PRODUCTION OF ETHANOL USING IMMOBILIZED YEAST CELLS

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CERTIFICATE

This is to certify that the work entitled **"PRODUCTION OF ETHANOL USING IMMOBILIZED YEAST CELLS"** submitted by **ESHA KHURANA (061732) and KUNAL CHOWDHARY (061563)** in partial fulfilment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Mrs. Runni Mukherjee

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Esha Khurana Esha.

And

Kunal Chowdhary Chowdhary.

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LIST OF ABBREVIATIONS

AIDS - Auto Immune Deficiency Syndrome

YPD - Yeast Peptone Dextrose

EDTA - Ethylene Diamine Tetra Acetic acid

PVA - Poly Vinyl Alcohol

Bp - Boiling point

ABSTRACT

Growing cells of *Saccharomyces cerevisiae* immobilized in calcium alginate gel beads were employed in flask reactors for continuous ethanol fermentation from sugar sources such as YPD media. Immobilized cells makes it easier for reuse of cells for fermentation. It becomes very difficult to separate normal cells after one fermentation and reuse them. Normal cells tend to die upon centrifugation and hence cannot be reused. Immobilization also makes it easier to handle the cells as the cell growth is retarded, the only problem is the regulation of oxygen (for aerobic conditions) for technical advances to be made in this field. Some improvements were made in order to avoid microbial contamination. The conditions were maintained for anaerobic fermentation using laboratory equipment. The estimation of ethanol is done using its property of oxidation by potassium dichromate. The amount of pure ethanol was estimated using standard graph and the values thus compared.

OBJECTIVES

- Production of ethanol using immobilized yeast cells.
- Comparison of production of ethanol using immobilized yeast cells and free yeast cells.
- Optimization of factors related to production of ethanol using immobilized yeast cells such as sugar content and amount of yeast.

CHAPTER 1

INTRODUCTION

Yeast

Yeast are unicellular fungi. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum *Ascomycetes*, class *Saccharomycetes* (also called *Hemiascomycetes*). The true yeasts are separated into one main order *Saccharomycetales*.

Yeasts are characterized by a wide dispersion of natural habitats. Common on plant leaves and flowers, soil and salt water. Yeasts are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites. The common "yeast infection" is typically Candidiasis is caused by the yeast-like fungus *Candida albicans*. In addition to being the causative agent in vaginal yeast infections *Candida* is also a cause of diaper rash and thrush of the mouth and throat.

Yeasts multiply as single cells that divide by budding (eg *Saccharomyces*) or direct division (fission, eg. *Schizosaccharomyces*), or they may grow as simple irregular filaments (mycelium). In sexual reproduction most yeasts form asci, which contain up to eight haploid ascospores. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores.

The awesome power of yeast genetics is partially due to the ability to quickly map a phenotype producing gene to a region of the *S. cerevisiae* genome. For the past two decades *S. cerevisiae* has been the model system for much of molecular genetic research because the basic cellular mechanics of replication, recombination, cell division and metabolism are generally conserved between yeast and larger eukaryotes, including mammals.

The most well-known and commercially significant yeasts are the related species and strains of *Saccharomyces cerevisiae*. These organisms have long been utilized to ferment the sugars of rice, wheat, barley, and corn to produce alcoholic beverages and in the baking industry to expand, or raise, dough. *Saccharomyces cerevisiae* is commonly used as baker's yeast and for some types of fermentation. Yeast is often taken as a vitamin supplement because it is 50 percent protein and is a rich source of B vitamins such as niacin, folic acid, riboflavin, and biotin.

In brewing, *Saccharomyces carlsbergensis*, named after the Carlsberg Brewery in Copenhagen, where it was first isolated in pure culture by Dr. Emil Christian Hansen (1842-1909) in 1883, is used in the production of several types of beers including lagers. *S. carlsbergensis* is used for bottom fermentation. *S. cerevisiae* used for the production of ales and conducts top fermentation, in which the yeast rise to the surface of the brewing vessel. In modern brewing many of the original top fermentation strains have been modified to be bottom fermenters. Currently the *S. carlsbergensis* designation is not used, the *S. cerevisiae* classification is used instead.

The yeast's function in baking is to ferment sugars present in the flour or added to the dough. This fermentation gives off carbon dioxide and ethanol. The carbon dioxide is trapped within tiny bubbles and results in the dough expanding, or rising. Sourdough bread, is not produced with baker's yeast, rather a combination of wild yeast (often *Candida milleri*) and an acid-generating bacteria (*Lactobacillus sanfrancisco* sp. nov). It has been reported that the ratio of wild yeast to bacteria in San Francisco sourdough cultures is about 1:100. The *C. milleri* strengthens the gluten and the *L. sanfrancisco* ferments the maltose.

The fermentation of wine is initiated by naturally occurring yeasts present in the vineyards. Many wineries still use natural strains, however many use modern methods of strain maintenance and isolation. The bubbles in sparkling wines is trapped carbon dioxide, the result of yeast fermenting sugars in the grape juice. One yeast cell can ferment approximately its own weight of glucose per hour. Under optimal conditions *S. cerevisiae* can produce up to 18 percent, by volume, ethanol with 15 to 16 percent being the norm. The sulphur dioxide present in commercially produced wine is actually added just after the grapes are crushed to kill the naturally present bacteria, molds, and yeasts.

The yeastlike fungus, *Candida albicans*, is commonly found in the mouth, vagina, and intestinal tract. *Candida* is a normal inhabitant of humans and normally causes no ill effects. However, among infants and individuals with other illness a variety of conditions can occur. Candidiasis of the mucous membranes of the mouth is known as thrush. Candidiasis of the vagina is called vaginitis. *Candida* also causes severe disease in persons with AIDS and chemotherapy patients.

Saccharomyces cerevisiae

Life cycle

There are two forms in which yeast cells can survive and grow: haploid and diploid. The haploid cells undergo a simple life cycle of mitosis and growth, and under conditions of high stress will generally simply die. The diploid cells (the preferential 'form' of yeast) similarly undergo a simple lifecycle of mitosis and growth, but under conditions of stress can undergo sporulation, entering meiosis and producing a variety of haploid spores, which can go on to mate.

Nutritional requirements

All strains of *Saccharomyces cerevisiae* can grow aerobically on glucose, maltose, and trehalose and fail to grow on lactose and cellobiose. However, growth on other sugars is variable. It was shown that galactose and fructose were two of the best fermenting sugars. The ability of yeasts to use different sugars can differ depending on whether they are grown aerobically or anaerobically. Some strains cannot grow anaerobically on sucrose and trehalose.

All strains can utilise ammonia and urea as the sole nitrogen source, but cannot utilise nitrate since they lack the ability to reduce them to ammonium ions. They can also utilise most amino acids, small peptides and nitrogen bases as a nitrogen source. Histidine, Glycine, Cystine and Lysine are however, not readily utilised. *S. cerevisiae* does not excrete proteases so extracellular protein cannot be metabolized.

Yeasts also have a requirement for phosphorus, which is assimilated as a dihydrogen phosphate ion, and sulfur, which can be assimilated as a sulfate ion or as organic sulfur compounds like methionine and cystine. Some metals like magnesium, iron, calcium, zinc also are required for good growth of the yeast.

Top-fermenting yeast

Saccharomyces cerevisiae is known as top-fermenting yeast, so called because during the fermentation process its hydrophobic surface causes the flocs to adhere to CO_2 and rise to the top of the fermentation vessel. It is one of the major types of yeast used in the brewing of ale, along with *Saccharomyces pastorianus* which is used in the brewing of lager. Top-fermenting yeasts are fermented at higher temperatures than lager yeasts and the resulting ales have a different flavor than the same beverage fermented with a lager yeast. "Fruity esters" may be formed if the ale yeast undergoes temperatures near 70 degrees Fahrenheit, or if the fermentation temperature of the beverage fluctuates during the process.

Lager yeast normally ferments at a temperature of approximately 40 degrees Fahrenheit, where ale yeast becomes dormant. Lager yeast can be fermented at a higher temperature normally used for ale yeast, and this application is often used in a beer style known as "steam beer".

Scientific Classification

Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Saccharomycotina
Class:	Saccharomycetes
Order:	Saccharomycetales
Family:	Saccharomycetaceae
Genus:	*Saccharomyces*
Species:	***S. cerevisiae***

Growth Media

Growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms or cells, or small plants like the moss *Physcomitrella patens*. There are different types of media for growing different types of cells.

There are two major types of growth media: those used for cell culture, which use specific cell types derived from plants or animals, and microbiological culture, which are used for growing microorganisms. The most common growth media for microorganisms are *nutrient broths* and agar plates; specialized media are sometimes required for microorganism and cell culture growth. Some organisms, termed *fastidious organisms*, require specialized environments due to complex nutritional requirements. Viruses, for example, are obligate intracellular parasites and require a growth medium composed of living cells. Basic yeast media is the YPD media.

YPD Medium (an enriched, non-selective yeast growth medium)

1. 10 g Yeast extract
2. 20 g Peptone
3. 20 g Dextrose
4. 1 L dH_2O
5. Adjust pH to 5.8 with approx. 50 µl 12 N HCl
6. Autoclave 30-45 minutes and cool to 65^0 C, then add 50 ml of sterile 40% glucose

YPD plates

Add 20 g agar before autoclaving

Ethanol production

Ethanol

Ethanol, C_2H_5OH, (also called Ethyl Alcohol) is the second member of the aliphatic alcohol series. It is a clear colourless liquid with a pleasant smell. Except for alcoholic beverages, nearly all the ethanol used industrially is a mixture of 95% ethanol and 5% water, which is known simply as 95% alcohol. Although pure ethyl alcohol (known as absolute alcohol) is available, it is much more expensive and is used only when definitely required.

Manufacture

There are two major industrial pathways to ethanol. Ethanol which is intended for industrial use is made by the first method, while ethanol intended for food use tends to be made by the second method.

Reaction of Ethene with Steam

Most of the ethanol used in industry is made, not by alcoholic fermentation, but by an addition reaction between ethene and steam.

$$\underset{\text{Ethene}}{C_2H_4} + \underset{\text{Steam}}{H_2O} \Longrightarrow \underset{\text{Ethanol}}{C_2H_5OH}$$

Alcoholic Fermentation

A solution of sucrose, to which yeast is added, is heated. An enzyme, invertase, which is present in yeast is added and this acts as a catalyst to convert the sucrose into glucose and fructose,

(invertase)

$$C_{12}H_{22}O_{11} + H_2O \quad ==> \quad C_6H_{12}O_6 + C_6H_{12}O_6$$

Sucrose **Glucose, Fructose**

The glucose, $C_6H_{12}O_6$, and fructose, $C_6H_{12}O_6$, formed are then converted into ethanol and carbon dioxide by another enzyme, zymase, which is also present in yeast.

(zymase)

$$C_6H_{12}O_6 \quad ==> \quad 2C_2H_5OH + 2CO_2$$

Glucose **Ethanol**

The fermentation process takes three days and is carried out at a temperature between 250°C and 300°C. The ethanol is then obtained by fractional distillation.

Absolute Ethanol

Whatever method of preparation is used, the ethanol is initially obtained in a mixture with water. The ethanol is then extracted from this solution by fractional distillation.

Although the boiling point of ethanol, 78.3°C, is significantly lower than the boiling point of water, 100°C, these materials cannot be separated completely by distillation. Instead, an azeotropic mixture (i.e. a mixture of 95% ethanol and 5% water) is obtained, and the boiling point of the azeotrope is 78.15°C. In a distillation, the most volatile material (i.e. the material that has the lowest boiling point) is the first material to distill from the distillation flask, and this material is the azeotrope of 95% ethanol which has the lowest boiling point. If an efficient fractionating column is used, there is obtained first 95% alcohol, then a small intermediate fraction of lower concentration, and then water. But no matter how efficient the fractionating column used, 95% alcohol cannot be further concentrated by distillation.

The separation of a mixture by fractional distillation occurs because the vapour has a different composition from the liquid from which it distils (i.e. the vapour is richer in the more volatile component). We cannot separate 95% alcohol into its components by

distillation, because here the vapour has exactly the same composition as the liquid; towards distillation, then, 95% alcohol behaves exactly like a pure compound.

A liquid mixture that has the peculiar property of giving a vapour of the same composition is called an azeotrope (i.e. a constant-boiling mixture). Since it contains two components 95% alcohol is a binary azeotrope. Most azeotropes, like 95% alcohol, have boiling points lower than those of their components, and are known as minimum-boiling mixtures. Azeotropes having boiling points higher than those of their components are known as maximum-boiling mixtures

Oxidation of Ethanol

Ethanol is oxidised

- with acidified Potassium Dichromate, $K_2Cr_2O_7$, or
- with acidified Sodium Dichromate, $Na_2Cr_2O_7$, or
- with acidified potassium permanganate, $KMnO_4$,

Uses

Ethanol is used

- in the manufacture of alcoholic drinks, e.g. Vodka
- as a widely used solvent for paint, varnish and drugs,
- in the manufacture of ethanal, (i.e. acetaldehyde), and ethanoic acid, (i.e. acetic acid),
- as a fuel (e.g. in Gasahol)
- as the fluid in thermometers, and
- in preserving biological specimens.

Immobilization

Immobilization of microbial cells in biological processes can occur either as a natural phenomenon or through artificial process. While the attached cells in natural habitat exhibit significant growth, the artificially immobilized cells are allowed restricted growth. Since the

time first reports of successful application of immobilized cells in industrial applications, several research groups world over have attempted whole-cell immobilization as a viable alternative to conventional microbial fermentations. Various immobilization protocols and numerous carrier materials were tried.

The cell immobilization process has also triggered interest in bioreactor design. Using immobilized cells, different bioreactor configurations were reported with variable success. The study on the physiology of immobilized cells and development of non-invasive measuring techniques have remarkably improved the understanding on microbial metabolism under immobilized state.

The industrial biotechnology processes using microorganisms are generally based on the exploitation of the cells in the fermentation medium during the process. The classical fermentations suffer from various constraints such as low cell density, nutritional limitations, and batch-mode operations with high down times. It has been well recognized that the microbial cell density is of prime importance to attain higher volumetric productivities. The continuous fermentations with free-cells and cell recycle options aim to enhance the cell population inside the fermenter.

However, the free-cell systems cannot operate under chemostatic mode that decouples specific growth rate and dilution rates. During the last 20–25 years, the cell immobilization technology, with its origins in enzyme immobilization, has attracted the attention of several research groups. This novel process eliminates most of the constraints faced with the free-cell systems. The remarkable advantage of this new system is the freedom it has to determine the cell density prior to fermentation. It also facilitates operation of microbial fermentation on continuous mode without cell washout.

The whole-cell immobilization process decouples microbial growth from cellular synthesis of favoured compounds. Since the early 70s, when Chibata's group announced successful o peration of continuous fermentation of l-aspartic acid, numerous research groups have attempted various microbial fermentations with immobilized cells. During these years, over 2500 research papers on various aspects of whole-cell immobilization have been published. Several comprehensive reviews as well as specialized reviews have been published on some important aspects of this field. There are also specialized monograms, and conference

proceedings pertaining to cell immobilization technology, which have excited microbiologists and bioengineers.

Rationale for whole-cell immobilization

Many processes have been practised traditionally, embodying the basic principle of microbial conversions offered by cells bound to surfaces. Waste treatment in trickling filters and ethanol oxidation to produce vinegar are but a few examples of such processes. Immobilization of cells is the attachment of cells or their inclusion in distinct solid phase that permits exchange of substrates, products, inhibitors, etc., but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. Therefore it is expected that the microenvironment surrounding the immobilized cells is not necessarily the same experienced by their free-cell counterparts.

Immobilization commonly is accomplished using a high molecular hydrophilic polymeric gel such as alginate, carrageenan, agarose, etc. In these cases, the cells are immobilized by entrapment in the pertinent gel by a drop-forming procedure. When traditional fermentations are compared with the microbial conversions using immobilized cells, the productivity obtained in the latter is considerably higher, obviously partly due to high cell density and immobilization-induced cellular or genetic modifications. Nevertheless, a few critical parameters such as the cost of immobilization, mass transport limitations, applicability to a specific end-product, etc. are to be carefully examined before choosing any particular methodology.

The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes. It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. The ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very high dilution rates, are few of the many advantages of immobilized cell systems.

The metabolically active cell immobilization is particularly preferred where co-factors are necessary for the catalytic reactions. Since co-factor regeneration machinery is an integral function of the cell, its external supply is uneconomical. There is considerable evidence to indicate that the bound-cell systems are far more tolerant to perturbations in the reaction environment and similarly less susceptible to toxic substances present in the liquid medium. The recent reports on higher retention of plasmid-bearing cells have further extended the scope of whole-cell immobilization to recombinant product formation. Another important advantage of immobilization, particularly in the case of plant cells, is the stimulation of secondary metabolite formation and elevated excretion of intracellular metabolites.

Immobilization methods

Many methods namely adsorption, covalent bonding, crosslinking, entrapment, and encapsulation are widely used for immobilization. These categories are commonly used in immobilized enzyme technology. However, due to the completely different size and environmental parameters of the cells, the relative importance of these methods is considerably different. The criteria imposed for cell immobilization technique usually determine the nature of the application.

Adsorption

This was apparently the first example of cell immobilization. Hattori and Furusaka reported the binding of *Escherichia coli* cells on to an ion exchange resin. Subsequently, a variety of microbial cells were immobilized by adsorption on different supports like kieselguhr, wood, glass ceramic, plastic materials, etc. Klein and Ziehr have reviewed the immobilization of microbial cells by adsorption. Since the adsorption phenomenon is based on electrostatic interactions (Van Der Waal's forces) between the charged support and microbial cell, the actual zeta potential on both of them plays a significant role in cell-support interactions. Unfortunately, the actual charge on support surfaces is still unknown and this limits the proper choice for microbial attachment. Along with charge on the cell surface, the composition of cell wall carrier composition will also play a predominant role. Cells of *Saccharomyces cerevisiae* and *Candida utilis* contain a -mannans in the cell wall. The cells of latter have a strong affinity to Cancanavalin-A-activated carrier. Carrier properties, other than zeta potential, will also greatly influence cell-support interaction. All glasses or ceramic

supports are comprised of varying proportions of oxides of alumina, silica, magnesium, zirconium, etc. which result in bond formation between the cell and the support. Several procedures of cell adsorption based on pH dependence are reported in the literature.

Covalent bonding

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is necessary. Cells of *S. cerevisiae* were immobilized by coupling silinized silica beads. The reaction requires introduction of reactive organic group on inorganic silica surface for the reaction between the activated support material and yeast cells. a -amino propyl triethoxy silane is generally used as the coupling agent. This inorganic functional group condenses with hydroxyl group on silica surface. As a result, the organic group is available for covalent bond formation on the surface of silica. Covalent bonding can also be achieved by treating the silica surface with glutaraldehyde and isocyanate.

A system of more general interest has been developed by Kennedy and Cabral, using inorganic carrier system. The addition of Ti^{4+} or Zr^{4+} chloride salts to water results in pH-dependent formation of gelatinous polymeric metal hydroxide precipitates wherein the metals are bridged by hydroxyl or oxide groups. By conducting such a precipitation in a suspension of microbial cells, the cells have been entrapped in the gel-like precipitate formed. In continuous operation, titanium hydroxide-immobilized cells of *Acetobacter* were employed to convert alcohol to acetic acid.

Cross linking

Microbial cells can be immobilized by cross-linking each other with bi- or multifunctional reagents such as glutaraldehyde, toluenediisocyanate. The toxicity of the chemicals used for cross-linking obviously imposes limitations for the general applicability of these procedures. Apart from chemical cross-linking, procedures employing physical processes, such as flocculation and pelletization, also benefit the immobilization techniques because of strong mutual adherence forces of some microbial cell cultures.

Entrapment

The most extensively studied method in cell immobilization is the entrapment of microbial cells in polymer matrices. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. Among the above matrices, polyacrylamide has been widely used by several workers. This gel was first used for immobilization of enzymes. Later this technique was successfully applied to immobilization of lichen cells.

As a rule, the entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate. The precise mode of entrapment of cells in polyacrylamide is critical for satisfactory retention of activity. The factors affecting the gel preparation are the content of acrylamide, the ratio of cells to acrylamide, and the size of the gel particles. While the former two influence the hardness of the resulting gel and the pore size of the microlattice in which the cells are entrapped, the third factor determines the activity, stability, and the pressure drop when packed in a reactor. The other procedures for network formation for cell entrapment are precipitation, ion exchange gelation, and polymerization . The precipitation techniques are exemplified by collagen, cellulose and carrageenan. By changing a parameter such as temperature, salinity, pH, etc. polymer precipitate can be prepared from a homogeneous solution of linear chain polymers. The networks are primarily formed by precipitation with salt solutions, ones that are constituted by secondary valency forces ranging from dispersion to hydrogen bonding. Network formation procedures where the cross-linking is established by ionic bonds between linear polyelectrolytes and multivalent cations have been extensively tried.

Entrapment of cells in alginate gel is popular because of the requirement for mild conditions and the simplicity of the used procedure. Several reports are available employing alginate gel. K-carrageenan is one of the earliest gel materials used for cell immobilization for continuous production of l-lactic acid by *Escherichia coli*. The immobilization procedure is similar to alginate, and several other groups have used this polysaccharide as a preferred gel matrix either alone or in combination with other gums because of the mild conditions required and good gel stability.

Similarly several other natural polymers such as agar, agarose, pectin and gelatin were also employed for cell immobilization. Casteillo employed gel as a carrier material for the immobilization of *Kluyveromyces fragilis* for b -galactadase activity and *E. coli* for penicillin acylase. The authors produced fibres instead of beads by direct extrusion in cold water. The fibres were further strengthened by treatment with 1.25% glutaraldehyde solution for 45 min.

The reversible network formed was affected by certain calcium-chelating agents like phosphates, Mg^{2+}, K^{+} and EDTA and the gel integrity was poor. In ethanol fermentation, where large quantities of CO_2 are produced, the gel spheres disintegrate due to CO_2 pressure in the bed. Many variations in the immobilization procedure were tried.

Some research groups in Japan have developed a novel immobilization procedure involving photo cross linkage resins. Nojima has reported glycolic cross linking with polyethylene. A prominent feature of such resins is that not only cells but also enzymes can readily be immobilized in their three-dimensional matrix, the size of which can be changed freely by adjusting the degree of polymerization of the polyethylene glycol molecules located between the two isophorone isocyanate molecules. Immobilized cells are produced by irradiating a mixture of such cells and the resin, using 300–400 nm wavelength from a lowpressure mercury lamp. By this method immobilized cell sheets of 50 cm width are prepared under aseptic conditions.

Modified methods of cell immobilization

Entrapment of microbial cells within the polymeric matrices is preferred by many researchers. Among the various methods, alginate gels have received maximum attention. There are several studies on the composition of alginate and their suitability for cell immobilization. In the recent years, the diffusional characteristics of the immobilized system are being studied to enhance our understanding on the microenvironment prevailing near the immobilized cells, to optimize the immobilization protocols, and to improve the stability of the gel beads by modifying the protocols.

According to Vorlop, drying of immobilized cell sphere greatly increases the compression stress. In certain cases the beads are hardened by glutaraldehyde treatment. Coating of immobilized biocatalyst gel particle with catalyst-free polymer is expected to be effective

toenhance the stability. This could either be carried out by single-step gelation of the double-layered droplet using twin-nozzle, or by two-step procedure. Yamagiwa tried the two-step preparation procedure by recoating the calcium alginate cell beads by plain alginate as a double layer to enhance the gel stability.

Gameiner carried out a comparative evaluation of both the gels and observed that pectate gel is less sensitive to chelating anions and chemical agents, and hence the mechanical stability improved considerably. The use of polyvinylalcohol (PVA) as a gel matrix has received attention in the recent past. Reports are available on the immobilization of various microbial cells in PVA either by freezing and thawing technique or gelling in saturated boric acid.

Incorporation of additional component into the gel matrix to improve the mechanical strength has been tried. Several components such as silica, sand, alumina, and various gums are generally used. In addition, the gel particles are further strengthened by treating with various cross-linking agents, such as glutaraldehyde.

APPLICATIONS OF IMMOBILIZATION

- Antibiotic production by immobilized microbial cells
- Organic acids production by immobilized cells
- Production of enzymes by immobilized cells
- Production of alcohols by immobilized cells
- Biotransformations by immobilized microbial cells

Co-immobilization

Though cell immobilization technology is mainly confined to potential monocultures, it is prudent to examine the benefits of mixed cultures under immobilized state to accelerate the fermentation processes.

There are several reports of co-immobilization of two or more cultures to derive the benefit of both cultures under monoculturing state. For example, direct alcoholic fermentation of soluble starch and dextrin uses amylotic yeasts *Saccharomyces diastaticus*, *Schwanniomyces castellii,* and *Endomycopsis fibuligera*.

Reilly and Scott reviewed several cases of mixed-culture fermentations to derive various biochemicals. Dincbas co-immobilized mixed cultures of plasmid-free and plasmid-containing *E.coli* HB101 in alginate matrix to study the stability of plasmid for long-time cultivation. This study has indicated that the stability of the plasmid-recombinant cells was enhanced in co-immobilized state. The co-immobilization of mixed cultures of algae and aerobic bacteria by encapsulation has solved the problems of oxygen limitation under high cell density conditions in the hydrogels.

Wikstrom have co-immobilized *Chlorella vulgaris* with *Providencia* sp. in agarose, and employed them in a photoreactor for the production of a -keto-isocaproic acid from l-leucine. It has been proved that the algae in the gel matrix acted as an *in situ* oxygen generator. In a similar study, *Cephalosporiumacrymonium*, an oxygen-consuming fungus, and *Chlorella pyrenoidosa*, an oxygen-generating alga, were co-immobilized for the cephalosporin C production. It was noticed that the co-immobilized microbial system enhanced the production of cephalosporin C by 370%. Similar co-immobilization protocols were worked out for *in situ* enzyme regeneration, complementation, multi-step biotransformation, exploitation of oxygen gradient in the polymer gel and photo-production of hydrogen.

Emerging trends

Whole-cell immobilization as a tool to intensify microbiological processes has been well established. Several examples of production of a variety of bio-chemicals by immobilized cells have been successfully demonstrated. Though initially our knowledge on physiology of immobilized cells was limited and hypothetical, the use of microelectrodes and development of non invasive techniques to study the immobilized cells under microenvironment have revealed significant information pertaining to metabolic structural alterations occurring in the cell under immobilized phase.

One of the difficulties experienced to evaluate various carriers, process conditions, and operating conditions is the non uniformity of reporting the information. For example, the volumetric productivity of the bioreactor system with immobilized cells can be determined by considering total volume of the reactor, or the void volume. But researchers calculate and report either one of them, which is sometimes misleading. It is necessary to evolve a common

protocol to assess the performance of a given system. Though a variety of carrier materials have been tried, there are very few reports comparing these in terms of their performance, long-term stability, and cost. The observations made with immobilized cells and altered morphology indicate the influence of anchorage on cell metabolism.

Perhaps this may lead to a separate study of solid state fermentation, which can be considered as microbial proliferation on solid surfaces, and its influence on bioprocess acceleration in some cases.

An important area of research requiring greater focus is the bioreactor design and its long-term operation. Except for a couple of experimental ventures, most of the experiments have been carried out on a very small scale, and hence very difficult to scale up. The future research should centre around not only for developing feasible microbiological processes with immobilized cells but also for carrying out extensive research in bioreactor design to solve some of the engineering problems, especially the ones that are connected with diffusional limitations. It is important to generate adequate data with larger systems for longer times to enable the design engineers to translate these results into commercial realities. It is also very important that future research should focus on microbial physiology under immobilized state to enrich our knowledge on process intensification. The recent reports on enhanced plasmid stability of genetically engineered microorganisms under immobilized conditions, and the viability of microbial cells over a period of 18 months under entrapped conditions, are few of the many potential new applications of immobilized cells.

CHAPTER 2

METHODS AND MATERIALS

Introduction

Revival of culture

The culture of *S.cerevisiae* is revived from the glycerol stock. This is done using liquid YPD media: Dissolve 10g of Yeast extract in 500ml water. Dissolve 20g of Peptone in the above solution. Dissolve 20g Dextrose in the above solution .Increase to 1000ml with water. Autoclave (for plates add 20g Agar).

Fig.1 revival of culture

Immobilization

Calcium alginate method

List of Reagents and Instruments

A. **Equipment:** balance, beakers, pipette, syringe and needle, spectrophotometer, magnetic stirrer

B. Reagents: growth medium, sodium alginate, calcium chloride, yeast culture

Immobilized cell preparation

Dissolve 1.5 g of sodium alginate in 50 ml of growth medium. Stir until all sodium alginate is completely dissolved. The final solution contains 3% alginate by weight. Thoroughly suspend about 8 g of wet cells in the alginate solution prepared in the previous step. Let air bubbles escape. Drip the yeast-alginate mixture from a height of 20 cm into 50 ml of crosslinking solution. (The crosslinking solution is prepared by adding an additional 0.05M of $CaCl_2$ to the growth media). The calcium crosslinking solution is agitated on a magnetic stirrer. Gel formation can be achieved at room temperature as soon as the sodium alginate drops come in direct contact with the calcium solution. Relatively small alginate beads are preferred to minimize the mass transfer resistance. A diameter of 0.5-2 mm can be readily achieved with a syringe and a needle. The beads fully harden in 1-2 hours.

Solutions used

- 50 ml sodium alginate solution (1.5g of sodium alginate in 50ml media)
- 50 ml crosslinking solution (.05M of calcium chloride in 50ml media)
- Cells used = 8 ml wet weight
- Media use – YPD media

Fig.2 immobilization of yeast cells

Fermentation :The fermentation process takes three days and is carried out at a temperature between 25^0C - 30^0C. The ethanol is then obtained by fractional distillation. Anaerobic conditions obtained by incubating at 27^0C in electrical incubator. No shaking is used and the flask is made airtight using cotton plugs and further covering it up with aluminum foil. The conditions are maintained until the whole process is completed. The culture is kept for three days after bubbling starts and after that the samples are collected.

Reuse of immobilized cells

Once the initial samples were collected, the remaining media was drained out and 100 ml of fresh media was added. The same steps and conditions of fermentation were followed as earlier. Sample was collected. Estimation of ethanol was carried out

Ethanol estimation

Potassium dichromate method

Alcohol estimation is done using potassium dichromate which acts as an oxidising agent. this oxidises primary alcohol to give carboxylic acid and aldehyde.

On reacting with secondary alcohols it gives ketones.

After the reaction, the orange color due to potassium dichromate changes to green.

Plotting the standard graph

Take (2,4,6,8) ml of absolute ethanol in different flasks and increase the volume of each flask to 100ml using distilled water. Take 1ml of each solution separately in aliquot and add 29ml of distilled water. Distill each sample at 78.5^0 C(bp of ethanol) and collect the distillate in a flask containing 25ml of potassium dichromate .Collect 25ml from the above flask (containing potassium dichromate and distillate).Incubate 25ml of each sample at 62.5^0 C. for 20 minutes in water bath.Cool the solution and mix with water in 1:1 ratio. Measure absorbance of sample at 600nm. Plot the standard curve.

S NO.	ALCOHOL % (ABSOLUTE)	AMOUNT (ML)	AMOUNT OF DW (ML)	TEMP. FOR DIST (^{0}C)	INCUBATION (^{0}C)	5 ML DISTILLATE AND 5 ML WATER	O.D AT 600NM
1	2	1	29	78.5	62.5 FOR 20 MINS		
2	4	1	29	78.5	62.5 FOR 20 MINS		
3	6	1	29	78.5	62.5 FOR 20 MINS		
4	8	1	29	78.5	62.5 FOR 20 MINS		
5	10	1	29	78.5	62.5 FOR 20 MINS		

Table 1. Standard plot

For the ethanol samples collected, 1ml of each sample was taken and the procedure was repeated.

Optimization of factors

For sugar content

In YPD production media different concentrations of sugar were added as according to the table:

Sno.	Yeast (ml wet wt)	Peptone(g)	Dextrose(g)	Water(ml)
1	8	2	2	100
2	8	2	1.5	100
3	8	2	2.5	100
4	8	2	3	100
5	8	2	3.5	100
6	8	2	4	100
7	8	2	4.5	100

Table 2 Optimization of sugar content

The cultures were allowed to grow under same circumstances.

Amount Of Yeast

Different concentrations of yeast were added to the YPD media in accordance to the following table:

Sno	Yeast(ml wet wt)	Peptone(g)	Dextrose(g)	Water(ml)
1	8	2	2	100
2	9	2	2	100
3	10	2	2	100
4	11	2	2	100
5	12	2	2	100
6	13	2	2	100

Table 3 Optimization of amount of yeast

All cultures were kept under similar conditions.

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CHAPTER 3

RESULTS AND DISCUSSIONS

S NO.	ALCOHOL % (ABSOLUTE)	AMOUNT (ML)	AMOUNT OF DW (ML)	TEMP. FOR DIST (^{0}C)	INCUBATION (^{0}C)	5 ML DISTILLATE AND 5 ML WATER	O.D AT 600NM
1	2	1	29	78.5	62.5 FOR 20 MINS		0.129
2	4	1	29	78.5	62.5 FOR 20 MINS		0.293
3	6	1	29	78.5	62.5 FOR 20 MINS		0.281
4	8	1	29	78.5	62.5 FOR 20 MINS		0.629
5	10	1	29	78.5	62.5 FOR 20 MINS		--------

Table 4. Standard Readings

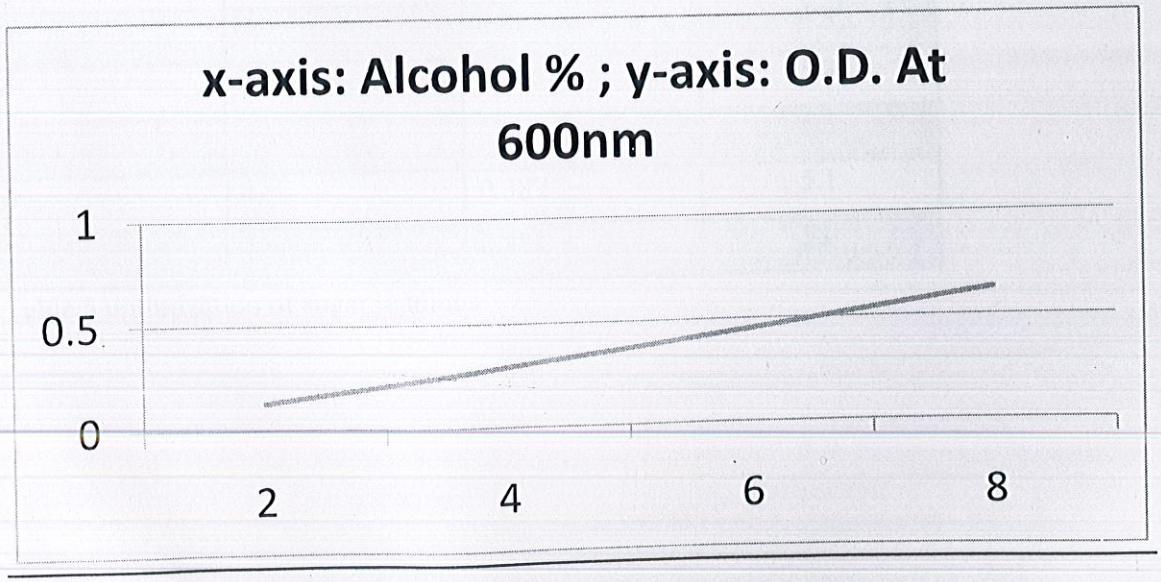

Fig 3. Standard graph

READINGS USING STANDARD GRAPH

- Free cells O.D = 0.311

  ethanol produced = 4.3%

- Immobilized cells O.D = 0.267

  ethanol produced = 3.4%

- Reused immobilized cells O.D = 0.223

  ethanol produced = 2.8%

OPTIMIZATION OF SUGAR CONTENT

Readings using standard graph plot

Sno	Dextrose(g)	O.D at 600nm	% ethanol content
1	2	0.265	3.4
2	1.5	0.220	2.6
3	2.5	0.323	4.4
4	3	0.396	5.3
5	3.5	0.402	5.5
6	4	0.387	5.1
7	4.5	0.381	5.0

Table 5 optimization of sugar readings

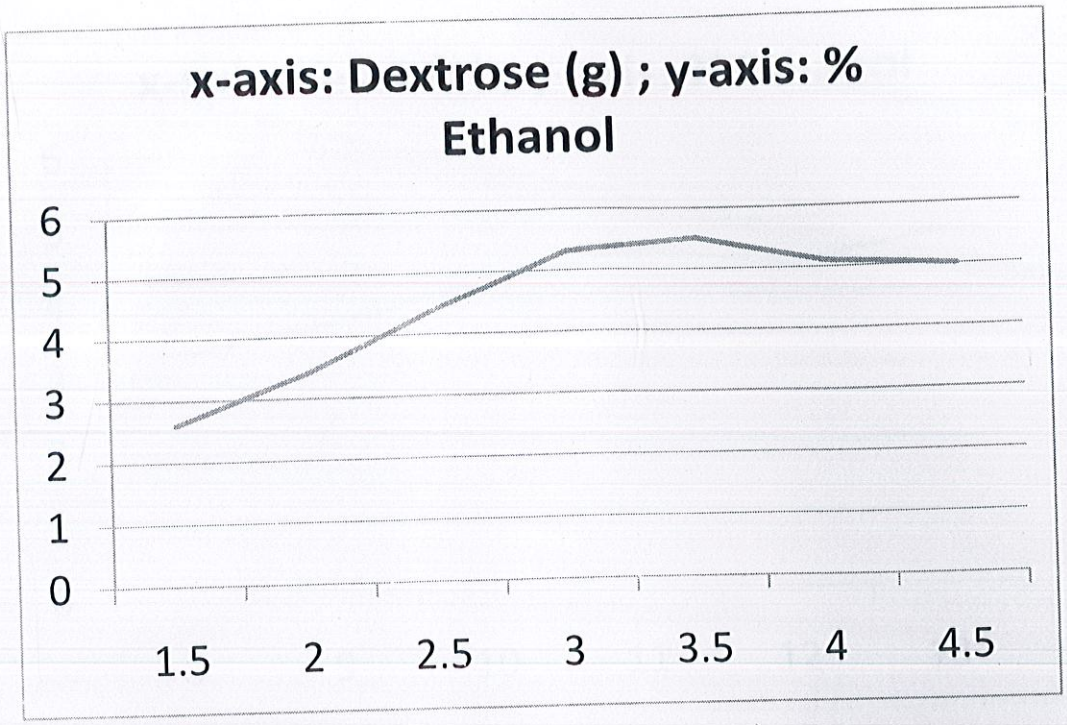

Fig.4.optimization of sugar

OPTIMIZATION OF AMOUNT OF YEAST CELLS

Sno	Yeast(g)	O.D at 600nm	% ethanol content
1	8	0.245	3.3
2	9	0.273	3.7
3	10	0.321	4.2
4	11	0.334	4.6
5	12	0.325	4.4
6	13	0.364	4.8

Table 6 optimization of yeast

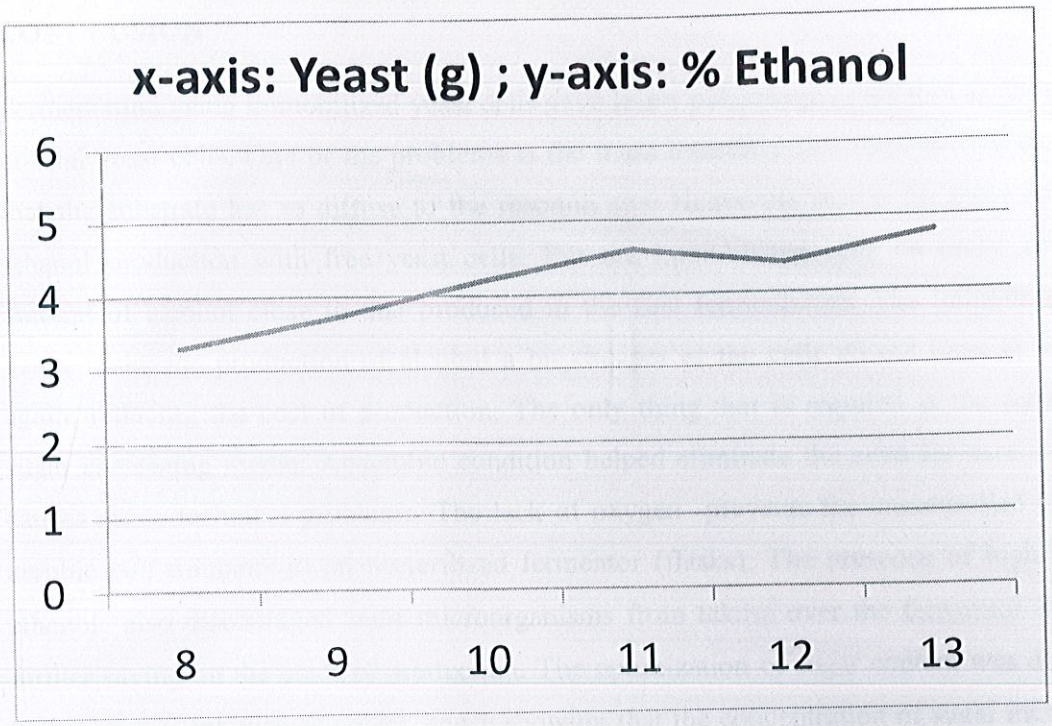

Fig.5.optimization of yeast

The optimum sugar content for ethanol production came out to be 3.5g in 100ml media.

The optimum amount of yeast was not found as the amount of ethanol produced kept on increasing.

CONCLUSION

Fermentation using immobilized yeast cells gave lesser percentage of alcohol as compared to normal yeast cells. One of the problems is the mass transfer resistance imposed by the fact that the substrate has to diffuse to the reaction site. Hence the reason for more amount of ethanol production with free yeast cells. But the immobilized cells on reuse gave some amount of alcohol close to that produced in the first fermentation. The immobilized cells hence make the production of alcohol a bit cheaper as the cells do not have to be grown again, reducing the cost of production. The only thing that is required is the substrate i.e. sugar containing media. Anaerobic condition helped eliminate the need for aeration, which causes many technical problems. The lack of oxygen prevents the uncontrolled growth of aerobic contaminants in an unsterilized fermentor (flasks). The presence of high levels of ethanol also discouraged most microorganisms from taking over the fermentor and hence further saving on the costs of production. The optimization of sugar content was done and a bell shaped graph was achieved, hence showing that the concentration of sugar increases the fermentation process to an extent but if in excess it leads to the decrease in production of ethanol. Optimized conditions help us achieve the maximum amount of ethanol from a sugar source and hence adding to the industrial benefits of the procedure. On the other hand the amount of yeast on increasing showed increased productions of ethanol.

Therefore, it can be concluded that the production of ethanol from immobilized cells if scaled up can be of great use to the industry.

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