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DEVELOPMENT OF SUITABLE BIO-CARRIER FOR CONTINUOUS ETHANOL FERMENTATION USING SUGARCANE BAGASSE

BY

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This is to certify that the thesis prepared by Wendemagegn Teshome, entitled: Development Of Suitable Bio-Carrier For Continuous Ethanol Fermentation Using Sugarcane Bagasse and submitted in partial fulfilment of the requirement for the degree of Master of Science (Chemical and Bio Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Declaration

I declare that this thesis entitled *"DEVELOPMENT OF SUITABLE BIO-CARRIER FOR CONTINUOUS ETHANOL FERMENTATION USING SUGARCANE BAGASSE*.'' has not been submitted in any form for another degree, diploma or an award at any university or other institution of the tertiary education. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and discussions. Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given. The work was under the guidance of Professor Belay Woldeyes instructor of AAiT in Addis Ababa University, School of Chemical and Bio Engineering.

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ABSTRACT

Cell immobilization has been proposed as an effective means of improving ethanol production. In this work, sugar cane bagasse as economically cheap and renewable was used as biofilm developmental carrier for saccharomyces cerevisiae ethanol production with cane molasses as a substrate. The aim of this thesis was to investigate the effect of cane preparation index (P.I.) and bagasse type; mill tandem bagasse (MTB) and cane diffuser bagasse (CDB) to cell retention then develop suitable bio-carrier for continuous ethanol production. The demand of ethanol per annum is increasing as the number of industries using ethanol as a solvent, fuel blending whereas distilleries production declined due to low fermentation efficiency. Hence the production of ethanol by fermentation technology has triggered us to investigate the best way and method which improve volumetric productivity. The CDB-immobilized bio-carrier was used for repeated batch fermentation, because CDB was with the highest cell retention from immobilization, the mass of cells retained on the carrier was quantified gravimetrically. From repeated batch fermentation overall results CDB of cane P.I. 87.5% was selected for continuous fermentation due to its highest stability. Continuous ethanol fermentation conducted in laboratory scale packed bed reactor and operating conditions (TRS and dilution rate) was optimized by using a central composite design (CCD) method. The optimum result which maximize ethanol volumetric productivity and ethanol yield were TRS 93.40 g/l, Dilution rate 0.91h-1 whereas the ethanol volumetric productivity and ethanol yield attained were 26.03g/lh and 0.474g/g respectively. The results have established the continuous ethanol fermentation with CDB at high cane P.I. (87.5%) with immobilized yeast cell reveals that the high dilution rate is favourable to the feed with low sugar concentration. For a high sugar concentration feed, the maximum sugar utilization can be achieved either by increasing bed height or by increasing number of columns.

Keywords: Immobilization, sugarcane bagasse, saccharomyces cerevisiae, cane preparation index, Biofilm, Continuous ethanol fermentation.

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Dedicated To My Dad!

He is The Strongest Inspiration in My Life.

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IV. Lists of Symbols and Acronyms

Acronym	Nomenclature
CDB	Cane Diffuser Bagasse
D	Dilution Rate
Ey	Percentage of the Theoretical Yield
FSF	Fincha Sugar Factory
H	water retention
ICR	Immobilized cell reactor
ICUMSA	International Commission for Uniform Method of Sugar Analysis
KSF	Kessem Sugar Factory
MSF	Metahara Sugar Factory
MTB	Mill Tandem Bagasse
P.I.	Preparation index
QP	Ethanol volumetric productivity
R	Cells retention
SCB	Sugar cane Bagasse
SU	Sugar Utilization
TRS	Total Reducing Sugar
W	Water content
WAH	Water content after hydration
WAI	water absorption index
Xi	Immobilized cells concentration
Xs	Free cells concentration
Xt	Total cells concentration
Yi	Immobilization Efficiency
Yp/s	Ethanol yield per consumed sugar

CHAPTER ONE

1. INTRODUCTION

1.1.Background

The traditional fermentation system uses freely suspended yeast cells to ferment a broth in a non-stirred batch reactor. In such setup, the reactor volume is gradually fermented. At the same time, yeast cells activity can be inhibited either by product or substrate concentration (Najafpour et al., 2004), and ultimately, yeasts are lost at the end of the batch process.

Recently, immobilized cells technology for alcoholic fermentation has been investigated because of its increased productivity, continuous production, reuse of the biocatalyst , reduced inhibition from the substrates or products, prolonging activity and stability of the biocatalyst, reducing fermentation time, reducing costs for equipment and energy demands, enhanced yields, ease to separate cell mass, reduce risk of contamination (Kourkoutas et al., 2004 ; Verbelen et al., 2006 , Razmovski and Vucurovic, 2012, Tang and Lee, 2013 and, Singh et al., 2013).

For bioethanol production application selection of suitable cell immobilization techniques needs some consideration like the cost of carrier material and the suitability of the applied techniques (Razmovski and Vucurovic, 2012).

Over the years, many different materials have been proposed for the development of carrier in whole cells immobilization, but the research on new materials and thus new immobilization approaches is still of great interest. The carrier materials which will be used for immobilization of cells have to have the following features like easily accessible, inexpensive, ample, less toxic to the cells, high mechanical strength, stability, and also to enhance diffusion of substrates and products should have core hollow space (Razmovski and Vucurovic, 2012; Genisheva et al., 2014).

Lignocellulosic biomass like sugarcane, sugar cane bagasse, sorghum bagasse, orange peel, corn husk, sugar beet pulp, have been researched before for bioethanol production and remarkable finding is registered by different researchers. Singh et al., (2013) reported that bioethanol production using immobilized cells on sugarcane bagasse was higher than using alginate and agar as a carrier. Sugarcane bagasse the byproduct of sugar processing, is potential to be used as a carrier for immobilized cells due to the porous of surface structure and the

availability in nature. The innovative solutions offered by sugar cane bagasse are due to the combined advantages the surface characteristics of carriers such as pore size, moisture content, hydrophilic properties (water retention rate), and water absorption index are the main factors that influence the efficiency of attachment, characteristics of immobilized yeast cell, and also their productivity (Yu et al., 2010; Razmovski and Vucurovic, 2012). Approximately, 5.4×10^8 dry tons of sugarcane bagasse was produced throughout the world (Singh et al., 2013). In Ethiopia production of sugarcane bagasse was about 3.2×10^6 ton per year (sugar corporation annual report, 2017/18).

Accordingly, sugar cane bagasse, mill and diffuser bagasse at different preparation index sources have been applied in this thesis for the development of bio-carrier in yeast cell immobilization for fermentation purposes. To demonstrate the effect of preparation index and bagasse type on immobilization cell retention analysis was conducted and for the selected top three highest cell retention carrier batch fermentation runs were performed at different preparation index to produce bioethanol using cane molasses substrates. Suitable carrier for continuous fermentation was selected from repeated batch fermentation and suitability test results, Saccharomyces cerevisiae, brewing Yeast were used in this study.

1.2. Statements of the Problem

In Ethiopian Sugar Corporation among the diversifications programs ethanol production having annual designed production capacity of 30 million litres using two ethanol plants, metehara and Fincha sugar factory. The ethanol domestic market is covered by these two sugar factories (Fincha and Metehara) with the combination production capacity of 30 million litres, but combined produced bioethanol in their annual production capacity at around 14.4 million litres (Sugar corporation annual report, 2017/18). Despite the presence of higher demands for ethanol in the country, these two bioethanol plants are producing below the plan due to low fermentation efficiencies. Conventional fermentation processes have some weaknesses. First, it commonly uses batch process like Fincha ethanol plant to easily control the fermentation process from microorganism contamination. Second, even continuous process like metehara ethanol plant the ethanol concentrations produced are so low because the accumulated produced ethanol and other inhibitors will poison microorganism while fermenting. The accumulation of dissolved product which is poisonous will slowly decrease or even cease microorganism growth. There is a process limit in conventional process such as ethanol inhibition, in ethanol concentration, specific microorganism growth and specific rate

production will decrease and cell density in a bioreactor will be low, therefore the sugar solution will not completely fermented; on an industrial scale, yield from continuous fermentation and free cells, varying from 4 to 8 g/L.h. In order to increase fermentation efficiency and ethanol volumetric productivity, we have to remove the inhibiting content or we need some mechanism to enhance the fermentation efficiency by intensification of fermentation process like immobilized cell technology. The aim of this research paper will be to study the capability of sugarcane bagasse, mill and diffuser bagasse separately as well as effect of cane preparation index for the development of carriers for yeast cell immobilization and optimize the operating conditions for continuous ethanol production.

1.3. Significance of the Study

In the world development of cost effective technologies and scalable research papers for ethanol production is a priority for many research centres, universities, and private firms and even the governments.

In these days the competitiveness of industries mainly depends on their own performance of utilizing all available resources by implementing scalable research out puts. Ethiopian government has emphasized on import-substituting industries are made to expand by being prioritized which is in line with the present scalable research proposal focus to fill the gaps on research and selecting, adapting and introducing appropriate technologies on ethanol production. Additionally, the implementation of this research will build new capacities in the domain of biochemical industrial processing specifically in the field of bio refinery and microbial biotechnology. These new capacities, built both in academic and sugar corporation, will not only yield efficient ethanol production but also promote and facilitate future breakthrough research and development in industrial biotechnology in Ethiopia.

1.4. Objectives

1.4.1. General Objective

The general objective of this research work were development of suitable bio-carrier for continuous ethanol fermentation using bagasse.

1.4.2. Specific Objective

- Physico-chemical characterization of sugar cane bagasse.
- Study the effect of cane preparation index and bagasse type in cell retention.
- Repeated Batch Fermentation using optimized sugar Cane Bagasse.
- Optimization of Continuous ethanol Fermentation.

1.5. Scope of the Study

This study is focused on the development of bio-carrier for yeast cells immobilization in sugar cane bagasse and their evaluation in the continuous ethanol production by using packed bed bioreactors, designing not included.

CHAPTER TWO

2. LITERATURE REVIEW

Ethanol, also known as ethyl alcohol, drinking alcohol or grain alcohol, is a flammable, colourless, mildly toxic chemical compound, and is best known as the alcohol found in alcoholic beverages. The most common meaning of fermentation is the conversion of a sugar into an organic acid or an alcohol. Fermentation occurs naturally in many foods and humans have intentionally used it since ancient times to improve both the preservation and organoleptic properties of food. However, the term "fermentation" is also used in a broader sense for the intentional use of microorganisms such as bacteria, yeast, and fungi to make products useful to humans (biomass, enzymes, primary and secondary metabolites, recombinant products, and products of biotransformation) on an industrial scale (Leona Paulova, 2013).

2.1. Types of Microorganisms used for Ethanol fermentation

Both yeast and bacteria are capable of efficiently converting sugars to ethanol by fermentation processes. The expansion of the ethanol industry requires the search for new and more efficient ethanologenic microorganisms. This section will highlight the varieties of bacteria and yeast that can be used for ethanol production.

A large number of bacteria are capable of ethanol production. But most of them produce other end products like butanol, isopropylalcohol, acetic acid, formic acid, arabitol, glycerol, acetone, methane, etc., as well as ethanol.

Yeast is the most commonly used microorganism for ethanol production by fermentation. The most widely used and popular biological agents of ethanol fermentations are yeasts of the genus Saccharomyces. There are certain unique properties of this genus that make it not only capable, but outstanding for ethanol production. Some of these properties are: fast growth rates, efficient glucose repression, efficient ethanol production and a tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels (Piskur *et* al., 2006). Some examples of yeasts used for ethanol production by fermentation are Saccharomyces cerevisiae, Schizosaccharomyces pombe, Saccharomyces uvarum, Kluyveromyces lactis, and Saccharomyces diastaticus. In fermentation of ethanol different types of yeast were used, but mainly Saccharomyces and klyueromyces marxianus are used for fermentation (Parkash, 2015).

Kluyeromyces marxians: As we know that during process of fermentation microorganisms were used to carry out the process, during that some yeast is used yeast have very much important in biotechnology Kluyeromyces Marxians has getting importance regarding ethanol production. It increases the production of ethanol from molasses or any sugar content raw material double. Due to that property it is used in many other countries like India, Brazil, and other European country (muenduen et al., 2006).

Due to it's applicably and availably it has many advantage in industrial and microbiological or we can say in biotechnology. Kluyeromyces Marxians has well known specie used for various fermentation processes.

Saccharomyces cerevisiae: Saccharomyces cerevisiae has getting interest regarding the conversion of sugary material or we can say that carbohydrates into ethanol. From previous year's researcher on working that plan to utilize the sugary material fully convertible into alcohol, because some side product also getting during process of fermentation due to this saccharomyces cerevisiae getting importance. In the world saccharomyces cerevisiae mostly used species for ethanol production, due to its availability. Saccharomyces cerevisiae has also an important application in baker yeast that's why it is researched also increase to make it most suitable for ethanol production and other fermentation process. Common bakery yeast it takes 20,000,000,000 (twenty billion) yeast cells to weigh one gram, or 1/28 of an ounce, of cake yeast. (Prasad et al., 2013).

Zymomonas mobilis: Zymomonas mobilis is not common microorganism regarding production of ethanol from molasses, but it can be utilize to produce more ethanol regarding other types yeast available for ethanol production. Zymomonas mobilis is needed to be more research regarding biotechnological application in process of fermentation. It is used in African industrial beverages because it was isolated for the beverages used in African industries. Regarding Zymomonas mobilis has many advantages due to its microbial activity. It takes more sugar and gives more ethanol regarding other yeast (Rafael and Ruhul et al., 2013).

2.2. Feed stock used for Ethanol Fermentation

Ethanol by fermentation processes can be produced from any and every material that contains sugars. To make ethanol production by fermentation an economically feasible process on an industrial scale, the use of inexpensive substrates and the maximization of substrate utilization and conversion are significant aspects. Lee et al. in 1995 reported that a variety of possible substrates have been studied for large-scale ethanol production. Some of them are corn residue

prehydrolysate, sugar beet molasses, sugar cane molasses, Jerusalem artichoke juice, cellulose, barley and cassava (Lee et al., 1995).

The raw materials used in ethanol production via fermentation are classified under three groups: sugars, starches, and cellulose materials. The sugars present in sugar cane, sugar beets, molasses, fruits, etc. can directly be converted to ethanol. Starches from potatoes, root crops and grains like wheat, corn, etc., need to be hydrolysed to simple fermentable sugars by the enzymes α-amylase and glucoamylase, before they can be converted to ethanol. Similarly, cellulose from wood and other agricultural residues must be converted to simple sugars by the action of mineral acids or cellulases. Mixed substrates such as glucose-xylose mixtures, glucose-fructose mixtures, and glucose-galactose mixtures have also been investigated (Lee et al., 1995).

In industry, molasses, a by-product of the sugarcane industry, is the most widely used and promising raw material for ethanol fermentation. Brazil is pioneer in large scale motor fuel ethanol production through the fermentation of sugar cane molasses by yeasts. Also in India molasses economically are widely used in alcohol industries. (Schweinitzer and Josenhans, 2010). In Ethiopia also studies indicated that ethanol production from sugar cane molasses is economical (milkesa, 2013).

General composition of cane molasses: as far as Molasses is an agricultural product and its composition varies with the variety of maturity of cane with the climate and soil condition. In addition processing condition in the sugar factory may also bring about changes in the composition of molasses. So, only typical values of main components of cane final molasses show in table 2-1.

Table 2-1 Typical Composition and properties of Final molasses

Sr. No	Parameter	Units	Concentration Range
1	Density of Molasses	kg/m3	1350
2	Colour	-	Dark Brown
3	Odor	-	Sugary
4	Total Solids	% w/w	73 – 80
5	Total Reducing Sugar %	w/w	48.5 – 52
6	Un Fermentable Sugar	% w/w	4.0 - 4.5
7	Fermentable Sugar %	w/w	44.5 - 47.5
8	F/N Ratio	-	1.46 - 1.56
9	Sludge %	w/w	1.96 - 3.1
10	Total Volatile Acidity	mg/lit	2500 – 4000
11	pH at 40°Bx -	-	4.6 - 5.0
12	Total Viable Count	cfu/gm	5.5x102 - 2.4x104

2.3. Medium Preparation

Fermentation and inoculation media includes feed stock preparation to nutrient supplementation to enhance to create favourable condition for yeast cell during culturing and fermentation process.

Feedstock preparation will vary with the feedstock. Molasses differs from other feedstock such as corn, sorghum, and potatoes etc. which have their carbohydrate content stored as starch which is usually precooked and hydrolysed into fermentable sugars. The carbohydrates in molasses are readily in the form of sugars and need no pre-treatment. Sucrose is the principal sugar contained in molasses and is readily fermentable either directly, or as its glucose and fructose components. Since molasses form a viscous fluid containing about 84% dissolved solids (sugars and non-sugars), a preliminary dilution is required in order to render the medium suitable for yeast. As a rule, this is about 18 – 20 degree Brix (Santos, 1990). The water used for dilution should be potable to avoid contamination by bacteria and wild yeast. Since the optimum pH for yeast activities lies in the range of 4 - 5, the acidity of the diluted syrup is adjusted for optimum yeast activities at pH 4.5 by adding 2 – 2.5 g H_2SO_4 per lire of dilute syrup (Santos, 1990).

The key to the development of improved fermentation process is the formulation of the culture medium. This requires the use of easily available alternative nutrients that meet the microbial requirements. Ruanglek et al. (2006) reported that the most significant factor for improving growth rates and ethanol productivities in synthetic media is the complex nitrogen supplement. Nitrogen should not be in the form of ammonium sulfate and liquid ammonia as they cause scaling (as calcium sulfate) and raising the pH that encourage bacterial contamination respectively. An excellent vehicle for these two nutrients is diammonium hydrogen phosphate; which is added the order of 100 g per m3 of dilute syrup (Santos, 1990).

2.4. Production of Ethanol from Cane Molasses

Fermentation of molasses is a two-step process, in the first step sucrose present in the molasses is hydrolysed to glucose and fructose; in the second step glucose and fructose is converted into ethanol and carbon dioxide. The enzymes invertase and zymase present in the yeast Saccharomyces cerevisiae acts as a catalyst for the first and second reaction respectively. Chemically this transformation for sucrose to alcohol can be approximated by the equation: -

$C_{12}H_{22}O_{11} + H_2O \xrightarrow{invetase} 2C_6H_{12}O_6$ …….....Equation 2-1
Sugar cane Glucose

$C_6H_{12}O_6 \xrightarrow[Zymase]{} 2C_2H_5OH + 2\,CO_2\uparrow$ ….…….....Equation 2-2
180g 2*46g + 2*44g
Glucose/Fructose Ethyl alcohol + Carbon dioxide.

Thus 180 gm. of sugars on reaction gives 92 gm of alcohol. Therefore, 1 MT of sugar gives 511.1 kgs of alcohol. The specific gravity of alcohol is 0.7934, therefore, 511.1 kg. Of alcohol is equivalent to 511.1 / 0.7934 = 644.19 litres of alcohol. During Fermentation of other by-products like glycerine, succinic acids etc. also are formed from sugars. Therefore, actually 94.5% total fermentable sugars are available for alcohol conversion. Thus, one MT of sugar will give only 644 x 0.945 = 608.6 litres of alcohol, under ideal condition theoretically (Ruhul et al., 2013).

The yeasts cannot sustain at a higher alcohol concentration of 10-15% which acts as an important rate limiting factor. The fermented wash produced as a product of reaction consists of ethanol, dissolved solids, suspended solids, unfermentable sugar, sludge etc. The produced fermented wash is then sent for distillation where the alcohol content of the wash is stripped of and ethanol of approximately 95.5% (w/w) concentration is recovered. Further concentration of ethanol using distillation is not possible because ethanol and water forms an azeotrope at this composition (95.6% ethanol). To concentrate further up to 99.99 % latest technology molecular sieve dehydration is used to separate ethanol and water (Patil et al., 2017)

Mode of operation can be batch or continuous, Traditional fermentation systems use freely suspended yeast cells in a batch bioreactor. The reactor is filled with unfermented medium (molasses medium) and the whole reactor volume (‘‘batch’’) is gradually fermented and subsequently removed from the reactor. By contrast, continuous fermentation systems have a continuous flow of unfermented medium into the fermenter and a corresponding continuous

flow of fermented product out of the system. In its simplest one-reactor incarnation, the continuous fermenter operates at steady state with a content that is equal to the finished product that flows out of the system. A relatively slow inflow and little internal heterogeneity between the points of in- and outflow in the reactor are needed to avoid direct mixing of the unfermented inflow and the finished product. Alternatively, a cascade of interconnected separate fermenters can be used to avoid a direct flow of unfermented medium into the near-finished product.

Continuous fermentation offers important advantages, such as higher conversion rates, faster fermentation rates, improved product consistency, reduced product losses and environmental advantages. An important aspect of continuous fermentation is the high volumetric efficiency, which is usually obtained by increased yeast cell concentrations in the reactor compared to traditional batch systems.

2.5. Immobilized Cell Systems

Immobilizing yeast cells on several support types can provide high cell densities in the bioreactor, which, in combination with high flow rates, leads to short residence times. These economic benefits are the driving force for a global research effort aimed at studying and implementing continuous fermenters. The first continuous fermentation system appeared in the 1960s, but few systems grew up to industrial scale, which is indicative of the many technical and qualitative pitfalls associated with this technology (Verbelen et al., 2006).

The development of immobilized-cell processes, using low-cost support and low operational (immobilization) cost would be desirable for economical production process (de Vasconcelos et al., 2004).

2.5.1. Cell Immobilization

Cell immobilization has been defined as the physical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free migration and exhibit hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use (Dervakos and Webb, 1991; Freeman and Lilly, 1998; Covizzi et al., 2007; Amim et al., 2010).

Immobilization is a natural phenomenon existing in the universe. Microorganisms in nature are irregularly distributed and often exist in biofilms. Biofilms are surface-attached microbial communities consisting of multiple layers of cells embedded in hydrated matrices (Kierek and

Karatan, 2005). Biofilms were first extensively studied during the 1940s but it was not, until the 1970s, appreciated that their formation occurs in almost all natural environments.

Current advancements in biotechnology have promoted the usage of immobilized cells for a wide range of applications. This increase in the number of applications of immobilized cell has allowed for an even wider range of research relating to the field of cell immobilization. Cell immobilization biotechnology is a multidisciplinary area shown to have an important impact on many scientific sub-disciplines, including biomedicine, pharmacology, cosmetology, food and agricultural sciences, beverage production, industrial waste treatment, and analytical applications (Abdelmajeed et al, 2012).

The concentration of cells in the immobilized cells system will be higher than the free ones. This is due to the biomass or solid substrate used as a carrier not only to give protection from extreme environmental conditions but also to provide nutrients to the cells. Kourkoutas et al. explained that the carrier for the immobilization of cells may act as a protective agent against the effects of physicochemical of pH, temperature, solvents, and even heavy metals on the fermentation medium. Genisheva et al. also reported that the minerals or proteins in the carrier dissolved in the fermentation medium and contributed to the improvement of the bioconversion process carried out by yeast cells. High cell densities will accelerate the consumption of sugar, thus increasing productivity and the yield of bioethanol during fermentation.

2.5.2. Methods of Cell Immobilization

Various immobilization methods are available to researchers and the nature of the application often dictates the choice. A thorough knowledge of the influence of immobilization on the fermentation performance and the parameters affecting the immobilization is vital to fine-tune the continuous process and reach an acceptable product quality (Verbelen et al., 2006).

Immobilization methods can be grouped as ‘‘passive” (using the natural tendency of microorganisms to attach to natural or synthetic surfaces and grow on them) and ‘‘active” (with the help of flocculants agents, chemical attachment and gel encapsulation) (Cohen, 2001; Moreno-Garrido, 2008). Generally, four categories of immobilization can be distinguished (Fig 2-1), based on the physical mechanism of the cell localization and the nature of support mechanisms: a) attachment to a surface, b) entrapment to a porous matrix, c) self-aggregation and d) containment behind a barrier.

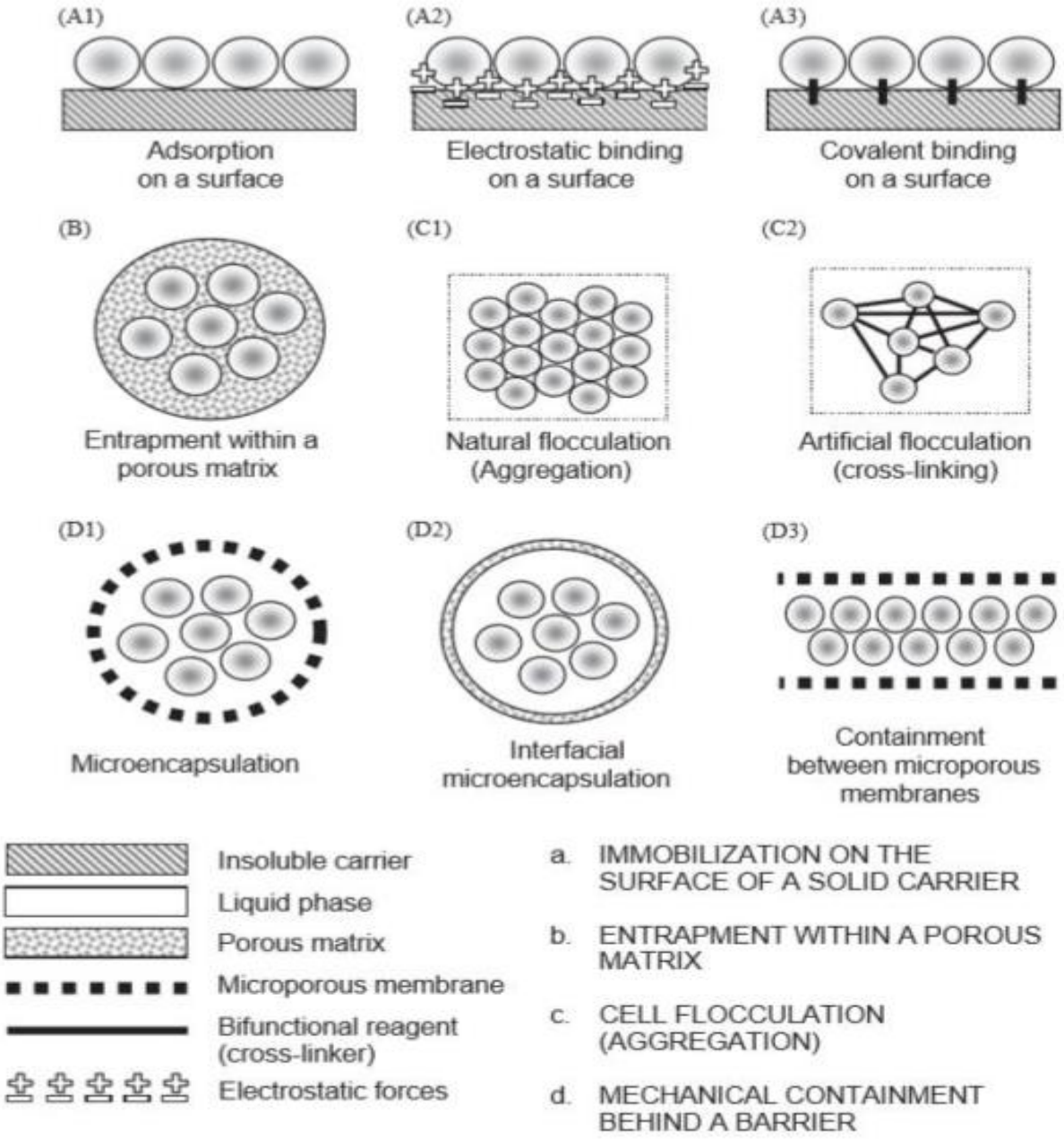


Figure 2-1 Basic methods of cells immobilization (Kourkoutas et al., 2004).

2.5.2.1. Attachment to a Solid Surface

This type of immobilization is carried out due to electrostatic forces or by covalent binding between the cell membrane and the surface. The thickness of the cells film usually ranges from 1 layer to 1 or more mm of cells (Kourkoutas et al., 2004). As there are no barriers between the cells and the solution, cell detachment and relocation is possible with potential establishment of equilibrium between adsorbed and freely suspended cells.

2.5.2.2. Entrapment to a Porous Matrix

Two types of cells entrapment are possible: a) cells are allowed to penetrate into the preformed porous matrix; b) the porous material is formed in situ into a culture of cells. Both entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into the surrounding medium, while still allowing transfer of mass, nutrients and metabolites. Among several different approaches described in literature the most useful for food processing is entrapment of cells within matrix of natural polymers (Nedovic et al., 2015).

2.5.2.3. Cells Self-Aggregation

The ability to form aggregates, the so called cell flocculation, is mainly observed in moulds, fungi and plant cells and can be considered as an immobilization technique. Cell flocculation has been defined as property of cells in suspensions to adhere in clumps and sediment rapidly (Jin and Speers, 1998). However, artificial flocculating agents or cross linkers can be used to enhance aggregation in cell cultures that do not naturally flocculate (Kourkoutas et al., 2004).

2.5.2.4. Containment behind a Barrier

This type of immobilization is appropriate when minimum transfer of cell free product and Compounds is required (Park and Chang, 2000).Containment of cells behind a barrier can be obtained either by use of microporous membranes or by microencapsulation or by cell immobilization onto an interaction surface of two immiscible solutions.

2.5.3. Carriers for Immobilization

The suitable carrier selection is one of the crucial decisions to be made in the course of preparation of the immobilization process. For biotechnology application the ideal carrier for immobilizing of cell culture should fulfil as many as possible the following requirements according to Abdelmajeed et al, A) High cell mass-loading capacity. B) Easy access to nutrient media. C) Simple nontoxic immobilization procedure. D) Optimum diffusion distance from flowing media to centre of support. E) Sterilization. F) Mechanical stability. G) Reusable. H) Easy separation of cells and carrier from media. I) Suitable for conventional reactor systems. J) Suitable for suspension as well as anchorage dependent cells. Other criteria, such as physical characteristics (porosity, swelling, compression, material and mean particle behaviour), as well as, possibility for microbial growth and solubility, are more application specific (Gorecka and Jastrzebska, 2011).

The carriers will be classified as inorganic material (zeolite, clay, anthracite, porous glass, activated char-coal, and ceramics) and organic polymers. Inorganic carriers will be selected to immobilize microorganisms because they can resist microbial degradation and are thermostable. The organic polymeric carriers are more abundant than inorganic carriers and can be natural and synthetic polymeric carriers (Cassidy et al., 1996; Verma et al., 2006)

Several synthetic (acrylamide, polyurethane, polyvinyl, resins) and natural polymer derivatives of algal polysaccharides (alginate, carrageenan, agar, agarose), and chitosan, an amino polysaccharide derived from chitin, has been experimentally used.

The immobilization matrixes more frequently used have been polymeric gels like alginate, where the cells are immobilized for entrapment into the gel. However, this method of immobilization is not too viable on an industrial scale, due to the high cost of raw material and the relative complexity of preparation of the biocatalysts (carrier-cell) with respect to the operative lifetime of the process (de Vasconcelos et al., 2004)

New carrier materials are constantly being developed; recent examples of interest include finding out a renewable and lignocellulosic biomass carrier which essentially is not synthetic, easy to use, cheaper and available naturally in abundance. The use of sugarcane (Saccharum officinarum) bagasse (SCB) for cell entrapment in several biotechnological processes has been described recently (Yu et al., 2007; Mohammadi et al., 2009) and has added a new dimension to the immobilization techniques. SCB is produced in large quantities in Ethiopia, and is a waste. The disadvantages associated with the use of conventional chemical polymeric matrices described may be overcome by the use of SCB as immobilization matrix: (a) the surfaces of SCB are highly porous which facilitates effective diffusion and solve the problem of mass transfer which occurs in the system of Ca-alginate, polyvinyl alcohol, chitosan, pectin, poly acrylamide, polyurethane etc.; (b) the highly porous SCB stalks al Lows cell growth and gas evolution which brings about mechanical disruption in the aforementioned immobilization system; (c) in comparison to some matrices such as polyacrylamide, SCB is nontoxic; (d) easily available and comparatively cheaper, thereby limiting the overall process cost (Santos et al., 2005). The cellulosic materials are regenerable, reusable, sterilizable on heat, biologically and chemically stable under different fermentation conditions and with adequate mechanical resistance (Branyik et al., 2005).

Sugarcane bagasse, a fibrous by product or residue obtained after crushing or diffuser extraction of the sugar cane, is a renewable and low cost lignocellulosic biomass. In 2018, the

production of sugarcane bagasse in Ethiopia was about 2.9 million tons per year (Ethiopian sugar corporation 2017/18 annual report).

The mechanism of cell adsorption into the carrier could be explained through the three stages of the process. First, the process of cell absorption into the porous carrier that was determined by the retention properties of material. Then the cells formed aggregates which adhered to each other. After the formation of aggregates, cell colonies attached to the carrier (Escobar et al., 2012). Vucurovic and Razmovski (2012) also explained that the adhesion of cells was depend on electrostatic interaction between positively charge of carrier binding site and negatively charge of cells surface. Attachment process was mediated by specific proteins on the cell surface i.e. adhesion or floculin. This protein could increase the hydrophobicity of the cell surface that triggers hydrophobic interactions between cells and abiotic surfaces (Lavoie et al., 2011).

Anita et al., (2016) reported 2.5 g of the sugarcane bagasse was used as a carrier to absorb 50 mL cells suspension, or known as biocatalyst. This was because a higher cell density inside the carrier can certainty cause an increase of sugar consumption, ethanol productivity, and ethanol yield. The best ratio between the carrier and the volume of cell suspension obtained in his research was 1:20 (w/v). This value was more efficient compared to other studies such as ratio sorghum bagasse: cell (1:2) (yu et al., 2007), orange peel: cell (1:4) (Plessas et al., 2007), and rice hull: cell (1:10) (Martini et al., 2010). For this study the ratio was used according to Anita 1:20 ratio, the best immobilization process bagasse was used by 2.5 g of bagasse as a carrier for 100 mg of cells in 50 mL of cells suspension. The best percentage of biocatalyst (carrier-containing cells) were used for bioethanol fermentation, namely 1% (w/v) or by 3 g to 300 mL of production medium (Anita et al., 2016).

Table 2-2 Examples of immobilized cell application in bioethanol production.

Substrate, c- source	Carrier	Strain	R, g/g	P (g/l)	Q_p (g/lh)	$Y_{p/s}$ (g/g)	References
Sugar cane bagasse	Sugar cane bagasse	S. cerevisiae	NA	NA	NA	0.44	Singh et al., 2013
Sugar cane bagasse	Ca-alginate	S. cerevisiae	NA	NA	NA	0.38	Singh et al., 2013
Sugar cane Bagasse	Agar-agar cubes	S. cerevisiae	NA	NA	NA	0.33	Singh et al., 2013
Glucose, sucrose	Sugar cane bagasse	S. cerevisiae	0.047		13.33, 13.00	0.48	(Escobar L.M.A. Ph.D. (c)., 2012)
cassava flour	Sugar cane bagasse	S. cerevisiae	0.04657	20	1.67	0.45	(Escobar L.M.A. Ph.D. (c)., 2012)
sugar beet molasses and thick juice	sugar beet pulp	S. cerevisiae	0.182	52.26	NA	0.446	(Vucurovic And Razmovski., 2012)
Glucose and sucrose	Sorghum bagasse	S. cerevisiae	0.6	96	16.68	0.48	Yu et al.,2007
Cane molasses	Alginate–loofah-matrix(ALM)	S. cerevisiae M 30	NA	81.4	NA	0.43	Phisalaphong et al
Glucose	Sugar cane bagasse	S. Cerevisiae	0.00541	23.95	1.24±0.01	NA	Anita et al., 2016
Sugar cane juice and cane molasses	Sugar cane pieces	S. cerevisiae	NA	89.73-77.13	2.48 - 2.62	NA	Liang et al., 2008
Grape	Sugarcane pieces	S. cerevisiae	NA	108	4.2	NA	Reddy et al., 2011
sugarcane molasses	Sugar cane stocks	S. cerevisiae	NA	NA	29.64	NA	De Vasconcelos et al., 2004
Sugar cane molasses	Sugar cane bagasse	S. Cerevisiae					This study

R, g cell retention dry cell weight/g dry mass of carrier; P, volumetric ethanol productivity; $Q_{p,}$ Ethanol volumetric productivity; Yp/s , Ethanol yield per consumed sugar; NA not available.

2.5.3.1. Sugar processing

Sugar processing begins when the cane plant arrives at the sugar mill. Rotating Knives, shredders, and crushers extract the juice from the cane. Heating the juice evaporates off excess water and condenses the juice into thick syrup. Sugar granules act as seed crystals when they are added to the syrup, making the dissolved sugar in the syrup crystalize. When as much sugar as possible has crystallized in the syrup, the mix is spun in a centrifuge, which separates the remaining syrup (now called molasses) from the raw sugar crystals. The fibrous residue of cane stalk left over after the crushing and extraction of juice from the sugar cane is called bagasse.

2.5.3.1.1. Cane Preparation

The objective of cane-preparation is to cut cane into short pieces for feeding the mills as also to rupture the cells, without extracting juice. The preparatory devices commonly employed and installed before the milling tandem.

Preparation index Percentage of cells opened to the total, which is indicative of the extent of cane preparation, is (a) 50-60% in the case of two sets of knives run in the direction of carrier, (b) 85-90% with a combination of knives and shredder, while for unigrator or fibrizor it would be 75-80%. In the case of knives run in opposite direction to the flow of cane, the
Preparation index can be 65-70% depending on whether one or both the sets are revolving in reverse direction. Preparation index is one of the most determining factor of juice extraction (peter, 2007).

2.5.3.1.2. Extraction

Milling

Juice extraction by milling is the process of squeezing the juice from the cane under a set mills using high pressure between heavy iron rollers. Those mills can have from 3 up to 6 rolls; every set of mills is called a tandem mill or mill train. To improve the milling extraction efficiency, imbibition water is added at each mill. Hot water is poured over the cane just before it enters the last mill in the milling train and is recirculated up to reach the first mill. The juice squeezed from this cane is low in sugar concentration and is pumped to the preceding mill and poured onto the cane just before it enters the rollers, the juice from this mill is the same way pumped back up the milling train. Mixed juice (that is to say cane juice mixed with the water introduced at the last mill) is withdrawn from the first and second mills and is sent for further processing. Milling trains typically have four, five or six mills in the tandem. To improve the milling

extraction performance before the cane reaches the first mill, knife and shredder preparation equipment is normally used.

Diffusion

Sugarcane diffusion is the process of extracting the sucrose from the cane with the use of imbibition but without the squeezing by mills. Shredded cane is introduced into the diffuser at the feed end, Hot water is poured over the shredded cane just before the discharge end of the diffuser. The hot water percolates through the bed of cane and removes sucrose from the cane. This dilute juice is then collected in a compartment under the bed of cane and is pumped to a point a little closer to the feed end of the diffuser and this dilute juice is allowed to percolate through the bed of cane. At this point the concentration of sucrose in the cane is higher than the concentration of sucrose in the dilute juice just mentioned and so sucrose diffuses from the cane to the juice, this now slightly richer juice is pumped back up the diffuser and the process is repeated, typically, 12 to 15 times (compared with the four to six times for the milling process)

2.5.4. Physical Properties of Sugar Cane Bagasse

Physical properties of sugarcane bagasse, the surface characteristics of the carrier, such as water content, water retention, water absorption index, and lignin content are the main factors that influence the efficiency of adsorption, the characteristics of immobilized yeast cells, and the productivity of product (Razmovski and Vucurovic, 2012 and De Vasconcelos et al., 2004).

As reported by Anita et al., The growth of microorganisms in the carrier depends on the availability of the water content, the water content increases after hydration. The water content of the sugarcane bagasse is in the range of water content in the solid-state fermentation system. For the microorganisms, the optimal water content requirement varies between 30-80% (w/w) (Chisti et al., 1999)

Water retention indicates the hydrophilic properties of materials. Materials with higher water retention indicates an ability to absorb more water (Razmovski and Vucurovic, 2012)

Generally, the lignin content of lignocellulosic biomass was about 15-40%. Lignocellulosic biomass is composed of cellulose, hemicelluloses, lignin, extractive, and inorganic materials. The cellulose chain is bound by hydrogen bonds and called micro fibril. These micro fibrils are attached to each other by hemicelluloses and covered by lignin. The lower the lignin content of the lignocellulosic biomass, the easier it will be for cells to immobilize. Genisheva *et al*. mentioned that specific regions, such as cellulose on a natural carrier, were more preferable for

yeast as a site to adhere to than a smooth structure. Cellulose component covered by lignin causes the cell wall to appear smoother (Yu et al., 2010)

The water absorption index (WAI) indicates the quantity of water that can be absorbed by materials. According to Anita et al., (2016) WAI of sugarcane bagasse is 8.58 ± 0.21 g/g and is higher compared to other materials (Table 2-3). Materials with high WAI are preferable as a carrier for cell immobilization, as they support the growth and development of cell microorganism, (Vucurovic and Razmovski, 2012; Mussatto et al., 2009)

Table 2-3 The Water Absorption Index (WAI) for Different Biomass used as Cell Immobilization Carrier.

Immobilization Carrier	Water Absorption Index (g/g)
Corn cobs	3.77
Sugar beet pulp	6.59
Dried sugar beet pulp	6.60
Loofa sponge	7.76
Coffee husk	8.30
Sugarcane bagasse	8.58

Source (Anita et al., 2016)

2.6. Reactors Used With Immobilized Cells

Reactors operating with immobilized cells have higher productivity and operational stability, as well as easier downstream processing. Another attractive advantage of the immobilized cell bioreactors, compared to the existing free cell fermenters, is the faster fermentation time. Because of this and other benefits, the immobilized cell bioreactors have been applied in many industrial processes, including beverage production. Choosing the proper reactor for use with immobilized cell systems depends on the type of immobilization, the type of the used support, mass transfer requirements and conditions of the process (Genisheva et al., 2014).

2.6.1. Continuous Reactors

The use of immobilized cell systems are, more attractive for using in continuous reactors. The continuous reactor is an “open reactor” where there is a constant inflow of nutrients and out flow of product (Fig. 2.2).

The main characteristic of the continuous reactor is the possibility of reaching a dynamic equilibrium, i.e. the system operates on steady-state basis. Continuous reactors are used widely in the food, pharmaceutical and chemical industries. For continuous production the most used reactors are the multiphase reactors, including packed bed reactor, fluidized bed reactor, bubble column and air-lift reactor (Verbelen et al., 2006). The multiphase rectors include three phases: solid (the support), liquid (the medium) and gas (air, or other).

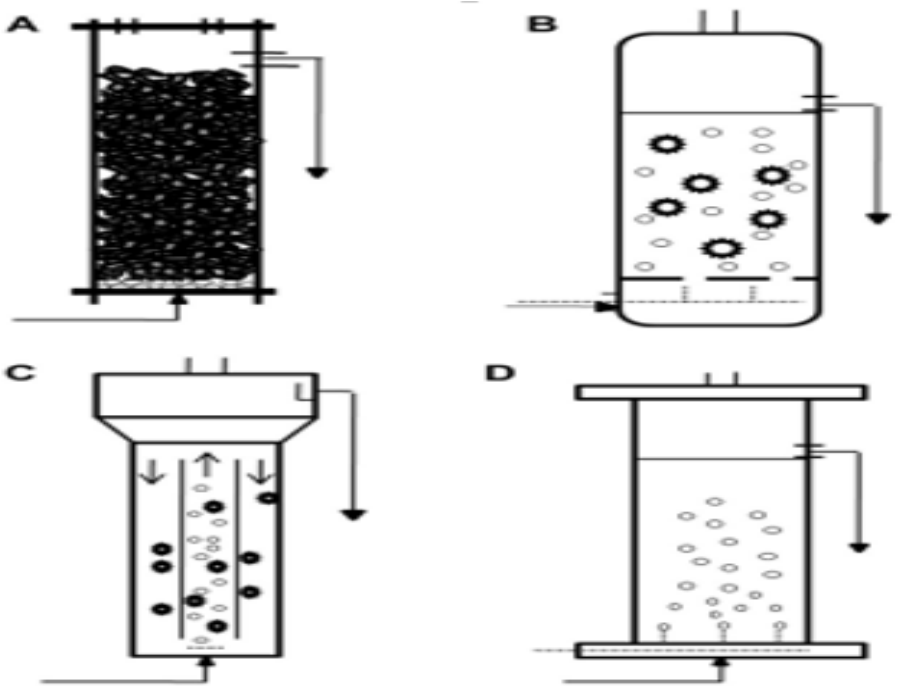


Figure 2-2 Immobilized cell reactor working in continuous mode

A. Packed Bed Reactor; B. Fluidized Bed reactor; C. Air Lift Reactor; D. Bubble column Reactor.

2.6.2. Packed Bed Reactor

Packed bed (Fig. 2-2A) or also known as fixed bed reactor is extensively used in the chemical, petrochemical and biotechnology industries. In this reactor type the immobilized cells are packed inside the reactor and a co-current of gas and fermentation media is passed up flow (flooded bed reactor) or down flow (trickle-bed reactor). Despite its simplicity, during the operation of a packed bed reactor the following drawbacks can take place: channelling, fouling, mass transfer limitations, difficulties in CO2 evacuation and compression of some support materials (Verbelen et al., 2006). Some application of packed bed reactor are tabulated below table 2-4. In this study work shop fabricated packed bed reactor according to (Najafpour, 2004)

was used for experimental purpose only designing not included the focus was on development of bio-carrier.

Table 2-4 Lab Scale bioreactors application for continuous fermentation

Reactor Type	Packing volume, %	Carrier Type	Total reactor volume	Operating parameters	References
Packed bed	70	Sugarcane bagasse	250 ml	Temp 30 ^{0}c PH 5.0	Escobar et al., 2012
Packed Bed	70	Sugarcane bagasse	150ml	Temp 30 ^{0}c PH 5.0	
Packed bed	50% porosity	Sugar cane stock	38.5 L	Temp 33 ± 1 ^{0}c PH 4.2	De Vasconcelos et al., 2004
Packed bed	NA	loofa-reinforced alginate	1 L	Temp 32 ± 1 ^{0}c PH 5.0	Bangrank et al., 2011
Packed bed	65 – 70 %	Calcium Alginate	1.41 L	Temp 32 ± 2 ^{0}c PH 5.0	Najafpour, 2004.
Packed bed	65-70%	Sugarcane bagasse	1.41 L	Temp 32 ± 2 ^{0}c PH 5.0	This study

In previous studies most of the time mill bagasse pre-treated as well as raw bagasse were investigated whereas diffuser bagasse and post processing cane preparation index were not seen studied in detail. The novelty of this work lies in the investigation of the effect of cane preparation index on cell retention and comparative study on mill tandem bagasse and cane diffuser bagasse. The optimal conditions (inoculum concentration, sugar concentration, PH and temperature) for the S. cerevisiae immobilization onto SCB was taken from previous studies. Further, the efficiency of immobilized yeast for repeated batch ethanol fermentation of sugar cane molasses was investigated with the aim to select suitable carrier for continuous ethanol fermentation. Finally optimization of continuous fermentation operating conditions was done using the selected bio-carrier.

CHAPTER THREE

3. MATERIALS AND METHODS

This chapter deals with the microorganisms, substrates, media and other chemicals required while performing the fermentation experiments. It also describes the experimental procedures performed. This study have two parts. In the first part Development of suitable bio carrier for yeast cell immobilization using bagasse is done. Here the study assess suitability of mill and cane Diffuser bagasse at different preparation index (comparative study) then best carrier is selected for continuous fermentation. The second part deal with application of selected carrier for continuous ethanol fermentation and optimized for total reducing sugar concentration and dilution rate. All chemicals used in thesis work are commercial grade.

3.1. Physico-Chemical Characterization of Sugar Cane Bagasse

Sugarcane bagasse was obtained from Fincha sugar factory (FSF), Cane diffuser and mill tandem bagasse with preparation index of 80-90% were collected and air dried then reserved at room temperature for next process. Prior to its use as a carrier, the lignin content, water content (W), water retention (H), and water absorption index (WAI) of bagasse were analyzed.

3.1.1. Materials

A. Samples

Cane diffuser and mill tandem bagasse with preparation index source of 80-90%.

B. Reagents

Tap water, Distilled water

C. Apparatus

Equipment's used for carrier preparation and characterization are sieve with ¾ and 4 in mesh, desiccators, Digital weighing balance, drying pan, moisture teller, jar bottle, centrifugation tube, centrifugation machine, beaker 100ml, glass rod, measuring cylinder.

3.1.2. Methods

3.1.2.1. Experimental procedures for Bagasse Preparation

Collect mill Tandem and Cane Diffuser bagasse separately while preparation index was analyzed and registered, then the sample was separated by standard Tayler sieve by passing 3/4in mesh (19,000 microns or 19mm) and retained on 4 mesh (4,760microns or 4.76mm) sieve that is 5mm to 20mm length bagasse will be collected. After separation and screening,

the bagasse was washed by distilled water until the wash water was clean and colorless. Finally the biomass was air-dried and kept in a desiccators at room temperature for further use (Singh et al., 2013).

3.1.2.2. Experimental Procedures for Water Content before and After Hydration (W)

Conducted according to MSF laboratory manual procedures as follow: 100 gm of the sample was weighed on a drying pan and Placed in a moisture teller at temperature of 127 ^{0}C (260 ^{0}F). Dried for 45 minutes until constant weight (The accepted weight loss after re drying for 5 minutes is not more than 0.1 gm.) Finally removed from the moisture teller and weighed quickly.

Expression of Result: Water content of bagasse =Weight loss after drying.

3.1.2.3. Experimental procedures for Determination of Water Retention (H)

Water retention was done by placing an amount of 2.5 g of the dry mass of sugarcane bagasse into the jar bottle containing 100 mL of distilled water and kept at room temperature for 24 hours. After 24 hours unabsorbed water was drained and weighed the hydrated bagasse. The water retention (H) was calculated as the grams of water retained per grams of the dry mass carrier:

$$H = \frac{\text{wt. Of bagasse after hydration} - \text{wt. Of bagasse before hydration}}{\text{Wt. of bagasse before hydration}}$$

3.1.2.4. Experimental procedures for Water Absorption Index (WAI)

The WAI was determined according to the methodology described by (Orzua et al., 2009). A Bagasse of 1.25 gm was weighed and added to 15 mL of distilled water in beaker. Then the suspension was mixed for 10 min in orbital shaker. The mixed suspension was placed into a 50 mL measured centrifugation tube and Run the Centrifuge machine at 3000 rpm for 10 min. Then supernatant was decanted and the gel weight was reported.

WAI was expressed as W g gel/g dry support

3.1.2.5. Experimental Procedures for Lignin Content

The method used for determining the lignin content was annexed in appendix-c.

3.2. Study the Effect of Cane P.I. And Bagasse Type in Cell Retention.

Yeast Saccharomyces cerevisiae isolated from source of yeast strain like yeast liquid culture, fermenters, yeast vessels, pre-fermenters was maintained on yeast extract peptone dextrose (YEPD) agar slant (glucose 20 g/l, yeast extract 10 g/l, peptone 20 g/l, and agar 25 g/l) and stored at 4 ^{0}c refrigerator, and sub cultured every 2 weeks (yadav et al., 2011).

3.2.1. Materials

A. Samples

One to two loop of 48-h yeast in Slant medium which was previously reserved at 4 0c in refrigerator, previously prepared 2.5g of hydrated Sugar cane bagasse (mill Tandem and cane diffuser bagasse at different preparation index source)

B. Reagents

Reagents used are commercial chemicals like distilled water, Glucose, yeast extract, peptone, KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2\ SO_4$, 10% $H_2\ SO_4$, isotonic solution (0.9% NaCl).

C. Apparatus

Apparatus used are for inoculum preparation for Immobilization and starter culture preparation were PH -meter, Erlenmeyer flask, pressure cooker, laminar flow chamber, Temperature regulated incubator, Digital Weighing Balance, Refrigerator, orbital shaker, Laboratory centrifuge machine, centrifuge tubes Flame equipment. , volumetric flasks having 1 liter capacity, refracto meter, Microscope with heamocytometer, spectrophotometer.

3.2.2. Design for Optimization of Cane P.I. And Bagasse Type In Cell Retention

Response surface methodology (RSM) is implemented as it is a collection of mathematical and Statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize the response. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results (Montgomery 2001). The optimum levels of the inoculum size, the initial sugar concentration and the incubation temperature during ethanol fermentation were taken from previous studies but the optimum level of preparation index (P.I.) and bagasse type were evaluated using user defined via the Design expert software. The experimental design matrices along with the response variables (Immobilized cell weight (g), Cells retention, R (g/g)) are summarized in Table below. The

factor levels are decided based on practical knowledge and the P.I. to be examined were as follows:

Bagasse type: Mill tandem bagasse (MTB) and Cane diffuser bagasse (CDB)
Cane Preparation index (P.I.): 80, 82.5, 85, 87.5 and 90% are selected by the software from the range of 80-90% and tabulated as follow: Response surface user defined was used.

Table 3-1 Response surface experimental design for optimization of bagasse type and P.I.

Std	Run	Factor		Response	
		P.I. (%)	Bagasse type	Immobilized cell weight (g)	Cells retention, R (g/g)
10	1	85.00	MTB	*	*
5	2	82.50	CDB	*	*
6	3	82.50	MTB	*	*
3	4	90.00	CDB	*	*
9	5	85.00	CDB	*	*
7	6	87.50	CDB	*	*
8	7	87.50	MTB	*	*
4	8	90.00	MTB	*	*
2	9	80.00	MTB	*	*
1	10	80.00	CDB	*	*

3.2.3. Methods

3.2.3.1. Experimental Procedures in Inoculum Preparation for Immobilization

For starter culture preparation, one to two loop of 48-h yeast was cultivated into 30 mL starter medium (Nikolic et al., 2009) that consist of (g/L): glucose, 10; yeast extract, 3; peptone, 3.5; KH_2PO_4, 1 ; $MgSO_4.7H_2O$, 1 ; $(NH_4)_2SO_4$, 1 ; pH 5, in 100 mL Erlenmeyer flask. The medium was adjusted to a pH 5 by adding 10% H_2SO_4 before sterilization. The flask was incubated in an orbital shaker (120 rpm) for 24 hours at room temperature (30 ± 2 °C). At the end of the incubation period, the starter culture was inoculated into the 270 mL production medium in 500 mL Erlenmeyer flask and then incubated under the same condition. After 24 hours, the cells were centrifuged aseptically at 3000 rpm for 10 minutes. As much as 100 mg of yeast cells (dry weight) were suspended in 50 mL sterilized 0.9% NaCl to be used as an inoculum for immobilization process (Razmovski and Vucurovic, 2012)

3.2.3.2. Experimental Procedures for Immobilization

Sugarcane bagasse as much as 2.5 g which was hydrated by adding 100 mL of distilled water and incubated for 24 h at room temperature (Vucurovic and Razmovski, 2012). After that, the liquid was decanted and the solids (bagasse) were autoclaved at 121 ^{o}C for 15 min in pressure cooker.

For immobilization process, flask that consist carrier was inoculated by transferring 50 mL of yeast suspension and incubated for 24 h at room temperature (30 ± 2 ^{0}C) in an orbital shaker at 120 rpm (Razmovski and Vucurovic, 2012). After immobilization, cell retention of whole cell biocatalyst/WCB (carrier containing cells) was measured according to Singh et al. (2013) and used for ethanol fermentation. The procedure for cell retention is discussed in Analytical method section 3.5 below.

3.3. Repeated Batch Fermentation using optimized Bagasse.

Due to simplicity the selection of carrier suitable for continuous fermentation was done using repeated batch fermentation as well as stability test for continuity was conducted at 3rd and 7th batch of fermentation.

Sugarcane molasses obtained from metehara sugar factory production process was used as fermentation medium as carbon source. The molasses will be pretreated with concentrated sulphuric acid to remove the sludge e.g., colloids, particles, sand etc. and also to kill organisms. The molasses analysis was done and reserved for the whole project work to be the same sample. For fermentation purpose the molasses will be used after the dilution with distilled water to give a total sugar concentration of 100 g/l, and pH will be adjusted to 5.5 by addition of 10% (v/v) H_2SO_4. All media will be sterilized by autoclaving at 121 ^{o}C for 30 min.

3.3.1. Materials

A. Sample

The samples used for repeated batch Fermentation and stability test were prepared biocatalyst (according to section 3.2; the optimized P.I. and bagasse type were used) and Fermentation media, fermentation media preparation is annexed in appendix-B.

B. Reagents

Fehling solution, methylene blue and 10% H_2 SO_4 for determination of residual sugar and yeast Count.

C. Apparatus

Apparatus used for this analysis were Erlenmeyer flask, pH meter, refracto meter, microscope with heamocytometer and Ebuillo meter, Spectrophotometer.

3.3.2. Design for Repeated Batch Fermentation.

After determination of bagasse type and Cane preparation index optimized result General Factorial according to the data were used for repeated batch fermentation optimization to yeast biomass and immobilization parameters. The factor levels are decided based on the above (section 3.2.2.) user defined response surface results.

The two factors optimized are cane preparation index and batch number:

P.I. 85, 87.5, 90 whereas batch number 1 to 7.

Table 3-2 General Factorial Experimental Design for Repeated Batch Fermentation

Std	Run	block	Factors		Response			
			P.I.	Batch No.	SU (%)	Qp (g/lh)	Yp/s (g/g)	Ey (%)
36	1	Block 1	P.I. 90	Batch 6	*	*	*	*
32	2	Block 1	P.I. 85	Batch 6	*	*	*	*
42	3	Block 1	P.I. 90	Batch 7	*	*	*	*
23	4	Block 1	P.I. 90	Batch 4	*	*	*	*
24	5	Block 1	P.I. 90	Batch 4	*	*	*	*
22	6	Block 1	P.I. 87.5	Batch 4	*	*	*	*
12	7	Block 1	P.I. 90	Batch 2	*	*	*	*
5	8	Block 1	P.I. 90	Batch 1	*	*	*	*
11	9	Block 1	P.I. 90	Batch 2	*	*	*	*
41	10	Block 1	P.I. 90	Batch 7	*	*	*	*
18	11	Block 1	P.I. 90	Batch 3	*	*	*	*
4	12	Block 1	P.I. 87.5	Batch 1	*	*	*	*
2	13	Block 1	P.I. 85	Batch 1	*	*	*	*
37	14	Block 1	P.I. 85	Batch 7	*	*	*	*
33	15	Block 1	P.I. 87.5	Batch 6	*	*	*	*
38	16	Block 1	P.I. 85	Batch 7	*	*	*	*
6	17	Block 1	P.I. 90	Batch 1	*	*	*	*
40	18	Block 1	P.I. 87.5	Batch 7	*	*	*	*
35	19	Block 1	P.I. 90	Batch 6	*	*	*	*
31	20	Block 1	P.I. 85	Batch 6	*	*	*	*
28	21	Block 1	P.I. 87.5	Batch 5	*	*	*	*
30	22	Block 1	P.I. 90	Batch 5	*	*	*	*
13	23	Block 1	P.I. 85	Batch 3	*	*	*	*
9	24	Block 1	P.I. 87.5	Batch 2	*	*	*	*
10	25	Block 1	P.I. 87.5	Batch 2	*	*	*	*
26	26	Block 1	P.I. 85	Batch 5	*	*	*	*
20	27	Block 1	P.I. 85	Batch 4	*	*	*	*
29	28	Block 1	P.I. 90	Batch 5	*	*	*	*
34	29	Block 1	P.I. 87.5	Batch 6	*	*	*	*
15	30	Block 1	P.I. 87.5	Batch 3	*	*	*	*
27	31	Block 1	P.I. 87.5	Batch 5	*	*	*	*
19	32	Block 1	P.I. 85	Batch 4	*	*	*	*
1	33	Block 1	P.I. 85	Batch 1	*	*	*	*
14	34	Block 1	P.I. 85	Batch 3	*	*	*	*
25	35	Block 1	P.I. 85	Batch 5	*	*	*	*
39	36	Block 1	P.I. 87.5	Batch 7	*	*	*	*
16	37	Block 1	P.I. 87.5	Batch 3	*	*	*	*
3	38	Block 1	P.I. 87.5	Batch 1	*	*	*	*
8	39	Block 1	P.I. 85	Batch 2	*	*	*	*
7	40	Block 1	P.I. 85	Batch 2	*	*	*	*
17	41	Block 1	P.I. 90	Batch 3	*	*	*	*
21	42	Block 1	P.I. 87.5	Batch 4	*	*	*	*

3.3.3. Design for Stability Test

Design for stability of the carrier for continuous fermentation were conducted using General factorial taking cane preparation index, P.I. and batch number as categorical factor the point where batch no 3 and 7 whereas P.I. were 85, 87.5 and 90% (According to section 3.2.2.) are tabulated below.

Table 3-3 General Factorial Design for Stability Taste

		Factors		Responses				
Std	Run	P.I. %	Batch no.	Cells retention, R (g/g)	Immobilized cells concentration, Xi (g/l)	Free cells concentration, Xs (g/l)	Total cells concentration, Xt (g/l	Immobilization Efficiency, Yi (%)
3	1	P.I. 87.5	B3	*	*	*	*	*
9	2	P.I. 87.5	B7	*	*	*	*	*
2	3	P.I. 85	B3	*	*	*	*	*
5	4	P.I. 90	B3	*	*	*	*	*
6	5	P.I. 90	B3	*	*	*	*	*
4	6	P.I. 87.5	B3	*	*	*	*	*
11	7	P.I. 90	B7	*	*	*	*	*
7	8	P.I. 85	B7	*	*	*	*	*
12	9	P.I. 90	B7	*	*	*	*	*
8	10	P.I. 85	B7	*	*	*	*	*
1	11	P.I. 85	B3	*	*	*	*	*
10	12	P.I. 87.5	B7	*	*	*	*	*

3.3.4. Methods

3.3.4.1. Experimental Procedures for Repeated Batch Fermentation.

The prepared biocatalyst will be used for the batch fermentation of the same fermentation Molasses medium as much as 300 mL, consist of (g/L) glucose, 100; yeast extract, 3; peptone, 3.5; KH_2PO_4, 2; $MgSO_4.7H_2O$, 2; $(NH_4)_2SO_4$, 1; pH 5 (Nikolic et al., 2009) was inoculated with equal biomass concentration of 1.09 ± 0.05 g/l, for free and immobilized cells, respectively in 1000 mL Erlenmeyer flask and covered by bubble traps using free (FC) and immobilized yeast cells (IC) onto bagasse (cane diffuser bagasse) were carried out in duplicate. The flask was incubated for 24 h at room temperature (30 ± 2 ^{0}C) in static condition. Medium sampling during the batch fermentation was carried out by taking aliquots (10 ml), for cell, sugar, pH, and total dissolved solids (TDS) concentration Analyses. At the end of the fermentation batch after 24hrs, the fermented liquid will be decanted and then another 300 ml of the fresh medium

will be added for the next fermentation batch (Vucurovic and Razmovski, 2012), seven repeated fermentation was done.

3.4. Optimization of Continuous Ethanol Fermentation

Optimization of the selected bio carrier operation condition in lab scale packed bed reactor for continuous ethanol fermentation was done. The Cane Diffuser bagasse (CDB) with 87.5% cane preparation index were selected for immobilization of yeast for the continuous fermentation as per batch fermentation results , this was because of its high porosity to adsorb the yeast cells, easy availability, natural source and stability at repeated batch Fermentation. The cells were adsorbed on bagasse with cell loading of 51mg/g of bagasse on dry basis with 60% adsorption efficiency.

Immobilized growing yeast cells were used to study the continuous fermentation and Figure below shows the schematic diagram of the experimental set-up. The Immobilized cell reactor was a packed bed Reactor, constructed with a nominal diameter of 5 cm, ID of 4.6 cm, 85 cm length and 3mm wall thickness stainless steel SS-316. The immobilized bagasse, biocatalyst were transferred to the column. About 65% of the column was packed. The extra space was counted for bed expansion by the fresh media. The void volume was measured by volume of distilled water pumped through the bed. The packed ICR column was used in continuous mode for the duration of fermentation. The fresh feed was fed in an up flow manner; while sugar and ethanol Concentration was monitored during the course of continuous fermentation. Stable system operation was achieved after the 12 hour operation, when the sugar concentration in the samples was stabilized. The samples were taken at the output of the reactor, once the system was in a stable state.

The medium at PH of 5.0 was fed to the ICR column from a feed tank located above the column by gravity. The experimental setup for the PBR with a packed volume of 65% (v/v) of the total reactor volume, 31.25% of packed volume is void volume, a total of 780 ml working volume. The temperature of the system was controlled at 32 ±2°C by passing cooling and reheating water inside the reactor jacket. At the top and bottom of fermenter screens were used to keep the bed packed as well as to prevent washout of the biocatalyst.

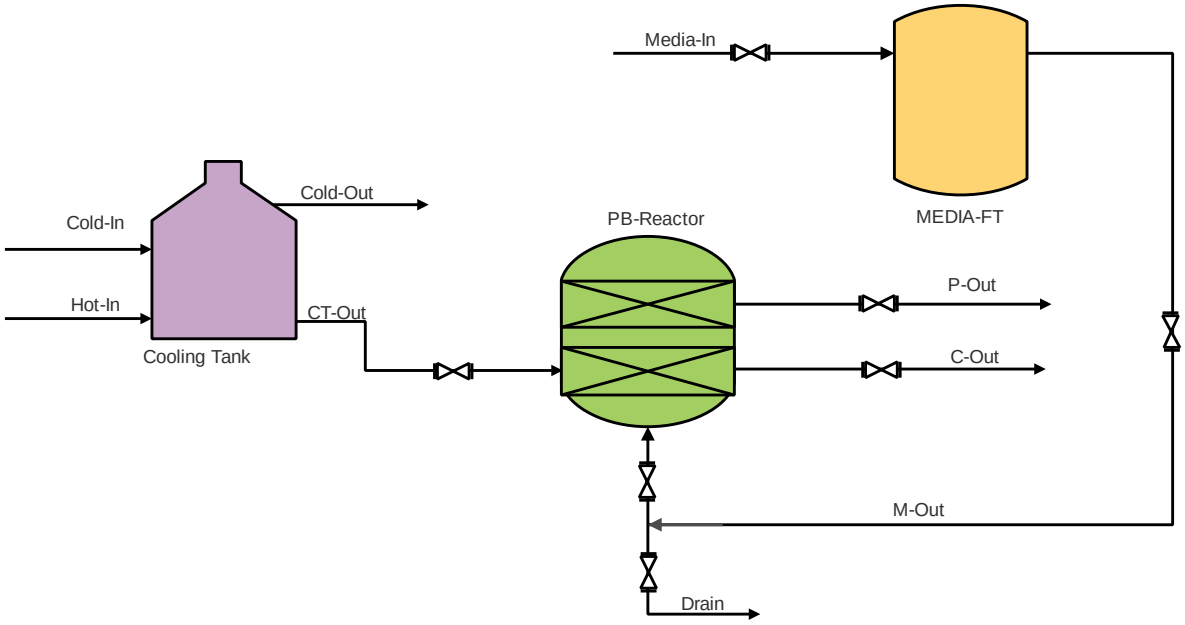


Figure 3-1 Schematic Diagram of Continuous Ethanol Fermentation Process in A Packed Bed Reactor.

3.4.1. Material

A. Sample

The samples for continuous fermentation analysis were taken online from reactor outlet, fermentation media preparation is annexed in appendix-B.

B. Reagents

Fehling solution, methylene blue and 10% H_2SO_4 for determination of residual sugar and yeast Count, process water and nutrients.

C. Apparatus

Apparatus used for this analysis were Erlenmeyer flask, pH meter, refractometer, microscope with heamocytometer, Ebuillo meter, Spectrophotometer, fermentation equipment was work shop manufactured packed bed reactor as mention previously according to (Najafpour, 2004).

3.4.2. Design for Optimization of Continuous Ethanol Fermentation

In continuous fermentation the batch selected immobilization material cane diffuser bagasse with cane preparation index 87.5 % were used. Here the Total reducing sugar concentration in molasses and dilution factor were optimized. According to data response surface was used for optimization.

Response surface methodology (RSM) is implemented as it is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest

is influenced by several variables and the objective is to optimize the response. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results (Montgomery 2001). In this portion of study sugar concentration total reducing sugar (TRS) and dilution rate (D) were taken as a factor to be studied and sugar utilization, SU (%), Ethanol volumetric productivity, QP (g/lh), Ethanol yield per consumed sugar, YP/s (g/g) and percentage of theoretical yield, Ey (%) were taken as a response variable. The factor levels are decided based on practical knowledge were as follows:

TRS ranges from 50g/l – 150g/l and Dilution 0.5- 1.5 h^{-1} were fed to the software where its selection were as below Table 3-4 for experimental run:

Table 3-4 Response Surface; Central Composite Design (CCD) For Continuous Fermentation

Std	Run	Block	Factors		Responses			
			TRS	D	Sugar utilization, SU (%)	Ethanol volumetric productivity, Qp (g/lh)	Ethanol yield per consumed sugar, Yp/s (g/g)	Percentage of the Theoretical Yield, Ey (%)
8	1	Block 1	100.00	1.71	*	*	*	*
5	2	Block 1	29.29	1.00	*	*	*	*
2	3	Block 1	150.00	0.50	*	*	*	*
9	4	Block 1	100.00	1.00	*	*	*	*
12	5	Block 1	100.00	1.00	*	*	*	*
13	6	Block 1	100.00	1.00	*	*	*	*
7	7	Block 1	100.00	0.29	*	*	*	*
4	8	Block 1	150.00	1.50	*	*	*	*
1	9	Block 1	50.00	0.50	*	*	*	*
6	10	Block 1	170.71	1.00	*	*	*	*
11	11	Block 1	100.00	1.00	*	*	*	*
10	12	Block 1	100.00	1.00	*	*	*	*
3	13	Block 1	50.00	1.50	*	*	*	*

3.4.3. Methods

3.4.3.1. Experimental Procedures for Continuous Fermentation.

Before Starting and Operating of the packed bed reactor was sterilized with sodium Hypochlorite solution (2% active chlorine) four days prior to the start of the fermentation experiment. Then thoroughly washed by hot water and sterilized by steam, in sterilized reactor the immobilized bagasse or biocatalyst were transferred to the column. About 65% of the column was packed. The fresh feed medium at PH 5.0, at different TRS and Dilution rate was

fed in an up flow manner; while cooling or reheating water circulating through the jacket to attain the reactor body temperature at 32 ± 2 ^{o}c. Stable system operation was achieved after the 12 hour operation, when the sugar concentration in the samples was similar. The samples were taken at the output of the reactor, once the system was in a stable state. Residual Sugar and ethanol Concentration was monitored during the course of continuous fermentation. At each designed dilution rate and TRS the system was run each 48-60 hrs.

3.5. Analytical Methods

Samples were analyzed for ethanol, sugar, and yeast biomass. The ethanol content was measured by using Ebuillo meter latest alcohol measuring equipment, It directly measure the alcohol content % volume /volume.

Cell biomass was determined gravimetrically after immobilization procedure according to Santos et.al. (2008) with slight modified by Vucurovic and Razmovski (2012), on the absorbance at 600 nm with a UV-visible spectrophotometer and converted to dry cell weight on standard curve. The concentration of immobilized cells, concentration of free cells, and efficiency of immobilization were determined and calculated according to Vucurovic and Razmovski (2012) and Santos et.al. (2008).

The ethanol productivity, ethanol yield, sugar conversion, and efficiency of sugar conversion to ethanol were calculated according to Singh et al., (2013).

3.5.1. Yeast Biomass Standard Curve

To perform dry weight experiments, the first step was to grow yeast cells in medium. Four 250 mL Erlenmeyer flasks were used and approximately 100 mL yeast medium containing 20 g/L glucose was poured into each of them. Four 2.0 mL centrifuge tubes containing frozen yeast cultures were taken from the freezer and thawed. Each centrifuge tube contained approximately 1.5 mL yeast solution. One centrifuge tube contents were emptied into each Erlenmeyer flask. The flasks were kept on an orbital shaker at room temperature for approximately 20 hours. The contents of all flasks were mixed. The optical density of this yeast solution was measured at 600 nm using a plastic cuvette. The spectrophotometer was zeroed using distilled water. Some of this yeast solution was diluted to get 5-6 readings between the maximum optical density measured and an optical density of 0.02. The total volume of the remaining yeast solution was recorded and it was then centrifuged (5000 RPM for 10 minutes) in about 6-8 centrifuge tubes, with approximately 40 mL in each tube. After centrifuging, the liquid above the pellet is decanted off. Ten mL of water was added to each tube and vortexed.

After the pellet dissolved in each tube, the tubes are centrifuged again at the same speed and for the same time. The liquid above the pellet in each tube was decanted off again and approximately 5 mL water was added to one of the tubes. This tube is vortexed until the pellet is resuspended. The solution from this tube is poured into the second tube and vortexed again to resuspend the second pellet. This procedure is repeated for all of the tubes. The final yeast solution is poured into a pre-weighed aluminum weigh-boat. This weigh-boat is kept in an oven at 65°C overnight. The weight of the dried biomass was measured and the dry biomass concentration was calculated by dividing the dry weight (in mg) by the total volume of yeast solution used (in mL). This concentration corresponds to the optical density of the original yeast solution. The optical densities were measured using a UV spectrophotometer at 600 nm wavelength. A graph of optical density verses dry weight concentration and cell count were plotted what we called it standard curve.

3.5.2. Determination of Immobilized Cell Mass.

The mass of cells immobilized onto the support particle was quantified gravimetrically after the immobilization procedure according to Santos et al. (2008) with slight modifications by Vucurovic v. Razmovski et.al (2012). The support with immobilized cells was placed on a filter paper to remove fluid. The sample without extra medium (1 g), was pressed out using a glass stick, the support was washed several times with 10 ml of distilled water to remove yeast cells and then dried at 105 ^{o}C up to constant weight. The dry weight of the immobilized cells in the collected liquid was calculated by measuring the optical density at 600 nm, using a spectrophotometer. The identical procedure was conducted using support particles recovered from the cell-free medium, as a control, in order to avoid any interference in optical density and weighing measurements. A previously constructed standard curve was used to relate the optical density measurements to dry cell concentration in the samples.

3.5.3. Evaluation of Immobilization Parameters

Cell retention onto the support (R, g/g) was calculated as the ratio of dry mass of cells immobilized in the support to the dry mass of the support. The concentration of immobilized cells (Xi, g/l) was calculated as the ratio of dry mass of cells immobilized in the support to the fermentation medium volume. The immobilization efficiency (Yi, %) was calculated as the ratio of Xi to the total (free plus immobilized) cells concentration (Xt, g/l) and multiplying by 100 (Santos et al., 2008).

3.5.4. Evaluation of Fermentation Parameters

Sugar utilization (Su, %) was calculated as the ratio of utilized sugar to the initial and multiplying by 100. The ethanol yield (Yp/s, g/g) was calculated as grams of ethanol produced per gram of utilized sugar. Also a percentage of the maximal theoretical ethanol yield (Ey, %) was estimated by the ratio of ethanol yield to the theoretical value of ethanol yield (0.51 g/g). The volumetric ethanol productivity (QP, g/l h) were calculated as grams of ethanol produced per liter per hour.

CHAPTER FOUR

4. RESULT AND DISCUSSION

4.1. Physico-chemical Characterization of Sugar Cane Bagasse

Physical properties of carrier such as water retention (H), water absorption index (WAI), and lignin content (L) were the main factor that influence adsorption of cells onto carrier and efficiency of immobilization process, as well as ethanol productivity (Razmovski and Vucurovic, 2012; Genisheva et al., 2014). Table 4.1 shows the physical properties of sugarcane bagasse Mill tandem (MTB) and cane diffuser (CDB) bagasse. The cane preparation index had non-significant effect on physical properties of the bagasse only bagasse type was considered which had significant effect.

The growth of microorganisms in the carrier depends on the availability of the water content. The initial water content of sugarcane bagasse was 7.76 % (w/w) and 7.81 % (w/w) MTB and CDB respectively. After adding 100 ml of distilled water and maintaining it for 24 hours, the water content of the carrier increased to 85.03% (w/w), 89.73 % (w/w) respectively. Another research reported that the initial water content of the maize stem ground tissue was 12.45% (w/w) and after the hydration process, the water content (WAH) increased to 97.34% (w/w) (Razmovski and Vucurovic, 2012). This indicates that the ability of the sugarcane bagasse to absorb water is higher than the maize stem ground tissue after the hydration process. Another report showed that the water content of hydrated fresh pressed sugar beet pulp and dried sugar beet pulp were 93.16 ± 0.04% and 93.13 ± 0.06% (Razmovski and Vucurovic, 2012). The water content of the sugar beet pulp was higher than the sugarcane bagasse. Nevertheless, the water content of the sugarcane bagasse is still in the range of water content in the solid-state fermentation system. For the microorganisms, the optimal water content requirement varies between 30-80% (w/w) (Chisti et al., 1999). Water affected the growth of microorganisms. Low water content reduced a nutrient solubility thus extending the lag phase of microorganisms. Conversely, a high water content reduced the porosity of substrate and the oxygen transfer into the substrate (Prior et al., 1992)

Water retention (H) was calculated as the ratio of water mass to the dry mass of the carrier after soaking for 24 hours. The value of water retention indicates the hydrophilic properties of materials (Razmovski and Vucurovic, 2012, Mussatto et al., 2009). The water retention of the sugarcane bagasse as a carrier MTB was 4.73 g/g and CDB was 8.0 g/g significant to each

other, CDB was more hydrophilic than MTB, and also indicating that the materials are more hydrophilic than maize stem ground tissue. Water retention of maize stem ground tissue was 0.85 g/g. Materials with higher water retention indicates an ability to absorb more water (Razmovski and Vucurovic, 2012).

Lignin content of lignocellulosic biomass was also important in selecting an appropriate substrate to be used as a carrier. Lignin, on lignocellulosic biomass, covered the hemicellulose and cellulose component causing cell wall appears smoother (Yu et al., 2010). This shows the diffuser operation reduced the lignin content that making it easier for the cells to immobilize on substrate. However, lignin was the component that gives stability and rigidity to the lignocellulosic biomass. Lignin content, that was too low, affected the strength of substrate as a carrier for cell immobilization (Plessas et al., 2007; Escobar et al., 2012). From the result we can say that the Diffuser operation decreased the lignin content of sugarcane bagasse, but also can maintain the strength of carrier.

In this study, bagasse type had a significant effect on water absorption index (P>0.05) (WAI), MTB was 8.53g/g whereas CDB was 8.92 g/g. Water absorption index (WAI) indicated the quantity of water that can be absorbed by the substrate. Sugarcane bagasse has a higher value of WAI than others such as fresh sugar beet pulp (6.59 g/g) (Vucurovic and Razmovski, 2012), coffee husk (7.76 g/g), and corn cobs (3.77 g/g) (Mussatto et al., 2009).

Table 4-1 Physico-chemical properties of sugarcane bagasse

Std	Run	Block 1	Factor	Response				
			Bagasse Type	W	WAH	H	L	WAI
1	3	Block 1	MTB	7.75	85.31	4.77	24.78	8.49
2	2	Block 1	MTB	7.77	84.75	4.69	24.81	8.57
3	4	Block 1	CDB	7.84	89.48	8.02	23.04	8.89
4	1	Block 1	CDB	7.79	89.98	7.97	23.17	8.94

W, was not significant but the rest were significantly different with bagasse type at 5% probability level.

Steam pretreatment was reported can remove and redistribute lignin on fiber surfaces thereby increasing the pore volume of the pretreated material (Jorgensen et al., 2007). Water in pretreatment process was absorbed into the carrier and then filled the pores of biomass. When steaming process occurs, the water evaporated rapidly and caused the changes of pores volume and fiber fragmentation (Chandel et al., 2009). The increasing of pore volume could enhance the material porosity that was indicated by the high value of water retention (Yi zheng et al.,

2009). In this study, bagasse type in other word diffuser operation showed better result on the improving of sugarcane bagasse physico-chemical properties than mill tandem operation.

4.2. Effect of Cane Preparation Index and Bagasse Type in Cell Retention

Optimization of cane Preparation index and bagasse type for immobilization was done by Response surface user define taking bagasse type as categorical factor and cane preparation index (P.I.) as numeric factors. The P.I. range was selected by actual data's in sugar processing technology. (80 – 90%) and the following data were generated.

Table 4-2 Immobilization properties

Std	Run	Block	Factors		Responses	
			P.I., %	Bagasse Type	Immobilized cell weight, Xi (g)	Cells retention, R (g/g)
10	1	block 1	85	MTB	0.0218	0.0087
6	2	block 1	82.5	MTB	0.0137	0.0055
2	3	block 1	80	MTB	0.0111	0.0044
4	4	block 1	90	MTB	0.0250	0.0100
1	5	block 1	80	CDB	0.0554	0.0221
7	6	block 1	87.5	CDB	0.1275	0.0510
3	7	block 1	90	CDB	0.1300	0.0520
5	8	block 1	82.5	CDB	0.0632	0.0253
9	9	block 1	85	CDB	0.1200	0.0480
8	10	block 1	87.5	MTB	0.0256	0.0102

4.2.1. Data Analysis for the Effect of Cane P.I. And Bagasse Type

Data were modeled by multiple regression analysis and the statistical significance of the terms was examined by analysis of variance for each response. The statistical analysis of the data were performed using design expert software 7.00. The adequacy of regression model was checked by R2, Adj R2, Pred R2, Adeq precision and F-test (Montgomery 2001). The significance of F value was judged at 95% confidence level. The degree of relationship between the variables was also checked by using correlation matrix of the Factors and response variables.

4.2.1.1. Data Analysis of response 1 Immobilized cell weight (g)

Response ranges from 0.0111088 to 0.13, ratio of max to min was 11.7024, and a ratio greater than 10 usually indicates a transformation is required, the best result was attained by natural log. Linear Model was suggested by the design program but when transformed to natural log main effect was selected. For this response to test for its adequacy and to describe its variation with independent variables. From ANOVA test in Table 4.3, the model F-value of 745.59 implies the model is significant. There is only a 0.01% chance that a "model F-value" this large could occur due to noise.

Table 4-3 ANOVA test for Immobilized cell weight (g), Xi

source	Sum of Squares	Df	Mean square	F value	p-value	
Model	7.82	5	1.56	745.59	< 0.0001	Significant
A-P.I	1.26	4	0.31	150.17	0.0001	
B bagasse Type	6.56	1	6.56	3127.29	< 0.0001	
Residual	8.390E-003	4	2.097E-003			
Cor Total	7.83	9				

The Model F-value of 745.59 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms.

Table 4-4 Post ANOVA statistics for Immobilized cell weight (g), Xi

Std. Dev.	0.046	R-Squared	0.9989
Mean	-3.19	Adj R-Squared	0.9976
C.V.%	1.44	Pred R-Squared	0.9933
Press	0.052	Adeq Precision	69.157

The "Pred R-Squared" of 0.9933 is in reasonable agreement with the "Adj R-Squared" of 0.9976. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. For this analysis ratio of 69.157 indicates an adequate signal. This model can be used to navigate the design space.

Diagnostic test for the responses

All diagnostic plots are also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.). For example fig 4.1 shows how precisely the immobilized cell weight is modeled, because all the points line up nicely and the deviation of points for immobilized cell weight from normality are insignificant. Similar results were observed for the remaining responses (Cell retention).

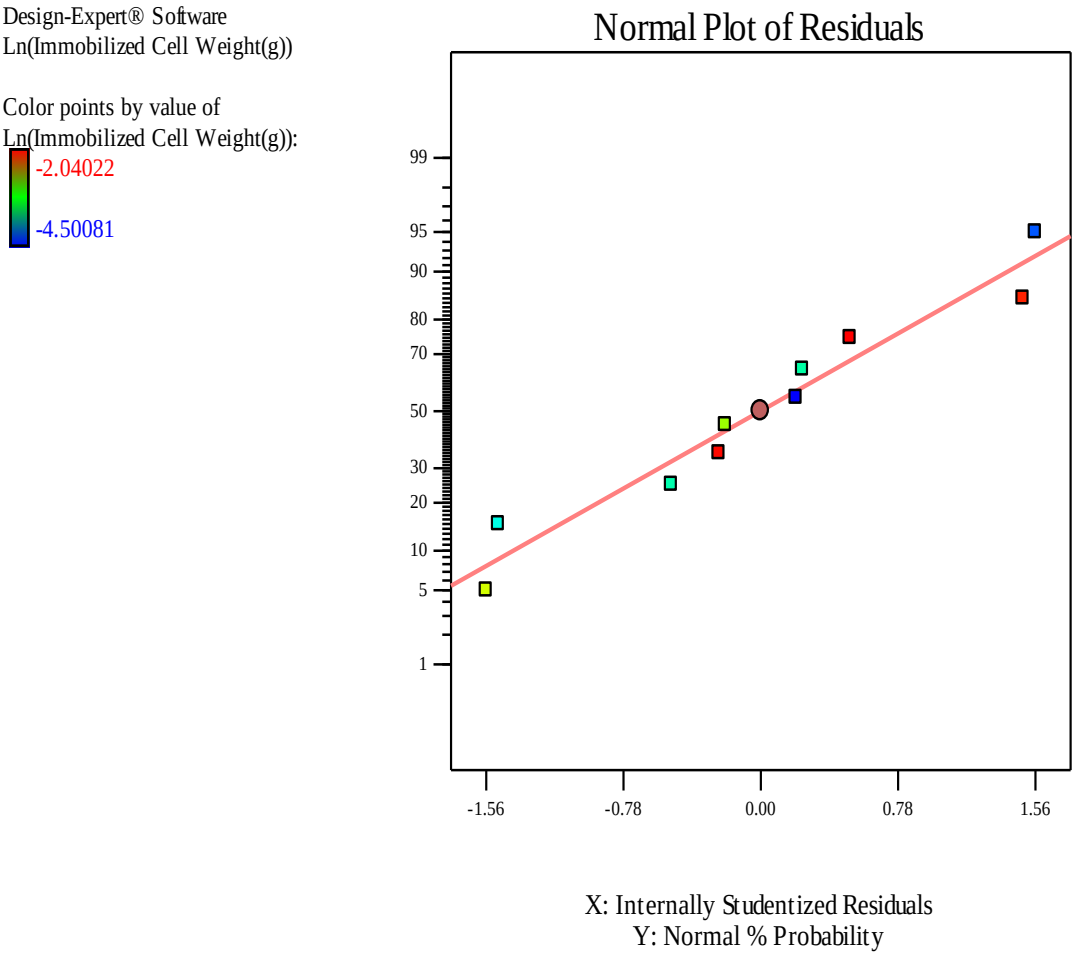


Figure 4-1 Normal plot of residual for immobilized cell weight

4.2.1.2. Data Analysis of Response-2 Cells Retention, R (g/g)

Cell retention, R (g/g) is mass of bagasse carrier times immobilized cell weight which have linear corelation with them , the software gave the same anlysis copy paste of the above.

4.2.1.3. Response Surface Plot Analysis

The effect of different treatment parameters on immobilized cell mass is given in Table 4.4, as well as for cell retention. To aid visualization of variation in responses with respect to processing variables, series of three dimensional Response surfaces were drawn for each response using design expert software 7.00

Response 1 and 2 below figure 4.2 and 4.3 immobilized cell mass and cell retention respectively. From the response surface plot graph 4.2 and 4.3 it is clearly seen that both immobilized cell mass and cell retention increases as preparation index increases for both bagasse type CDB as well as MTB but intensity of inreament was different. For the case of cane diffuser bagasse the increament was bigger than mill tandem bagasse.

The retention of S. cerevisiae cells onto CDB carrier is significantly different ($P<0.05$) than MTB (Table 4.2 and 4.3). The highest immobilized cell of 0.1275g of dry cell weight that is cell retention of 51mg dry cell weight /g of dry carrier weight was obtained by using CDB at 87.5% P.I. where as the highest in MTB was only 0.0102 g of dry cell weight was obtained at 87.5% P.I. This finding was supported by the results of Yu et al.,2007 who reported that if the total mass of the carrier was equal, the large pieces had more intact stalk cells than the small ones, and more yeast cells could be immobilized on every unit. The cells of the large sorghum pieces were large, long and porous. The cell retention was due to the action of capillary forces during immobilization process, which pulled the cells closer to the surface and through the channels where they could be entrapped or attached, and multiplied (Liang et al., 2008).

The cane diffuser bagasse carrier was very hydrophilic so that cells can easily absorb into the carrier. Yeast cells penetrated into pores of carrier and be embedded. Interaction between cells and carrier surfaces allowed the immobilization process to occur. The process of cell adsorption into the carrier occurred not only by natural entrapment mechanisms but also by physical or chemical adhesion between cells and carrier surface. Natural entrapment occurred due to the surfaces of carrier that were very rough and porous (Verstrepen and Klis, 2006). Physical adhesion was due to the hydrophobic interactions or Van der Waals forces, while chemical adhesion occurred as a result of covalent, hydrogen, and ionic bonds between cells and carrier (Verbelen et al., 2006; Verstrepen and Klis, 2006; Plessas et al., 2007).

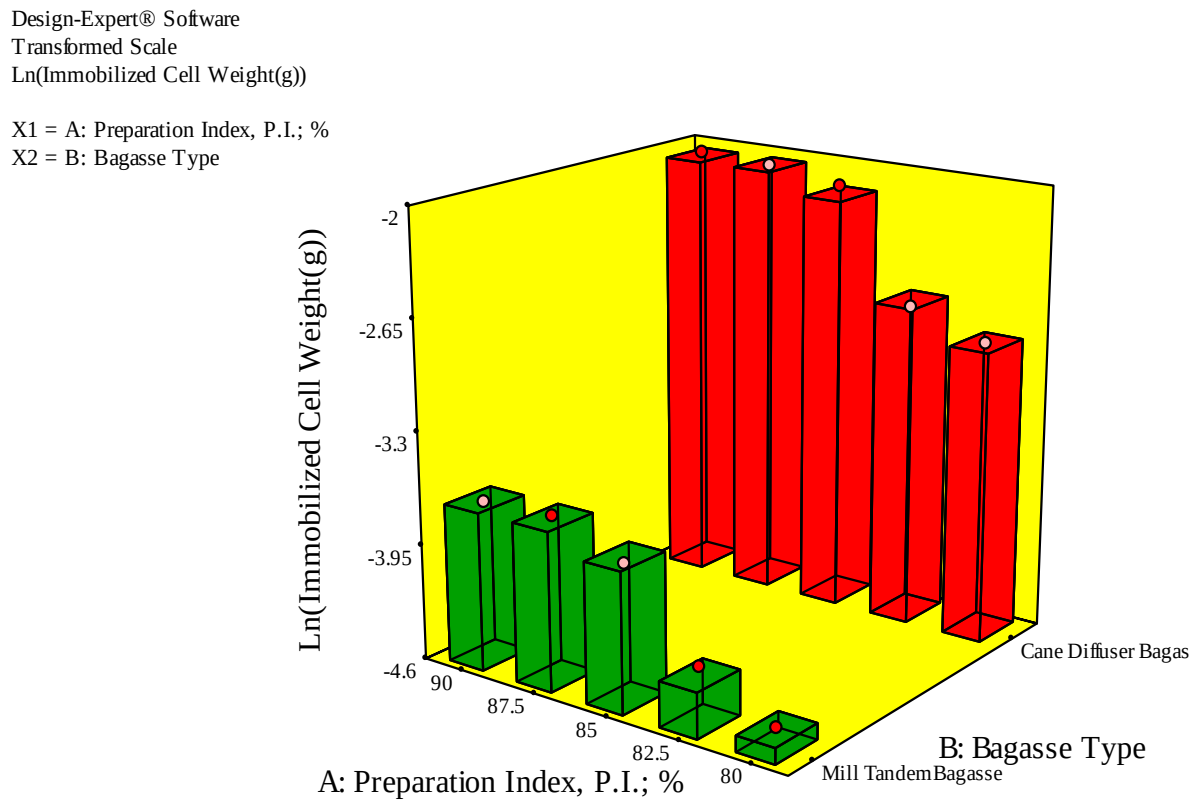


Figure 4-2 Response surface plot for immobilized cell weight(g)

The negative value was due to the system, Response ranges from 0.0111088 to 0.13, ratio of max to min was 11.7024, and a ratio greater than 10 usually indicates a transformation is required as suggested by the design expert software, the best result was attained by natural log.

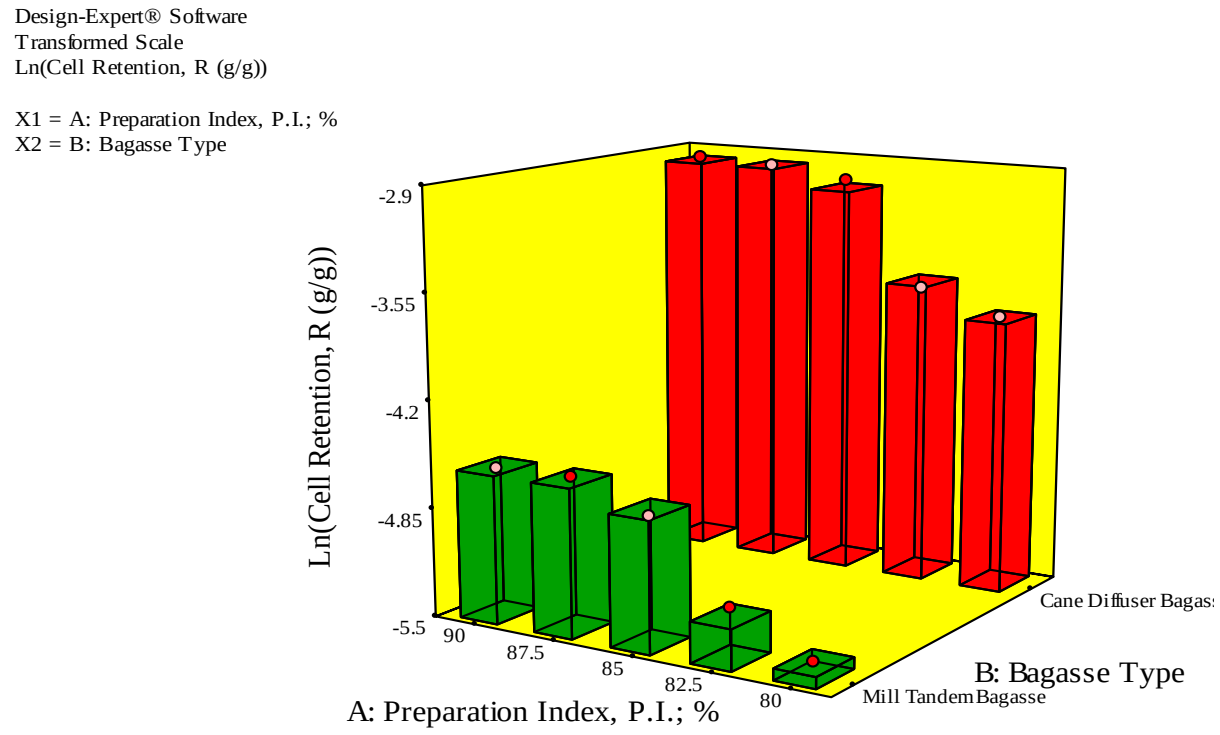


Figure 4-3 Response surface plot for cell retention, R

4.2.1.4. Optimization Solution for P.I. and Bagasse Type for R

According to the software the optimum cane P.I. and cane bagasse type was selected and tabulated as follows

Table 4-5 Solutions for 10 combinations of categoric factor levels

No.	P.I	Bagasse Type	Ln(Xi)	Ln(R)	Desirability	
1	87.5	Cdb	-2.053	-2.970	0.995	Selected
2	90	Cdb	-2.055	-2.971	0.994	
3	85	Cdb	-2.2164	-3.080	0.950	
4	82.5	Cdb	-2.717	-3.634	0.725	
5	80	Cdb	-2.887	-3.803	0.656	
6	87.5	Mtb	-3.673	-4.590	0.336	
7	90	Mtb	-3.674	-4.591	0.336	
8	85	Mtb	-3.784	-4.700	0.291	
9	82.5	Mtb	-4.337	-5.253	0.066	

From this optimization numerical Solution CDB with 87.5% P.I. was recommended to be selected. when we see the top three results all are CDB with desirablity of 0.995, 0.994, 0.950 respectively. For repeated batch fermentation in selection of carrier for continuous fermentation we used the top three only.

4.3. Repeated Batch Fermentation using optimized sugar Cane Bagasse

In the above section 4.2. the effects of cane Preparation index and bagasse type in cell retention was assessed and Optimization was done by Response surface user define taking bagasse type as categorical factor and cane preparation index (P.I.) as numeric factors. From optimization numerical Solution CDB with 87.5% P.I. was recommended to be selected. When we see the top three results all are CDB with desirability of 0.995, 0.994, 0.950 respectively. For fermentation we used the top three only. The ability of the CDB carrier for repeated batch fermentation as well as stability test in other way suitability for continuous fermentation was assessed and results are discussed here under. Repeated Batch Fermentation by Immobilized Bio Carrier

The parameter of seven repeated batch fermentation by CDB immobilized at 87.5, 90, 85 by general factorial with two replication 42 experimental result are tabulated as follow:

Table 4-6 Parameter of seven repeated batch fermentation by CDB carrier

Std	Run	Block	Factor		Response			
			A:P.I., %	B:Batch no	SU, %	Qp, g/lh	Yp/s, g/g	Ey, %
1	27	Block 1	P.I. 85	Batch 1	96.33	0.93	0.2306	45.13
2	6	Block 1	P.I. 85	Batch 1	96.27	0.92	0.2291	44.84
3	35	Block 1	P.I. 87.5	Batch 1	96.63	0.96	0.2389	46.76
4	19	Block 1	P.I. 87.5	Batch 1	96.23	0.95	0.2374	46.47
5	1	Block 1	P.I. 90	Batch 1	96.80	1.03	0.2549	49.88
6	41	Block 1	P.I. 90	Batch 1	96.23	1.02	0.2548	49.85
7	26	Block 1	P.I. 85	Batch 2	88.97	1.02	0.2765	54.10
8	17	Block 1	P.I. 85	Batch 2	89.07	1.04	0.2815	55.09
9	15	Block 1	P.I. 87.5	Batch 2	89.37	1.07	0.2868	56.12
10	28	Block 1	P.I. 87.5	Batch 2	89.23	1.05	0.2836	55.51
11	31	Block 1	P.I. 90	Batch 2	90.13	1.11	0.2958	57.88
12	18	Block 1	P.I. 90	Batch 2	90.40	1.13	0.3002	58.74
13	21	Block 1	P.I. 85	Batch 3	93.47	1.18	0.3022	59.14
14	37	Block 1	P.I. 85	Batch 3	93.37	1.19	0.3068	60.03
15	12	Block 1	P.I. 87.5	Batch 3	92.67	1.19	0.3091	60.49
16	13	Block 1	P.I. 87.5	Batch 3	93.23	1.19	0.3055	59.79
17	4	Block 1	P.I. 90	Batch 3	92.57	1.14	0.2948	57.70
18	7	Block 1	P.I. 90	Batch 3	91.90	1.13	0.2953	57.78
19	23	Block 1	P.I. 85	Batch 4	95.47	1.47	0.3707	72.54
20	33	Block 1	P.I. 85	Batch 4	95.80	1.53	0.3843	75.20
21	30	Block 1	P.I. 87.5	Batch 4	92.57	1.56	0.4037	79.00
22	29	Block 1	P.I. 87.5	Batch 4	92.47	1.57	0.4067	79.59
23	42	Block 1	P.I. 90	Batch 4	92.30	1.65	0.4281	83.77
24	39	Block 1	P.I. 90	Batch 4	92.57	1.66	0.4294	84.03
25	24	Block 1	P.I. 85	Batch 5	92.53	1.65	0.4270	83.56
26	32	Block 1	P.I. 85	Batch 5	92.30	1.51	0.3920	76.71
27	20	Block 1	P.I. 87.5	Batch 5	92.57	1.62	0.4191	82.02
28	10	Block 1	P.I. 87.5	Batch 5	92.43	1.63	0.4232	82.81
29	5	Block 1	P.I. 90	Batch 5	90.43	1.69	0.4492	87.90
30	9	Block 1	P.I. 90	Batch 5	91.20	1.64	0.4324	84.61
31	2	Block 1	P.I. 85	Batch 6	90.13	1.50	0.4005	78.38
32	3	Block 1	P.I. 85	Batch 6	89.67	1.53	0.4097	80.17
33	40	Block 1	P.I. 87.5	Batch 6	93.30	1.66	0.4260	83.37
34	34	Block 1	P.I. 87.5	Batch 6	92.97	1.73	0.4463	87.35
35	16	Block 1	P.I. 90	Batch 6	89.67	1.47	0.3946	77.23
36	25	Block 1	P.I. 90	Batch 6	89.30	1.56	0.4185	81.89
37	8	Block 1	P.I. 85	Batch 7	89.17	1.43	0.3844	75.22
38	14	Block 1	P.I. 85	Batch 7	88.80	1.44	0.3904	76.41
39	38	Block 1	P.I. 87.5	Batch 7	93.30	1.86	0.4796	93.86
40	11	Block 1	P.I. 87.5	Batch 7	93.40	1.93	0.4961	97.08
41	36	Block 1	P.I. 90	Batch 7	89.10	1.40	0.3767	73.71
42	22	Block 1	P.I. 90	Batch 7	88.90	1.36	0.3668	71.78

4.3.1. Data Analysis for Repeated Batch Fermentation

4.3.1.1. Data Analysis for Response 1

Factorial model was selected by the design program for this response. All statistical analysis including ANOVA test, Post ANOVA statistics, normal plot of residuals etc. were done for sugar utilization, SU % in repeated fermentations. All the tests indicated that the model is statistically acceptable. The Model F-value of 186.69 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, AB are significant model terms.

Table 4-7 Post ANOVA Statistics for sugar utilization SU %

Std. Dev.	**0.26**	**R-Squared**	**0.9944**
Mean	92.22	Adj R-Squared	0.9891
C.V. %	0.28	Pred R-Squared	0.9776
PRESS	5.72	Adeq Precision	40.827

The "Pred R-Squared" of 0.9776 is in reasonable agreement with the "Adj R-Squared" of 0.9891. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. For this analysis ratio of 40.827 indicates an adequate signal. This model can be used to navigate the design space.

Diagnostic Test for the Response 1 sugar utilization SU, %

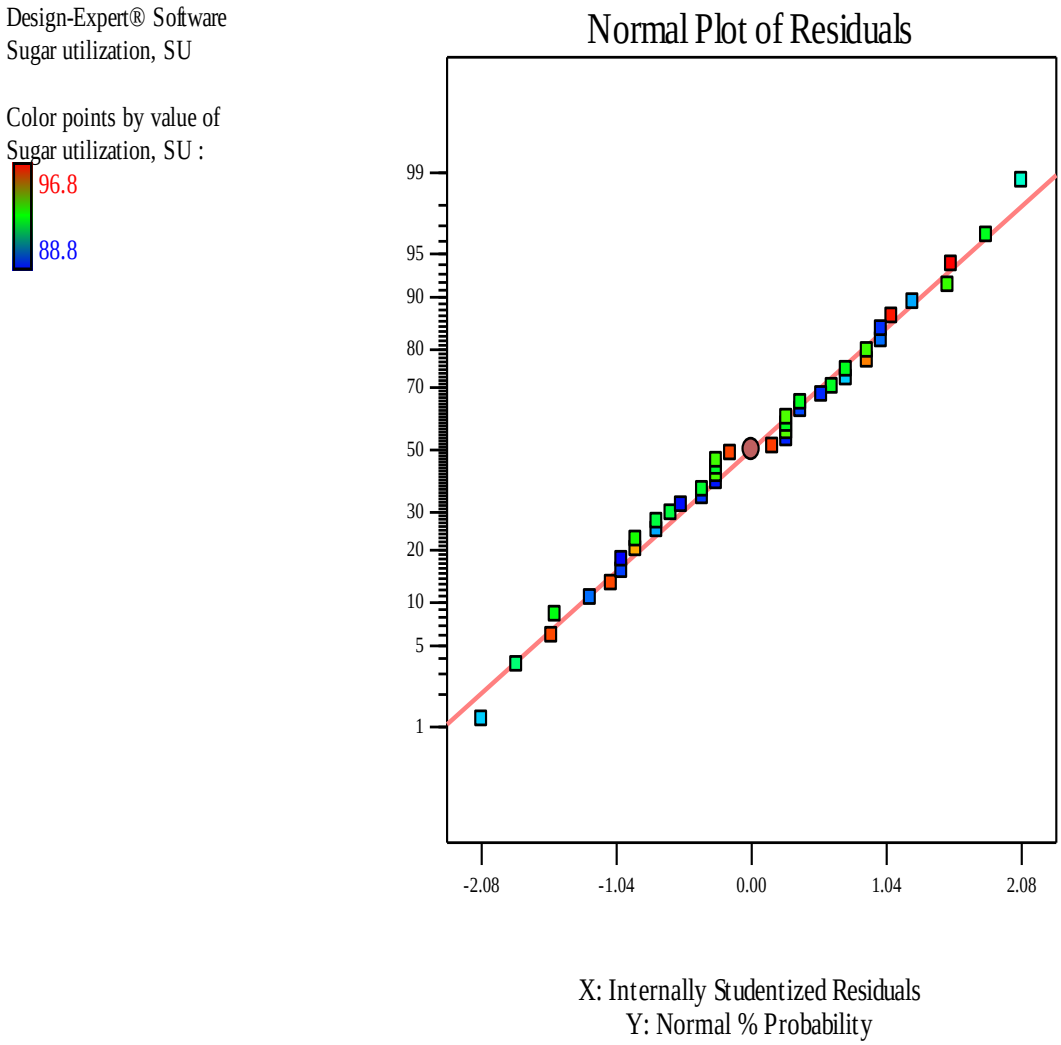


Figure 4-4 Normal plot for sugar utilization

4.3.1.2. Data Analysis for Response 2, 3, And 4

For response 2, 3, and 4 (QP, Yp/s and Ey respectively) all required post ANOVA statistics (F-value, Prob > F, R-Squared, Adj R-Squared, Pred R-Squared, Adeq Precision etc.) and Responses diagnostic plots were also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.) found in good agreement.

4.3.1.2.1. Response Surface Plot Analysis

Repeated batch fermentation responses as shown below Figure 4-5 to 4-8 the following findings are observed.

When we observe sugar utilization , SU from figure 4-5 at first batch the sugar utilization was high more than 96% at all the given preparation index, the highest with 90% preparation index(P.I.). The result confirmed the highest cell retention (Table 4-2) with it maximized the sugar utilization as well as decreasing in initial sugar level was due to the absorption of some sugar in to carrier. (Escobar et al., 2012) found the carriers could absorb some of sugars in the culture medium, reducing the amount of sugars available to be converted into ethanol and therefore decreasing the rate of fermentation. However, the absorption of sugar into carrier could reach a saturation point which no more absorption occurs and the concentration of sugar in the medium remained stable.

The control medium and control biocatalyst were used in this study. Control medium contains a medium with initial sugar 1000 g/L without biocatalyst, while control bio carrier contains a medium with initial sugar 100 g/L and a carrier without yeast cells. From control bio-carrier data, it can be seen that the initial sugar of control of biocatalysts also decreased to around 54 g/L and remained constant until the end of fermentation.

At the second batch the sugar utilization decreased then again increased finally except for P.I. 87.5% which increased and became stable the rest sugar utilization declined gradually. This shows wash out of immobilized cells as the batch number increases declined fermentation efficiency.

Walsh et al. (1993) assumed that a microbial population grows in a heterogeneous way, multiplying more quickly at the surface, given its easy access to the substratum and other nutrients to the detriment of cells located inside the calcium alginate sphere. Eventually, a dense external biomass layer is formed around a dispersedly populated nucleus. The authors attributed this heterogeneous cell distribution inside the support to the diffusion effects of the substratum and the product through the gel particles, which contributes decisively to the alteration of intra-support cell viability. Given the internal and complex structures of this natural support, the distribution of cells on the sugar-cane bagasse would also be heterogeneous.

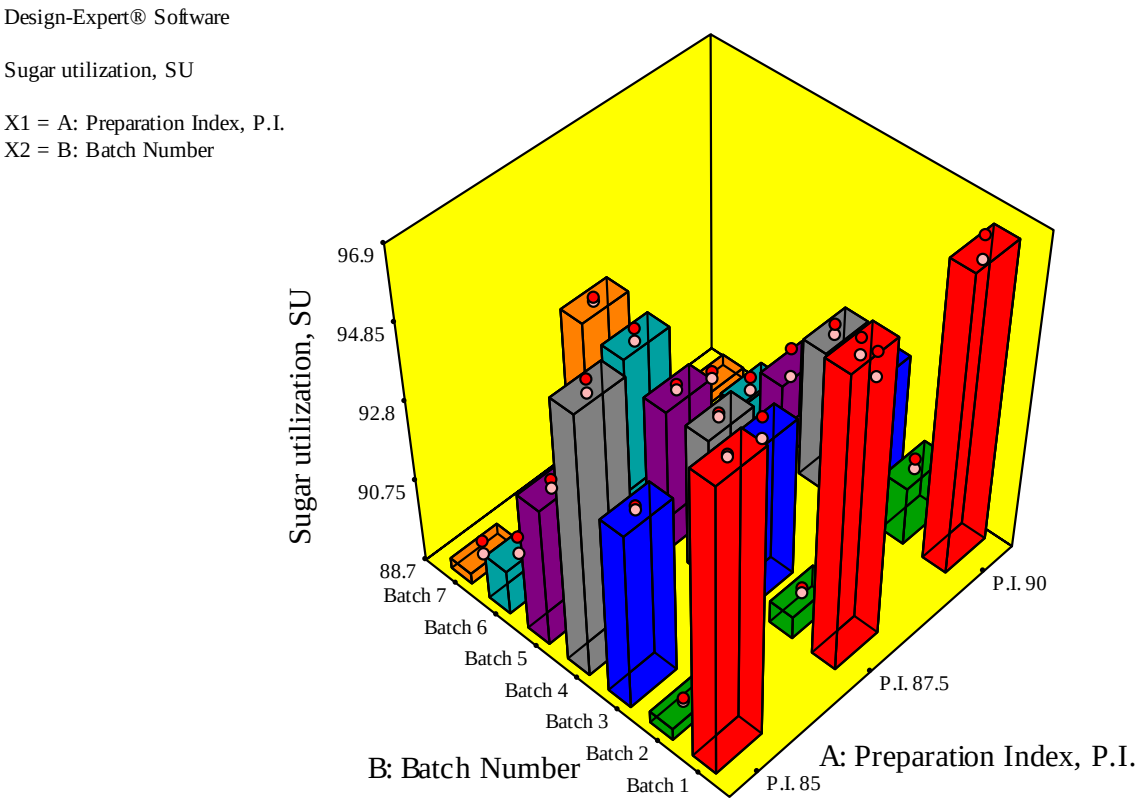


Figure 4-5 Sugar Utilization SU, % Response Graph

The Ethanol volumetric productivity, Qp; Ethanol yield per consumed sugar, Yp/s and Percentage of the Theoretical Yield, Ey at the first batch was too low this was as discussed above due to sugar was absorbed by the bagasse carrier rather converting to alcohol, conversion started after the bagasse carrier was saturated by sugar then after increment was observed by all samples even if after 3rd batch P.I. 90% was stable for 4th and 5th batch but after 5th batch declined this result was in agreement with sugar utilization decrease as the immobilized cell detach from the carrier, this shows un stable property of 90% P.I. CDB whereas 87.5 % P.I. CDB increased gradually and sustained as can be seen from figure 4-6 to 4-8. At the 7th batch Ethanol volumetric productivity, QP (g/lh) of 1.9 g/lh, Ethanol yield per consumed sugar, Yp/s (g/g) 0.488 g/g equal to 95.49 % Percentage of the Theoretical Yield was achieved, which is considerable result.

This result was confirmed by cell count at fermented broth as well absorbance measure and standard curve reading data by taking the 3rd and 7th batch as can be seen below as stability test section 4.3.2.

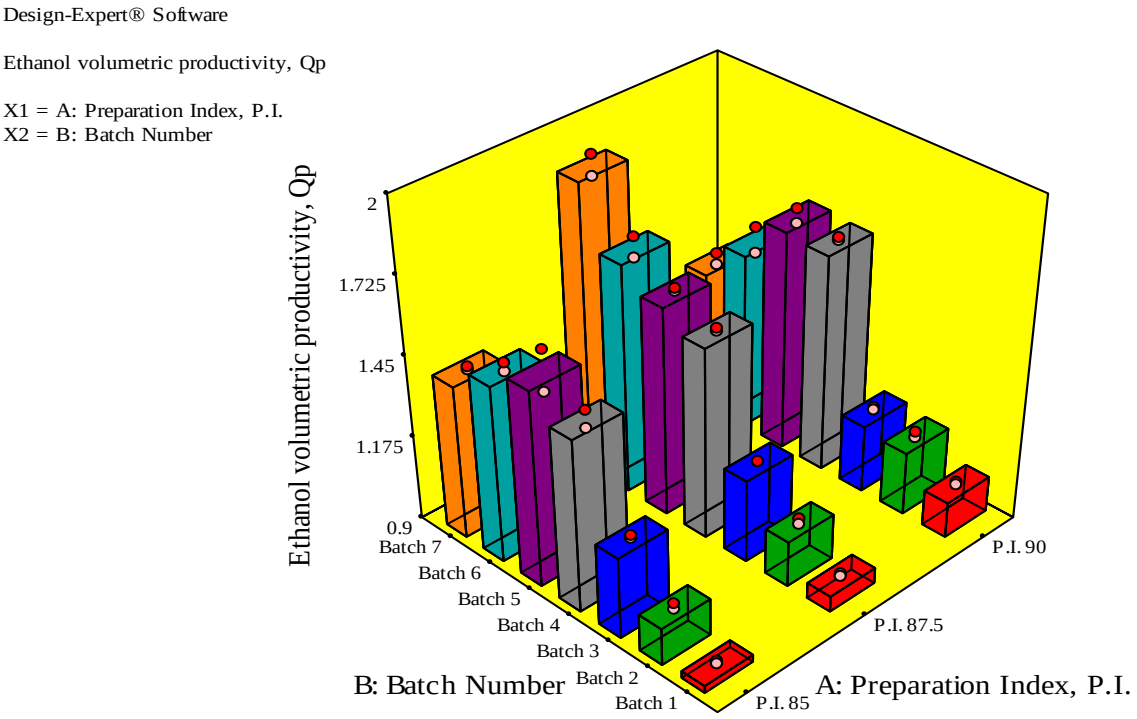


Figure 4-6 Ethanol volumetric productivity, Qp (g/lh) response graph.

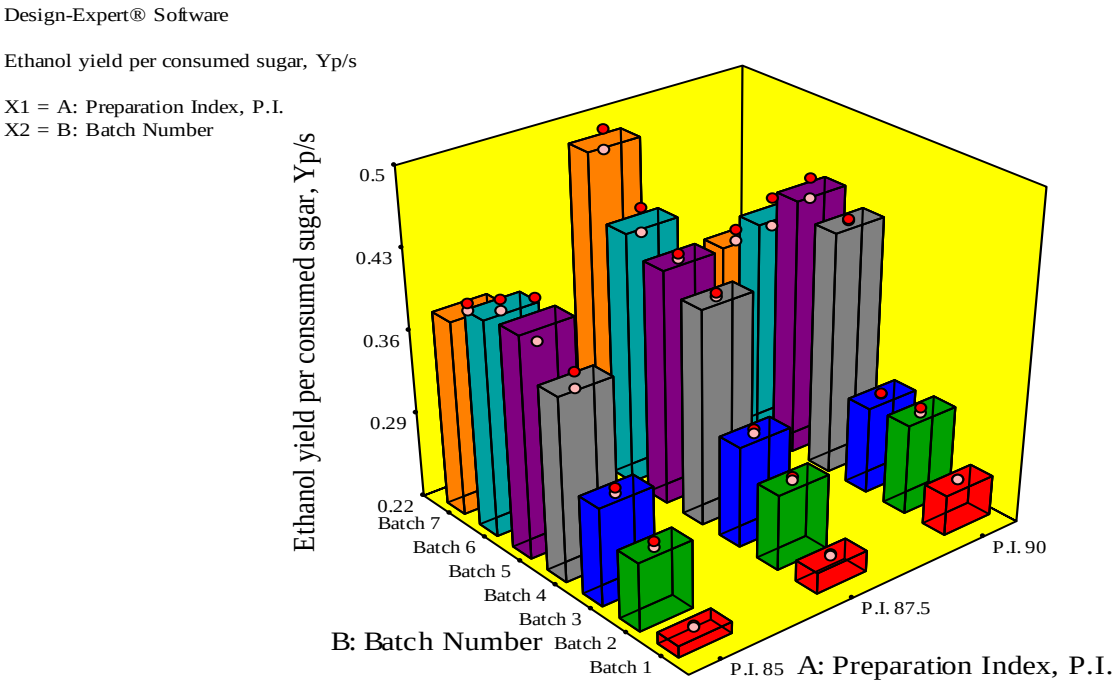


Figure 4-7 Ethanol yield per consumed sugar, Yp/s (g/g) response graph

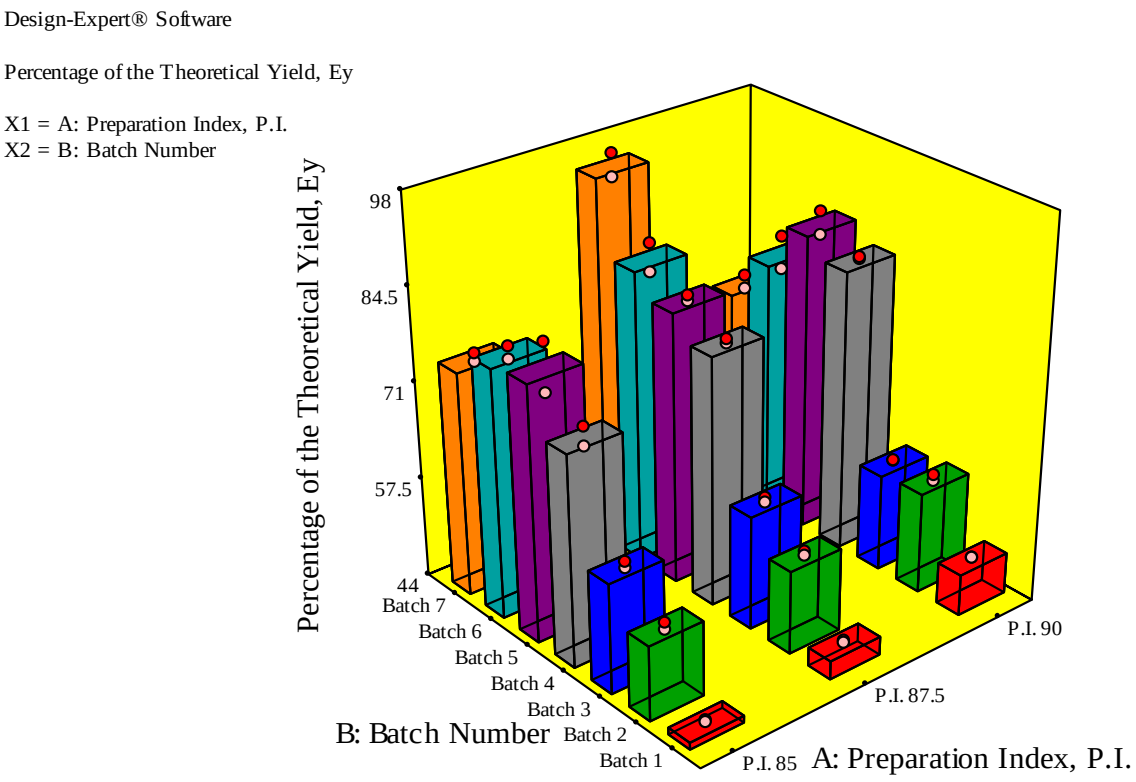


Figure 4-8 Percentage of the Theoretical Yield, g (%) Response Graph

4.3.2. Data Analysis for Stability Test

The stability of the bagasse carrier was tested at third and seventh batch of repeated batch fermentation for Cells retention (R), Immobilized cells concentration (Xi), free cells concentration (Xs), Total cells concentration (Xt) and Immobilization Efficiency (Yi) results are table 4-8 as follows:

Table 4-8 Stability Test Result of Repeated Batch Fermentation

Std	Run	Block	Factors		Responses				
			A:P.I. %	B:Batch no	R, g/g	Xi, g/l	Xs, g/l	Xt, g/l	Yi, %
3	1	Block 1	P.I. 87.5	B3	0.0579	0.4824	0.0588	0.5412	89.13
9	2	Block 1	P.I. 87.5	B7	0.0734	0.6118	0.0063	0.6181	98.98
2	3	Block 1	P.I. 85	B3	0.0409	0.3410	0.1183	0.4593	67.74
5	4	Block 1	P.I. 90	B3	0.0594	0.4951	0.0055	0.5005	98.91
6	5	Block 1	P.I. 90	B3	0.0606	0.4963	0.0067	0.5017	98.91
4	6	Block 1	P.I. 87.5	B3	0.0572	0.4764	0.0581	0.5345	88.03
11	7	Block 1	P.I. 90	B7	0.0401	0.3342	0.1277	0.4619	72.35
7	8	Block 1	P.I. 85	B7	0.0590	0.4913	0.0587	0.5500	89.33
12	9	Block 1	P.I. 90	B7	0.0396	0.3297	0.1260	0.4557	71.38
8	10	Block 1	P.I. 85	B7	0.0569	0.4744	0.0567	0.5310	86.24
1	11	Block 1	P.I. 85	B3	0.0449	0.3738	0.1296	0.5034	74.25
10	12	Block 1	P.I. 87.5	B7	0.0734	0.6118	0.0063	0.6181	98.98

Data were analyzed by factorial modeled and the statistical significance of the terms was examined by analysis of variance for each response. The statistical analysis of the data and three dimensional plotting were performed using design expert software (7.00). The adequacy of regression model was checked by R2, Adj R2, Pred R2, Adeq precision and F-test (Montgomery 2001). The significance of F value was judged at 95% confidence level. For all responses checked and in good agreement including Responses diagnostic plots were also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.) found in good agreement. The basic tool to explain stability. The degree of relationship between the variables was also checked by using interaction curve of the Factors and response variables and discussed here under.

4.3.2.1. Model graph of Responses R, Xi, Xs, Xt and Yi

The yeast biomass and immobilization parameters (R, Xi, Xs, Xt and Yi) were evaluated after the third and seventh batches and presented in Table 4-8. At the end of third and seventh batches for each media it can be concluded that immobilized cells were able to grow and multiply. The cells remained strongly adsorbed to the carrier surface even after the successive washings between fermentation batches for P.I. 87.5 % where for gradually decreases even if at batch three was the highest with 90% P.I. Figure 4-9 and 4-10 model graph of responses depicts this.

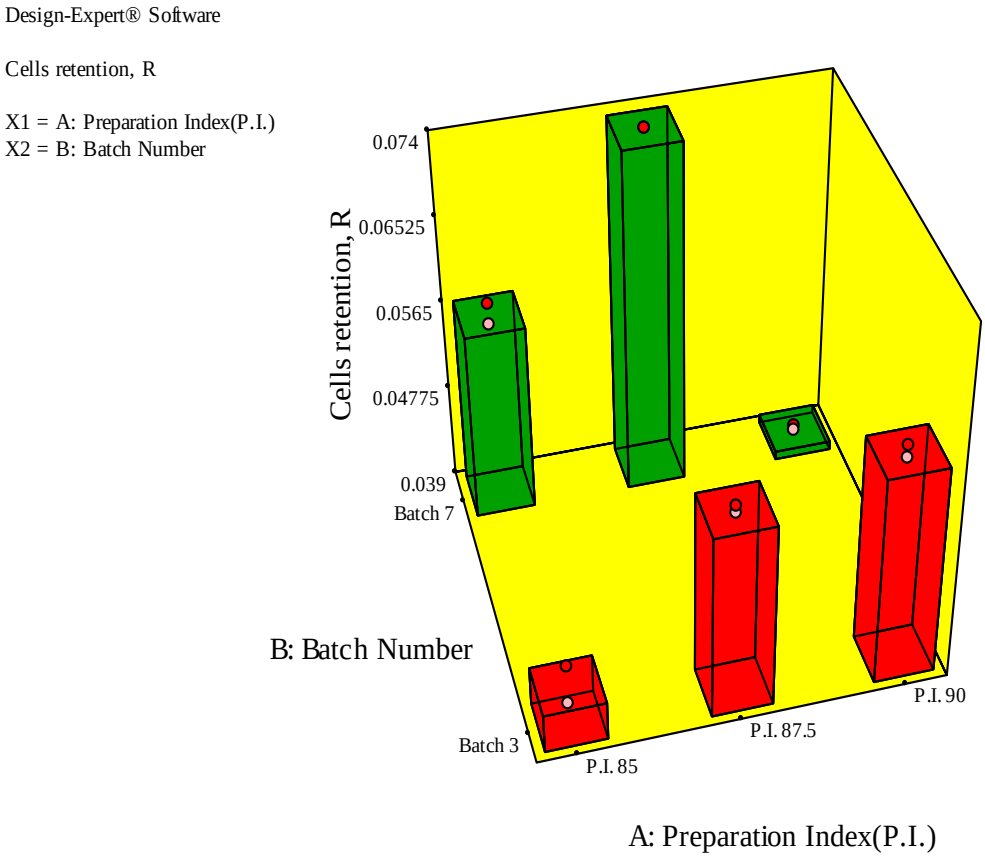


Figure 4-9 Model Graph for Cell Retention, R; (g/g)

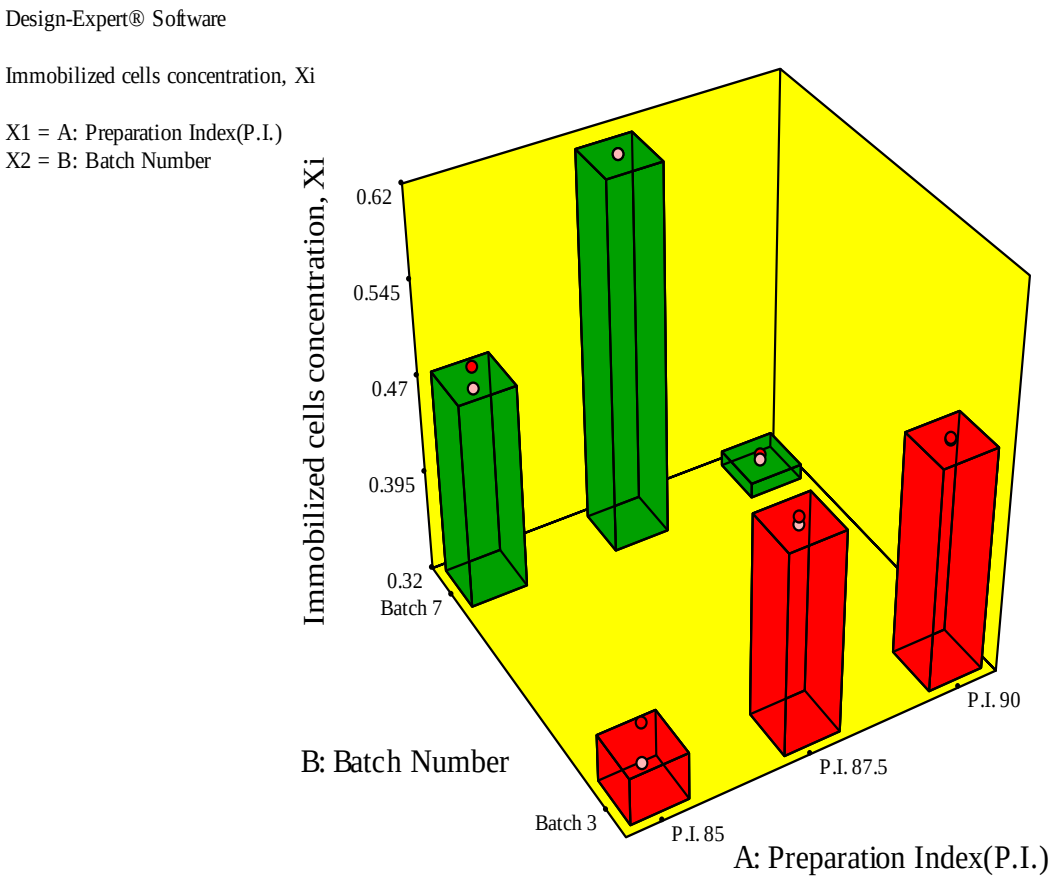


Figure 4-10 Model graph of Immobilized cells concentration, Xi (g/l)

The free cell increases with P.I. 90% which shows cell detachment from the carrier and maximized in free cell system then the immobilization efficiency declined, it was not stable. From figure 4-9 to 4-13 we can observe P.I.87.5% was with highest immobilization efficiency 98.98% at 7th batch which increased from 89.13% at the 3rd batch. This shows the immobilized cells could be reused several time. The ability of CDB with 87.5% P.I. immobilized cells used in repeated batch fermentation for at least seven successive cycles without any loss of ethanol production efficiency indicates that CDB immobilized cells can be potentially used for industrial ethanol production as well as in continuous fermentation (Nuanpeng et al., 2018)

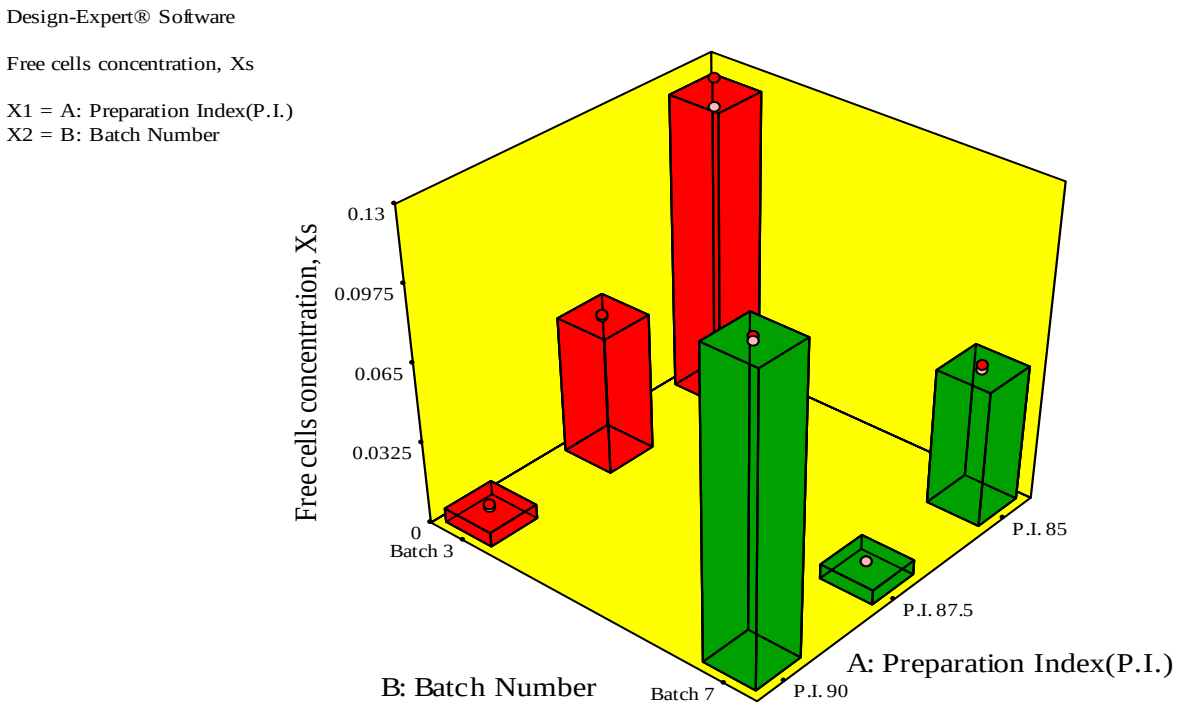


Figure 4-11 Model Graph of Free cells concentration Xs (g/l)

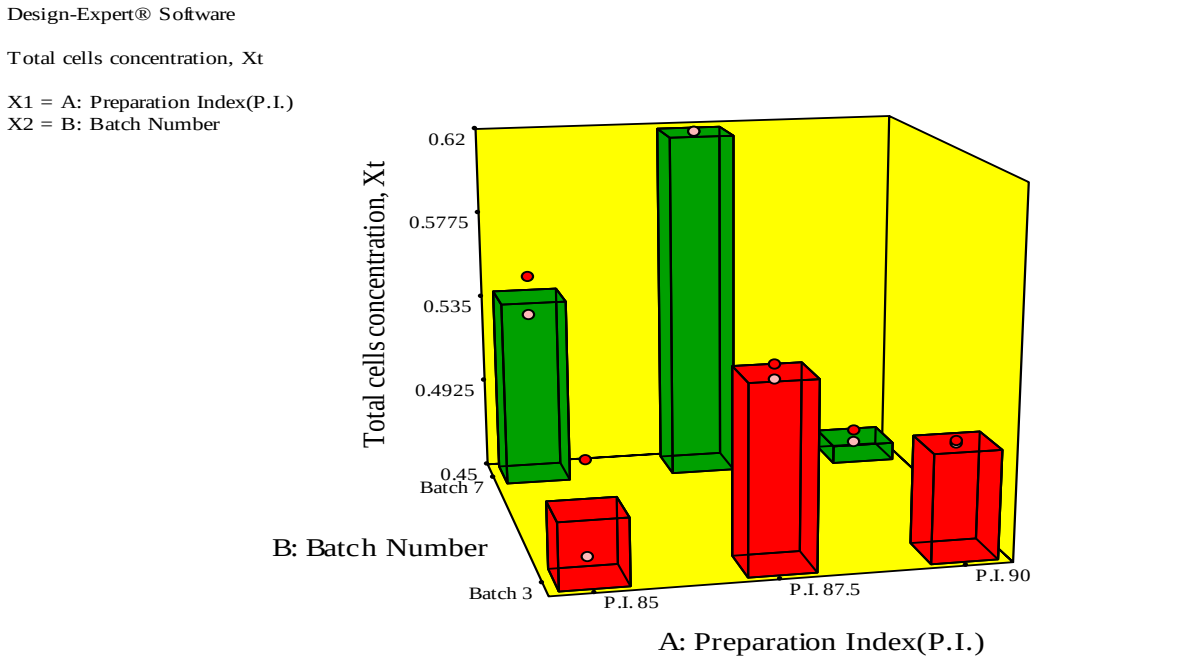


Figure 4-12 Model Graph of Total cells concentration Xt (g/l)

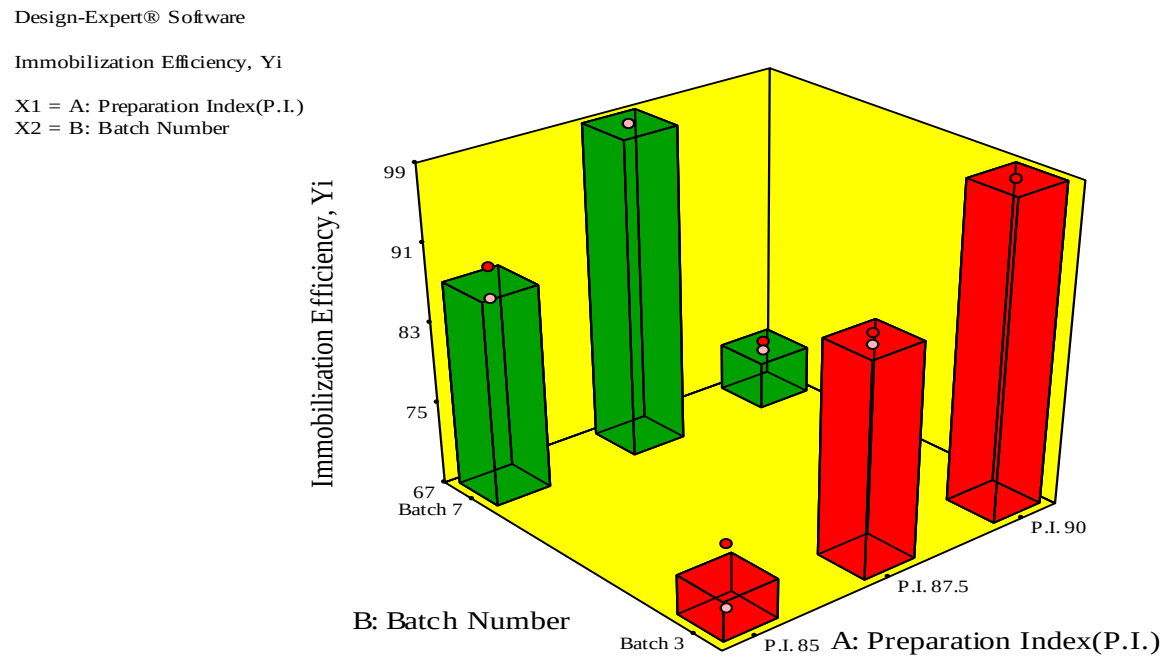


Figure 4-13 Model Graph of Immobilization Efficiency, Yi (%)

4.4. Optimization of Continuous Ethanol Fermentation

Continuous ethanol fermentations using cane molasses in a 1.41-L packed-bed reactor was performed at a temperature of 32 ± 2°C and an initial pH of 5.0. The initial Total reducing sugar concentrations (TRS) and dilution rate were optimized. The effects of dilution rate and TRS on ethanol concentration and ethanol productivity are presented in response Figures below:

The optimum levels of the dilution rate (D) and the total reducing sugar concentration (TRS) during ethanol fermentation immobilized cells were evaluated using a Central Composite Design (CCD) via the Design expert software. The experimental design matrices along with the response variables (ethanol concentration and ethanol productivity) are summarized in Table 4-9

Table 4-9 Optimization results of continuous fermentation

Std	Run	Block	Factors		Responses			
			A:TRS	B: D	SU, %	Qp, g/lh	Yp/s, g/g	Ey, %
8	1	Block 1	100.00	1.71	38.1	22.6009	0.3469	67.8865
5	2	Block 1	29.29	1.00	82.98	11.3115	0.4654	91.0763
2	3	Block 1	150.00	0.50	55.89	18.9928	0.4531	88.6693
9	4	Block 1	100.00	1.00	58.98	27.3195	0.4632	90.6458
12	5	Block 1	100.00	1.00	59.84	28.3761	0.4742	92.7984
13	6	Block 1	100.00	1.00	58.85	26.7002	0.4537	88.7867
7	7	Block 1	100.00	0.29	80.99	10.9734	0.46721	91.4305
4	8	Block 1	150.00	1.50	28.64	19.2225	0.2983	58.3757
1	9	Block 1	50.00	0.50	90	10.377	0.4612	90.2544
6	10	Block 1	170.71	1.00	35.98	21.1536	0.3444	67.3973
11	11	Block 1	100.00	1.00	58.33	25.846	0.4431	86.7123
10	12	Block 1	100.00	1.00	58.64	28.2762	0.4822	94.364
3	13	Block 1	50.00	1.50	59.98	19.438	0.4321	84.5597

4.4.1. Data Anlysis for Optimization of Continuous Ethanol Fermentation

Data were modelled by multiple regression analysis and the statistical significance of the terms was examined by analysis of variance for each response. The statistical analysis of the data and three dimensional plotting were performed using design expert software (7.00). The adequacy of regression model was checked by *R*2, Adj R2, Pred R2, Adeq precision and F-test (Montgomery 2001). The significance of F value was judged at 95% confidence level.

4.4.1.1. Data Analysis for Response1: sugar utilization, SU (%)

Linear model was suggested by the design program for this response to test for its adequacy and to describe its variation with independent variables. From ANOVA test in Table below, The Model F-value of 3060.19 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms.

Table 4-10 ANOVA for Response Surface Linear Model, SU, %

Source	Sum of squares	Df	Mean square	F value	p-value Prob > F	
Model	3913.6	2	1956.8	3060.19	< 0.0001	Significant
A-TRS	2175.3	1	2175.3	3401.89	< 0.0001	
B-D	1738.31	1	1738.31	2718.49	< 0.0001	
Residual	6.39	10	0.64			
Lack of Fit	5.11	6	0.85	2.66	0.1813	not significant
Pure Error	1.28	4	0.32			
Cor Total	3920	12				

The "Lack of Fit F-value" of 2.66 implies the Lack of Fit is not significant relative to the pure error. There is a 18.13% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 4-11 Post ANOVA Statistics for Sugar Utilization SU, %

Std. Dev.	0.8	R-Squared	0.9984
Mean	59.02	Adj R-Squared	0.998
C.V. %	1.35	Pred R-Squared	0.9967
PRESS	12.75	Adeq Precision	162.6

The "Pred R-Squared" of 0.9967 is in reasonable agreement with the "Adj R-Squared" of 0.9980. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. For this case the ratio of 162.600 indicates an adequate signal. This model can be used to navigate the design space.

4.4.1.2. Data Analysis for Response 2: Ethanol Volumetric Productivity, Qp (g/lh)

Quadratic model was suggested by the design program for this response to test for its adequacy and to describe its variation with independent variables. From ANOVA test in Table below, The Model F-value of 46.44 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Table 4-12 ANOVA for Response Surface Quadratic Model, QP (g/lh)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	507.00	5	101.40	46.44	< 0.0001	Significant
A-TRS	62.27	1	62.27	28.52	0.0011	
B-D	82.78	1	82.78	37.92	0.0005	
AB	19.50	1	19.50	8.93	0.0203	
A^2	203.69	1	203.69	93.29	< 0.0001	
B^2	183.35	1	183.35	83.98	< 0.0001	
Residual	15.28	7	2.18			
Lack of Fit	10.70	3	3.57	3.11	0.1508	not significant
Pure Error	4.59	4	1.15			
Cor Total	522.28	12				

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 3.11 implies the Lack of Fit is not significant relative to the pure error. There is a 15.08% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 4-13 Post ANOVA Statistics for Ethanol volumetric productivity, QP (g/lh)

Std. Dev.	1.48	R-Squared	0.9707
Mean	20.81	Adj R-Squared	0.9498
C.V. %	7.10	Pred R-Squared	0.8406
PRESS	83.24	Adeq Precision	18.688

The "Pred R-Squared" of 0.8406 is in reasonable agreement with the "Adj R-Squared" of 0.9498. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 18.688 indicates an adequate signal. This model can be used to navigate the design space.

4.4.1.3. Data Analysis for Response 3: Ethanol Yield Per Consumed Sugar, Yp/S (G/G)

Quadratic model was suggested by the design program for this response to test for its adequacy and to describe its variation with independent variables. From ANOVA test in Table below, The Model F-value of 49.55 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Table 4-14 ANOVA for Response Surface quadratic Model, Yp/s (g/g)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.041	5	8.216E-003	49.55	< 0.0001	Significant
A-TRS	0.012	1	0.012	73.87	< 0.0001	
B-D	0.016	1	0.016	94.50	< 0.0001	
AB	3.950E-003	1	3.950E-003	23.82	0.0018	
A^2	5.411E-003	1	5.411E-003	32.64	0.0007	
B^2	5.001E-003	1	5.001E-003	30.16	0.0009	
Residual	1.161E-003	7	1.658E-004			
Lack of Fit	1.844E-004	3	6.147E-005	0.25	0.8568	not significant
Pure Error	9.762E-004	4	2.441E-004			
Cor Total	0.042	12	8.216E-003			

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 0.25 implies the Lack of Fit is not significant relative to the pure error. There is a 85.64% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 4-15 Post ANOVA Statistics for Ethanol yield per consumed sugar, Yp/s (g/g)

Std. Dev.	0.013	R-Squared	0.9725
Mean	0.43	Adj R-Squared	0.9529
C.V. %	3.00	Pred R-Squared	0.9328
PRESS	2.837E-003	Adeq Precision	20.402

The "Pred R-Squared" of 0.9328 is in reasonable agreement with the "Adj R-Squared" of 0.9529. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. For this case ratio of 20.402 indicates an adequate signal. This model can be used to navigate the design space.

4.4.1.4. Data Analysis for Response 4: Percentage of the Theoretical Yield, Ey, (%)

Quadratic model was suggested by the design program for this response to test for its adequacy and to describe its variation with independent variables. From ANOVA test in Table below, The Model F-value of 49.55 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Table 4-16 ANOVA for Response Surface Quadratic Model, Ey, (%)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1573.16	5	314.63	49.55	< 0.0001	significant
A-TRS	469.04	1	469.04	73.87	< 0.0001	
B-D	600.04	1	600.04	94.50	< 0.0001	
AB	151.28	1	151.28	23.82	0.0018	
A^2	207.24	1	207.24	32.64	0.0007	
B^2	191.53	1	191.53	30.16	0.0009	
Residual	44.45	7	6.35			
Lack of Fit	7.06	3	2.35	0.25	0.8568	not significant
Pure Error	37.39	4	9.35			
Cor Total	1617.61	12				

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 0.25 implies the Lack of Fit is not significant relative to the pure error. There is a 85.68% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 4-17 Post ANOVA Statistics for Percentage of the Theoretical Yield, Ey, (%)

Std. Dev.	2.52	R-Squared	0.9725
Mean	84.07	Adj R-Squared	0.9529
C.V. %	3.00	Pred R-Squared	0.9328
PRESS	108.64	Adeq Precision	20.402

The "Pred R-Squared" of 0.9328 is in reasonable agreement with the "Adj R-Squared" of 0.9529. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. For this

case ratio of 20.402 indicates an adequate signal. This model can be used to navigate the design space.

4.4.2. Equation for Optimization of Continuous Ethanol Fermentation

I. Sugar utilization, SU (%)

Final SU Equation in Terms of Coded Factors:

SU = +59.02 - 16.49 * A - 14.74 * B ……...……Equation 4-1

Final SU Equation in Terms of Actual Factors:

SU =+121.47630 - 0.32980* TRS - 29.48140 * D ……..……Equation 4-2

II. Ethanol volumetric productivity, Qp (g/lh)

Final Qp Equation in Terms of Coded Factors:

Qp = +27.30 +2.79 * A +3.22 * B - 2.21 * A * B -5.41 * A^2 - 5.13 B^2……...Equation 4-3

Final Qp Equation in Terms of Actual Factors:

Qp = -35.72083 +0.57700* TRS +56.33543* D -0.088314 * TRS * D -2.16445E -003 * TRS^2 -20.53522 * D^2 …….Equation 4-4

III. Ethanol yield per consumed sugar, Yp/s (g/g)

Final Yp/s Equation in Terms of Coded Factors:

Yp/s = +0.46 - 0.039 * A - 0.044* B - 0.031* A * B - 0.028* A^2 - 0.027* B^2…Equation 4-5

Final Yp/s Equation in Terms of Actual Factors:

Yp/s = +0.28547 + 2.70650E - 003 * TRS + 0.25177* D - 1.25700E - 003 * TRS * D - 1.11563E - 005* TRS^2 - 0.10725 * D^2……..…….Equation 4-6

IV. Percentage of the Theoretical Yield, Ey (%)

Final Ey Equation in Terms of Coded Factors:

Ey = +90.66 - 7.66 * A - 8.66 * B - 6.15 * A * B - 5.46 * A^2 -5.25 * B^2…... Equation 4-7

Final Ey Equation in Terms of Actual Factors:

Ey = +55.87690 + 0.52949 * TRS + 49.25518 * D - 0.24599 * TRS * D - 2.18322E-003 *TRS^2 - 20.98875 * D^2 …….Equation 4-8

4.4.3. Diagnostic Test for the Responses

All diagnostic plots are also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.). For example fig below shows how precisely the sugar utilization SU (%) is modelled, because all the points line up nicely and the deviation of points for sugar

utilization SU, (%) from normality is insignificant. Similar results were observed for the remaining responses (QP, Yp/s, and Ey)

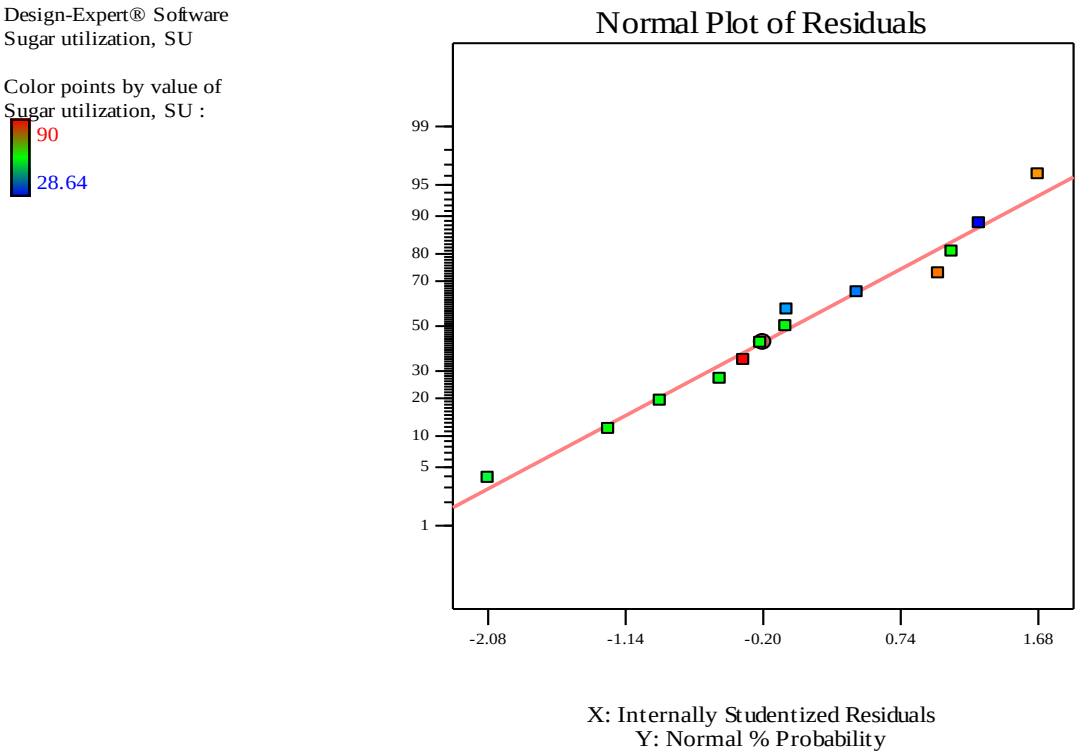


Figure 4-14 Normal plot of residual for sugar utilization

4.4.4. Optimization Solution for Continuous Ethanol Fermentation Conditions

Optimization solution to maximize the following responses: Ethanol volumetric productivity (QP), Ethanol yield per consumed sugar (Yp/s), Percentage of the Theoretical Yield (Ey) whereas sugar utilization cannot be decided because it depends on the condition. The factors dilution rate and total reducing sugar in range.

Only one solution was suggested by design expert 7.0.0 given below.

Solutions Number	TRS	D	Qp	Yp/s	Ey	Desirability	
1	93.40	0.91	26.0315	0.474376	92.8329	0.927	Selected

1 Solutions found

We cane use equation 4.2 and determine the sugar utilization and substituting in equation 4.4 and confirm Qp = 26.0315 g/lh.

SU =+121.47630 - 0.32980* TRS - 29.48140 * D

Substituting TRS = 93.40 g/l and D = 0.91 h^{-1} we got SU = 63.845%

Then substiting in equation 4.4

Qp = -35.72083 +0.57700* TRS +56.33543* D -0.088314 * TRS* D -2.16445E -003 * TRS^2 -20.53522 * D^2 , we got Qp = 26.0315 g/lh confirmed!

4.4.5. Response surface plot analysis for Optimization of Continuous Fermentation

The effect of different treatment parameters on responses was given in Table 4-9 optimization results of continuous fermentation. To aid visualization of variation in responses with respect to processing variables, series of three dimensional Response surfaces were drawn for each response using design expert software 7.0.0.

4.4.5.1. Model Graph for Responses

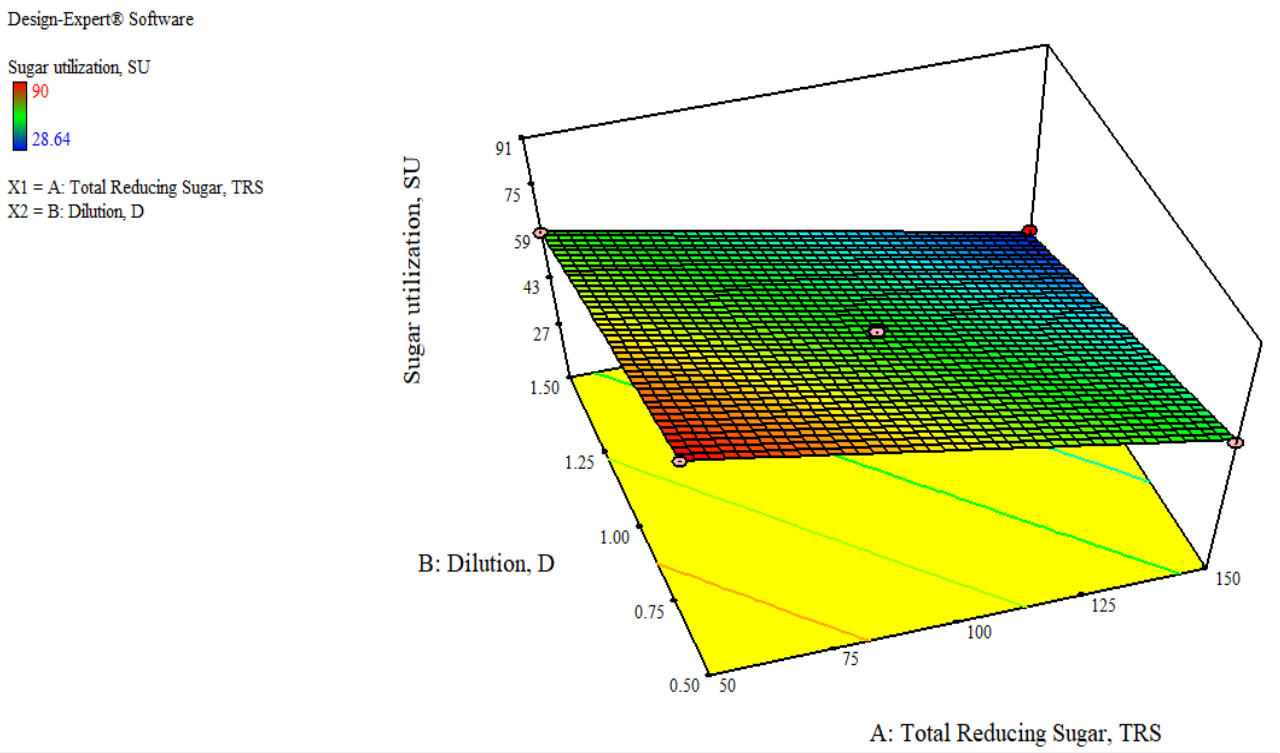


Figure 4-15 Response surface graph for sugar utilization.

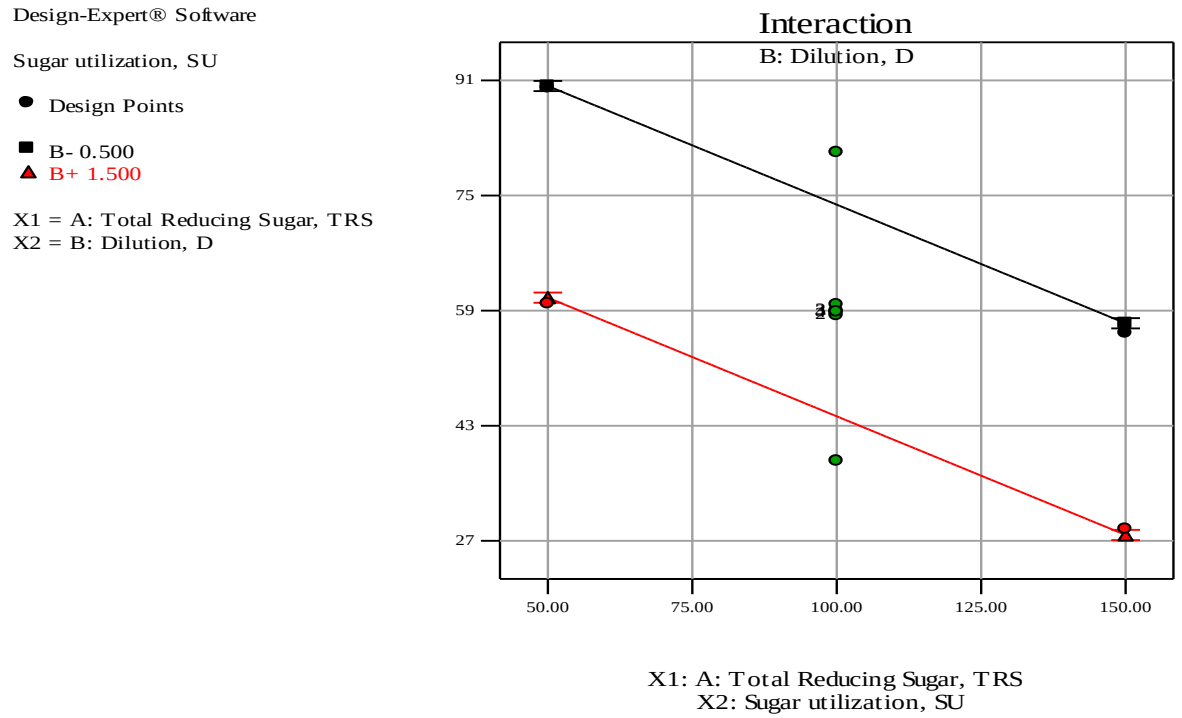


Figure 4-16 Interaction graph for sugar utilization, SU

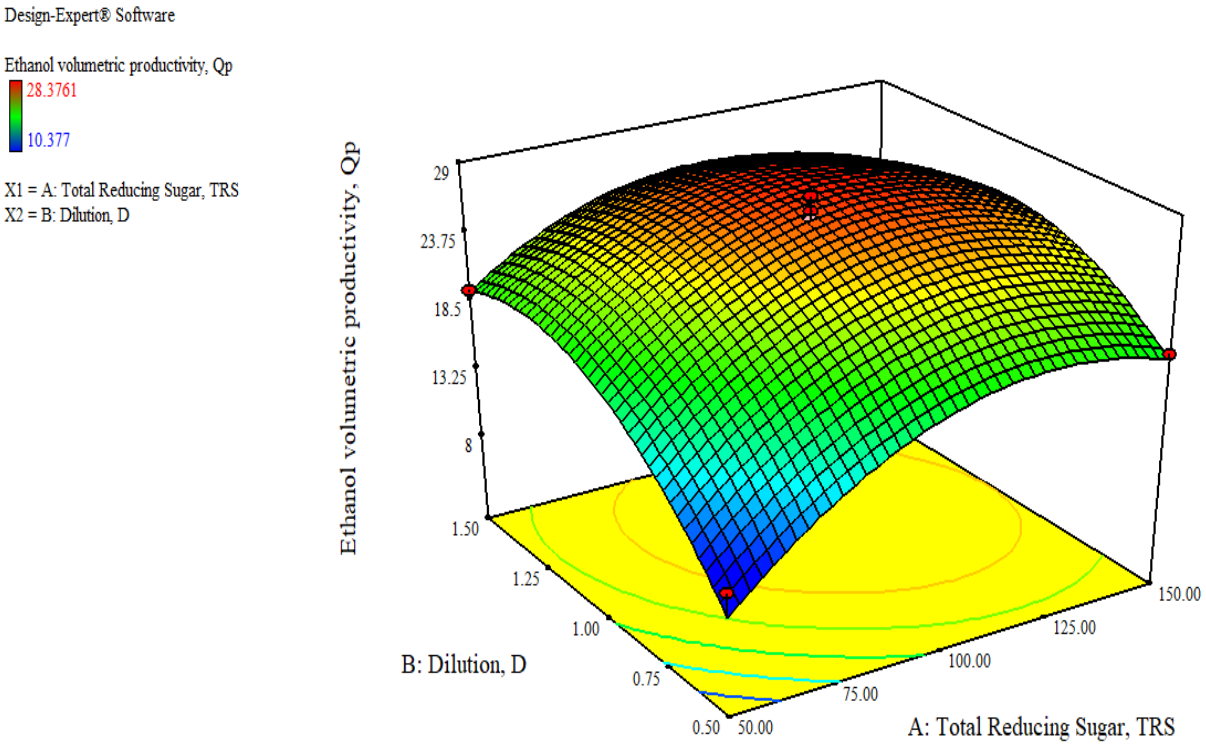


Figure 4-17 Response graph For Ethanol Volumetric Productivity, QP

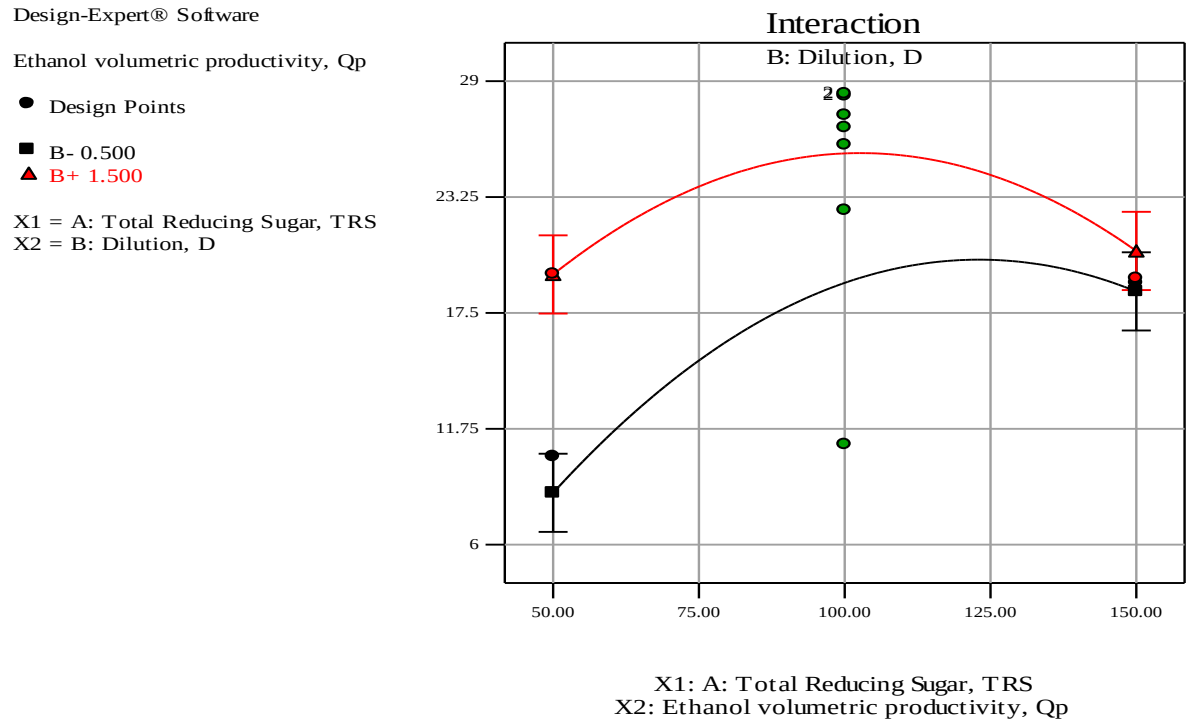


Figure 4-18 Interaction graph Ethanol Volumetric Productivity, QP

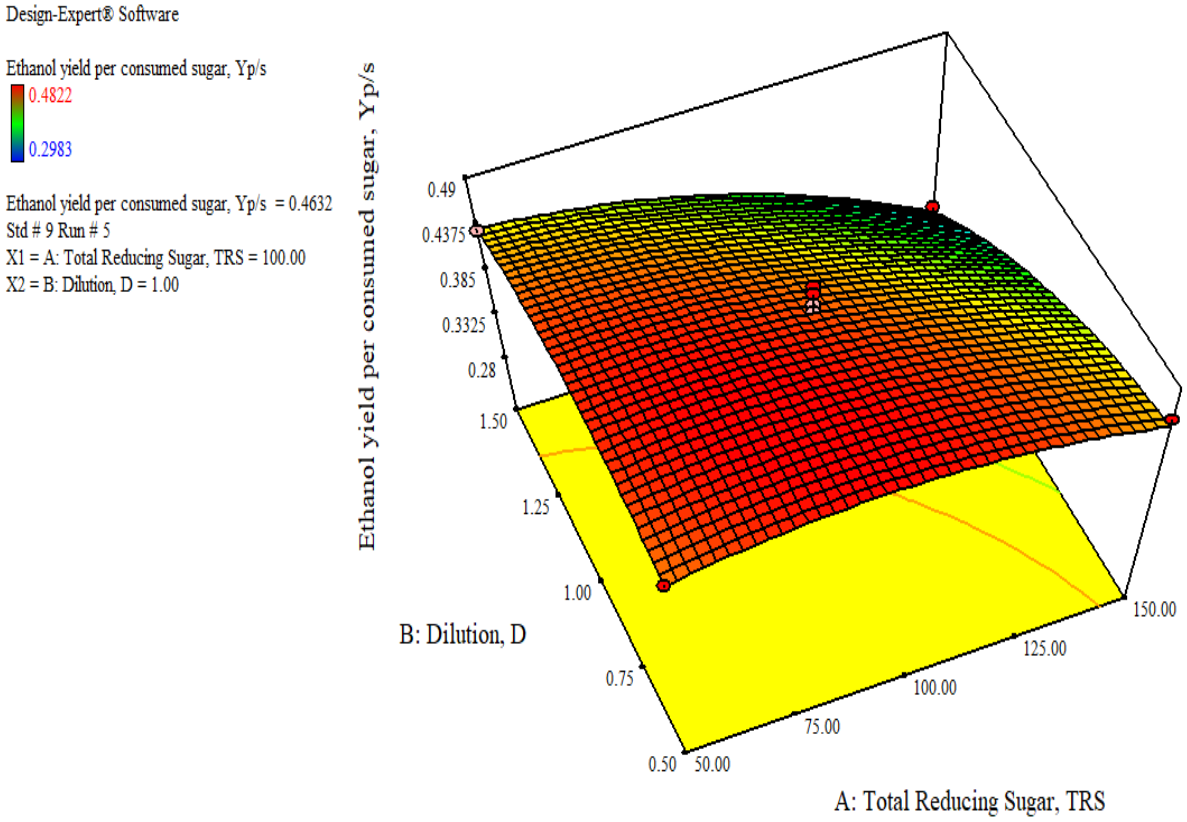


Figure 4-19 Response graph for Ethanol yield per consumed sugar, Yp/s (g/g)

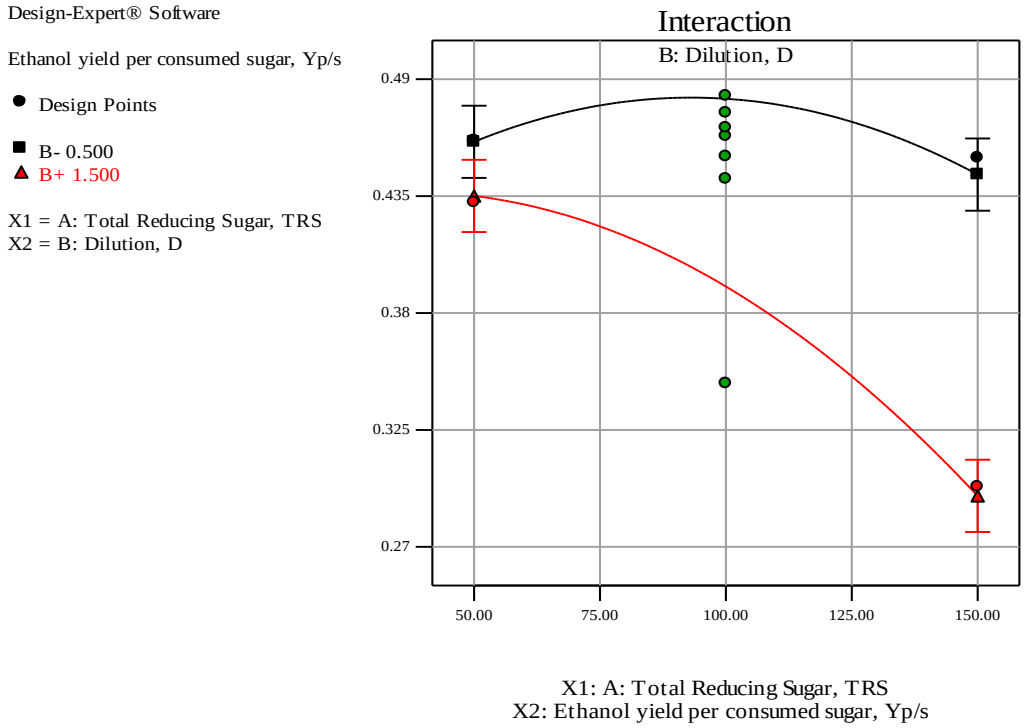


Figure 4-20 Interaction graph for Ethanol yield per consumed sugar, Yp/s (g/g)

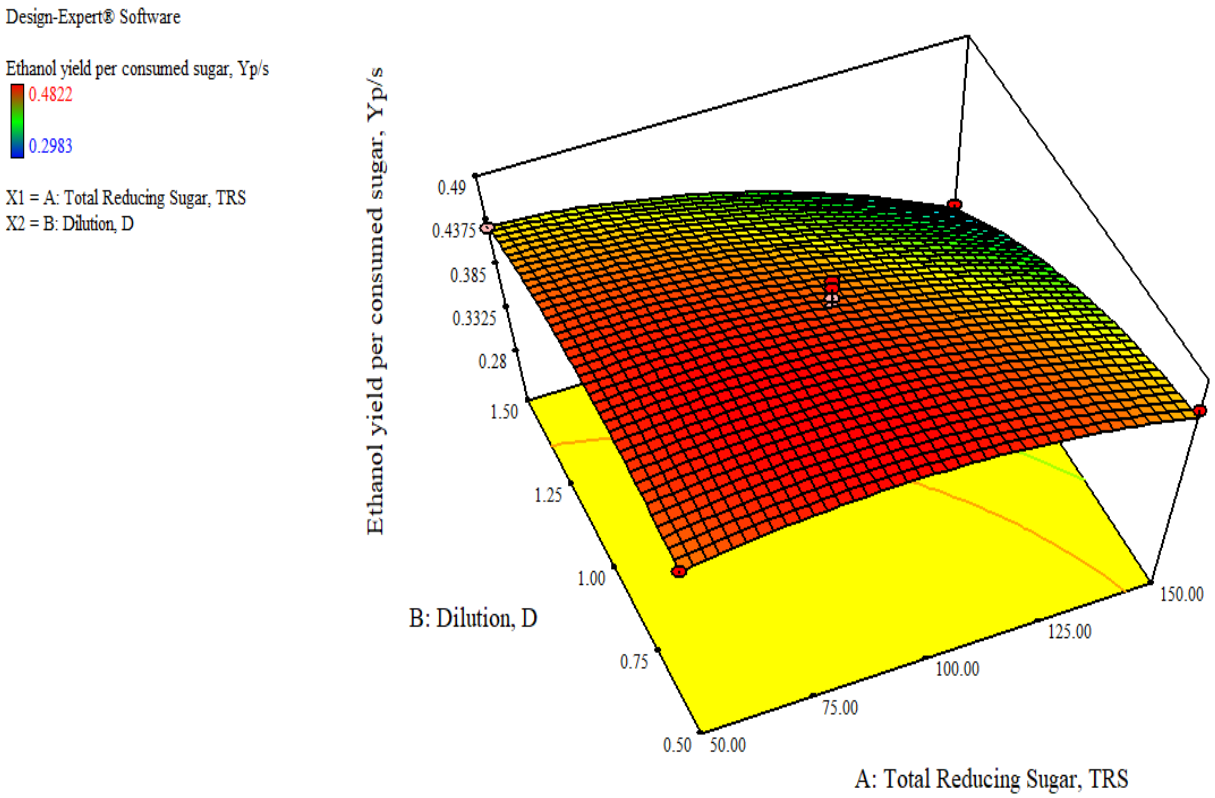


Figure 4-21 Response surface graph for Percentage of the Theoretical Yield, Ey

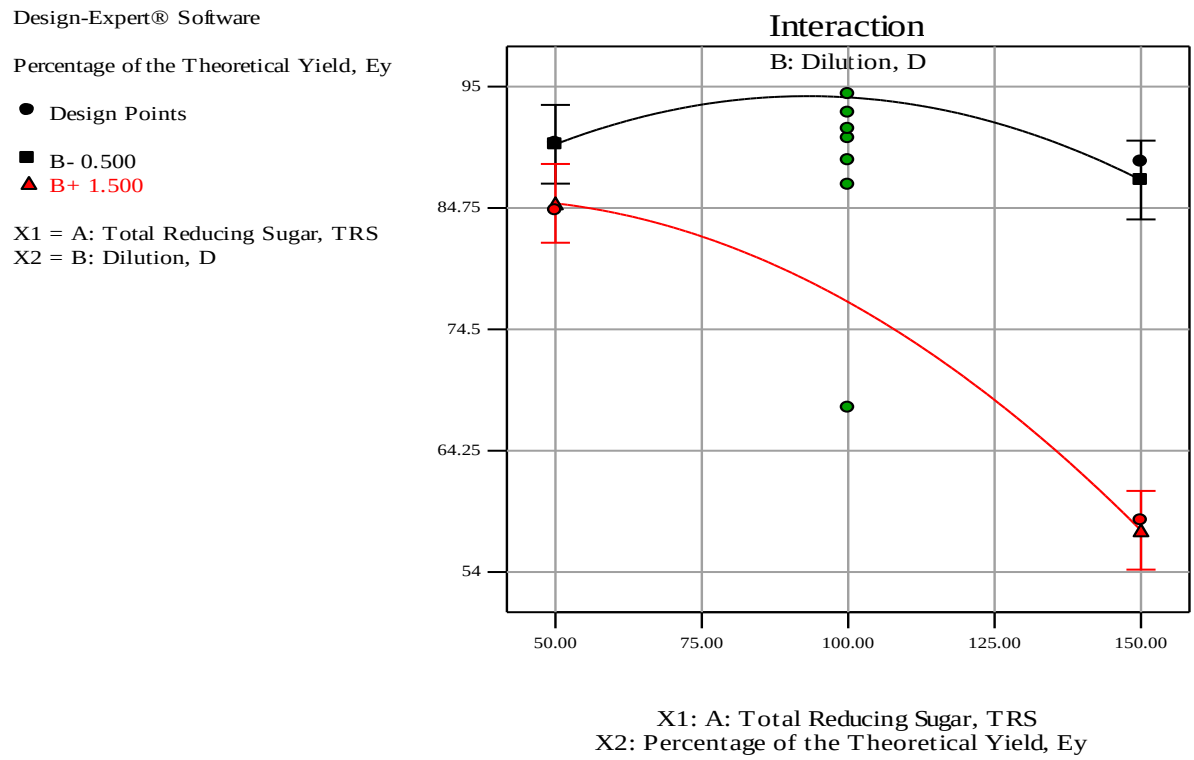


Figure 4-22 Interaction graph for Percentage of the Theoretical Yield, (Ey)

4.4.5.2. Effect of Dilution Rate and Total Reducing Sugar Concentrations

The effect of Dilution rate and total reducing sugar concentration depicted by response and interaction graphs above Figure 4-15 to 4-22 are discussed as under.

4.4.5.2.1. Effect of Dilution Rate (D)

At a dilution rate of 0.5 h^{-1}, 37.98 g/l ethanol concentration and 18.99 g/lh volumetric productivity were obtained in the outlet with an ethanol yield of 88.67 % of its theoretical yield at 0.4531g/g. At a dilution rate of 1.0 h^{-1}, 27.304 g/l ethanol concentration was obtained in the outlet with an ethanol yield of 90.66 % of its theoretical yield 0.4633g/g. At a dilution rate of 1.5 h^{-1}, 12.96 g/l ethanol concentration and volumetric productivity19.44 g/lh were obtained in the outlet with an ethanol yield of 84.56 % of its theoretical yield 0.4321g/g.

The ethanol productivity could be increased when the dilution rate was increased from 0.5 to 1.0 h^{-1} but the ethanol concentration and sugar utilization decreased significantly whereas theoretical yield almost at steady state. The ethanol concentration of 37.98 g/l with 55.89 % sugar utilization was achieved at a dilution rate of 0.5 h-1. The ethanol concentration decreased up to 12.82 g/l with 28.64 % sugar utilization when dilution rate was increased up to 1.5 h^{-1}. The volumetric productivity 22.601 g/lh was achieved at a dilution rate of 1.7 h^{-1}. The ethanol

concentration was decreased due to less interaction of glucose molecule with the immobilized yeast or low hydraulic retention time. The higher ethanol productivity was achieved as compared to 1.93 g/lh in this study in repeated batch fermentation; 1.71 and 3.7 g /lh in a batch fermentation and continuous fermentation with cell recycle, respectively using free cells of the same strain (Kumar et al., 2009b). Thus, the ethanol fermentation using immobilized yeast is a better option.

Ozmihci and Kargi (2008a) reported the ethanol concentration of 10.5 g/l and volumetric productivity of 0.58 g/lh with 63% sugar utilization at a dilution rate of 0.057 h-1 on feeding 50 g/l sugar concentration in cheese whey powder solution by Kluyveromyces marxianus (DSMZ 7239) in a packed column bioreactor. Yu et al. (2007) reported the volumetric productivity of 16.68 g /lh with ~55% sugar utilization at a dilution rate of 0.3 h-1 on feeding 200 g/l sugar concentration using immobilized S. cerevisiae on sorghum bagasse. In the present study, we could achieve the highest ethanol concentration with the highest ethanol productivity and maximum sugar utilization at a dilution rate of 1.0 h^{-1} as compared to reported literature.

The theoretical Ethanol yield, Yp/s (g/g) was stable or slight effect up to 1.0 h^{-1} dilution rate whereas dilution rate increase above 1.0 it decreased by increasing dilution rate or increased by increasing hydraulic retention time; at 0.5 h-1 it was 0.4612g/g somewhat stable up to 1.0 h^{-1} dilution 0.4633 g/g then decreased to 0.2983 g/g at 1.5 h-1 . Ozmihci and Kargi (2008a) reported that the ethanol yield was decreased by increasing dilution rate or increased by increasing hydraulic retention time on fermenting cheese whey powder by Kluyveromyces marxianus (DSMZ 7239) in a packed column bioreactor.

As shown in Table 4-9, the volumetric productivity could be increased when dilution rate was increased from 0.5 to 1.0 h^{-1} whereas the ethanol concentration was declined consistently due to decrease in sugar utilization at high flow rate, the maximum attained in this study was 27.304g/lh at a dilution rate of 1.0h^{-1} with 58.93% sugar utilization. De Vasconcelos et al. (2004) reported maximum 29.64 g/lh volumetric productivity at a dilution rate of 0.83 h-1 with 74.61% sugar utilization using immobilized Fleischmann yeast cells in sugarcane stalks.

4.4.5.2.2. Effect of Total Reducing Sugar (TRS)

The medium with varying total reducing sugar concentrations from 50 to 150 g/l was fed into the column at dilution rate 0.29 to 1.71 h^{-1}. At a feed TRS concentration of 50 g/l, 20.75 g/l ethanol concentration was obtained with an Ethanol yield per consumed sugar of 0.4612 g/g and 90.25 % of its Percentage of the Theoretical Yield. At a feed TRS concentration of 150, 37.98 g/l ethanol concentration was obtained with a percentage of the theoretical ethanol yield

of 88.67 % with slight decrease but at 0.29 h-1 dilution rate 37.84 g/l ethanol concentration is attained. The ethanol concentration, ethanol volumetric productivity could be increased when TRS concentration in feed was increased 50 -100 g/l but slight decrement up to 150 g/l but it dropped above 150 g/l TRS, the sugar utilization decreased considerably. The ethanol concentration of 37.98 g/l with 55.89 % sugar utilization was achieved on feeding TRS concentration of 150 g/l as compared to 20.75 g/l with 90 % sugar utilization on feeding TRS concentration of 50 g/l.

The volumetric productivity of 18.993 g/lh was achieved on feeding TRS concentration of 150 g/l but at 100g/l TRS 27.304 g/lh was attained the maximum of this study. The sugar utilization decreased on increasing TRS concentration in feed due to high ratio of glucose to cell mass concentration (S/X) or sugar uptake limit. Ozmihci and Kargi (2008b) reported that the ethanol concentration increased when sugar concentration was increased in feed from 50 to 100 g/l and decreased when sugar concentration was further increased due to lower sugar utilization. They obtained the ethanol concentration of 22.5 g/l at feed sugar concentration of 100 g/l in cheese whey powder solution by Kluyveromyces marxianus in a packed column bioreactor.

In the present study, the ethanol concentration increased by increasing TRS concentration up to optimum point of 93.40 g/l TRS, Dilution of 0.91 h -1 , Ethanol volumetric productivity of 26.022 g/lh, Ethanol yield per consumed sugar, 0.4744 g/g and Percentage of the Theoretical Yield 92.84% and sugar utilization of 63.845 %.

Above this up to 150 g/l TRS, with slight decrement but above 150 g/l it dropped. Love et al. (1998) reported maximum ethanol concentration 46 to 48 g/l on feeding glucose concentration of 100 g/l with ethanol productivity of 4.8 g /lh at a dilution rate of 1 h-1 by Kluyveromyces marxianus IMB3 immobilized on mixed alginate and kissiris whereas Gough and McHale (1998) reported ethanol concentration of 34 g/l on feeding glucose concentration of 120 g/l with ethanol productivity of 5.1 g/lh at a dilution rate of 1.5 h-1 by the same strain immobilized on alginate.

The ethanol yield on feeding TRS at any concentration was almost same up to 100g/l TRS. Therefore, no effect was observed of TRS concentration in feed on ethanol yield but after 100g/l decline critically this was due to non-sugar or unfermentable sugar percentage increment. Ozmihci and Kargi (2008b) reported that the ethanol yield was decreased by increasing feed sugar on fermenting cheese whey powder by Kluyveromyces marxianus in a

packed column bioreactor. The ethanol concentration and volumetric productivity significantly increased when feed glucose concentration was increased from 50 to 100 g/l whereas at 150 g/l feed glucose concentration slightly increase in ethanol concentration and volumetric productivity were observed. Thus, the immobilization on sugarcane bagasse chips is favorable for low feed sugar concentration.

4.4.6. Validation Experiments

In order to verify the optimization results, triplicate experiments were conducted under predicted conditions by the developed model. The model predicted the volumetric productivity was 26.03 g/lh and Ethanol yield per consumed sugar 0.4744 g/g at TRS of 93.4 g/l and dilution rate of $0.91h^{-1}$. These are found to be optimum condition for maximum percent of the response variables. The triplicate average experimental value obtained at these conditions were volumetric productivity was 25.90 g/lh and Ethanol yield per consumed sugar was 0.472 g/g (the difference between the two conditions are less than 0.50 %) and results were closely in agreement with the result obtained from the model and hence validated the findings of the optimization.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATION

5.1. Summary and Conclusions

Based on the results obtained in the analysis of this work, the following conclusions can be deduced.

- When we compare the two types of bagasse- the cane diffuser bagasse (CDB) had better physical properties of carrier, i.e. water retention and water absorption index to 8.0 g/g and 8.92 g/g, respectively whereas mill tandem bagasse MTB 4.73g/g and 8.53g/g respectively . Moreover, the CDB increased the cell retention up to 52 mg/g whereas with mill tandem bagasse (MTB) it was 10.05 mg/g that is five times higher with CDB.
- The preparation index do not have significant effect with physical properties with the same bagasse type. Whereas considerable effect was observed with cell retention, cell retention increases with P.I. increases with different magnitude with bagasse type.
- In mill tandem bagasse the highest cell retention with low P.I. (82.5%) was 5.5 mg/g whereas with highest P.I. (90%) 10 mg/g was recorded. In cane diffuser bagasse the lowest cell retention was 25.3 mg/g which was 2.5 time more than mill tandem bagasse with highest P.I. The highest in cane diffuser bagasse was 52mg/g, 51mg/g with 90% and 87.5% P.I. respectively.
- In repeated batch fermentation cell retention increases as batch number increases but after 3^{rd} batch the highest P.I. showed poor performance or low immobilization efficiency it was 98.91% at 3^{rd} batch then it declined to 71.38% immobilization efficiency at 7^{th} batch repeated fermentation with on average 60 mg/g and 40 mg/g cell retention respectively.
- The optimum and the stable P.I. was 87.5% with on average 57.55 mg/g and 73.4 mg/g cell retention at 3^{rd} and 7^{th} cycle respectively.
- The results obtained have allowed us to establish the potential these cane diffuser bagasse with 87.5 % P.I. have as carriers in the immobilization of cells for the continuous production of ethanol.
- Continuous ethanol fermentation using packed bed was carried out Thus, ethanol fermentation with cane diffuser bagasse at high P.I. (87.5%) with immobilized yeast S. cerevisiae reveals that the high dilution rate is favourable to the feed with low sugar concentration. For a high sugar concentration feed, the maximum sugar utilization can be achieved either by increasing bed height or by increasing number of columns.

5.2. Recommendation

- The use of the cell immobilization system on sugar cane bagasse can improve the bioethanol production with the increasing number of cells in the carrier. However, modification of sugar cane bagasse as a carrier through the mill and cane diffuser operation needs to be further investigated, as it may reveal the influence of changes in the physical properties of sugar cane bagasse to cell adhesion as well as bioethanol production.
- We have obtained better yields than the batch processes, currently being used on an industrial level. Additionally, we have obtained better yields than continuous alcoholic fermentations performed with free cells and fermentations performed with cells immobilized on other materials. However, an industrial implementation of this process requires further optimizations that allow for one to increase the amount of cells immobilized on the carriers, as well as improvements in the operational conditions of the process. In this way, the sugars provided in the reactor input could be fully consumed, increasing the yield and the productivity furthermore this study will be starting point for further studies.

REFERENCES

A. Singh, P. Sharma, A. K. Saran, N. Singh, and N. R. Bishnoi, "Comparative study on ethanol production from pretreated sugar cane bagasse using immobilized Saccharomyces cerevisiae on various matrices," Renewable Energy, vol. 50, pp. 488–493, 2013.

Abdelmajeed N.A., Khelil O.A. and Danial E.N. Immobilization technology for enhancing bio-products industry. African Journal of Biotechnology. Vol. 11(71), pp. 13528-13539, 4 September, 2012.

Amim Jr J, Petri DFS, Maia FCB, Miranda PB (2010). Ultrathin cellulose ester films: preparation, characterization and protein immobilization. Quím. Nova. 33:2064-2069.

Anita S.H., Mangunwardoyo W. and Yopi. Sugar cane Bagasse as a Carrier for the Immobilization of Saccharomyces cerevisiae in Bioethanol Production. Makara J. Technol. 20/2 (2016), 73-81.

ASTM D1106–96 (2013) Standard Test Method for Acid-Insoluble Lignin in Wood

Bangrak p., Limtong S. and Phisalaphong M. 2011. Continuous ethanol production using immobilized yeast cells entrapped in loofa reinforced alginate carriers. Brazilian Journal of Microbiology 42: 676-684.

Behera S., Mohanty C. and Ray C., (2012), Ethanol fermentation of sugarcane molasses by zymomonas mobilis mtcc 92 immobilized in luffa cylindrica l. Sponge discs and ca-alginate matrices, Brazilian Journal of Microbiology: 1499-1507

Cassidy MB, Lee H, Trevors JT (1996). Environmental applications of immobilized microbial cells: a review. J. Ind. Microbiol. 16:79-101.

Chandel, A.K., Narasu, M.L., Chandrasekhar, G., Manikyam, A., and Rao L.V., (2009), Use of Saccharum spontaneum (Wild Sugarcane) as Biomaterial for Cell Immobilization and Modulated Ethanol Production by Thermotolerant Saccharomyces cerevisiae VS3, Bioresources Technology, 100, pp. 2404-2410.

Cohen, Y., 2001. Biofiltration-the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. Bioresour Technol 77, 257-274.

Covizzi LG, Giese EC, Gomes E, Dekker RFH, Silva R (2007). Immobilization of microbial cells and their biotechnological applications. Semina: Ciências Exatas e Tecnológicas. 28:143-160.

De Vasconcelos JN, Lopes CE, de França FP (2004). Continuous ethanol production using yeast immobilized on sugar-cane stalks. Braz. J. Chem. Eng., 21: 357-65.

Dervakos GA, Webb C (1991). On the merits of viable-cell immobilisation. Biotechnol. Adv. 9: 559-612.

E.Hugot (1986) Hand book of cane sugar engineering, 3rd edition, Elesevier science Publisher company INC. The Netherlands.

Escobar, L.M.A., Alvarez, U.S., and Penuela, M., (2012), Yeast Immobilization Wastes for Ethanol Production in Packed Bed Bioreactor, Revista Facultad de Ingenieria University of Antioquia, 62, pp. 66-76.Ethiopian sugar corporation annual report, 2017/18 campaign year.

Freeman A, Lilly MD (1998). Effect of processing parameters on the feasibility and operational stability of immobilized viable microbial cells. Enzyme Microb. Technol. 23: 335-345.

Gorecka E, Jastrzebska M (2011). Immobilization techniques and biopolymer carriers. Biotechnol. Food Sci. 75:65-86.

Jin, Y.L., Speers, R.A., 1998. Flocculation of Saccharomyces cerevisiae. Food Res. Int. 31, 421–440.

Jorgensen, H., Kristensen, J.B., and Felby, C., (2007), Review: Enzymatic Conversion of Lignocellulose into Fermentable Sugars: Challenges and Opportunities, Biofuels Bioproduct and Biorefining, 1, pp. 119-134.

Kierek K, Karatan E (2005). Biofilm development in bacteria. Adv. Appl. Microbiol. 57:79-84.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R., Koutinas, A., 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review". Food Microbiol 21, 377-397.

Kunduru, Mahipal Reddy, "Development of a biofilm bioreactor for enhanced ethanol production" (1994). Retrospective Theses and Dissertations. 11280. https://lib.dr.iastate.edu/rtd/11280

Lebaka Veeranjaneya Reddy, Lebaka Prasannanjaneya Reddy, Young-Jung Wee and Obulam Vijaya Sarathi Reddy (2011) Production and Characterization of Wine with Sugarcane Piece Immobilized Yeast Biocatalyst. Food Bioprocess Technol 4:142-148.

Lee, Y., Lee, W. G., Chang, Y. K. and Chang, H. N. (1995) Modelling of Ethanol Production by Saccharomyces cerevisiae from a Glucose and Maltose Mixture. Biotechnol. Lett. 17, 791-796.

Lei Liang,yuan-ping zhang,Li Zhang, ming-jun zhu,shi-zhong liang and yu-nan huang (2008) study of sugarcane pieces as yeast support for ethanol production from sugarcane juice and molasses. Ind Microbiol Biotechnol. Ltt. 35:1605-1613.

Leona paulova, Petra patakova, and Tomas Branyik (2013) "Advanced Fermentation Process" https://www.researchgate.net/publication/259193501

Milkessa T (2013) Financial and Economic Analysis of Ethanol Production from Sugar Molasses: a Case Study of an Integrated Sugar Plant in Ethiopia. In a Book of proceeding of the Tenth International Conference on the Ethiopian Economy. Edited by Demirew G, Getenet A, Worku G. Published by Ethiopian Economic Association.

Mohammadi, A., Enayatzadeh, M., Nasernejad, B., 2009. Enzymatic degradation of anthracene by the white rot fungus Phanerochaete chrysosporium immobilized on sugarcane bagasse. J. Hazard. Mater. 161, 534e537.

Montgomery, D.C. (2001). Design and Analysis of Experiments, fourth ed., John Wiley and Sons, New York.

Moreno-Garrido, I., 2008. Microalgae immobilization: current techniques and uses. Bioresour Technol 99, 3949-3964.

Muenduen P, Nuttapan S, Wiwut T (2006) Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation. Biochemical Engineering Journal 28: 36-43

Mussatto, S.I., Aguilar, C.N., Rodrogues, L.R., and Texeira, J.A., (2009), Fructooligosaccharides and β- Fructofuranosidase Production by Aspergillus japonicus Immobilized on Lignocellulosic Materials. Journal of Molecular Catalysis B: Enzymatic, 59, pp. 76-81.

Najafpour G., Younesi H. and Ku Ismail K, (2004), Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae. Bioresource Technology 92: 251–260

Nedović, V., Gibson, B., Mantzouridou, T.F., Bugarski, B., Djordjević, V., Kalušević, A., Paraskevopoulou, A., Sandell, M., Šmogrovičová, D., Yilmaztekin, M., 2015. Aroma formation by immobilized yeast cells in fermentation processes. Yeast 32, 173–216.

Nuanpeng S., hanonkeo S., Klanrit P., Thanonkeo P (2018). Ethanol production from sweet sorghum by Saccharomyces cerevisiae DBKKUY-53 immobilized on alginate-loofah matrices. BJM-372.

Olbrich, H., 1963. The Molasses. Biotechnologie-Kampe GmbH (2006).

Orzua, M.C., S.I. Mussatto, J.C. Contreras-Esquivel, R. Rodriguez and H. de la Garza et al., 2009. Exploitation of agro industrial wastes as immobilization carrier for solid-state fermentation. Ind. Crops Products, 30: 24-27. DOI: 10.1016/j.indcrop.2009.02.001

Park, J.K., Chang, H.N., 2000. Microencapsulation of microbial cells. Biotechnol Adv 18, 303–319.

Parkash A (2015) Modeling of Ethanol Production from Molasses: A Review. Ind Chem 3: 108. doi: 10.4172/2469-9764.1000108.

Patil N.P. and Patil V.S. "Fuel Ethanol from Cane Molasses: A Review of Feed stocks, Technologies, Opportunities and Challenges", J. Nat. Prod. Resour. - Volume 3 Issue 1 (2017) 104–110.

Paul, F., and P. M. Vignais. 1980. Photophosphorylation in bacterial chromatophores entrapped in alginate gel: improvement of physical and biochemical properties of gel beads with barium as the gel inducing agent. Enzyme Microb. Technol. 2:281-286.

Peter Rein. (2007). Cane Sugar Engineering Verlas Dr. Albert Bartens Kg - Berlin 2007.

Phisalaphong M, Budiraharjo R, Bangrak P, Mongkolkajit J, Limtong S. Alginate-loofa as carrier matrix for ethanol production. J Biosci Bioeng. 2007;104:214–217.

Plessas, S., Bekatorou, A., Kountinas, A.A., Soupioni, M., Banat, I.M., and Marchant, R., (2007), Use of Saccharomyces cerevisiae Cells Immobilized on Orange Peel as Biocatalyst for Alcoholic Fermentation, Bioresources Technology, 98, pp. 860865.

Prasad S, Anoop S, Joshi H (2007) Ethanol as an alternative fuel from agricultural, industrial and urban residues resources. Conservation and Recycling 50: 1-39.

Prior, B.A., Du Preez, J.C., and Rein, P.W., (1992), Environmental Parameters in Solid Substrate Cultivation, editor H.W. Doelle, D.A. Mitchell and C.E. Rolz, Elsevier Applied Science, London, pp. 65- 85.

Rafael R, Francisco F, Rubens F, Aline C (2013) Kinetics of ethanol production from sugarcane bagasse enzymatic hydrolysate concentrated with molasses under cell recycle. Bioresource Technology 130: 351-359.

Razmovski, R. and Vucurovic, V., (2012), Bioethanol Production from Sugar Beet Molasses and Thick Juice Using Saccharomyces cerevisiae Immobilized on Maize Stem Ground Tissue, Fuel, 92, pp. 1-8.

Ruanglek, V., Maneewatthana, D. and Tripetchkul, S. (2006) Evaluation of Thai agroindustrial wastes for bio-ethanol production by Zymomonas mobilis. Process Biochem. 41, 1432-1437.

Ruhul A, Saquib H, Sarker M (2013) Simulation of Ethanol Production by Fermentation of Molasses. Journal of Engineering 1: 69-73.

Santos, J.C., Pinto, I.R., Carvalho, W., Mancilha, I.M., Felipe, M.G., Silva, S.S., 2005. Sugarcane bagasse as raw material and immobilization support for xylitol production. Appl. Biochem. Biotechnol. 121e124, 673e683.

Schweinitzer, T. and Josenhans, C. 2010. Bacterial energy taxis: a global strategy? Arch Microbiol. J Bacteriol 170, 5507 - 5511.

T. Brányik, A. Vicente, P. Dostálek, J. Teixeira. "Continuous Beer Fermentation Using Immobilized Yeast Cell Bioreactor Systems". Biotechnology Progress. Vol. 21. 2005. pp. 653-663.

Tang, P.D.P., Lee, V.V.M., 2013. Fermentation performance of free and immobilized yeast on cork (Sonneratia caseolaris) root – application of immobilized yeast to repeated batch ethanol fermentation. Int Food Res J 20, 1813-1817.

Training Manual on manufacturing of Ethyl alcohol from cane molasses by Ethiopian Sugar Corporation Research and Training Directorate, Wonji-Shoa.

Verbelen, P.J., De Schutter, D.P., Delvaux, F., Verstrepen, K.J., and Delvaux, F.R. 2006. Immobilized yeast cell systems for continuous fermentation applications. Biotechnol.Lett. 28:1515–1525.

Verma M, Brar SK, Blais JF, Tyagi RD, Surampalli RY (2006). Aerobic biofiltration processes-advances in wastewater treatment. Pract. Period. Hazard Toxic Radioact. Waste Manage. 10:264-276.

Verstrepen, K.J. and Klis, F.M., (2006), Micro Review: Flocculation, Adhesion, and Biofilm Formation in Yeasts, Molecular Microbiology, 60(1), pp. 5-15

Vesna Vucurovic, Radojka Razmovski. Ethanol Fermentation Of Molasses By Saccharomyces Cerevisiae Cells Immobilized Onto Sugar Beet Pulp. BIBLID: 1450-7188 (2012) 43, 325-333.

Vucurovic, V. and Razmovski, R.N., (2012), Sugar Beet Pulp as Support for Saccharomyces cerevisiae Immobilization in Bioethanol Production, Industrial Crops and Products, 39,:pp.128-134.

Walsh, P.K., Brady, J.M. and Malone, D.M., Determination of the Radial Distribution Saccharomyces cerevisiae Immobilized in Calcium Alginate Gel Beads, Biotechnology Techniques, 7(6), 435-440 (1993).

Y. Chisti, In: M.C. Flickinger, S.W. Drew (Eds.), Encyclopedia of Bioprocess Technology: Fermentation, Biocatalyst, and Bioseparation, vol. 5, Wiley, New York, 1999, p.2446

Yacob Gebreyohannes Hiben (2013). Long-term Bioethanol Shift and Transport Fuel Substitution in Ethiopia.

Yu, J., Yue, G., Zhang, J., Zhang, X., and Tan, T., (2010), Immobilization of Saccharomyces cerevisiae to Modified Bagasse for Ethanol Production. Renewable Energy, 35, pp. 1130-1134.

Yu, J., Zhang, X., Tan, T., 2007. "A novel immobilization method of Saccharomyces cerevisiae to sorghum bagasse for ethanol production". J. Biotechnol. 129, 415 - 420.

Zheng, Y., Pan, Z. and Zhang, R., (2009), Overview of Biomass Pretreatment for Cellulosic Ethanol Production, International Journal of Agricultural and Biological Engineering, 2(3), pp. 51-68.

APPENDICES

Appendix -A: Yeast Preparation and Culturing

1. Yeast Preparation and Culturing

1.1. Technique for Yeast Purification and Isolation

To isolate and purify the yeast strain following procedure is used:

In case the liquid culture from which we wish to isolate the yeast is heavily contaminated by bacteria, acidify it to 2.5 to 3.5 PH for 2 hour retention time then use for isolation by plating method. Antibiotics like benzyl penicillin - 3ppm, tetracycline - 12 ppm, streptomycin - 3 ppm can be used.

Requirements:

Inoculation Wire, Conical Flask 500 Ml, Tubes 50 Ml Capacity, Source Of Yeast Strains Yeast Liquid Culture (Fermenters, Yeast Vessels, Pre-fermenters) Dry Yeast, Ripen Grapes, Fruits Etc., Petre Dishes, Cotton Roll, Test Tube Stand , Inoculation Chamber, Pressure Cooker, Aluminium Foil, Distilled Water, Ethanol

Preparation of Solid Media for Plating/Slant Preparation

COMPOSITION:

Yeast Extract: 0.3%; Peptone: 0.5%; Agar Agar: 2.0%; Dextrose: 1%; Sterilized molasses lab culture solution as medium

PROCEDURE-

Weigh the above solid media components to prepare 150 ml solid media in a 500 ml conical flask in pre sterilized and cooled lab culture molasses media. Maintain PH 4.2 to 4.5 with Ortho-phosphoric acid or sulphuric acid. Shake well and close the mouth of the conical flask with cotton plug; shake well and slowly boil the solution for 5 mints taking care of vigorous foaming which may be harmful. Then put into pressure cooker for 30 minutes at 121 degree centigrade and 20 psi pressure in pressure cooker. Remove the flask at room temperature and again warm media on water bath to melt it into liquid state so that it can be poured whenever required.

Then pour the media while hot into petre dishes up to $1/3^{rd}$ depth or 3-4 mm layer in and under flame and cover the petredish with upper petredish in and under flame. All this transfer should be done in laminar flow chamber taking care that top roof of laminar flow chamber is protected

by aluminum sheet from inside to protect the plastic sheet from melting due to heat. Cool the petre dishes. After cooling, the media will solidify and the petredish will be ready for plating.

1.2. Dilution of Yeast Sample

Take 10 pre-sterilized test tubes (50 ml capacity each) ,fill each test tube with 25 ml distilled water and then cotton plug and sterilize in autoclave for at least 30 minutes at 121 ^{0} centigrade and 20 psi pressure, then allow to cool while in pressure cooker.

Now take 1 ml yeast sample from yeast sample from which yeast cells/strain has to be isolated and proceed as under flame/laminar flow chamber.

1. Add 1 ml yeast sample to test tube No.1 and shake well.
2. Then transfer 1 ml diluted sample from test tube No 1 to test tube No.2, shake well.
3. Then transfer 1 ml diluted sample from test tube No 2 to test tube No.3, shake well.
4. Then transfer 1 ml diluted sample from test tube No 3 to test tube No.4, shake well.
5. Then transfer 1 ml diluted sample from test tube No 4 to test tube No 5, shake well.
6. Then transfer 1 ml diluted sample from test tube No 5 to test tube No 6, shake well.
7. Then transfer 1 ml diluted sample from test tube No 6 to test tube No 7, shake well.
8. Then transfer 1 ml diluted sample from test tube No 7 to test tube No 8, shake well.
9. Then transfer 1 ml diluted sample from test tube No 8 to test tube No 9, shake well.
10. Then transfer 1 ml diluted sample from test tube No 9 to test tube No10, shake well.

1.3. Plating Method

Now transfer 2-3 ml of diluted yeast solution from test tube no-1, 3,5,7,8 and 10 to 6 petredish respectively in laminar flow chamber under flame. Spread the liquid over solid media and pour out the excess liquid carefully with partial covering of upper petredish. Put all six petredish in inverted position at 32-33 degree centigrade in yeast chamber for 3-4 days. After 3-4 days cream colored 1 to 3 mm diameter yeast colonies developed.

1.4. Preservation of Yeast Strain: Slant Preparation on Solid Media

PROCEDURE-

Weigh solid media components as given in yeast purification and isolation procedure to prepare 250 ml solid media in a 1000 ml conical flask in pre sterilized and cooled lab culture molasses media. Maintain PH 4.2 to 4.5 with Orthophosphoric acid or sulphuric acid. Shake well and close the mouth of conical flask with cotton plug shake well and slowly boil the solution for 5 mints taking care of vigorous foaming which may be harmful. Then put into autoclave for 30 minutes at

121^0 centigrade and 20 psi pressure in autoclave or pressure cooker. Remove the flask at room temperature and again warm media on water bath to melt it into liquid state so that it can be poured whenever required. Then pour the media while hot into 25 test tubes (50 ml capacity each) in and under flame. All this transfer should be done in laminar flow chamber taking care that top roof of laminar flow chamber is protected by aluminum sheet from inside to protect the plastic sheet from melting due to heat. Then while hot media in test tubes, put test tubes in an inclined position as given in following figure and allow solidifying and cooling to 32 ^{o} centigrade.

Now choose healthy colony of yeast and put it on wire loop previously sterilized on flame and transfer to the solid media in test tube as shown in the following figure. All this operation must be done in and under flame.

Now put the yeast strain inoculated test tube at 32 degree centigrade in laminar flow chamber for 3 days till yeast is grown on solid media and then preserve the yeast slant so made in refrigerator at less than 4 degree centigrade.

The procedure can be repeated by inoculating the yeast strain from slant into liquid media of desired brix/alcohol concentration and temperature and again plating and preserving to solid media in test tubes.

Appendix- B: Analytical Procedures and Experimental Runs

I. Determination of acid soluble and acid insoluble lignin in biomass

ASTM D 1106-96 standard procedure was adopted in the determination of Lignin in biomass samples. Biomass sample of 0.3g was added to 3 ml of 72 % of H2SO4, the solution was mixed well and incubated for 60 minutes at 30°C. Then it was diluted with 84 ml of distilled water to 4% concentration. It was then autoclaved at 121°C for 1 h. The solution was then filtered and the liquid filtrate obtained was the acid soluble lignin and carbohydrates. The residue contains the acid insoluble lignin, and then it was dried until constant weight was noted after which it was placed in a muffle furnace in a silica crucible at 575±25°C. The ash obtained was weighed and the lignin was calculated using the following expressions, the oven dry weight (ODW) extractives free sample was calculated as,

$ODW = [(Weight_{air\text{-}dry}) \times (\% \text{ Total solids})] / 100$

The weight percent acid insoluble residue (AIR) was calculated using the following Expression,

$\%AIR = [(Weight_{(Crucible+AIR)}) - Weight_{(crucible)}] \times 100 / (ODW \text{ sample})$

Weight of the acid soluble lignin (AIL) on an extractives free basis was derived From the formula,

$\%AIL = [(Weight_{(Crucible+AIR)}) - Weight_{(crucible)}] - [(Weight_{(Crucible+ash)}) - Weight_{(crucible)}] - [Weight_{Protein}] \times 100 / (ODW \text{ sample})$

Where, weight of protein was the amount of protein present in the acid insoluble residue. The amount of acid soluble lignin (ASL) on an extractive free basis,

$\%ASL = \{[UV_{abs} \times \text{volume of filtrate} \times \text{dilution}] / [(\varepsilon) \times ODW_{(sample)} \times \text{Path length}]\} \times 100$

Where, UVabs reading was the absorption reading displayed in the spectrometer, the average ultra violet visual absorbance for the sample at appropriate wave length of 560nm. ε was the absorptivity of the biomass at specific wavelength, was 25nm and the path length was the path length of ultra violet visual cell in cm, which was 1cm. The dilution was derived as,

$Dilution = [(\text{Volume of sample}) - (\text{Volume of diluting sample})] / (\text{Volume of sample})$

Total amount of lignin on an extractives free basis was calculated as,

$$\%Lignin_{(Extractives\ free)} = \%AIL + \%\ ASL$$

The total lignin obtained or received from the analysis was estimated using the Formula,

$$\%Lignin_{(as\ received)} = \%Lignin\ (Extractives\ free) \times [(100 - \%extractives)/ 100]$$

II. Determination of Total Reducing Sugar (TRS) in Final Molasses.

Take 50 gram Molasses Sample and dissolve in 450ml of water and check brix by hydrometer. Then make up volume to 500ml and mix well. Now take 10ml in 100ml volumetric flask and add 5ml of HCL and 20ml of water. Then heat in water bath to 69 degree for (10-15min).Now cool it to normal temperature and neutralize with 5N NaOH solution. Use phenolphthalein indicator 2-3 drops. End point colour will be pink. Now make up its volume to 100ml with water. Now titrate with 5ml of Fehling solution A and 5ml of Fehling solution B, and add 20-25ml of water using methylene blue indicator. End point just brick red colour appears.

Calculation:-

$$\text{TRS\%} = \frac{\text{Feling Factor} * \text{Dilution} * 100}{\text{Titration value in ml}}$$

Determination of Fehling Factor

Prepare 1% w/v solution of dextrose (pre dried at 105 degree centigrade for 1hour) then titrate this solution with 10ml Fehling solution (5ml Fehling A+5ml Fehling B) add 15ml water using methylene blue (0.5% in water) as indicator. Add solution through burette while Fehling solution on boiling. A brick red colour appears at the end point. Fehling factor = Titration value in ml/100

III. Determination of Unfermentable Sugars in Molasses

Take 50 gram molasses add 20 gram dry yeast then make up volume to 500ml with water and keep for 24 hours at 32 degree centigrade, then after fermentation is complete centrifuge and titrate the solution with 10ml Fehling solution (5ml Fehling A+5ml Fehling B) and 15ml water using methylene blue (0.5%in water) as indicator. Add solution through burette while Fehling solution in boiling. A brick red colour appears at the end point.

$$\text{Unfermentable sugars\%} = \frac{Feling\ Factor * Dilution * 10 * 100}{Titration\ value\ in\ ml}$$

Fermentable sugars = T.R.S – U.F.S

IV. Determination of Residual Sugar in Fermented Wash.

Take 25ml sample of Fermented Wash and put it in 100ml volumetric flask. Fill to mark with distilled water and shake well. Take 5ml of Fehling's solution "A" and 5ml of Fehling's solution "B" in 150ml Erlenmeyer flask and add 10-15ml of distilled water to the Erlenmeyer flask. Put the Erlenmeyer flask on a hot stove and run about 5ml of the fermented wash from

the burette to the Erlenmeyer flask. When the solution starts boiling drop about 4 drops of methylene blue and start titrating until the blue colour changes to a faint pink or brick red.

Calculation

$$\text{Residual Sugar \%} = \frac{Feling\ Factor * Dilution}{Burette\ Reading}$$

V. Alcohol Determination by Ebuliometry

Ebuliometry is the most encountered procedure for determination of the alcohol content of aqueous solutions. In principle, the analysis is based on the Ragout's low relationship of boiling point depression:

Where: P = h.X1

P = Vapour Pressure of Solution

H = Proportionate constant

X1 = Mole fraction of solvent

Thus, the vapour pressure of a solute (ethanol) in a solution will vary in a regular manner as a function of its concentration. As the concentration of the solute (alcohol) in the wine increases, the boiling point is reached at a temperature that is low relative to the boiling point of pure water. Ebuliometry involves a measurement of the boiling point depression caused by the presence of alcohol in a wine sample. Mechanically, the boiling point of the sample is measured relative to the boiling point of pure water and the difference is related to percent ethanol.
The alcohol content was displayed directly.

Appendix –C: Yeast Counting by Heamocytometer

Take 1ml sample in a 100ml measuring cylinder and add 1ml methylene blue solution; Then makeup volume to 100ml by distilled water. Mix well. Take heamocytometer slide and put a drop of diluted yeast culture solution on the heamocytometer and cover with a cover slip. The viable yeast cells remains transparent and dead cells becomes dark blue.

- To count the cells within an area, switch over to the 10X objective lens with the 40X objective lens, frame up the counting area of one of the 25 large squares, and take a count. Use your hand-held counter to keep an accurate tally of the cells.

- ✓ To Count the number of yeast cells found inside grid 1, 2, 3, 4 and 5 and sum up.
- ✓ Total number of yeast cell is obtained considering the sum of yeast cell found in chamber 1-5 and dilution factor as follows: Yeast Count = total cell in grid(1-5)*5*DF*10^4

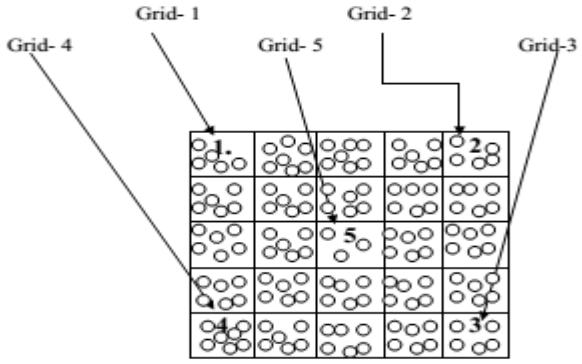


Figure D-0-1 cell count by Heamocytometer

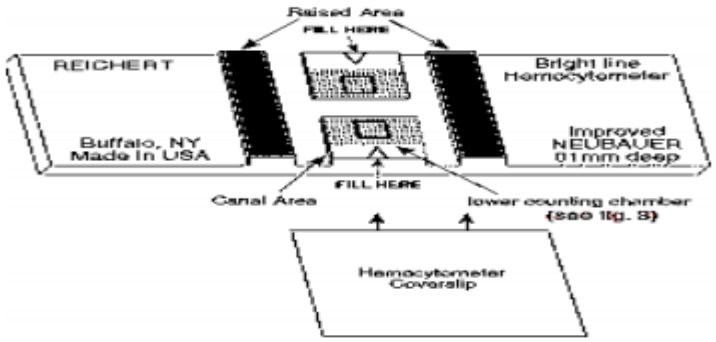
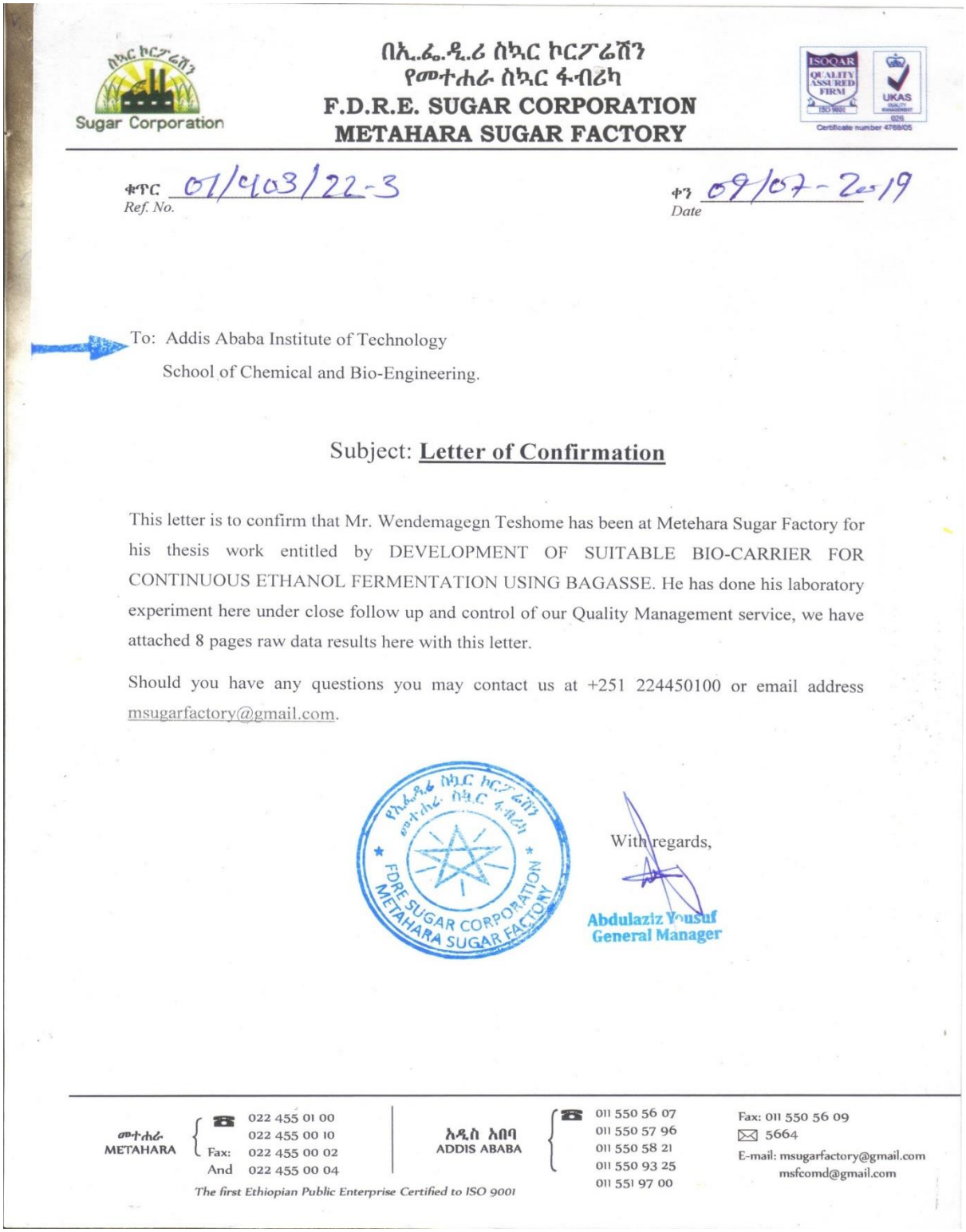


Figure D -2 Heamocytometer showing counting chamber and filling point

Source: Fal Allen, 1994- A practical guide to laboratory and quality control procedures for small scale brewers.

Appendix- D: Raw data and standard Curves



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F.D.R.E. SUGAR CORPORATION
METAHARA SUGAR FACTORY

Sugar Corporation

ISOQAR QUALITY ASSURED FIRM ISO 9001 — UKAS QUALITY MANAGEMENT 0016 — Certificate number 4768/05

ቁጥር Ref. No. 01/403/22-3

ቀን Date 09/07-2019

To: Addis Ababa Institute of Technology
School of Chemical and Bio-Engineering.

Subject: **Letter of Confirmation**

This letter is to confirm that Mr. Wendemagegn Teshome has been at Metehara Sugar Factory for his thesis work entitled by DEVELOPMENT OF SUITABLE BIO-CARRIER FOR CONTINUOUS ETHANOL FERMENTATION USING BAGASSE. He has done his laboratory experiment here under close follow up and control of our Quality Management service, we have attached 8 pages raw data results here with this letter.

Should you have any questions you may contact us at +251 224450100 or email address msugarfactory@gmail.com.

FDRE SUGAR CORPORATION METAHARA SUGAR FACTORY

With regards,

Abdulaziz Yousuf
General Manager

መተሐራ METAHARA ☎ 022 455 01 00, 022 455 00 10, Fax: 022 455 00 02, And 022 455 00 04

አዲስ አበባ ADDIS ABABA ☎ 011 550 56 07, 011 550 57 96, 011 550 58 21, 011 550 93 25, 011 551 97 00

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Sugar Corporation	METEHARA SUGAR FACTORY	Ref.No. Date:
Title	Physical properties of sugarcane bagasse (CDB)	Page: 1/1

Run	Block	Factor	Responses				
		A:P.I.	Water content, w %	Water content after hydration, WAH %	Water Retention, H g/g	Lignin content, %	Water absorption index, WAI g/g
1	Block 1	P.I. 80	7.8	89.98	7.98	23.19	8.93
2	Block 1	P.I. 85	7.77	89.97	7.96	23.2	8.91
3	Block 1	P.I. 87.5	7.78	89.95	7.96	23.16	8.95
4	Block 1	P.I. 82.5	7.79	89.98	8	23.11	8.88
5	Block 1	P.I. 90	7.76	89.89	7.79	23.23	8.99
6	Block 1	P.I. 80	7.8	89.87	7.93	23.54	9
7	Block 1	P.I. 87.5	7.77	89.97	7.98	23.24	9.01
8	Block 1	P.I. 90	7.788	89.87	8.01	23.22	8.94
9	Block 1	P.I. 85	7.89	88.99	8.012	23.09	8.97
10	Block 1	P.I. 82.5	7.798	88.97	8	23.24	8.95

Physical properties of sugarcane bagasse(composite sample)							
Run	Block	Factor	Responses				
		Bagasse Type	Water content, w %	Water content after hydration, WAH %	Water Retention, H g/g	Lignin content, %	Water absorption index, WAI g/g
1	Block 1	MTB	7.79	89.98	7.97	23.17	8.94
2	Block 1	MTB	7.77	84.75	4.69	24.81	8.57
3	Block 1	CDB	7.75	85.31	4.77	24.78	8.49
4	Block 1	CDB	7.84	89.48	8.02	23.04	8.89

Temesgen Kassahun
Quality Management
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METHAARA SUGAR FACTORY

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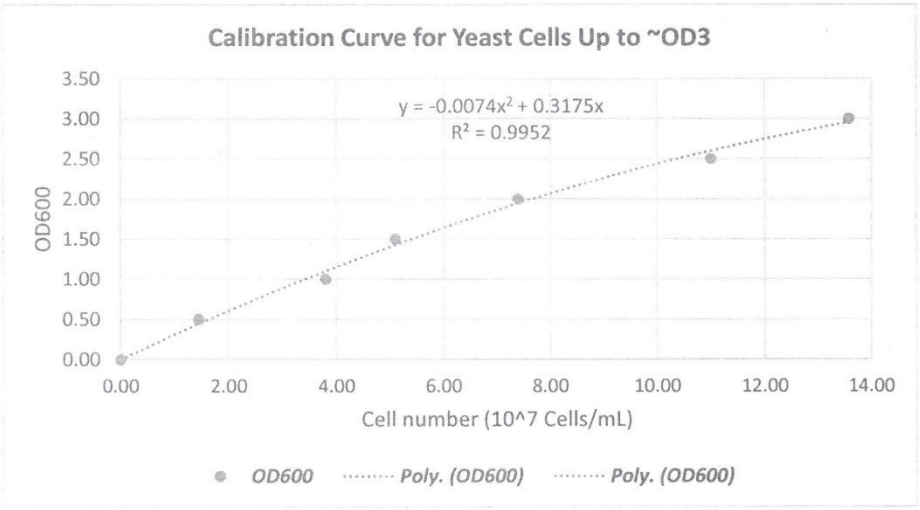
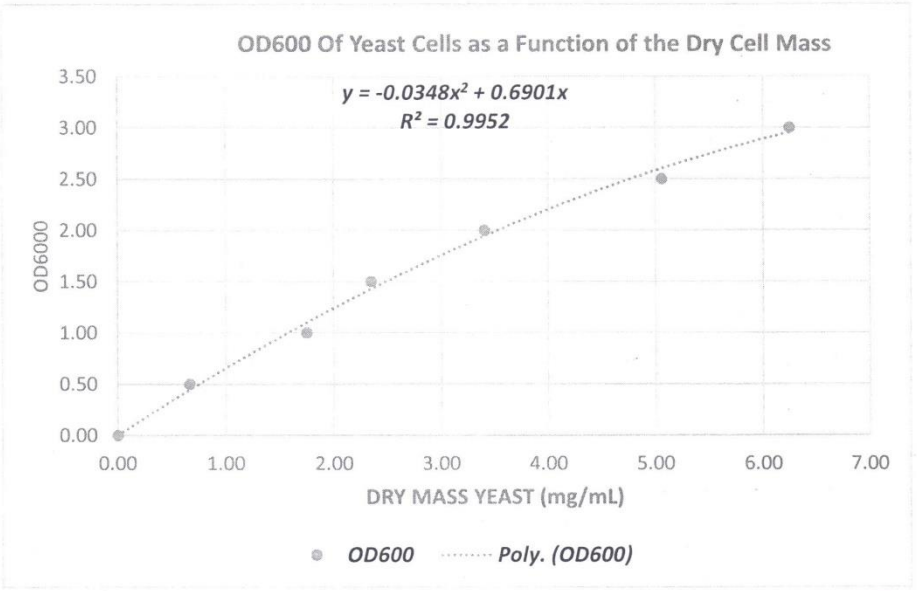
Sugar Corporation	METEHARA SUGAR FACTORY	Ref.No. Date
Title	Calibration Curve	Page 1/1





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Sugar Corporation	METEHARA SUGAR FACTORY	Ref.No.
Title	Immobilization properties	Date:
		Page: 1/1

Run	Factors			Responses						
	P.I., %	Bagasse Type	OD600 (carrier Filtrate)	Dry mass of cell from Filtrate (mg/Ml) from standard graph	Control dry mass (cell free medium, mg)	control Filtrate Absorbance	washed carrier dry mass (mg)	Dry mass of cell from Carrier (mg)	Total cell retention in the sample (mg)	Cell Retention mg/mg
1	85	MTB	0.1021	0.1986	28.50	For Calibration	28.55	0.0496	0.2482	0.0087
2	82.5	MTB	0.0677	0.1316	28.31		28.33	0.0232	0.1549	0.0055
3	80	MTB	0.0576	0.1120	28.33		28.34	0.0138	0.1259	0.0044
4	90	MTB	0.1103	0.2145	28.60		28.67	0.0715	0.2860	0.0100
5	80	CDB	0.2674	0.5202	27.00		27.08	0.0777	0.5979	0.0221
6	87.5	CDB	0.5050	0.9825	27.52		27.94	0.4211	1.4035	0.0510
7	90	CDB	0.5495	1.0690	27.41		27.77	0.3563	1.4253	0.0520
8	82.5	CDB	0.2690	0.5233	26.90		27.06	0.1563	0.6796	0.0253
9	85	CDB	0.4881	0.9496	27.10		27.45	0.3512	1.3008	0.0480
10	87.5	MTB	0.1166	0.2268	28.45		28.51	0.0640	0.2908	0.0102

METHAARA SUGAR FACTORY

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Sugar Corporation	METEHARA SUGAR FACTORY	Ref.No. Date:
Title	Parameters of seven repeated batch fermentation by CDB carrier results	Page: 1/2

Std	Block	Factors		Responses			
		P.I.	Batch No.	Initial Sugar (10%), g/l	Alcohol % (V/V)	Residual Sugar (g/l)	Sugar utilization, Ys (%)
1	Block 1	P.I. 85	Batch 1	100	2.8	1.1	96.33
2	Block 1	P.I. 85	Batch 1	100	2.78	1.12	96.27
3	Block 1	P.I. 87.5	Batch 1	100	2.91	1.01	96.63
4	Block 1	P.I. 87.5	Batch 1	100	2.88	1.13	96.23
5	Block 1	P.I. 90	Batch 1	100	3.11	0.96	96.80
6	Block 1	P.I. 90	Batch 1	100	3.09	1.13	96.23
7	Block 1	P.I. 85	Batch 2	100	3.1	3.31	88.97
8	Block 1	P.I. 85	Batch 2	100	3.16	3.28	89.07
9	Block 1	P.I. 87.5	Batch 2	100	3.23	3.19	89.37
10	Block 1	P.I. 87.5	Batch 2	100	3.19	3.23	89.23
11	Block 1	P.I. 90	Batch 2	100	3.36	2.96	90.13
12	Block 1	P.I. 90	Batch 2	100	3.42	2.88	90.40
13	Block 1	P.I. 85	Batch 3	100	3.56	1.96	93.47
14	Block 1	P.I. 85	Batch 3	100	3.61	1.99	93.37
15	Block 1	P.I. 87.5	Batch 3	100	3.61	3.61	87.97
16	Block 1	P.I. 87.5	Batch 3	100	3.59	3.59	88.03
17	Block 1	P.I. 90	Batch 3	100	3.44	2.23	92.57
18	Block 1	P.I. 90	Batch 3	100	3.42	2.43	91.90
19	Block 1	P.I. 85	Batch 4	100	4.46	1.36	95.47
20	Block 1	P.I. 85	Batch 4	100	4.64	1.26	95.80
21	Block 1	P.I. 87.5	Batch 4	100	4.71	2.23	92.57
22	Block 1	P.I. 87.5	Batch 4	100	4.74	2.26	92.47
23	Block 1	P.I. 90	Batch 4	100	4.98	2.31	92.30
24	Block 1	P.I. 90	Batch 4	100	5.01	2.23	92.57
25	Block 1	P.I. 85	Batch 5	100	4.98	2.24	92.53

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METHAARA SUGAR FACTORY

Sugar Corporation	METEHARA SUGAR FACTORY	Ref.No. Date:
Title	Parameters of seven repeated batch fermentation by CDB carrier results	Page: 2/2

Continued

Std	Block	Factors		Responses			
		P.I.	Batch No.	Initial Sugar (10%), g/l	Alcohol % (V/V)	Residual Sugar (g/l)	Sugar utilization, Ys (%)
26	Block 1	P.I. 85	Batch 5	100	4.56	2.31	92.30
27	Block 1	P.I. 87.5	Batch 5	100	4.89	2.23	92.57
28	Block 1	P.I. 87.5	Batch 5	100	4.93	2.27	92.43
29	Block 1	P.I. 90	Batch 5	100	5.12	2.87	90.43
30	Block 1	P.I. 90	Batch 5	100	4.97	2.64	91.20
31	Block 1	P.I. 85	Batch 6	100	4.55	2.96	90.13
32	Block 1	P.I. 85	Batch 6	100	4.63	3.1	89.67
33	Block 1	P.I. 87.5	Batch 6	100	5.01	2.01	93.30
34	Block 1	P.I. 87.5	Batch 6	100	5.23	2.11	92.97
35	Block 1	P.I. 90	Batch 6	100	4.46	3.1	89.67
36	Block 1	P.I. 90	Batch 6	100	4.71	3.21	89.30
37	Block 1	P.I. 85	Batch 7	100	4.32	3.25	89.17
38	Block 1	P.I. 85	Batch 7	100	4.37	3.36	88.80
39	Block 1	P.I. 87.5	Batch 7	100	5.64	2.01	93.30
40	Block 1	P.I. 87.5	Batch 7	100	5.84	1.98	93.40
41	Block 1	P.I. 90	Batch 7	100	4.23	3.27	89.10
42	Block 1	P.I. 90	Batch 7	100	4.11	3.33	88.90
43	Free cell (Control)			100	3.56	3.5	88.33
				100	3.61	3.38	88.73

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METHAARA SUGAR FACTORY

Sugar Corporation

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Stability Test Results

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Run	Block	Factor			Responses					
		P.I., %	Batch No.	OD600 Reading of sample filtrate	Dry mass of cell from Filtrate (mg/Ml) from standard graph	Control dry mass (cell free medium, mg)	For Calibration control Filtrate Absorbance	washed carrier dry mass	Dry mass of cell from Carrier (mg)	Total cell retention in the sample (mg)	Cell Retention mg/mg
1	Block 1	P.I. 87.5	B3	0.5979	1.16	28.71		29.21	0.4986	1.66	0.0579
2	Block 1	P.I. 87.5	B7	0.6264	1.22	28.72		29.61	0.8898	2.11	0.0734
3	Block 1	P.I. 85	B3	0.5025	0.98	28.10		28.28	0.1725	1.15	0.0409
4	Block 1	P.I. 90	B3	0.5278	1.03	28.81		29.49	0.6846	1.71	0.0594
5	Block 1	P.I. 90	B3	0.5398	1.05	28.83		29.53	0.6972	1.75	0.0606
6	Block 1	P.I. 87.5	B3	0.6069	1.18	28.73		29.19	0.4617	1.64	0.0572
7	Block 1	P.I. 90	B7	0.5229	1.02	28.86		29.00	0.1400	1.16	0.0401
8	Block 1	P.I. 85	B7	0.5627	1.09	28.13		28.69	0.5639	1.66	0.0590
9	Block 1	P.I. 90	B7	0.5215	1.01	28.85		28.98	0.1267	1.14	0.0396
10	Block 1	P.I. 85	B7	0.5385	1.05	28.14		28.69	0.5542	1.60	0.0569
11	Block 1	P.I. 85	B3	0.5442	1.06	28.10		28.30	0.2017	1.26	0.0449
12	Block 1	P.I. 87.5	B7	0.6290	1.22	28.74		29.63	0.8862	2.11	0.0734

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Stability Test Results

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Run	Factor			Responses				
	Block	P.I., %	Batch No.	Cell Retention mg/mg	Xi, mg/Ml	Fermentation medium OD600	Dry mass of free cell ,Xs (mg/Ml) from standard graph	XT, g/l	Yi, %
1	Block 1	P.I. 87.5	B3	0.0579	0.4824	0.0302	0.0588	0.5412	89.13
2	Block 1	P.I. 87.5	B7	0.0734	0.6118	0.0032	0.0063	0.6181	98.98
3	Block 1	P.I. 85	B3	0.0409	0.3410	0.0608	0.1183	0.4593	74.25
4	Block 1	P.I. 90	B3	0.0594	0.4951	0.0028	0.0055	0.5005	98.91
5	Block 1	P.I. 90	B3	0.0606	0.5051	0.0034	0.0067	0.5117	98.70
6	Block 1	P.I. 87.5	B3	0.0572	0.4764	0.0299	0.0581	0.5345	89.13
7	Block 1	P.I. 90	B7	0.0401	0.3342	0.0656	0.1277	0.4619	72.35
8	Block 1	P.I. 85	B7	0.0590	0.4913	0.0302	0.0587	0.5500	89.33
9	Block 1	P.I. 90	B7	0.0396	0.3297	0.0648	0.1260	0.4557	72.35
10	Block 1	P.I. 85	B7	0.0569	0.4744	0.0291	0.0567	0.5310	89.33
11	Block 1	P.I. 85	B3	0.0449	0.3738	0.0666	0.1296	0.5034	74.25
12	Block 1	P.I. 87.5	B7	0.0734	0.6118	0.0032	0.0063	0.6181	98.98

METEHARA SUGAR FACTORY

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Run	Total reducing sugar (TRS)	Dilution, D (h^-1)	Alcohol % (v/v)	Residual Sugar(g/g)	Sugar Utilization, Ys (%)
1	50	0.5	3.35	3.90	90.00
9	150	0.5	6.14	51.61	55.89
3	50	1.5	2.09	15.61	59.98
6	150	1.5	2.07	83.49	28.64
10	29.29	1	1.83	3.89	82.98
11	170.71	1	3.42	85.25	35.98
13	100	0.29	6.11	14.83	80.99
5	100	1.71	2.14	48.28	38.10
8	100	1	4.41	32.00	58.98
2	100	1	4.57	32.26	58.64
7	100	1	4.18	32.50	58.33
12	100	1	4.59	31.32	59.84
4	100	1	4.31	32.10	58.85

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