Process optimization for batch culture of *Saccharomyces cerevisiae*

A Thesis
By

Zarifa Murshed Raceme
Student No: MS 110711
Session: 2010 - 2011

Submitted To

Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University in partial fulfillment of the requirements for the degree of Masters of Science (M. Sc.) in Biotechnology and Genetic Engineering.

Biotechnology and Genetic Engineering Discipline
Life Science School, Khulna University
Khulna – 9208, Bangladesh

September 2013

Process Optimization for Batch Culture of *Saccharomyces cerevisiae*

A Thesis
By

Zarifa Murshed Raceme
Student No: MS-110711
Session: 2010 - 2011

Approved as to style and contents by:

Supervisor

.....

Dr. Sheikh Md. Enayetul Babar
Professor & Head
Biotechnology and Genetic
Engineering Discipline
Khulna University, Khulna

Co - Supervisor

.....

Md. Ahedul Akbor
Senior Scientific Officer
Analytical Research Division
BCSIR Laboratories,
Dhaka-1205

.....

Dr. Sheikh Md. Enayetul Babar
Chairman
Examination Committee
Biotechnology and Genetic Engineering Discipline
Khulna University
Khulna, Bangladesh

Biotechnology and Genetic Engineering Discipline
Life Science School, Khulna University
Khulna – 9208, Bangladesh

Dedicated To……

My Venerated

Mother (Nasima Sultana)

Father (Hassan Murshed)

Elder Sister (Dr. Jereen Murshed)

And Husband (Engr. S.M. Hasan Ibna Mizan)

…….who sacrificed a lot for me!

Acknowledgement

First of all, I would like to express my humble gratitude to Almighty Allah for enabling me to complete the onerous job of this thesis.

I am heartily thankful to my supervisor, **Dr. Sheikh Md. Enayetul Babar,**Professor, and Head of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the thesis work. Without his supervision and constructive assistance, this study would not have been possible. There cannot be any writing form, which can fully express my adoring to him.

I also express my deepest gratitude and immense to co-supervisor **Md. AhedulAkbor,** Senior Scientific Officer, Analytical Research Division, BCSIR Laboratories, Dhaka – 1205 for his kind permission, cordial cooperation and valuable suggestions in thesis work in Science Laboratory.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

The Author

DECLARATION

This thesis work reports the original research work of the author, except as otherwise used. The material presented has not been submitted either in whole or in a part for a degree from this or any other university. The findings of the research has not been / will not be published anywhere without the concurrence of the concerned supervisor.

Zarifa Murshed Raceme.

ZarifaMurshed Raceme
Student No: MS 110711
Session: 2010 – 2011
September, 2013

CONTENTS

List of Tables and Figures

Chapter One

Introduction

1. Introduction

1.1 Background

Saccharomyces cerevisiae better known as baker's yeast; has been domesticated thousands of years ago. It is used in baking, brewing and wine making. *S. cerevisiae* is also an important model system in modern biology and medicine. It reproduces quickly, and large numbers of cells can be grown in culture in a very small space, in the same way as bacteria can be grown.

Many different types of processes for ethanol fermentation have been proposed including batch fermentation, continuous fermentation, continuous fermentation with cell recycling, fed-batch and repeated-batch culture. The batch culture with the intermittent addition of glucose and without the removal of fermentation broth is one of the most common methods for the production of ethanol. One advantage of this process is the reduction of substrate inhibition. A high concentration of sugar in fermentation medium inhibits growth and ethanol production. Other advantages of this process are higher productivity, higher dissolved oxygen in the medium, decreased fermentation time and reduced toxic effects of the medium components, which are present at high concentrations.

Though there exist many parameters like optical density, pH, incubation time, incubation temperature, etc., the important separation parameters are considered to be optical density, incubation temperature, and pH, all of which can have important effects. As an optimizing approach, the Box–Behnken design has so far been employed with moderate method optimizations.

Box–Behnken designs are the response surface methods (RSM) that can be used in optimization for analyzing biochemicals. Response surface methodology is a very useful statistical tool to optimize multiple variables for predicting the best performing conditions by using a minimum number of experiments. The Box–Behnken design is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial design. In this design, the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or nearly rotatable) and require three levels of each factor. The designs have limited capability for orthogonal blocking compared to central composite designs (CCD). The Box–Behnken design investigates every possible combination of two factors out of all factors, with the selected two factors set to the extreme limits while the other factors are set to zero. After investigating every combination, a center point run is added. This experimental design is useful for estimating linear effects, quadratic effects, and all linear two-way interactions.

1.2 Justification of study

Growth parameters: lag time (time to adapt to the environmental change), maximum relative growth rate (kinetics of growth), and stationary phase OD increment (related to the efficiency of growth), developments are important initial steps towards large-scale analysis of mutants based on rigorous statistical grounds. However, more analytical tools need to be put in place before the methodology becomes fully operational. It is the aim of this thesis to address several issues related to growth curve modeling and growth parameter estimation. We hope that the results we obtain will contribute to establishing of a rigorous modeling basis that will facilitate the phenotypic analysis of *S. cerevisiae*.

Due to a rapid depletion of the world's petroleum reserves and its rising prices day by day, new sources of hydrocarbons must be found to supply chemical and energy needs. In this context, ethanol fermentation offers promising alternative as it can be produced from various sources of raw materials. In view of increasing importance of ethanol, as an alternative source for chemicals and liquid fuel, a great deal of research interest in ethanol fermentation has been generated in the last two.

In view of the anticipated shortage of the traditional supplies of fossil fuels there is a great deal of interest in production of ethanol as an alternative biofuel in recent years. The present report describes the characterization of potential yeast from ferments capable of producing ethanol. Ethanol production by the selected yeasts was highest at pH 6.0 and 37°C in molasses medium with initial sugar concentration of 3%. In conical flask as fermenter, the yeast produced ethanol. This study further revealed that indigenous yeast isolates could be used to benefit the fuel ethanol, spirit and industrial alcohol industries.

1.3 Objectives

The specific objectives of this research work were as follows:

1. To study batch growth of *Saccharomyces cerevisiae.*
2. To optimize growth parameters for batch growth using Box-Behnken design.

Chapter Two

Literature Review

2. Literature review

2.1 Overview

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi, with 1,500 species currently described estimated to be only 1% of all fungal species. Most reproduce asexually by mitosis, and many do so via an asymmetric division process called budding. Yeasts are unicellular, although some species with yeast forms may become multicellular through the formation of a string of connected budding cells known as pseudohyphae, or false hyphae, as seen in most molds. Yeast size can vary greatly depending on the species, typically measuring 3–4 μm in diameter, although some yeast can reach over 40 μm.

By fermentation the yeast species *Saccharomyces cerevisiae* converts carbohydrates to carbon dioxide and alcohols - for thousands of years the carbon dioxide has been used in baking and the alcohol in alcoholic beverages. It is also extremely important as a model organism in modern cell biology research, and is one of the most thoroughly researched eukaryotic microorganisms. Researchers have used it to gather information about the biology of the eukaryotic cell and ultimately human biology. Other species of yeast, such as *Candida albicans*, are opportunistic pathogens and can cause infections in humans. Yeasts have recently been used to generate electricity in microbial fuel cells, and produce ethanol for the biofuel industry.

Yeasts do not form a single taxonomic or phylogenetic grouping. The term "yeast" is often taken as a synonym for *Saccharomyces cerevisiae*, but the phylogenetic diversity of yeasts is shown by their placement in two separate phyla, the Ascomycota and the Basidiomycota. The budding yeasts ("true yeasts") are classified in the order Saccharomycetales
Yeasts are chemoorganotrophs, as they use organic compounds as a source of energy and do not require sunlight to grow. Carbon is obtained mostly from hexose sugars, such as glucose and fructose, or disaccharides such as sucrose and maltose. Some species can metabolize pentose sugars like ribose, alcohols, and organic acids. Yeast species either require oxygen for aerobic cellular respiration (obligate aerobes), or are anaerobic, but also have aerobic methods of energy production (facultative anaerobes). Unlike bacteria, there are no known yeast species that grow only anaerobically (obligate anaerobes). Yeasts grow best in a neutral or slightly acidic pH environment.

Yeasts vary in what temperature range they grow best. For example, *Leucosporidium frigidum* grows at -2 to 20 °C (28 to 68 °F), *Saccharomyces telluris* at 5 to 35 °C (41 to 95 °F) and *Candida slooffi* at 28 to 45 °C (82 to 113 °F). The cells can survive freezing under certain conditions, with viability decreasing over time.

Yeasts are generally grown in the laboratory on solid growth media or in liquid broths. Common media used for the cultivation of yeasts include potato dextrose agar (PDA) or potato dextrose broth, Wallerstein Laboratories nutrient (WLN) agar, yeast peptone dextrose agar (YPD), and yeast mould agar or broth (YM). Home

brewers who cultivate yeast frequently use dried malt extract (DME) and agar as a solid growth medium. The antibiotic cycloheximide is sometimes added to yeast growth media to inhibit the growth of *Saccharomyces* yeasts and select for wild/indigenous yeast species. This will change the yeast process.

The appearance of white, thready yeast, commonly known as kahm yeast, is often a byproduct of the lactofermentation (or pickling) of certain vegetables, usually the result of exposure to air. Although harmless, it can give pickled vegetables a bad flavour and so must be removed regularly during fermentation.

Yeasts are able to grow in foods with a low pH, (5.0 or lower) and in the presence of sugars, organic acids and other easily metabolized carbon sources. During their growth, yeasts metabolize some food components and produce metabolic end products. This causes the physical, chemical, and sensible properties of a food to change, and the food is spoiled. The growth of yeast within food products is often seen on their surface, as in cheeses or meats, or by the fermentation of sugars in beverages, such as juices, and semi-liquid products, such as syrups and jams.

2.2 Growth in Liquid or Solid Medium

Yeast can be grown in either liquid medium or on the surface of a solid agar plate. Yeast cells will grow on a minimal medium containing dextrose (glucose) as a carbon source and salts that supply nitrogen, phosphorus, and trace metals. Yeast cells grow much more rapidly in the presence of rich medium that contains reagents such as yeast extract. These provide many of the metabolites that the cells would synthesize when growing under minimal growth conditions. During log-phase growth in rich medium, yeast cells divide once approximately every 90 min. Early log phase is the period when cell densities are <107 cells/mL. Mid–log phase is the period when densities are between 1 and 5 · 107 cells/mL. Late log phase occurs when cell densities are between 5 · 107 and 2 · 108 cells/mL. The rich medium yeast extract, peptone, dextrose (YPD) is most commonly used for growing yeast under nonselective conditions.

Autoclaving is usually carried out for 15 min at 15 lb/in.2 but should be increased when larger volumes are prepared. Minimal medium, also known as synthetic defined (SD) medium, supports the growth of yeast, which has no nutritional requirements. It contains yeast nitrogen base, ammonium sulfate, and dextrose. Minimal medium is commonly used when testing the mating type of yeast cells using specific mating tester strains (of both mating types). Minimal medium is most often used as a basal medium to which mixtures of amino acids and nucleoside precursors are added. Yeast can grow on solid medium. For all plates, agar is added to a final concentration of 2.0% (20 g/L). To prevent agar breakdown during autoclaving, it is possible (although not necessary) to add a pellet of sodium hydroxide (per liter) to the medium-agar suspension. Care should be taken to avoid autoclaving the medium-agar suspensions longer than necessary, because this will cause agar breakdown leading to "soft" plates.

Wild-type yeast grows well at 30°C with good aeration and glucose as a carbon source. Erlenmeyer flasks work well for growing liquid cultures, and baffled-bottom flasks are good but not necessary. Although small cultures may be grown in culture tubes, in many cases the cells will settle out from suspension.

For optimal aeration and growth, the medium should constitute no more than 20% of the total volume of the flask, and growth should be carried out in a shaking incubator at 250–300 rpm. On solid YPD medium at 30°C, single may be seen after 24 h, but generally growth for at least 48 h is required prior to picking of colonies or replica plating. Growth on dropout medium is approx 50% slower than that observed in YPD.

2.3 Growth cycle of yeast

The cell division of yeast occurs by budding in which a daughter is initiated as an outgrowth from the mother cell, followed by nuclear division, cell-wall formation, and finally cell separation. The sizes of haploid1 and diploid2 cells vary with the phase of growth and from strain to strain. Yeast cell grows in three main phases– the lag phase, the exponential phase and the stationary phase. When a culture of yeast cells is inoculated in a fresh growth medium, they enter a brief lag phase where they are biochemically active but not dividing.

The lag phase refers the initial growth phase, when number of cells remains relatively constant prior to rapid growth, also referred as adaptation time. During this phase the individual cells are actively metabolizing, in preparation for cell division. The cells usually activate the metabolic pathways to make enough of the essential nutrients to begin active growth. From literature it is seen that the duration and extent of this phase depends on firstly the initial population size and secondly environmental conditions like temperature, pH, alcohol, oxygen, salt concentration, nutrients etc. Once the cell starts actively metabolizing, they begin DNA replication and shortly after the cells divide. This begins the second phase of growth called the exponential phase of growth. This is the period in which the cells grow most rapidly. The time it takes the culture to double is called generation time. This exponential phase depends on several factors: the organism itself, the growth medium, and the temperature are all important factors in determining the generation time. The third phase in growth of yeast is stationary phase when metabolism slows and the cells stop rapid cell division.

The factors that cause cells to enter stationary phase are related to change in the environment typically caused by high cell density. Figure 2.1 illustrates how the yeast cell grows in three main phases.

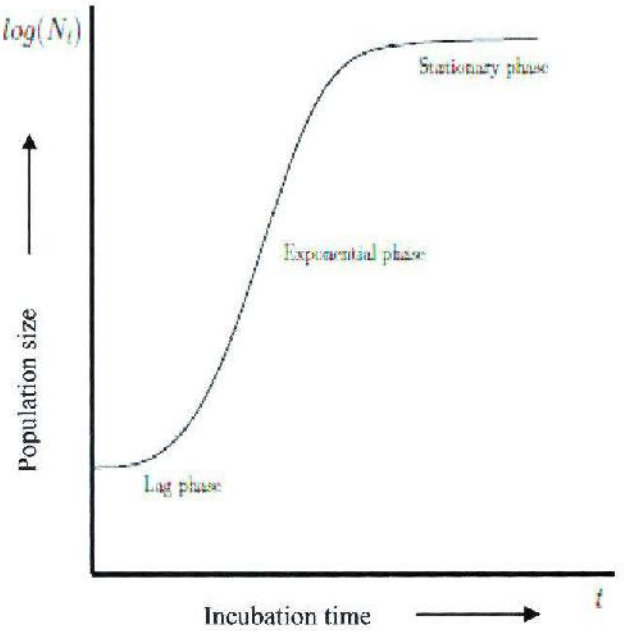


Figure 2.1 A typical logarithmic yeast growth curve where N_t is the population size at time t.

2.4 Yeast Fermentation

Baker's yeast is the common name for the strains of yeast commonly used as a leavening agent in baking bread and bakery products, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol. Baker's yeast is of the species *Saccharomyces cerevisiae*, which is the same species commonly used in alcoholic fermentation, and so is also called brewer's yeast.

Fermentation can be defined as an energy yielding process where yeast converts organic molecules (such as sugar) into energy, carbon dioxide or/and ethanol depending on the respiration pathway. Yeast can respire in anaerobically and aerobically. However, yeast gets more energy from aerobic respiration, but in the absence of oxygen it can continue to respire anaerobically, though it does not get as much energy from the substrate. Yeast produces ethanol when it respires anaerobically and ultimately the ethanol will kill the yeast.

$C_6H_{12}0_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 + 2\ ATP$
$C_6H_{12}0_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 16\text{-}18\ APT$

When the feed substrate to the reactor is not monosaccharide e.g. sucrose ($C_{12}H_{22}O_{11}$), yeast enzyme cause glycosidic bond to break in a process called hydrolysis

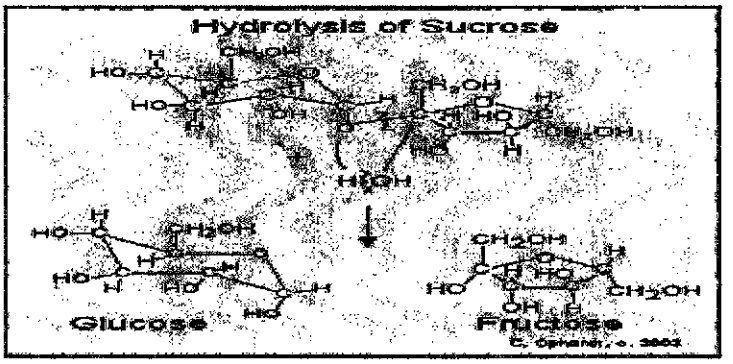


Figure 2.2 Glucose formation pathway.

In ideal fermentation process in which the growing cells are consuming the substrate (sugars), and producing more cells according to the following scheme.

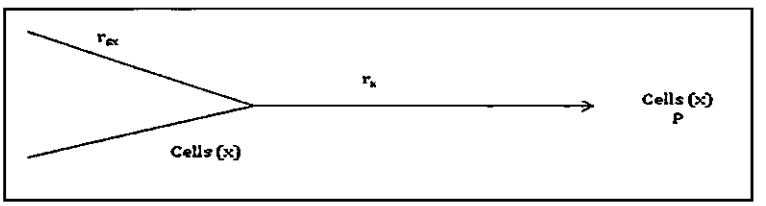


Figure 2.3 Reaction pathway to produce ethanol.

r_{sx} = rate of substrate consumption

r_x = rate of cell growth

s = substrate concentration

x = cell concentration

P = ethanol concentration (in anaerobic case)

Assume all yeast generated is attributable only to sugar complete consumption. Conservation of mass requires that the remaining product be equimolar amounts CO_2 and ethanol

2.5 Determination of Cell Density

Optical density (absorbance), OD, is a widely used concept in the estimation of the total number of cells present in a culture. It is a measure of the turbidity of the culture. A cell suspension looks cloudy (turbid) to the eye because cells scatter the light passing through the suspension. The more cell material is present, the more the suspension scatters the light and the more turbid it will be. Optical density can be measured with a spectrophotometer; a device that passes light through a cell suspension and detects the amount of unscattered light that goes through. Since the cell sizes affect the absorption capacity, the OD measurements are never perfectly proportional to the number of cells or to the cell mass. This error affects even the measurements done in the exponential phase since the cell size distribution in a culture depends on the age distribution which in turn depends on the rate of growth. For the sake of simplicity in the sequel, we choose to ignore this problem, both in the calibration and in the interpretation of the data.

The approximate number of cells in a culture can be determined with a spectrophotometer by measuring the optical density (OD) at 630 nm. Cultures should be diluted such that the observed reading (OD_{630}) is <1.0. In this range, an OD_{630} = 1.0 is approximately equal to 3 · 107 cells/mL. However, there is strain variability in this measurement, or it may be affected by over expression of a particular gene product within a strain (such as two-hybrid bait)

2.6 Gas chromatography (GC)

Gas chromatography (GC), is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC helps in identifying a compound.

In gas chromatography, the *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (an homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a *gas chromatograph* (or "acrograph", "gas separator").

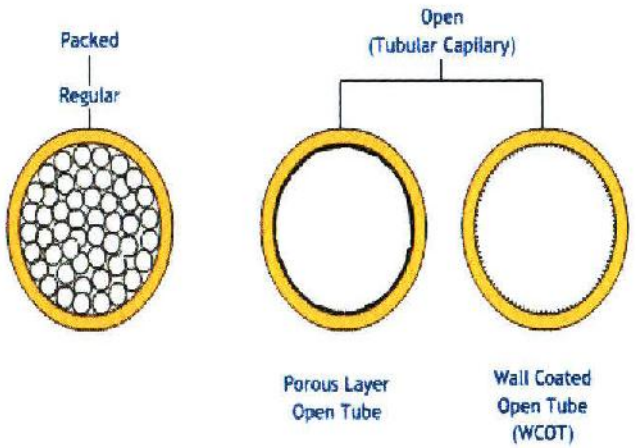


Figure 2.4 Type of GC tube

The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the retention time of the compound. The comparison of retention times is what gives GC its analytical usefulness.

Recommended stationary phases for various sample types

Compound to be separated	Types of stationary phases used	
gases	alumina, silica gel, zeolites (molecular sieves) porous polymers	Gas:solid
nonpolar liquids	methylsiloxanes	Gas:liq
PCBs, petrochemical samples	phenylmethylsiloxanes, polysiloxane carboranes	
herbicides/pesticides, pharmaceuticals	phenyl polysilphenylene siloxanes	
sugars	cyanopropylphenyl methylsiloxanes	
free fatty acids, alcohols	polyethylene glycols	
alcohols, amines	phenylmethylsiloxanes (>50% phenyl)	

Figure 2.5 A gas chromatograph with a headspace sampler

2.7 Gas chromatography analysis

Principle of gas chromatography: The sample solution injected into the instrument enters a gas stream which transports the sample into a separation tube known as the "column." (Helium or nitrogen is used as the so-called carrier gas.) The various components are separated inside the column.

A simple GC system consists of:

1. Gas source (with pressure and flow regulators)
2. Injector or sample application system (sample inlet)
3. Chromatographic column (with oven for temperature control)
4. Detector & computer or recorder

A typical GC system used is shown below (*a gas chromatograph*)

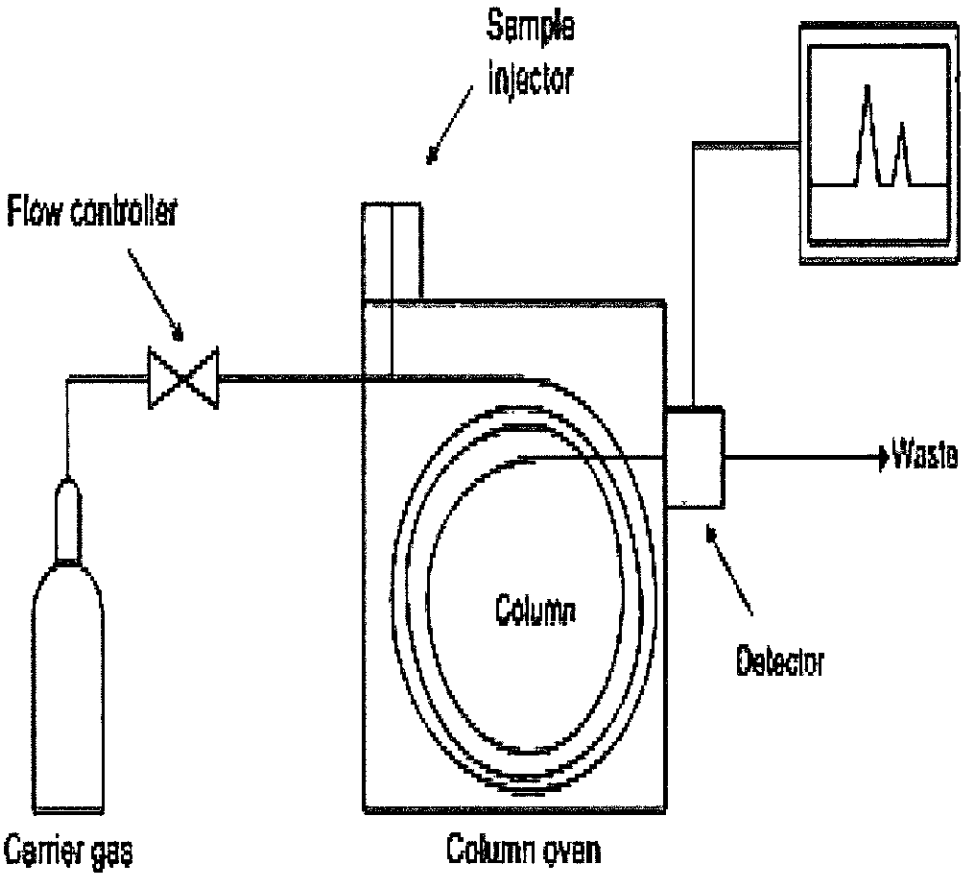


Figure 2.6 Component parts of GC structure

A gas chromatograph is a chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph uses a flow-through narrow tube known as the *column*, through which different chemical constituents of a sample pass in a gas stream (carrier gas, *mobile phase*) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the *stationary phase*. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (*retention time*). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, column length and the temperature.

In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a microsyringe (or, solid phase microextraction fibers, or a gas source switching system). As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of the analyte molecules either onto the column walls or onto packing materials in the column. The rate at which the molecules progress along the column depends on the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times (retention time).GC can be applied to the separation of any compound that is either naturally volatile (i.e., readily goes into the gas phase) or can be converted to a volatile derivative. A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are identified (qualitatively) by the order in which they emerge (elute) from the column and by the retention time of the analyte in the column. To measure a sample with an unknown concentration, a standard sample with known concentration is injected into the instrument. The standard sample peak retention time (appearance time) and area are compared to the test sample to calculate the concentration.

The following devices are common types of GC detectors:

1. Thermal Conductivity Detector (TCD)
2. Flame Ionization Detector (FID)
3. Nitrogen-phosphorus Detector
4. Electron Capture Detector (ECD)
5. Mass Spectrometers (discussed later in the course)

The choice of detector will depend on the analyte and how the GC method is being used (i.e., analytical or preparative scale).

Flame Ionization Detector (FID)

This detector is similar in general arrangement to the flame thermocouple detector except that the thermocouple is replaced by a collecting grid. An EMF may be

applied across the same and changes in its resistance determined. Presence of an eluted vapor is indicated by a large fall in the resistance due to the ionization of organic molecules in the flame. A suitable design is shown in Figure 2.5. A hypodermic needle serves as a jet and a piece of gauge servos as the collecting grid which is placed at about 1 cm above the jet.

The flame may be produced by using a mixture of hydrogen and air. Nitrogen can be used as a carrier gas. The purpose of a FID is to detect compounds as they exit out of the gas chromatography column. The compounds that are exiting the column are in the gas phase. Detection is done by first turning the gas compounds into ions and electrons and then collecting the charged molecules. As these charged materials are collected, they make a current which is measured. The larger the current the more charged compounds are being collected.

Flame ionization detectors are used most often for organic compounds. This is because organic compounds contain lot of carbon atoms which easily turn into ions. Reduced carbons (carbons with no oxygen bonds) make the most ions. Other carbons such as alcohols (C-O-H) and carbonyls (C=O) do not make as many ions. Many times organic samples being tested are not pure. The samples often contain impurities such as water (T-feO), carbon dioxide (CO_2), sulfur dioxide (SO_2), nitrous oxides (NO), and noble gases. The FID does not detect these impurities because they do not burn. This makes the FID a very useful detector because the samples being tested do not have to be purified before being tested. FID can be used to detect very small amounts of material, as small as 10 g/s. It is also accurate over a large range of molecule amounts (a range of over 10). One problem with FID is that it destroys the sample being tested. Any sample used in gas chromatography with an FID cannot be used for other measurements afterwards. Also, a lot of fuel is required as well as very exact equipment to get exact results.

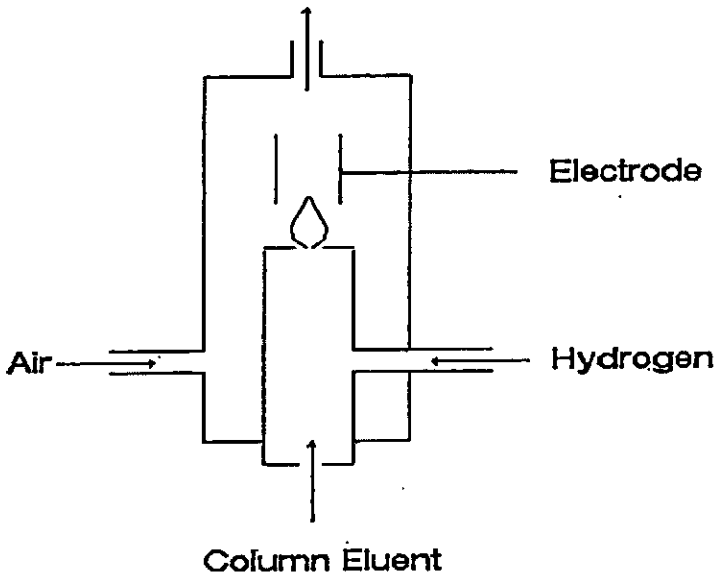


Figure 2.7 Structure of FID

2.8 Gas Chromatography Mass Spectrometry (GC-MS)

Gas Chromatography Mass Spectrometry (GC-MS) is a technique for the analysis and quantization of organic volatile and semi-volatile Gas Chromatography (GC) is used to separates mixtures into individual components using a temperature-controlled capillary column. Smaller molecules with lower boiling points are travel down the column more quickly than larger molecules with higher boiling point.

Mass spectrometry (MS) is used to identify the various components from their mass spectra. Each compound has a unique or near unique mass spectrum that can be compared with mass spectral databases and thus identified. Through use of standards, quantization is also possible compounds. The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off of) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by

breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio.

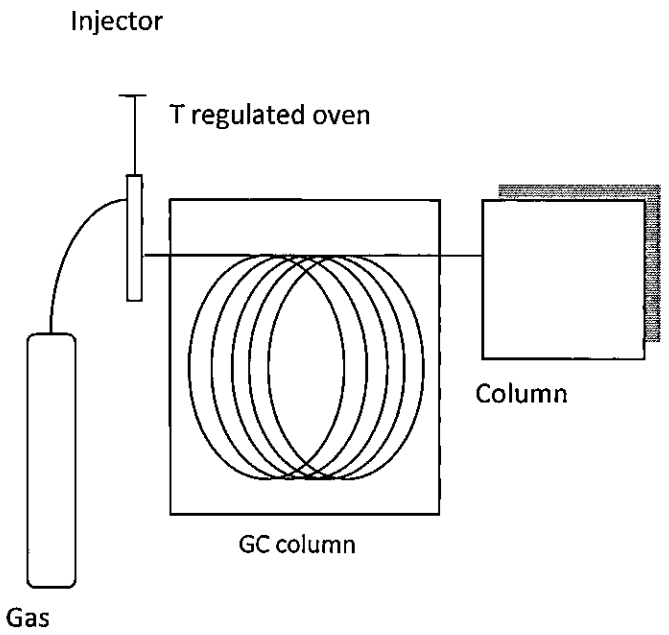


Figure 2.8: GC Schematic diagram

There two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. Flame ionization detector) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (i.e. have the same retention time), which results in two or more molecules that co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore, when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it typically lends to increased certainty that the analyte of interest is in the sample.

Mass spectrometer detectors:

The most common type of mass spectrometer (MS) associated with a gas chromatograph (GC) is the quadrupole mass spectrometer, sometimes referred to by the Hewlett-Packard (now Agilent) trade name "Mass Selective Detector" (MSD). Another relatively common detector is the ion trap mass spectrometer. Additionally one may find a magnetic sector mass spectrometer, however these particular instruments are expensive and bulky and not typically found in high-throughput service laboratories. Other detectors may be encountered such as time of flight (TOP), tandem quadrupoles (MS-MS) (see below), or in the case of an ion trap MSn where n indicates the number mass spectrometry stages.

Gas Chromatography - spectrometric System for sample analysis:

Many ethanol analyses have done with GC since impurities in ethanol are basically volatile as well as ethanol itself. A sample is vaporized in headspace. The sample vapor is sent to column, each component in sample is separated depending on its physical and chemical property. The end of column the amount of each compounds are measured by a detector.

The following components are also required

- Detector: Flame Ionization Detector (FID),Mass Spectrometer (Shimadzu GCMS-QP2010 Ultra, Japan)
- The analytical column is an Rtx-5 & Rxi-5ms (Restek) capillary column (30-m x 0.25-mm) i.d., 0.25-µm film thickness.
- Data Station: The data station was capable of storing and reintegrating chromatographic data and also of determining peak areas using a forced baseline projection.
- Autosampler: An autosampler (Shimadzu AOC-20 i+s, Japan) capable of making 1 to 4 µl injections is recommended.
- Disposable pipets: Pasteur
- Microsyringes: 10- µl, 100- µl, 250- µl, 500- µl, 1000- µl
- Temperature programming: Injection temperature 180 °C; detector temp. 280 °C; column temp. initially 40 °C for 5 min. heat to 120 °C at rate 3 °C/min and keep for 5

min; carrier gas flow 1 ml/min; sample injection volume 0.5 µl; injection mode; spit; spit ratio 1:100.

A typical GC chromatogram of alcoholic beverage is shown in following figure

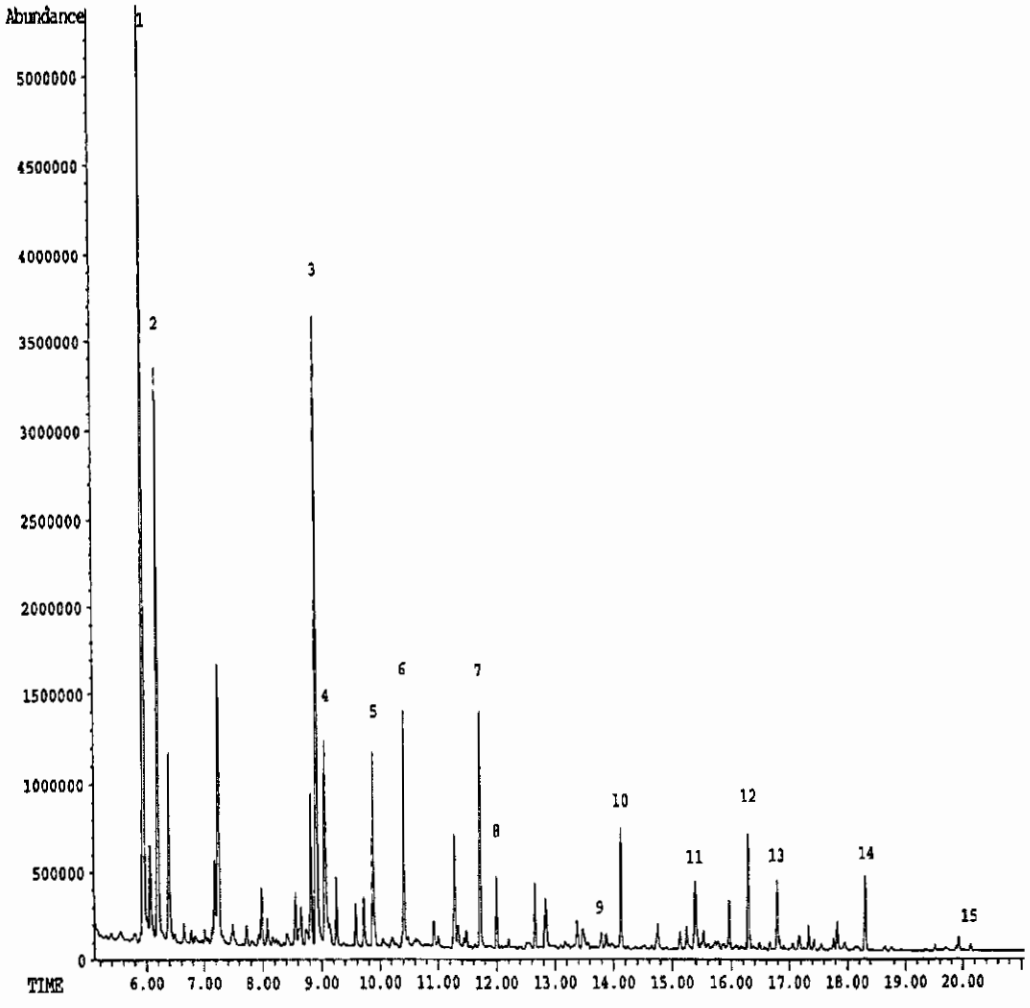


Figure 2.9 Gas Chromatogram

2.9 Box-Behnken design

The Box-Behnken design is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial design. In this design the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require 3 levels of each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs.

Examples of Box-Behnken designs are given:

Table 2.1 Structure of Box-Behnken Designs for Three Factors

Replication	X_1	X_2	X_3
1	-1	-1	0
1	1	-1	0
1	-1	1	0
1	1	1	0
1	-1	0	-1
1	1	0	-1
1	-1	0	1
1	1	0	1
1	0	-1	-1
1	0	1	-1
1	0	-1	1
1	0	1	1
3	0	0	0

For three factors, the Box-Behnken design offers some advantage in requiring a fewer number of runs. For 4 or more factors, this advantage disappears.

The aim of this study was to investigate the combined influence of 3 independent variables in the compression coated tablet of mesalamine for ulcerative colitis. A 3-factor, 3-level Box-Behnken design was used to derive a second order polynomial equation and construct contour plots to predict responses. The independent variable selected was: temperature in compression Glucose% ($X1$), pH ($X2$) and incubation temperature ^{0}C ($X3$). Fifteen batches were prepared and evaluated for percent of ethanol produced (Ye), OD of yeast cell (Yc). Transformed values of independent and dependent variables were subjected to multiple regressions to establish a full-model second-order polynomial equation. The computer optimization process and contour plots predicted the levels of independent variables $X1$, $X2$, and $X3$.

While Identifying desirable properties for a response surface design following sides are main concern:

Satisfactory distribution of information across the experimental region.
Fitted values are as close as possible to observed values.
Good lack of fit detection.
Internal estimate of error.
Constant variance check.
Transformations can be estimated.
Suitability for blocking.
Sequential construction of higher order designs from simpler designs
Minimum number of treatment combinations.
Good graphical analysis through simple data patterns.
Good behavior when errors in settings of input variables occur.

Based on these properties regarding optimization process is designed.

Chapter Three

Materials and Methods

3. Materials and Methods

This research work was carried out in Bioprocess Technology laboratory of Biotechnology and Genetic Engineering discipline, Khulna University, Khulna, Bangladesh and Analytical research division, Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh.

3.1 Materials

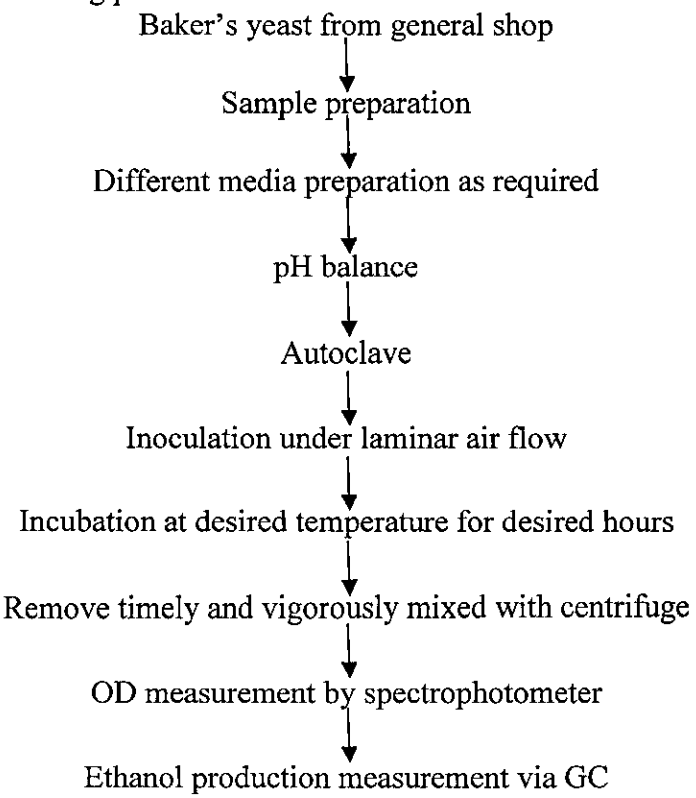
All the chemicals that were used in the process running were good in quality and instruments were operated in proper way.

Baker's yeast (Grocery Shop, Khulna)
YPD media (Dextrose, Yeast Extract, Peptone) from *Sigma Aldrich*, USA
Distill Water- prepared in laboratory
Absolute ethanol from TEDIA, USA
Electric Balance
Measuring cylinder
pH meter
Autoclave
Micropipette
GC-MS
GC
HT3
Glass road, biker, screw cap bottle, etc from Pyrex

3.2 Methods

Working Procedure:

The laboratory working procedure was as follows



3.2.1 Experimental Procedure

Media preparation

Yeast growth favorable media preparing procedure is as follows

Dissolve 10g of Yeast extract in 500ml water

↓

Dissolve 20g of Peptone in the above solution

↓

Dissolve 20g Dextrose in the above solution

↓

Volume to 1000ml with water

↓

pH balance

↓

Autoclave

Suspension

To prepare a suspension of bakers yeast 1gm yeast was added in 50ml distil water. Then it was properly mixed.

Inoculation

Under laminar air flow 180 µl yeast suspension were added in 30 ml media. And immediately screw capped to prevent aeration.

Fermentation

Just after inoculation the screw capped bottles were placed in incubator at 33°C for experimented time.

Desirable media preparation:

For 3%Glucose contained media: 1000ml H_2O contained 10gm yeast extract, 20gm peptone and 30gm dextrose. Likewise, 2% Glucose contained media means 1000ml H_2O with 10gm yeast extract, 20gm peptone and 20gm dextrose. Thus 1% Glucose contained media has 10gm dextrose with others.

3.2.2 Statistical design

Box-Behnken design

The Box-Behnken design is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial matrix. In this design the treatment combinations are at the mid-points of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require three levels of each factor. Table 3.1 represents structure of Box-Behnken design. These designs

have limited capability for orthogonal blocking compared to the central composite designs (Box and Behnken, 1960).

The geometry of this design suggests a sphere within the process space such that the surface of the sphere protrudes through each face with the surface of the sphere tangential to the mid-point of each edge of the space. For three factors, the Box-Behnken design offers some advantage in requiring a fewer number of runs.

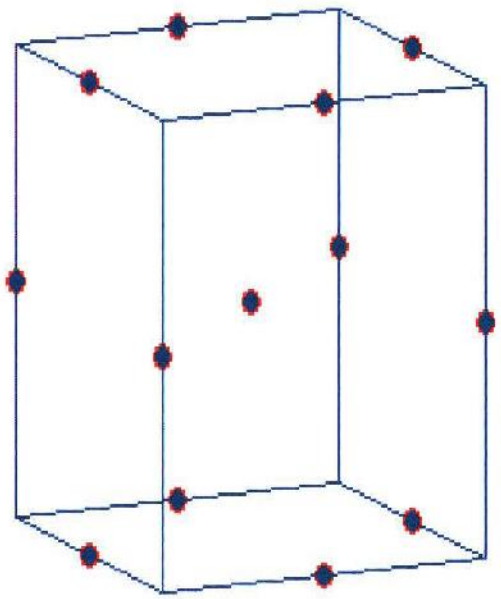


Figure 3.1: Box-Behnken designs for three factors.

Sample preparation

The experimental design used for this study was the Box–Behnken design. This design helps in investigating linear, quadratic, and cross product effects of three factors, each varied at three levels, and also include three center points for replication. According to figure 3.1 these three factors were Glucose% (X_1), pH(X_2), Incubation temperature ^{0}C (X_3) and the three levels are high, middle, and low which are designated as +1, 0, and –1, respectively. The variables (factors) and their levels are presented in Table.

Table-3.1: Levels of variables chosen for Box–Behnken study

	Level		
variables	+1	0	-1
Glucose% (X_1)	3%	2%	1%
pH (X_2)	7.0	6.0	5.0
Incubation temperature ^{0}C (X_3)	36	30	24

Table-3.2: Input parameters for Box–Behnken experimental design

Run No	Glucose%	pH	Incubation temperature (^{0}C)
1	1	5	30
2	1	7	30
3	3	5	30
4	3	7	30
5	1	6	24
6	1	6	36
7	3	6	24
8	3	6	36
9	2	5	24
10	2	5	36
11	2	7	24
12	2	7	36
13	2	6	30
14	2	6	30
15	2	6	30

Two responses namely, optical density Y(c), ethanol production Y(e) migration time (t), and resolution (R), were tested in this study. The quadratic response equation was used as follows:

$$Y=A_0A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_1X_3 + A_6X_2X_3 + A_7X_1^2 + A_8X_2^2 + A_9X_3^2$$

Where A_0 the regression coefficient, A_1–A_3 are the linear coefficients, A_4–A_6 the crossproduct coefficients, and A_7–A_9 are the quadratic coefficients. The regression analysis and statistical significance were carried out using Microsoft Excel software.

Surface plots and contour plot were developed using the same software along with SigmaPlot 12.

3.3 Properties of measurements

Optical density

A spectrophotometer is a device used to measure light at a specific wavelength. It consists of two parts: a spectrometer and a photometer. The spectrometer provides light at a specific wavelength. The photometer measures how intense the light is. By calculating the amount of light that a solution is able to absorb and applying Beer's Law, the spectrophotometer can determine the concentration of a colored solution. For yeast cell count absorbance was at 630nm.

There is a way to measure the concentration of some unknown solution using the spectrophotometer by generating a standard curve: a graph of absorbance vs. concentration for standard solutions whose yeast concentration are known. We then compare the absorbance of an unknown solution (of the same yeast) to that curve. Essentially, we are "calibrating" the spectrophotometer to measure the concentration of the molecule we're interested in.

To make a standard curve, 10 different solutions with known concentrations (called standards). You can come up with these easily by taking a single solution of known concentration and making several different dilutions of it. Then, measure the absorbance of each known solution and draw a graph, plotting absorbance vs. concentration.

Program of GC

The method is the collection of conditions in which the GC operates for a given analysis. Method development is the process of determining what conditions are adequate and/or ideal for the analysis required. Conditions which can be varied to accommodate a required analysis include inlet temperature, detector temperature, column temperature and temperature program, carrier gas and carrier gas flow rates, the column's stationary phase, diameter and length, inlet type and flow rates, sample size and injection technique. Depending on the detector(s) installed on the GC, there may be a number of detector conditions that can also be varied. Some GCs also include valves which can change the route of sample and carrier flow. The timing of the opening and closing of these valves can be important to method development.

Used program of GC is given here where analysis time was 60 minutes.

SPL1

Temperature 280℃
Injection mode Splt
Sampling time 1 minute
Pressure 35.0 kPa

Holding time 0
Totals program time 0
Carrier gas He
Row control mode: pressure
Pressure: 35.5 kPa
Total flow 39.0 ml/min
Linear velocity 18.4 cm/sec
Purge flow: 3.0 ml/min
Split ratio 35.0
Column information: RTX-5
Length 35.0 m
Inner diameter: 0.32 mmID
Film thickness: 0.250 m

Column
Temperature 40℃
Equilibration time 3.0 min
Total program time 45min
Temperature 40 ℃ holding 20 min
Increase 10 ℃ till 240℃ holding 5.0 min
Column information: RTX-5
Serial number 1044406
Installation date 12/02/27
Column maximum temperature 350℃

FID1
Temperature 280℃
Sampling rate 40msec
Stop time 60.0 min
Flow 30
Holding time 0
Total program time 0

Program of HT3:

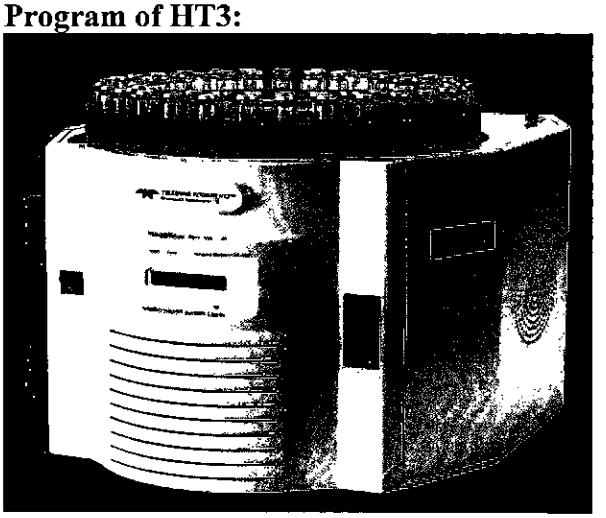


Figure 3.2: Used HT3

The HT3™ Automated Headspace Analyzer incorporates traditional static headspace with the option to perform dynamic headspace. In the static set up, a sample is placed in a vial and then delivered to the autosampler. Once in the auto sampler, the vial is loaded into a platen for heating, upon reaching the final heat time it is then mixed for a set period of time. Using an electronic mass flow controller the static vial pressure is recorded and the sample is pressurized to a user-defined set point. Next, the sample is passed through a fixed volume loop to another user-defined final pressure set point. The loop containing the sample is then placed in line with the GC column for separation and detection.

Used method is:
Residual ethanol method
Constant heat time ON
GC cycle time 50.0 min
Valve oven temperature 105℃
Transfer line temperature 105℃
Standby flow rate 50ml/min
Platen/sample temperature: 80℃
Platen temperature equilibrium time 1.0min
Sample equilibrium time 45.0min
Mixer : ON
Mixing level: level 5
Mixing time: 5.0 min
Mixer stabilize time: 0.50 min
Pressurize: 10 PSIG
Pressurize time: 0.50min
Pressurize equilibrium time: 0.20min
Loop fill pressure: 5 PSIG
Loop fill time 2.0min
Inject time: 1.0min

Chapter Four

Result and Discussion

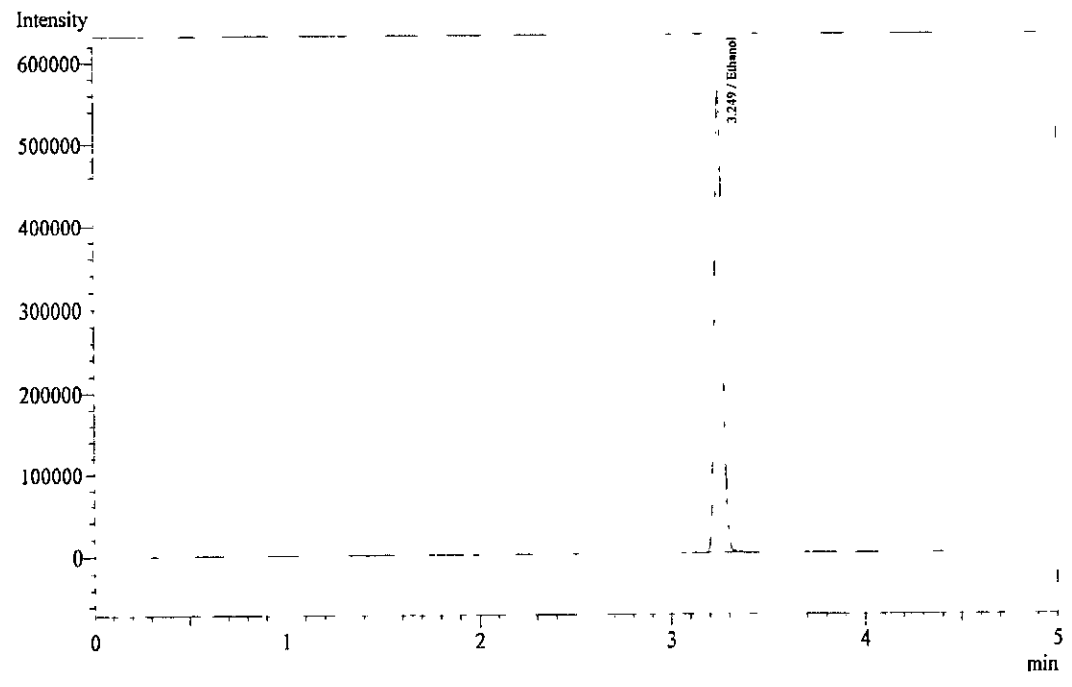
4. Result and Discussion

4.1 Analysis and detection

Microorganisms as biocatalysts are widely used in fermentation processes where a product and biomass are obtained from the fermentable sugars. These biologically active organisms have significant contributions in beverage and food industries. *Saccharomyces cerevisiae* is budding yeast known as a baker yeast or brewer yeast. This microorganism is most often used in the fermentation process and obtains energy from various carbon sources. Yeasts are the most common microorganisms for ethanol fermentation. Among the yeast kingdom, *S. cerevisiae* is one of the well known ethanol producers. Ethanol is an essential chemical which is used as a raw material for a vast range of applications including chemicals, fuel (bioethanol), beverages, pharmaceuticals and cosmetics. *S. cerevisiae* has short germination time and is easily cultured in large scale processes. The rapidly growing *S. cerevisiae* ensured the ethanol production in batch culture with the limited substrate concentration; the ethanol production with high substrate concentrations and without substrate inhibition was reported by the immobilized cells.

4.1.1 Calculation of ethanol production

Ethanol production was calculated in %. GC gave the print out result of ethanol in area.



Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	3.249	16447733	556456	0.000	ppm		1	Ethanol
Total		16447733	556456					

Figure 4.1 A chromatographic area of ethanol

$$\text{Ethanol \%} = \frac{\text{Actual area of sample} \times \text{Standard Value}}{\text{Area of standard}}$$

Table 4.1 Records from GC

Actual Area of Sample	Area of Standard	Ethanol %
7670770	1644375	0.46415302
14305760	1693524	0.84050956
27222330	1644375	1.64720446
27262710	1644375	1.64964783
9711600	1693524	0.57058784
18019660	641662	1.39009817
35842440	1693524	2.10585901
29950920	1693524	1.7597132
5313260	641662	0.40988304
18812760	850372	1.09508735
22091320	850372	1.28593173
13021240	850372	0.75796402
19677320	850372	1.14541323
19411980	850372	1.12996784
14650200	641662	1.13016651

Actual area of sample = Sample area-Blank area
Area of standard varies with time and ethanol concentration.
Standard value measured regularly. Here used two values are as follows:

99.5% 0.05ml used to make 50ml standard of values $= \frac{0.05 \times 99.5}{50} = 0.0995\%$

99.5% 0.05ml used to make 100ml standard of values $= \frac{0.05 \times 99.5}{50} = 0.0495\%$
Blank area was 3358.

4.1.2 Yeast cell concentration

Firstly OD was measured from known Concentration. Then an equation (y=21.965x+0.0211) was developed by using these data. Using this equation, concentrations were calculated by measured OD.

Table 4.2 Relation of cell concentration with OD

Known Concentration (µg/ml)	OD_{630}	Concentration from Equation (µg/ml)	OD_{630}
0.0009	0.025	0.008383	0.205
0.001	0.053	0.001321	0.05
0.003	0.084	0.008337	0.204
0.006	0.16	0.000592	0.034
0.009	0.229	0.003736	0.103
0.01	0.249	0.012301	0.291
0.02	0.43	0.004738	0.125
0.03	0.706	0.010661	0.255
0.04	0.885	0.008519	0.208
0.05	1.124	0.022005	0.504
		0.009704	0.234
		0.007654	0.189
		0.001549	0.055
		0.001686	0.058
		0.001412	0.052

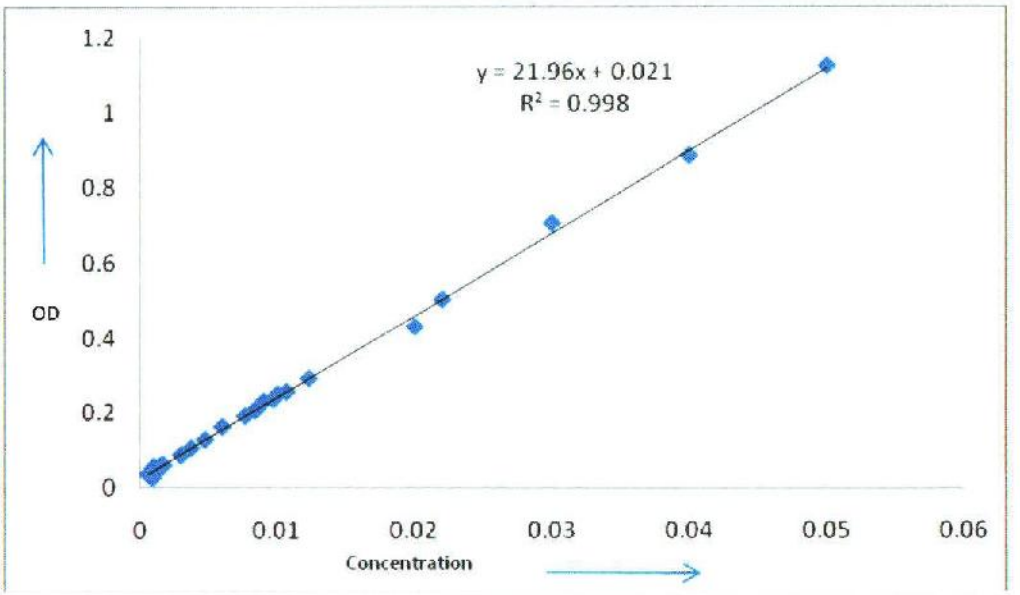


Figure 4.2 Concentration vs OD

4.2 Finding favorable parameters

Yeast growth pattern observed on different pH, glucose amount, and incubation temperature. Best result giving parameters were examined finally. Here are some growth patterns of yeast:

Table 4.3 Parameters Calculations

Incubation time (hour)	A		B		C	
	pH 7, Glucose 3% at 24^{0}C		pH 5, Glucose 1% at 36^{0}C		pH 6, Glucose 2% at 30^{0}C	
	OD_{630}	Ethanol %	OD_{630}	Ethanol %	OD_{630}	Ethanol %
1	0.1	1.05436206	0.1	1.00436206	0.1	1.08436206
5	0.13	1.069013729	0.13	1.089013729	0.13	1.129013729
10	0.18	1.12781206	0.15	1.11751326	0.15	1.12983016
15	0.2	1.132604237	0.2	1.122705252	0.2	1.130664254
20	0.25	1.139856777	0.23	1.141836234	0.23	1.142846287
25	0.33	1.167735059	0.33	1.163415542	0.33	1.165536099
30	0.4	1.180685099	0.4	1.185145289	0.4	1.182355802
35	0.46	1.25008235	0.46	1.25388624	0.46	1.25226154
40	0.5	1.295316457	0.5	1.292561475	0.5	1.293516626
50	0.55	1.30882774	0.55	1.32842624	0.55	1.35852872
60	0.6	1.383074589	0.6	1.383573144	0.6	1.583033879
70	0.7	1.474867169	0.77	1.472892329	0.77	1.674845269
80	0.8	1.487446967	0.8	1.484562664	0.8	1.687267478
90	0.85	1.493773511	0.75	1.495672615	0.75	1.88691566
100	0.88	1.44691566	0.75	1.48882774	0.75	1.893773511

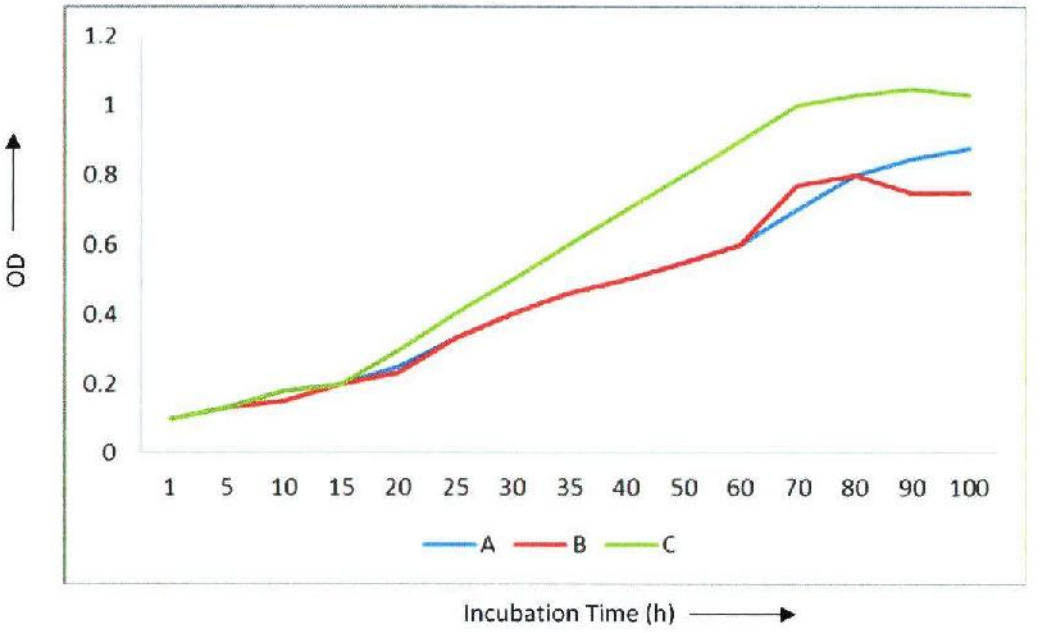


Figure 4.3 Different growth pattern of yeast at different condition. A: pH 7, Glucose 3% at 24^0C, B: pH 5, Glucose 1% at 36^0C, C: pH 6, Glucose 2% at 30^0C.

After examine all the patterns; most suitable is found in pH 5 to 7, Glucose 1 to 3% at 24^0C to 36^0C. Low glucose was primary obstacle of yeast cell growth. pH was minor factor here. As acidic condition prohibits microbial growth, basic pH initiates other organisms' growth that will inhibit yeast cell growth and interfere in ethanol production. Moreover, low incubation produce low ethanol and excess incubation initiate cell decline phase first rather than ethanol production.

Table 4.4 Range of independent variables in the experimental design

	Level		
Variables	+1	0	-1
Glucose% (X_1)	3%	2%	1%
pH (X_2)	7.0	6.0	5.0
Incubation temperature (X_3)	36	30	24

The range and levels of experimental variables investigated in this study were presented in Table 3.1 the central values (zero level) chosen for experimental design were: Glucose% 2 %-(X_1), pH (6.0)-(X_2) and Incubation temperature (30^0C)-(X_3). The results of Box-Behnken design experiments for studying the effects of three independent variables, viz., Glucose%, pH, Incubation temperature on ethanol production by yeast fermentation are presented in Table 4.4 These values were used for analysis of regression where Microsoft office excel tool "data analysis" was used. 95% confidence level was kept.

Table 4.5 The Box-Behnken design matrix employed for three independent variables (Glucose%, pH, Incubation temperature) with observed values (Optical density and Ethanol Production).

Run No	Glucose%	pH	Incubation temperature(^{0}C)	OD	Ethanol (%)
1	1	5	30	1.025	0.464153
2	1	7	30	0.25	0.84051
3	3	5	30	1.02	1.647204
4	3	7	30	0.17	1.649648
5	1	6	24	0.515	0.570588
6	1	6	36	1.455	1.390098
7	3	6	24	0.625	2.105859
8	3	6	36	1.275	1.759713
9	2	5	24	1.04	0.409883
10	2	5	36	2.52	1.095087
11	2	7	24	1.17	1.285932
12	2	7	36	0.945	0.757964
13	2	6	30	0.275	1.145413
14	2	6	30	0.29	1.129968
15	2	6	30	0.26	1.130167

From regression analysis all nine coefficients are used in making the response equation. The second order polynomial equations for each response were found as follows:

$$Y(c)=19.83313+0.675625X1-2.97X2-0.73615X3-0.01875X1X2-0.01208X1X3-0.07104X2X3-0.055X1X1+0.39625X2X2+0.020764X3X3 \quad (1)$$

$$Y(e)=0.179755+0.006156X1-0.02706X2-0.00671X3-0.00017X1X2-0.00011X1X3-0.00065X2X3-0.0005X1X1+0.00361X2x2+0.000189X3X3 \quad (2)$$

Where Y (c) and Y (e) = Optical density of Yeast cell and Ethanol Production. X_1, X_2 and X_3 are coded values for Glucose%, pH, Incubation temperature respectively.

4.3. Some general characteristics of ethanol based on which HT3 was used with GC to separate ethanol from sample

Ethanol C_2H_6O, also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colorless liquid. Boiling point: 78.37 °C, Density: 789.00 kg/m³, Melting point: -114 °C. It was examined through that the collected samples were ethanol.

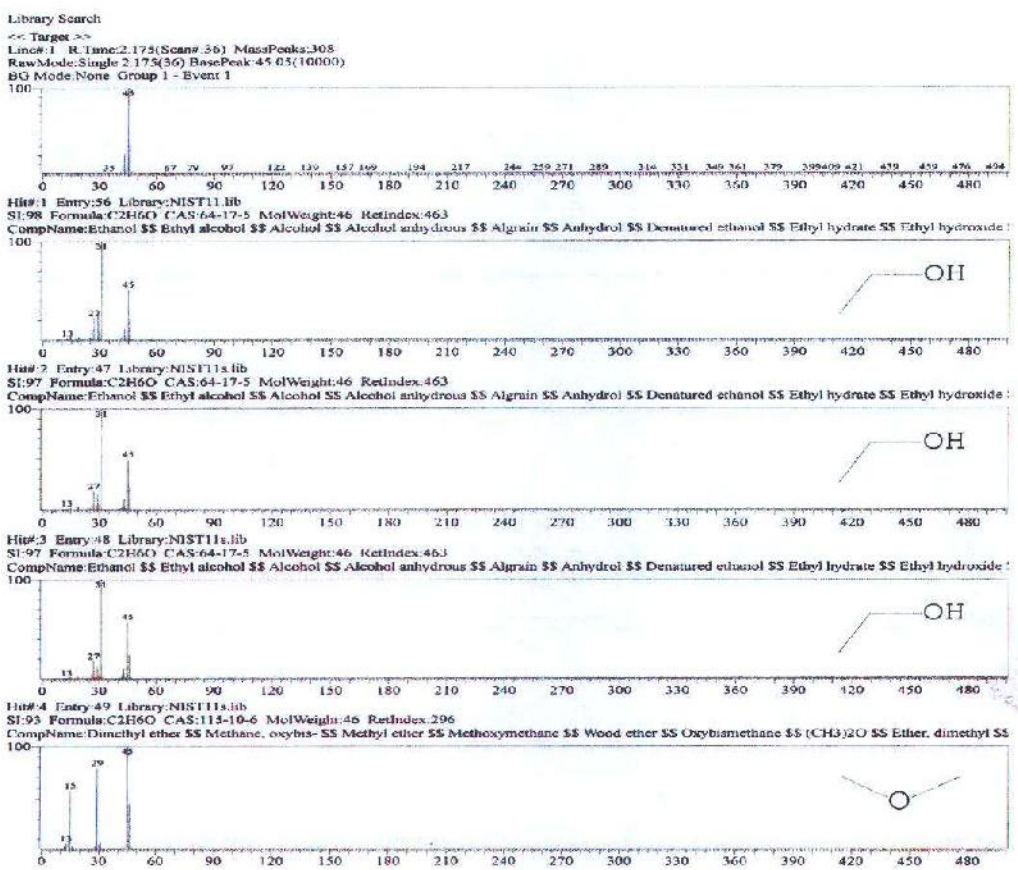
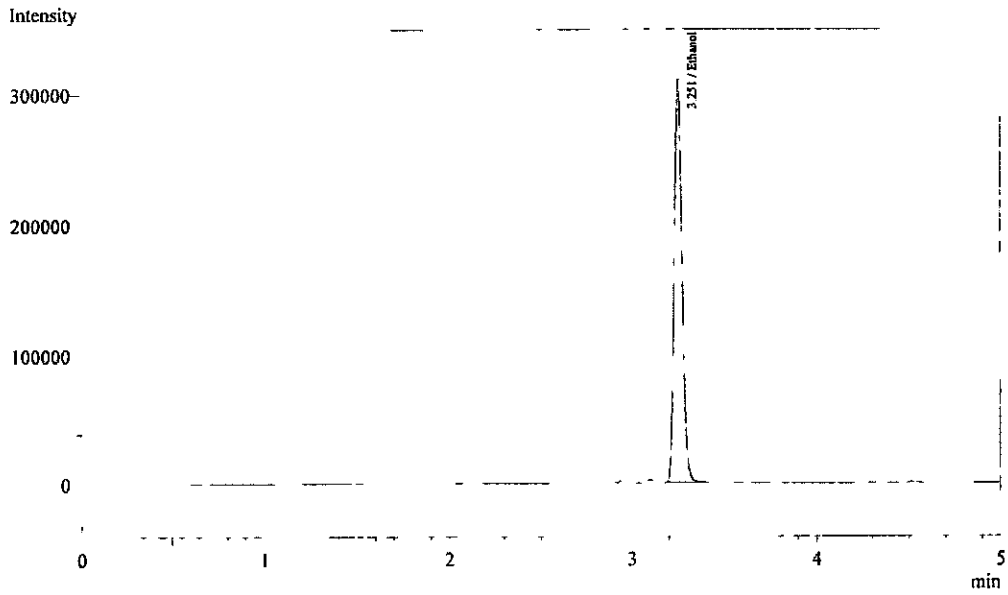


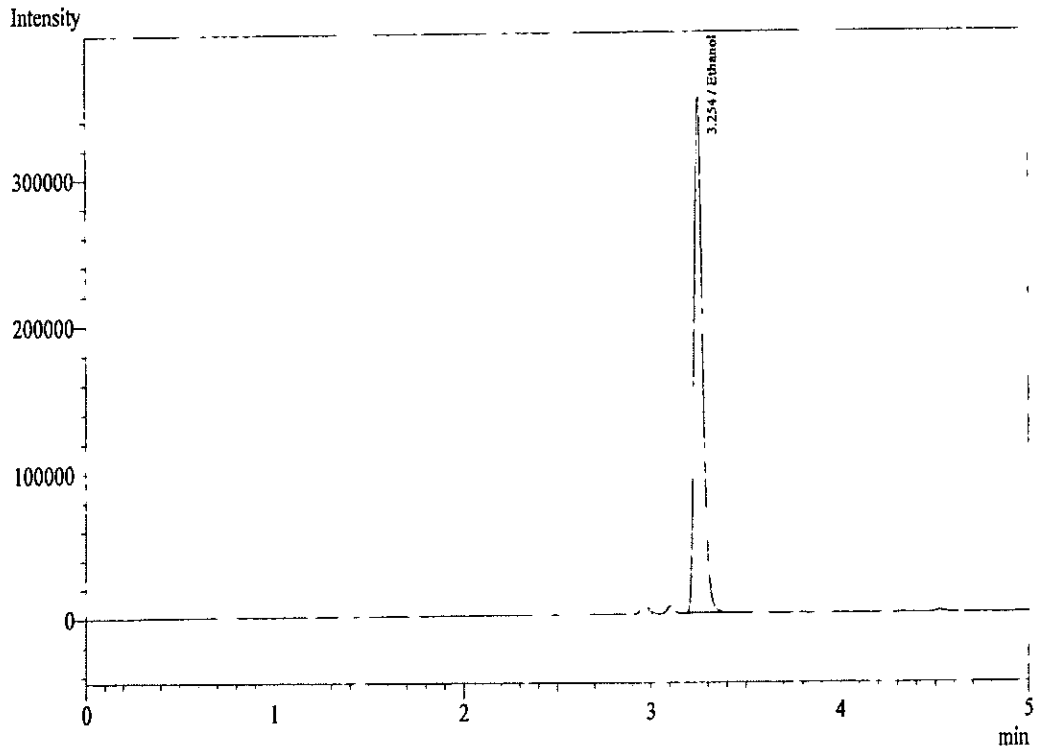
Figure 4.4: Ethanol Peak detected by GC-MS

GC-MS was used to assure the product i.e., ethanol. After that ethanol production amount was detected via GC.



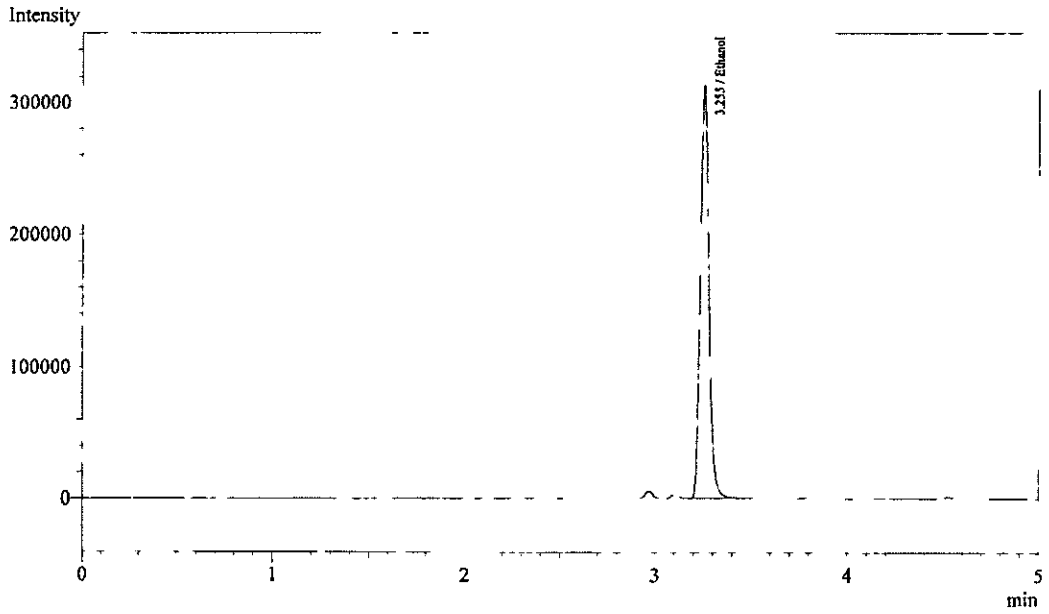
a

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	3.251	943996	310576	0.000	ppm		1	Ethanol
Total		943996	310576					



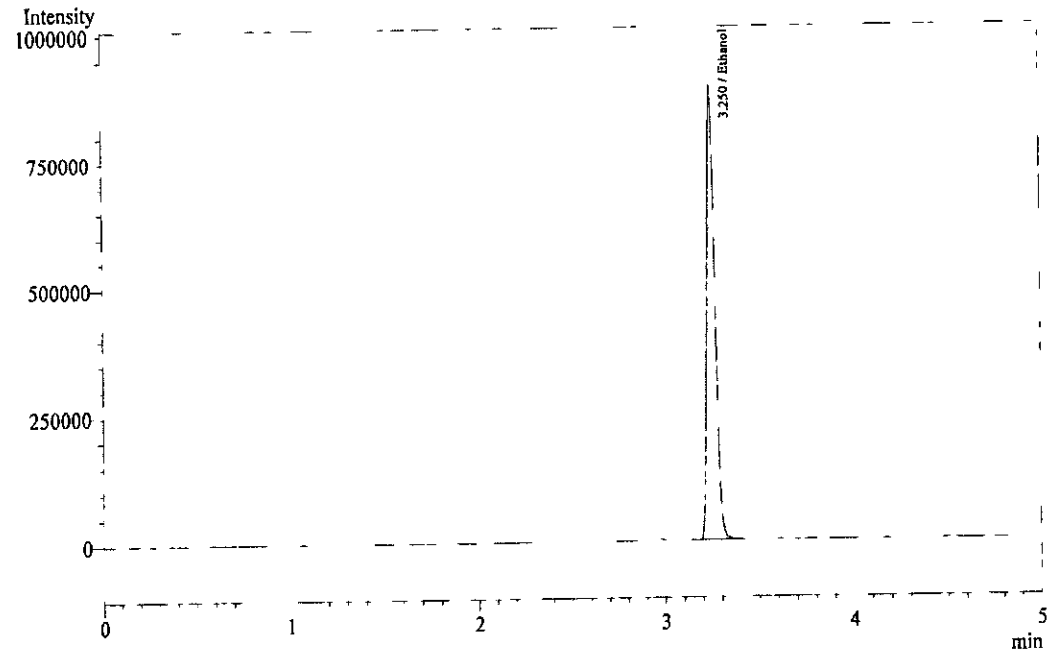
b

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	3.254	1107924	350848	0.000	ppm		1	Ethanol
Total		1107924	350848					



Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	3.255	987224	311940	0.000	ppm		1	Ethanol
Total		987224	311940					

c



Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	3.250	2725591	891945	0.000	ppm		1	Ethanol
Total		2725591	891945					

d

Figure 4.5: Some GC result of ethanol production. a: ethanol peak area 943996, b: ethanol peak area 1107924, c: ethanol peak area 987224, d: ethanol peak area 2725591. All were at 3.25 retention time.

From the above picture (Figure 4.5) ethanol retention time was 3.25. They show the ethanol production rate through area. These were raw data from which blank area had to reduce. Final area was multiply with dilution time to get actual ethanol amount.

Table 4.6 Regression coefficient and corresponding probability values (*p*-values) for specific responses (Optical density of Yeast cell and Ethanol Production)

Parameter (coefficient)	OD		Ethanol Production	
	coefficient	*p*-value	coefficient	p-value
Constant(A_0)	19.83313	9.91E-05*	0.179755	0.000102*
$X_1(A_1)$	0.675625	0.054613	0.006156	0.054613
$X_2(A_2)$	-2.97	0.000827*	-0.02706	0.000827*
$X_3(A_3)$	-0.73615	7.43E-05*	-0.00671	7.43E-05*
$X_1X_2(A_4)$	-0.01875	0.565121	-0.00017	0.565121
$X_1X_3(A_5)$	-0.01208	0.063152	-0.00011	0.063152
$X_2X_3(A_6)$	-0.07104	3.35E-05*	-0.00065	3.35E-05*
$X_1X_1(A_7)$	-0.055	0.143301	-0.0005	0.143301
$X_2X_2(A_8)$	0.39625	5.82E-05*	0.00361	5.82E-05*
$X_3X_3(A_9)$	0.020764	2.56E-06*	0.000189	2.56E-06*

*Most significant factors and interaction effects (*p*-value <0.05)

The calculation of regression analysis also gives the value of the determination coefficient R^2 represented in table 4.7. All the values are over 0.90, which indicates that all factors are well related to the response.

Table 4.7 R^2 values for the ANOVA analysis of the three response output

	OD	Ethanol Production
R^2 value	0.996	0.996

From equation (1) - (2) predicted values of the three responses were obtained.

Figure 4.7 represents the effect of glucose amount. pH and incubation temperature on cell density and ethanol production. When incubation time was constant maximum OD was at 3% glucose and pH6. If pH is constant, then maximum cell growth is in 3% glucose at 30^0C incubation zone. Likewise, for pH and incubation temperature variation highest cell density is at 6pH and 32^0C incubation. In case of ethanol production, peak area is in pH 5.5 and 3% glucose where incubation temperature was constant. When pH is constant, maximum ethanol will be produced in 3% glucose and 32^0C incubation. For pH 5.5 and 28^0C incubation time this production is also in top.

4.5 Method Validation

The method was validated by randomly selecting a few values from among the combinations. Validation results are presented in Table 4.8.

Table 4.8 Predicted and experimental values

Run No.	Glucose %	pH	Incubation Temperature (^{0}C)	OD		Ethanol Production	
				Predicted	Experimental	Predicted	Experimental
1	1.5	6.0	33	1.0830	1.089114	0.007778	0.00814
2	2.5	5.5	30	1.0448	1.038443	0.007467	0.007824
3	3.0	6.5	28	0.3118	0.466759	0.002544	0.002602
4	2.5	7.0	35	0.0209	0.013918	0.009484	0.009128
5.	2.0	6.5	24	1.0352	1.053337	0.007928	0.008054

It was found that the experimental efficiency was almost close to predicted efficiency value, within about ±5% limit.

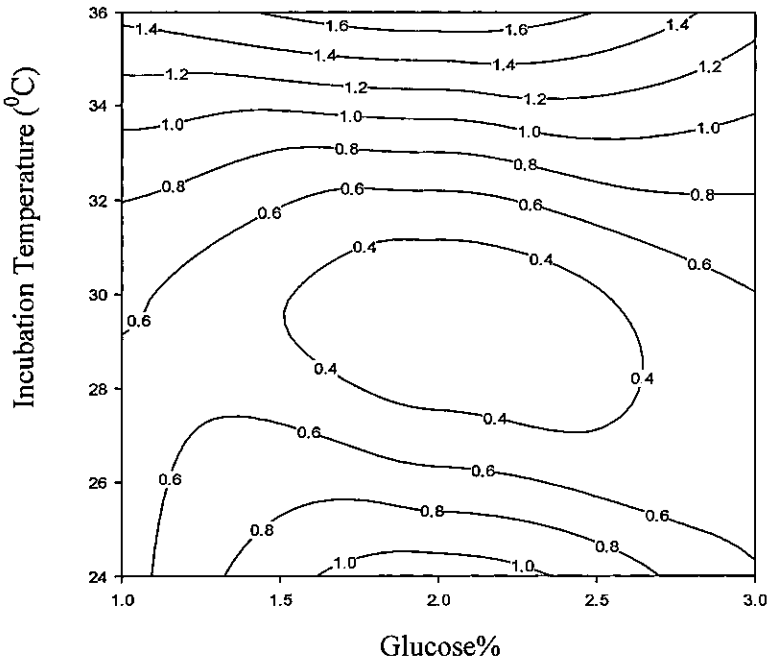


Figure 4.8 Effects of Glucose concentration and Incubation temperature on ethanol production at constant pH.

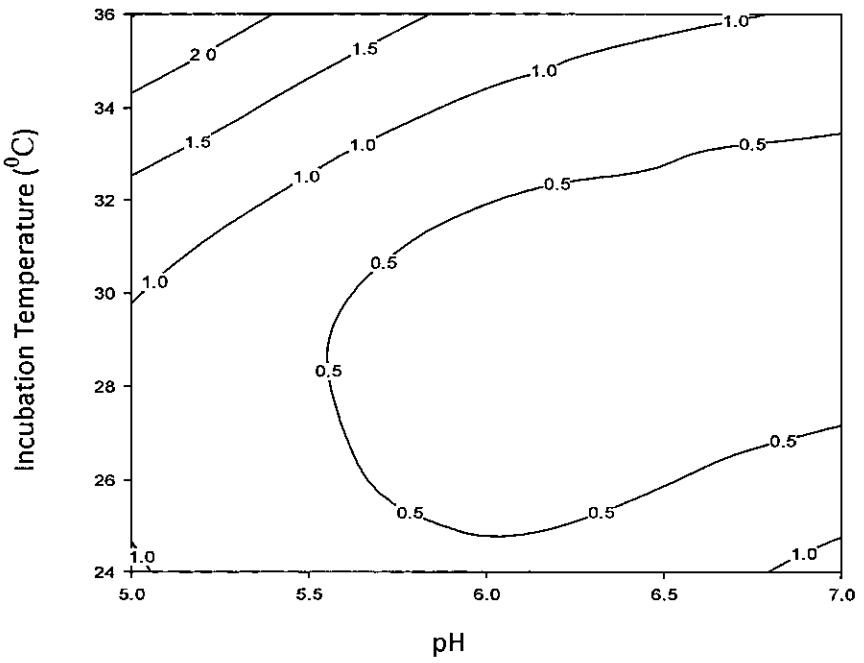


Figure 4.9: Effect of Incubation temperature and pH on ethanol production at constant glucose concentration.

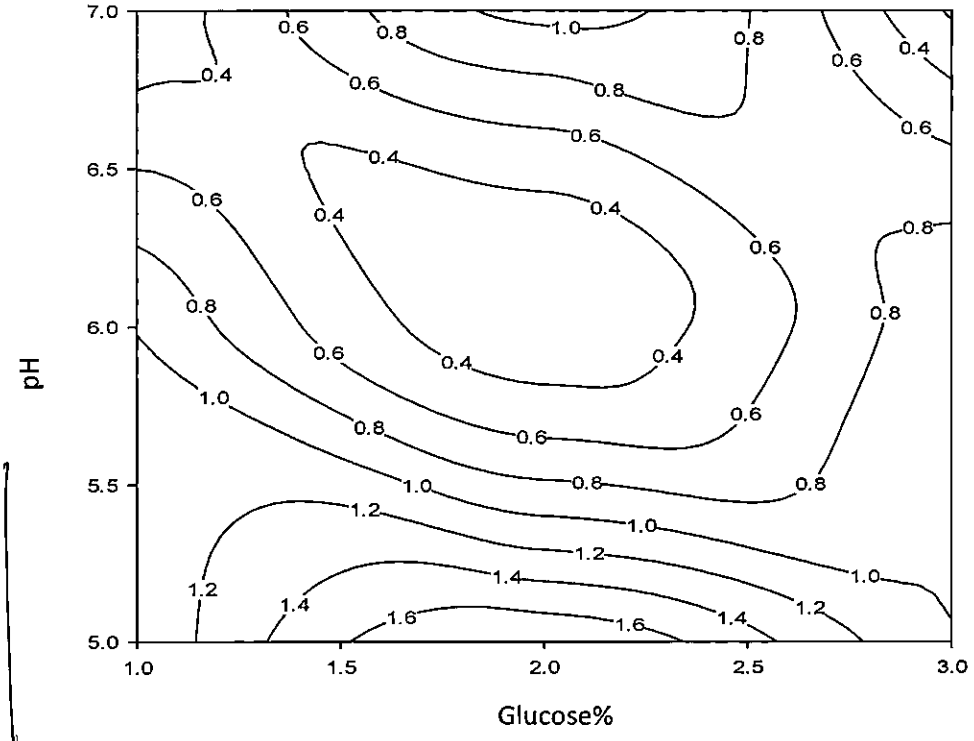


Figure 4.10: Effect of Glucose concentration and pH on ethanol production at constant incubation temperature.

From figure 4.8-4.10, ethanol production increased with higher incubation temperature and higher glucose amount, and also vice versa. But, it is constant at 2% glucose and 30^0C incubation (Figure 4.8). Again, ethanol rate also rose with incubation time where pH was 4.5. In spite of that, from figure 4.10, 2% glucose and pH6 create a zone of constant ethanol production and it decreases with pH lower than 5.0, like as glucose amount of 1.5%. Higher pH levels may well have aided in the denaturing of the enzyme that help yeast to ferment. The optimal pH would have a faster rate of reaction. Under low pH, high glucose concentration and temperatures around 30°C, ethanol production was highest.

The fact behind this distribution of efficiencies was due to glucose concentration and incubation temperature manly. The method was validated by randomly selecting a few values from among the combinations. Validation results are presented in Table 4.8. It was found that the experimental efficiency was almost close to predicted efficiency value, within about ±5% limit. It was also observed that R^2 values were over 0.99. It can thus be inferred that efficiency varies if any of the factors are varied.

Chapter Five

Conclusion

5. Conclusion and Recommendation

Using ethanol as an alternative to gasoline provides several key benefits which is relatively low-cost. Overall, ethanol is considered to be better for the environment than gasoline. Ethanol-fueled vehicles produce lower carbon monoxide and carbon dioxide emissions, and the same or lower levels of hydrocarbon and oxides of nitrogen emissions. 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 production supports farmers and creates domestic jobs. And because ethanol is produced domestically, from domestically grown crops, it reduces dependence on foreign oil and increases the nation's energy independence.
Ethanol is widely considered a way to reduce greenhouse gases from fossil fuel use and thereby reduce human-caused global warming.

The Box–Behnken design can be a useful approach to determine the optimum conditions for maximum production. This study developed coefficients of a quadratic equation for each kind of responses and used them in designing a valid predictive. The mathematical correlation among Glucose amount, pH and Incubation temperature was established using the quadratic equation obtained from the design, which required only a few experiments. The optimum condition was found when a 3% glucose media of pH 6.0 at 30^0C of an incubation period over 100 hours was used. These parameters produced maximum efficiency as well as good resolution. Using various random conditions, the ethanol production rate was also validated, resulting in experimental efficiency values varied within ±5% of the predicted values. Results obtained in this work show that change in values of fermentation temperature, pH and glucose concentration effect on ethanol and cell growth of yeast.

Ethanol is used in many different products ranging from perfumes to explosives. The most popular use of ethanol alone is in the automotive fuel industry. There are cars that run off 100% ethanol or fuel mixtures containing part gasoline and part ethanol also known as gasohol. It is mixable in all proportions with water and most organic solvents. Ethanol is made by fermenting and then distilling starch and sugar crops -- maize, sorghum, potatoes, wheat, sugar-cane, even cornstalks, fruit and vegetable waste. One of the advantages of producing ethanol is very little is wasted. With sugar cane, for example, the leftover pulp is utilized in power plants as a surprisingly efficient fuel to produce electricity. To date, corn has been the raw material of choice for ethanol producers in the U.S. In the Midwest, there are millions of acres planted in corn already. In a fermentation tank, yeast gradually turns the sugars into alcohol that is separated from the water by distillation. Further works can also be done here. Some ethanol companies have tried to reduce the amount of fossil fuels they need for the process.

References

6. Reference

1. Al-Judaibi,A.A., Effect of some fermentation parameters on ethanol production from beet molasses by *Saccharomyces cerevisiae,* American Journal of Agricultural and Biological Sciences **6** (2), 2011, 301-306.

2. Asaduzzaman,Md., Standardization of Yeast Growth Curves from Several Curves with Different Initial Sizes, CHALMERS, 2007, 1-57.

3. A.G. Harrison, Chemical ionization mass spectrometry, CRC Press, USA, 1983.

4. Arroyo-López,F.N., Orlić,S., Querol,A., Barrio,E., Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae, S. kudriavzevii* and their interspecific hybrid, International Journal of Food Microbiology 131 (2009) 120–127.

5. Babar,S Md. E, Song,E.J., Hasan,Md.N. and Yoo,Y.S., Experimental design optimization of the capillary electrophoresis separation of leucine enkephalin and its immune complex, J.Sep. Sci. (2007), **30**, 2311-2319.

6. Banks, W. A., Kastin, A. J., Am. J. Physiol. (Endocrinol. Metab. 22) 1990, 259, E1 – E10.

7. Box, G. E. P. and Behnken, D. W., Technometrics 1960, **2**, 455 – 475.

8. Bodea, A. and Leucuta, S. E., Drug Dev. Ind. Pharm. (1998), **24**, 145 – 155.

9. Brewer,L.W., Gas chromatography, Chapter 21, 1-20.

10. Bergman,L.W., Growth and Maintenance of Yeast, Methods in Molecular Biology, Vol. **177**, 9-14.

11. Cheng,N.G., Masitah Hasan.M., Kumoro,A.C., Ling,C.F. and Tham,M., Production of Ethanol by Fed-Batch Fermentation, 2009, Pertanika J. Sci. & Technol. 17 (**2**): 399 – 408.

12. Cocharn, W. G. and Cox, G. M., Experimental Designs, 2nd Edn., Wiley, New York 1992.

13. E. de Hoffmann, V. Stroobant, Mass spectrometry, principles and applications, John Wiley &
Sons, UK, 2002.

14. F.J. Santos, M.T. Galceran, J. Chromatogr. A, 1000 (2003) 125.

15. G.C. Mattern, G.M. Singer, J. Louis, M. Robson, J.D. Rosen and J. Agric. Food Chem., 38 (1990), 402.

16. H.M. McNair, G.L. Reed, J. Microcol. Sep., 12 (2000) 352.

17. Hows, M. E. P., Perrett, D., Kay, J., J. Chromatogr. 1997, 768, 97–104.

18. Jana Hajšlová,J., Cajka,T., Gas chromatography–mass spectrometry (GC–MS), Food Toxicants Analysis, 2007, 1-55.

19. J. Dallüge, R.J.J. Vreuls, D.J. van Iperen, M. van Rijn, U.A.Th. Brinkman, J. Sep. Sci, 25 (2002) 608.

20. J. Dallüge, J. Beens, U.A.Th. Brinkman, J. Chromatogr. A, 1000 (2003) 69.

21. J. Dallüge, R.J.J. Vreuls, J. Beens, U.A.Th. Brinkman, J. Sep. Sci., 25 (2002) 201.

22. J. Barker, Mass spectrometry, John Wiley & Sons, UK, 1999.

23. Mirsky.N., Chinkov.N., A simple rapid gas-chromatography flame-ionization-detector (GC-FID) method for the determination of ethanol from fermentation processes, African Journal of Biotechnology Vol. 11(15), 2012, 3612-3616.

24. Marianna Pap,M., Lončar,M.B., Belovari,T., Cell Biology and Histology, Biotechedu, 2009, 7-121.

25. M. Adahchour, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, TrAC-Trend Anal Chem, 25 (2006)
438.

26. M. Adahchour, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, TrAC-Trend Anal Chem, 25 (2006)
540.

27. M. Guilhaus, D. Selby, V. Mlynski, Mass Spec. Rev., 19 (2000) 65.

28. Myers, R. H., Response Surface Methodology: Process and Product Optimization, Wiley, New York 1995.

29. Nagaragan, G., Natarajan, K., World J. Microbiol. Biotechnol. 1999, 15, 197 – 203.

30. Prasad,S., Anoop Singh,A., Joshi, H.C., Ethanol as an alternative fuel from agricultural, industrial and urban residues, Resources, Conservation and Recycling 50 (2007) 1–39.

31. Reeves,E.G.M., Louisiana,B.S., Kinetic analysis of *Kluyveomyces Marxlanusv* yeast strain, The Department of Biological and Agricultural Engineering, State University, 2001, May 2004, 40-60.

32. R.G. Cooks and K.L. Busch, J. Chem. Educ., 59 (1982) 926.

33. Suresh Babu, C. V., Chung, B. C., Lho, D. S., Yoo, Y. S., J. Chromatogr. A 2006, 1111, 133 – 138.

34. Schütz, M., Gafner, J., 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. Journal of Applied Bacteriology 19, 253–257.

35. Serra, A., Strehaiano, P., Taillandier, P., 2005. Influence of temperature and pH on *Saccharomyces bayanus* var. uvarum growth; impact of a wine yeast interspecific hybridization on these parameters. International Journal of Food Microbiology 104, 257–265.

36. Sorensen, B.J., Jakobsen, M., 1997. The combined effects of temperature, pH and NaCl on growth of Debaryomyces hansenii analyzed by flow cytometry and predictive microbiology. International Journal of Food Microbiology 34, 209–220.

37. Swinnen, I.A.M., Bernaerts, Kdens, E.J.J., Geeraerd, A.H., Van Impe, J.F., 2004. Predictive modelling of the microbial lag phase: a review. International Journal of Food Microbiology 94, 137–159.

38. T.D. Bucheli, R.C. Brändli, J. Chromatogr. A, 1110 (2006) 156.

39. Wen-jun,G., Yu-ping,Z., Yi-Jun,Z., Guang-ri,XU., Xin-jun,W., Kwang-pill,L., Optimization strategies for separation of sulfadiazines using Box-Behnken design by liquid chromatography and capillary electrophoresis , J. Cent. South Univ. Technol. (2007)02−0196−06.

40. W.G. Fong, H.A. Moye, J.N. Seiber, J.P. Toth (eds), Pesticide residues in food: methods, techniques, and regulations, John Wiley & Sons, UK, 1999.

Appendices

Appendices

Appendix 1: Data Input for regression analysis of yield
Microsoft office Excl-2010 was used for regression analysis.

Operating procedure:

Start
↓
Microsoft Excel
↓
Tool
↓
Data analysis
↓
Input Y range (all values of Y column)
↓
Input X range (all values of X1, X2, X3, X1X2, X1X3, X2X3, X1X1, X2X2, X3X3)
↓
Confidence level 95%
↓
Output range (any place was clicked near input data at the same sheet)
↓
Residuals

As for example, following data was used for regression analysis of yield:

Glucose%	pH	Incubation Temperature ^0C	X1X2	X1X3	X2X3	X1X1	X2X2	X3X3	OD	Ethanol %
a	b	c	ab	ac	bc	aa	bb	cc	Y©	Y(E)
1	5	30	5	30	150	1	25	900	1.025	0.464153
1	7	30	7	30	210	1	49	900	0.25	0.84051
3	5	30	15	90	150	9	25	900	1.02	1.647204
3	7	30	21	90	210	9	49	900	0.17	1.649648
1	6	24	6	24	144	1	36	576	0.515	0.570588
1	6	36	6	36	216	1	36	1296	1.455	1.390098
3	6	24	18	72	144	9	36	576	0.625	2.105859
3	6	36	18	108	216	9	36	1296	1.275	1.759713
2	5	24	10	48	120	4	25	576	1.04	0.409883
2	5	36	10	72	180	4	25	1296	2.52	1.095087
2	7	24	14	48	168	4	49	576	1.17	1.285932
2	7	36	14	72	252	4	49	1296	0.945	0.757964
2	6	30	12	60	180	4	36	900	0.275	1.145413
2	6	30	12	60	180	4	36	900	0.29	1.129968
2	6	30	12	60	180	4	36	900	0.26	1.130167

Appendix 2: Summary output of regression analysis

Regression Statistics		
	OD	Ethanol%
Multiple R	0.998318	0.998318
R Square	0.996638	0.996638
Adjusted R Square	0.990587	0.990587
Standard Error	0.06092	0.000555
Observations	15	15

Appendix 3: Anova (Yield)

OD	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	9	5.501137	0.611237	164.6985	1.21E-05
Residual	5	0.018556	0.003711		
Total	14	5.519693			

Ethanol %	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	9	0.000457	5.07E-05	164.6985	1.21E-05
Residual	5	1.54E-06	3.08E-07		
Total	14	0.000458			

Appendix 4: Coefficients of X variables

X Variables	Coefficients (OD)	Coefficients (Ethanol %)
Intercept (A_0)	19.83313	0.179755
X Variable 1 (A_1)	0.675625	0.006156
X Variable 2 (A_2)	-2.97	-0.02706
X Variable 3 (A_3)	-0.73615	-0.00671
X Variable 4 (A_4)	-0.01875	-0.00017
X Variable 5 (A_5)	-0.01208	-0.00011
X Variable 6 (A_6)	-0.07104	-0.00065
X Variable 7 (A_7)	-0.055	-0.0005
X Variable 8 (A_8)	0.39625	0.00361
X Variable 9 (A_9)	0.020764	0.000189

Appendix 5: Determination of predicted value

Coefficients (A1, A2, A3.....) and their corresponding X (X_1, X_2, X_3.....) variables were used for construction of 2nd order polynomial equation. It was used for determination of predicted values. Total 15 predicted values were obtained for each response. General equation was as follows-

$Y= A+A_1\times X_1+A_2\times X_2+A_3\times X_3+A_4\times X_1X_2+A_5\times X_1X_3+A_6\times X_2X_3+A_7\times X_1X_1+A_8\times X_2X_2+ A_9\times X_3X_3$

Equation, 1 and 2 represent 2nd order polynomial equation for OD and ethanol production respectively.

Y(c)=19.83313+0.675625X1-2.97X2-0.73615X3-0.01875X1X2-0.01208X1X3-0.07104X2X3-0.055X1X1+0.39625X2X2+0.020764X3X3..... (1)

Y(e)=0.179755+0.006156X1-0.02706X2-0.00671X3-0.00017X1X2-0.00011X1X3-0.00065X2X3-0.0005X1X1+0.00361X2x2+0.000189X3X3.....(2)

Where, Y(c) and Y(e) = Predicted value of yeast cell and ethanol production respectively. X1, X2 and X3 are coded values for glucose amount, pH and incubation temperature respectively.