.60 - 7.43A - 2.27B + 0.53AB - 14.47A^2 - 6.07B^2$**

Where,

A= pH

B= substrate concentration

Y= Ethanol concentration.

Matrix designs of combination of variables conducted by central composite analysis

Run	Factor 1 A:pH	Factor B:temp (^{o} C)	Response 1 ethanol g/L
1	7.21	31.00	36
2	5.80	31.00	76.4
3	5.80	31.00	76.4
4	5.80	18.27	52
5	5.80	43.73	47
6	4.80	40.00	57.8
7	4.39	31.00	59.7
8	5.80	31.00	76.4
9	5.80	31.00	76.4
10	6.80	22.00	44.7
11	4.80	22.00	58.2
12	6.80	40.00	42
13	5.80	31.00	76.4

Table 12 : representing the combined effect of two factors i.e. pH and temperature in 13 combinations. Standard order 2 represents the maximum ethanol concentration.

Run	Factor 1 A= temp ^{o}C	Factor 2 B= substrate g	Response 1 Ethanol g/L
1	22.00	140.00	57
2	31.00	188.28	61
3	18.27	160.00	52
4	40.00	140.00	53.5
5	31.00	160.00	74.6
6	31.00	160.00	74.6
7	43.73	160.00	47
8	31.00	160.00	74.6
9	31.00	131.72	68
10	31.00	160.00	74.6
11	40.00	180.00	50
12	31.00	160.00	74.6
13	22.00	180.00	55

Table 13: representing the combined effect of two factors i.e. temperature and substrate concentration in 13 combinations. Standard order 5 represents the maximum ethanol concentration.

Run	Factor A:pH	Factor B:substrate g	Response 1 ethanol g/L
1	5.80	160.00	74.6
2	5.80	160.00	74.6
3	5.80	160.00	74.6
4	5.80	160.00	74.6
5	5.80	160.00	74.6
6	5.80	188.28	61
7	4.80	180.00	57
8	7.21	160.00	36
9	6.80	140.00	48.2
10	4.39	160.00	59.7
11	6.80	180.00	43
12	5.80	131.72	68
13	4.80	140.00	60.1

Table 14: representing the combined effect of two factors i.e. pH and substrate concentration in 13 combinations. Standard order 1 represents the maximum ethanol concentration

ANOVA for Response Surface Quadratic Models

Analysis of variance of quadratic model (i)

ANOVA and regression coeffcients are listed in **Table 15.** The model F value of 310.89 implies that model is significant. There is only 0.01% chance that value this large could occur due to noise. Values of "Prob> F" less than 0.050 indicate model terms are significant. In this case linear factors (A, B), quadratic factors (A^2, B^2) are significant terms. Values greater than 0.100 indicate model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model rejection may improve the model. Both the quadratic and linear effect of temperature and pH are significant. The effect of pH is more significant than temperature. These data

analysis also validate the inference that can be drawn from 3-D contour plots as shown in figure 18 which represents the effect of pH and temperature on ethanol production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob < F
Model	**2707.84**	**5**	**541.57**	**310.89**	**< 0.0001**
significant					
A-pH	***493.24***	***1***	***493.24***	***283.15***	***< 0.0001***
B-temp	***12.93***	***1***	***12.93***	***7.42***	***0.0296***
AB	***1.32***	***1***	***1.32***	***0.76***	***0.4125***
A^2	***1320.00***	***1***	***1320.00***	***757.76***	***< 0.0001***
B^2	***1166.63***	***1***	***1166.63***	***669.71***	***< 0.0001***
Residual	**12.19**	**7**	**1.74**		
Lack of Fit	***12.19***	***3***	***4.06***		
Pure Error	***0.000***	***4***	***0.000***		
Cor Total	**2720.03**	**12**			

Table 15: Analysis of variance of quadratic model (Partial sum of squares type III)

The "Pred R-Squared" of 0.9681 is in reasonable agreement with the "Adj R-Squared" of 0.9923 (**Table 16**).

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 43.110 indicates an adequate signal (**Table 16**). This model can be used to navigate the design space.

Std. Dev.	1.32	R-Squared	0.9955
Mean	**59.95**	Adj R-Squared	0.9923
C.V. %	**2.20**	Pred R-Squared	0.9681
PRESS	**86.71**	Adeq Precision	43.110

Table 16: Standard deviation and correlation coefficients

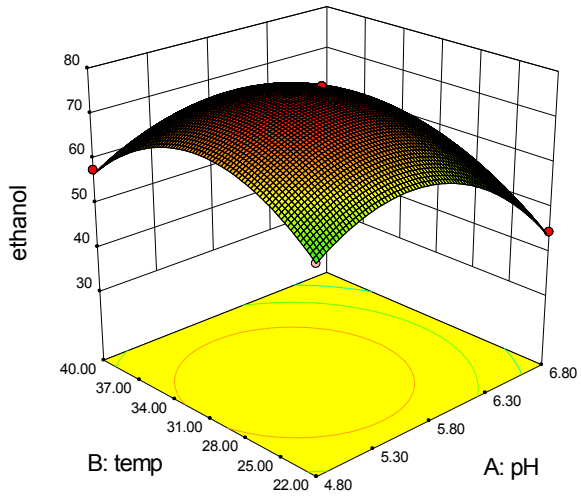


Figure 18: Response surface and contour plot showing the effect of pH and temperature on ethanol production

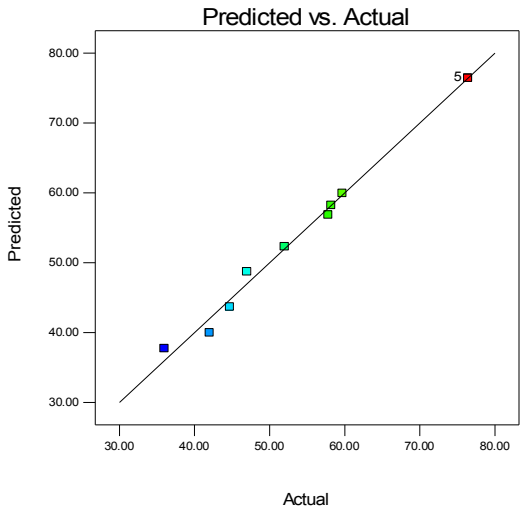


Fig. 19: Predicted vs. Actual response of experimental run under Central Composite Design.

Analysis of variance of quadratic model (ii)

ANOVA and regression coeffcients are listed in **Table 17.** The model F value of 89.23 implies that model is significant. There is only 0.01% chance that value this large could occur due to noise. Values of "Prob> F" less than 0.050 indicate model terms are significant. In this case both the linear factors A, B and quadratic factors (A^2, B^2) are significant terms. Values greater than 0.100 indicate model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model rejection may improve the model. The quadratic effect of temperature and substrate is more prominent than linear effect. It can also be concluded from the table that effect of temperature is more pronounced than substrate concentration. These data analysis also validate the inference that can be drawn from 3-D contour plots as shown in fig 20 which represents the effect of pH and temperature on ethanol production.

Source	Sum of Squares	df	Mean Square	F Value	pvalue Prob < F
Model	**1415.36**	**5**	**283.07**	**89.23**	**< 0.0001**
significant					
A-temp	***30.31***	***1***	***30.31***	***9.55***	***0.0175***
B-substrate	***29.64***	***1***	***29.64***	***9.34***	***0.0184***
AB	***0.56***	***1***	***0.56***	***0.18***	***0.6863***
A^2	***1236.33***	***1***	***1236.33***	***389.73***	***< 0.0001***
B^2	***236.55***	***1***	***236.55***	***74.57***	***< 0.0001***
Residual	**22.21**	**7**	**3.17**		
Lack of Fit	***22.21***	***3***	***7.40***		
Pure Error	***0.000***	***4***	***0.000***		
Cor Total	**1437.57**	**12**			

Table 17: Analysis of variance of quadratic model (Partial sum of squares type III)

The "Pred R-Squared" of 0.8902 is in reasonable agreement with the "Adj R-Squared" of 0.9735.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 24.310 indicates an adequate signal (**Table 18**). This model can be used to navigate the design space.

Std. Dev.	1.78	R-Squared	0.9846
Mean	**62.81**	Adj R-Squared	0.9735
C.V. %	**2.84**	Pred R-Squared	0.8902
PRESS	**157.91**	Adeq Precision	24.310

Table 18: Standard deviation and correlation coefficients

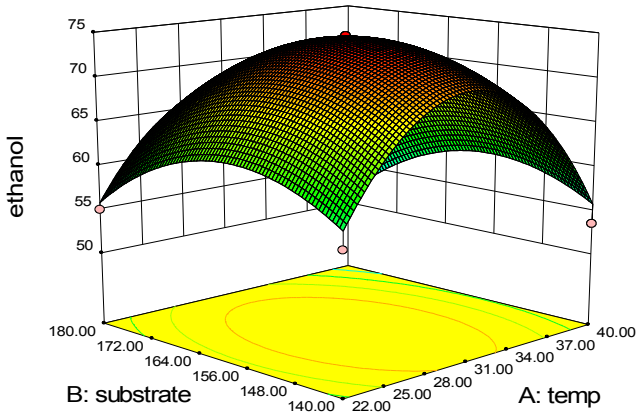


Fig. 20: Response surface and contour plot showing the effect of temperature and substrate concentration on ethanol production.

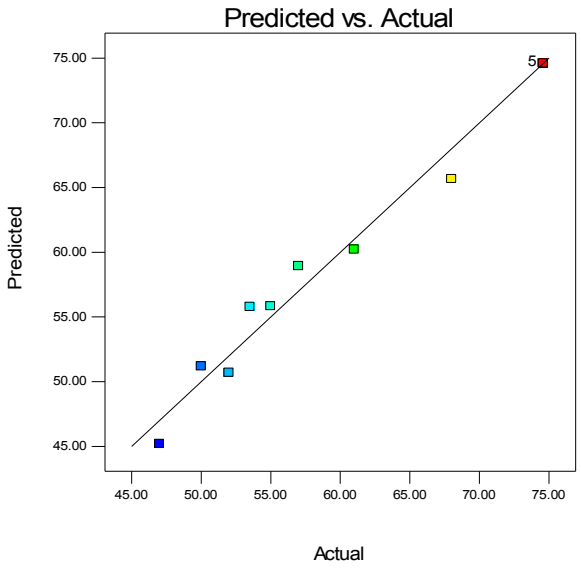


Fig 21: Predicted vs. Actual response of experimental run under Central Composite Design.

Analysis of variance of quadratic model (iii)

ANOVA and regression coeffcients are listed in **Table 19.** The model F value of 69.71 implies that model is significant. There is only 0.01% chance that value this large could occur due to noise. Values of "Prob> F" less than 0.050 indicate model terms are significant. In this case both the linear factors A, B and quadratic factors (A^2,B^2) are significant terms. Values greater than 0.100 indicate model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model rejection may improve the model. The quadratic effect of temperature and substrate is more prominent than linear effect. It can also be concluded from the table that effect of temperature is more pronounced than substrate concentration. These data analysis also validate the inference that can be drawn from 3-D contour plots as shown in figure 22 which represents the effect of pH and temperature on ethanol production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	**2050.95**	**5**	**410.19**	**69.71**	**< 0.0001**
significant					
A-pH	***441.30***	***1***	***441.30***	***74.99***	***< 0.0001***
B-substrate	***41.40***	***1***	***41.40***	***7.04***	***0.0328***
AB	***1.10***	***1***	***1.10***	***0.19***	***0.6782***
A^2	***1442.50***	***1***	***1442.50***	***245.13***	***< 0.0001***
B^2	***256.73***	***1***	***256.73***	***43.63***	***0.0003***
Residual	**41.19**	**7**	**5.88**		
Lack of Fit	***41.19***	***3***	***13.73***		
Pure Error	***0.000***	***4***	***0.000***		
Cor Total	**2092.14**	**12**			

Table 19: Analysis of variance of quadratic mode (Partial sum of squares Type III)

The "Pred R-Squared" of 0.8600 is in reasonable agreement with the "Adj R-Squared" of 0.9662.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 23.849 indicates an adequate signal (**Table20**). This model can be used to navigate the design space.

Std. Dev.	**2.43**	**R-Squared**	**0.9803**
Mean	**62.00**	Adj R-Squared	0.9662
C.V. %	**3.91**	Pred R-Squared	0.8600
PRESS		Adeq Precision	23.849
292.92			

Table 20: Standard deviation and correlation coefficients

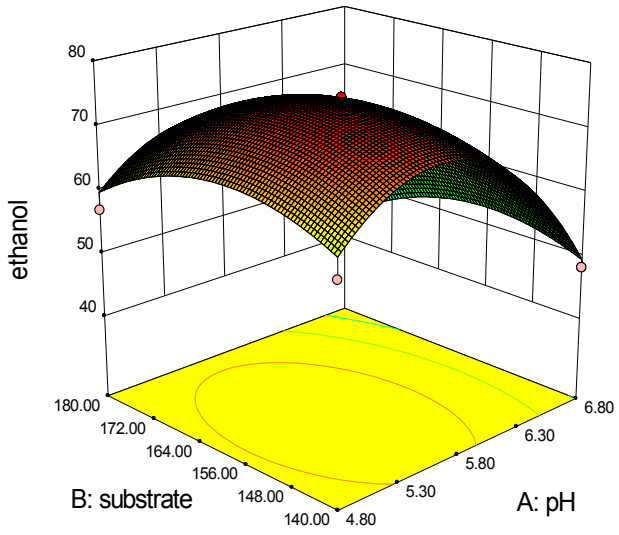


Fig 22: Response surface and contour plot showing the effect of pH and substrate concentration on ethanol production

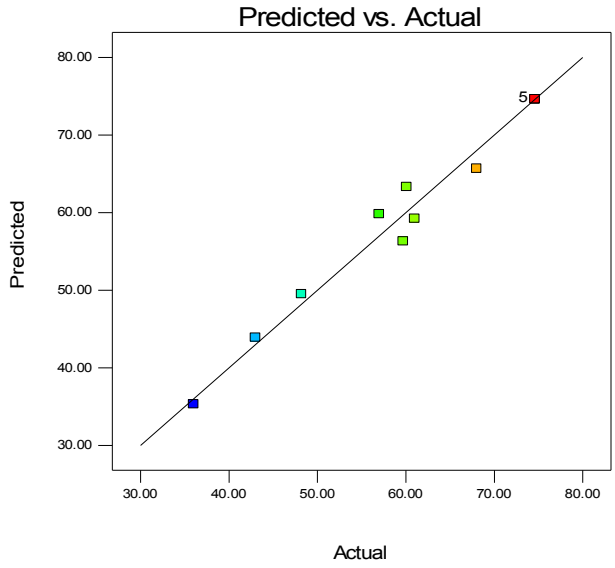


Fig 23: Predicted vs. Actual response of experimental run under CCD.

4.2 Discussion

In the present study, the 2^3 factorial central composite designs (CCD) of RSM using design expert software (trial version 8.0.7.1, STATE EASE, USA) was applied to optimize the conditions of pH, temperature and substrate concentration. The CCD enables to locate the correct values of temperature; pH and substrate concentration for maximum ethanol production .CCD was successfully used to optimize the key factors that influence the final ethanol concentration in fermentation of corn flour using the SSF method. The main advantages of applying multi factorial experiments are that such an approach considers the interaction between the non linear natures of the response in short experiments.

Three design matrixes were made by CCD, thirteen batches for each matrix were run in a 3 L fermentor. The first matrix designs conducted by CCD include combined effect of variables, temperature and pH while the substrate concentration remained constant at 160 g. The second matrix design conducted by CCD includes combined effect of variables temperature and substrate concentration at constant pH 5.8. The third matrix design conducted by CCD includes combined effect of variables pH and substrate concentration at constant temperature 31^{O}C. The maximum ethanol production (74.6 g/L) was achieved at combination – pH 5.8, temperature 31^{O}C.However, the minimum ethanol concentration (36 g/L) was obtained at pH 7.21 and temperature 31^{O}C in the first quadratic model. The maximum ethanol production (74.6g/L) was achieved at temperature 31^{O}C and substrate concentration 160 g. The minimum ethanol production (47 g/L) was found to be at temperature 43.7 ^{O}C and substrate concentration 160 g in the second quadratic model. The maximum ethanol production (74.6 g/L) in this experiment was found to be at pH 5.8 and substrate concentration 160 g. The minimum ethanol production (36 g/L) was reported at pH 7.21 and substrate concentration 160 g in the third quadratic model.

In the first quadratic model, the effect of pH is more significant than temperature. In the second quadratic model, the quadratic effects of both the variables were more significant than the linear effects. However, the linear effect of temperature was a slightly more than substrate concentration on ethanol production. The effect of pH was found to be more profound than substrate concentration in the third quadratic model. It can be

established that the change in pH has more profound effect on ethanol production followed by temperature and substrate concentration.

The values of adjusted R^2 in all the three quadratic models were high (0.9923), (0.9735) and (0.9662) which are the supporters to high significance of the models. The coefficient of variation (CV) indicates the degree of precision with which the treatment is compared. Usually, higher the value of CV, lower is the consistency of the experiment (Ghosh and Swaminathan, 2003). Here, the values of CV (2.20%), (2.84%) and (3.91%) indicates the reliability of the experiments performed. Adequate precision, is a measure of signal to noise ratio (43.550), (24.310) and (23.849) indicates a better precision and reliability of the experiments carried out. A ratio greater than 4 is desirable. In the present study, the ratio of 43.550, (24.310) and (23.849) indicates an adequate signal to use the models for prediction purposes (Montgomery, 2001).

The maximum ethanol production (78 g/L) during the first experiment i.e. by changing one variable at a time was found to be at conditions- pH 5.5, temperature 35^{o}C. It was found from the statistical analysis of the results that ethanol concentration was effected more by pH followed by substrate and temperature. This due to the fact that at high pH yeast cannot survive, rather than ethanol it produces acids.

5. Conclusion

This study concluded that ethanol concentration is greatly affected by the parameters such as pH, temperature and substrate concentration. The outcome of this study has undoubtedly indicated that RSM is an effective method for optimization of fermentation process. Central composite design confines the number of experiments. Therefore, smaller and less time consuming experimental designs could generally be sufficient for the optimization of many processes. The adequacy of all the models was satisfactory as correlation coeffients were (0.9923) (0.9735) (0.9662). The optimum conditions as stated by further numerical analysis of the responses using the Design Expert Software revealed that the maximum ethanol production was achieved at pH 5.8, temperature 31^{O}C and substrate concentration 160g.This additional proved that all the models were significant at 99% significance level.

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