Semester – V

17MBU514A MICROBIAL BIOTECHNOLOGY - PRACTICAL (3H – 1C)

Instruction Hours / week: L: 0 T: 0 P: 3 Marks: Internal: **40** External: **60** Total: **100**

End Semester Exam: 6 Hours

SCOPE

To impart knowledge on applications of microorganisms in various fields.

OBJECTIVE

- To make students understand the aspects of industrial, soil, environmental, agricultural microbiology.

EXPERIMENTS

1. Study yeast cell immobilization in calcium alginate gels.
2. Study enzyme immobilization by sodium alginate method.
3. Pigment production from fungi (*Trichoderma* / *Aspergillus* / *Penicillium*).
4. Isolation of xylanase or lipase producing bacteria.
5. Study of algal Single Cell Proteins.

SUGGESTED READINGS

1. Ratledge, C and Kristiansen, B. (2001). Basic Biotechnology, 2nd edition, Cambridge University Press.
2. Demain, A. L and Davies, J. E. (1999). Manual of Industrial Microbiology and Biotechnology, 2nd edition, ASM Press.
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4. Opinion in Biotechnology, 12, 195–201.
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7. Glazer AN and Nikaido H (2007) Microbial Biotechnology, 2nd edition, Cambridge University Press.
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9. Stanbury PF, Whitaker A, Hall SJ (1995) Principles of Fermentation Technology 2nd edition, Elsevier Science.
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Experiment 1

IMMOBILIZATION OF YEAST CELLS BY CALCIUM-ALGINATE ENTRAPMENT METHOD

Introduction

The term 'immobilization' was first proposed at the first enzyme engineering conference in 1971. Immobilization often causes a dramatic change in the apparent measuring parameter of the enzyme catalyzed by Michalis – Menton constant, temperature optima, pH optima, and effect of inhibitors may be changed when an enzyme in immobilized. The degree and nature of these changes not only depends on the immobilization but also on the enzyme reaction.

There are various methods available for immobilization of enzyme

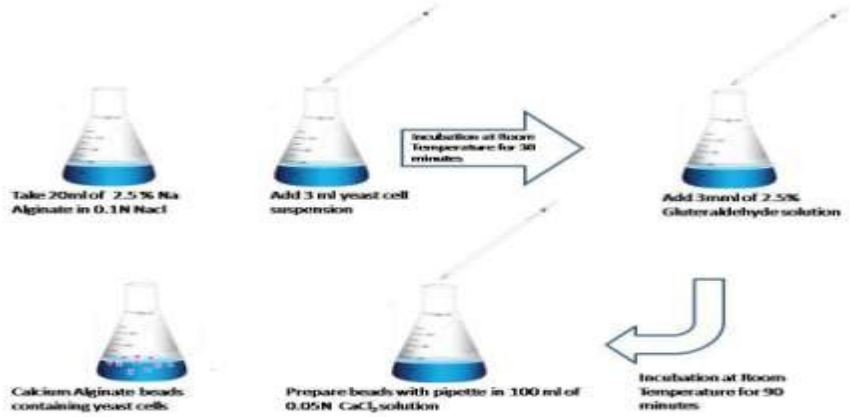
- Absorption
- Covalent binding
- Cross matching
- Micro encapsulation
- Polymerization
- Gel entrapment

Advantages of enzyme- immobilization

- Stability of the enzyme immobilization increases even in adverse condition.
- Resistance of enzyme molecules against metal ions and other inhibitors can also be increased.
- Enzyme can be used repeatedly and continuously for the conversion of substrate into products.

- In immobilized condition enzyme can be stored for longer time.

Procedure for the preparation of immobilized cell in calcium alginate



Requirement

- Yeast potato Dextrose (YPD)/ Potato Dextrose Agar (PDA) medium (for cultivation of Yeast cells)
- Sodium alginate solution (2.5% w/v in 0.1% NaCl)
- Calcium Chloride ($CaCl_2$) solution (0.05 N)
- Gluteraldehyde (2.5% v/v)
- Sucrose solution (1% w/v)

Procedure for immobilization of Yeast Cells

- Grow the yeast cells in YPD/PDA medium
- Keep it in shaker for 24hours at 100 rpm
- Filter out the yeast cells with the help of Whattman filter paper
- Take 20 ml sodium alginate solution and add 3 ml yeast cell in it. Mix properly and incubate at room temperature for 30 minutes then add 3 ml of gluteraldehyde solution incubate at room temperature for 90 minutes With the help of 10 ml pipette, drop wise add this mixture into the beaker containing 100 ml $CaCl_2$ solution

- Filter out the beads with the help of normal filter paper
- Wash the beads 2-3 times with sterile Distilled water
- Load these beads into thoroughly washed glass column/ beaker
- Add 50ml of sterile 1% sucrose solution
- After every 30 minutes interval collect the 1 ml of sample and estimate the amount of glucose by using Dinitrosalilcylic acid method (DNSA) method

Procedure for Estimation of Glucose by Dinitrosalilcylic acid method (DNSA) method

- Prepare a standard solution of carbohydrate (here glucose) having concentration of 1.0 mg/ml
- Take different volumes of glucose solution like 0.5, 1.0, 1.5 & 2.0 ml etc. into various tubes previously labeled as S_1, S_2, S_3, S_4 etc. respectively
- One tube should be labeled as blank
- Now, take three tubes and labels as U1, U2 & U3 & pipette out 3 same volume of sucrose solution from reaction mixture from above experiment in this tube
- Add distilled water in all tubes in such a way that the total volume will be 2.0 ml

- Add 2.0 ml of DNSA reagent in all tubes. Mix it properly by reversing the tubes or by using magnetic stirrer
- Keep all the tubes in boiling water bath for 10 minutes. Then allow it to cool down
- Take absorbance at 540 nm (using green filter) and plot a standard curve
- Calculate out the concentration of glucose produced in the reaction mixture of above experiment

Result

Increase in the concentration of glucose from the sucrose with respect totime by Active yeast cell (Invertase) indicates that Yeast Cells immobilized and they are viable

Reference

- *Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino& Natalie Sherman*
- *Bacteriological Techniques By F.J. Baker*
- *Introduction to Microbial Techniques By Gunasekaran*
- *Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold ; . Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu*

Experiment 2

ENZYME IMMOBILIZATION PROTOCOL

ENTRAPMENT IN ALGINATE GEL

Aim: Immobilization of enzyme using sodium alginate (Preparation of strong gel using entrapment

Method

Alginate, commercially available as alginic acid, sodium salt, commonly called sodium alginate, is a linear polysaccharide normally isolated from many strains of marine brown seaweed and algae, thus the name *algi*nate. The copolymer consists of two uronic acids: D-mannuronic acid (M) and L-guluronic acid (G). Because it is the skeletal component of the algae it has the nice property of being strong and yet flexible.

Alginic acid can be either water soluble or insoluble depending on the type of the associated salt. The salts of sodium, other alkali metals, and ammonia are soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium. The alginate polymer itself is anionic (i.e., negatively charged) overall. Polyvalent cations bind to the polymer whenever there are two neighboring guluronic acid residues. Thus, polyvalent cations are responsible for the cross-linking of both different polymer molecules and different parts of the same polymer chain. The process of gelation, simply the exchange of calcium ions for sodium ions, is carried out under relatively mild conditions. Because the method is based on the availability of guluronic acid residues, which will not vary once given a batch of the alginate, the molecular permeability does not depend on the immobilization conditions. Rather, the pore size is controlled by the choice of the starting material.

$2\ Na(Alginate) + Ca^{++} \text{-------}> Ca(Alginate)_2 + 2\ Na^{+}$

The ionically linked gel structure is thermostable over the range of 0-100ºC; therefore heating will not liquefy the gel. However, the gel can be easily redissolved by immersing the alginate gel in a solution containing a high concentration of sodium, potassium, or magnesium. Maintaining sodium:calcium <= 25:1 will help avoid gel destabilization. In fact, it is recommended by alginate vendors to include 3mM calcium ions in the substrate medium. On the other hand, citrate or phosphate pH buffers cannot be effectively used without destabilizing the alginate gel.

Alginate is currently widely used in food, pharmaceutical, textile, and paper products. The properties of alginate utilized in these products are thickening, stabilizing, gel-forming, and film-forming. Alginate polymers isolated from different alginate sources vary in properties. Different algae, or for that matter different part of the same algae, yield alginate of different monomer composition and arrangement. There may be sections of homopolymeric blocks of only one type of monomer (-M-M-M-) (-G-G-G-), or there may be sections of alternating monomers (-M-G-M-G-M-). Different types of alginate are selected for each application on the basis of the molecular weight and the relative composition of mannuronic and guluronic acids. For example, the thickening function (viscosity property) depends mainly on the molecular weight of the polymer; whereas, gelation (affinity for cation) is closely related to the guluronic acid content. Thus, high guluronic acid content results in a stronger gel.

List of Reagents and Instruments

A. Equipment

- Beakers
- Graduated cylinder
- Balance
- Pipets
- Syringe

B. Reagents

- Alginic acid, sodium salt

- $CaCl_2$
- Enzyme

Procedures

1. Dissolve 30g of sodium alginate in 1 liter to make a 3% solution. See Note 1.
2. Mix approximately 0.015 g of enzyme with 10 ml of 3% (wt.) sodium alginate solution. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness. See Note 2.
3. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M $CaCl_2$solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 0.5-2 mm in diameter. Other shapes can be obtained by using a mold whose wall is permeable to calcium ions. Leave the beads in the calcium solution to cure for 0.5-3 hours.

Notes

1. Sodium alginate solution is best prepared by adding the powder to agitated water, rather than vice versa, to avoid the formation of clumps. Prolonged stirring may be necessary to achieve the complete dissolution of sodium alginate. After sodium alginate is completely dissolved, leave the solution undisturbed for 30 minutes to eliminate the air bubbles that can later be entrapped and cause the beads to float.
2. Although not necessary, the beads may be hardened by mixing some amines in the sodium alginate solution and cross-linking with glutaraldehyde.

Discussions

Because of the mild conditions needed for gelation, calcium alginate is also widely used for cell immobilization.

References

1. S. Ohlson, P.-O. Larsson, and K. Mosbach, Steroid transformation by living cells immobilized in calcium alginate, *European J. Appl. Microbiol. Biotechnol.,* **7,** 103, 1979.
2. J. Vaija, et al., *Appl. Biochem. Biotechnol.,* **7,** 51, 1982.
3. J. M. Lee and J. Woodward, *Biotech. Bioeng.,* **25,** 2441, 1983.

Experiment 3

PIGMENT PRODUCTION FROM FUNGI

Aim:

Pigment Production from Fungi (*Aspergillus* / *Penicillium*).

Introduction

There is an ever growing demand of eco-friendly/ non toxic dyes especially for food colours and child textiles. Natural dye producing microbes offer a viable alternative to natural vegetable and harmful synthetic dyes. Fungi are reported as potential biological source of natural pigments like anthraquinones.

Coloured substances known as dyes are used to impart colour to variety of material. The use of such synthetic dyes in dying industry results in dye containing waste water which increases the environmental pollution. Some of these dyes have potential potent carcinogen posing serious health hazard.The use of food colorants as additives in the food industry is a significant factor for both food manufacturers and consumers in determining the acceptability of processed food,

Many consumers are likely to be unaware of the exotic sources of some of the currently authorized so-called natural colorants. Fungi are reported as potent pigment producing microorganisms (Babitha et al., 2007). The importance of pigments such as anthraquinone, anthraquinone carboxylic acids, pre-anthraquinones extracted from filamentous fungi are already known. The application of these fungal pigments in dyeing of cotton, silk and wool has been reported in several studies.

Material and Methods

Isolation and identification of fungi

Collect the Sample from domestic fridge.

Purify the isolates store on PDA as spore stock at -20 C for further studies.

Identification can be done microscopically by slide culture method and wet mount.

Cultivation, extraction and purification of fungal pigments

Cultivate the isolated fungi individually on defined mineral salts- glucose medium contains (per liter of deionized water): glucose 30 g; 1.0 g (NH4)2SO4; 0.5 g MgSO4.7H2O; 1.4 g K2HPO4; 0.6 g KH2PO4; 0.8 mg ZnSO4.H2O; 0.8 mg FeCl3.6H2O; 0.8 mg NaMoO4.2H2O; 0.4 mg MnSO4 2H2O; 0.08 mg CuSO4.5H2O; and pH 5.6 in flask.

Iinoculate all flasks by a mycelial disk by using cork borer (12 mm diameter) from PDA culture with isolates .

Keep it for growth at 37º C in the dark as stationary cultures for 4 6 week .

After an incubation period of 6 week, the mycelium can be harvested, and the supernatant can be filtered in a sterilized Whatt man s filter paper. Later, two volumes of 95% (v/v) ethanol was added to culture broth according to the following procedure:

(i) After dilution with about 60% of the solvent volume needed, the resulting mixture can be kept on the rotary shaker at 180 rpm at 30 C for 30 min

(ii) The ethanolic mixture was centrifuged at 3780 rpm for 15 min

(iii) Once the supernatant had been recovered, the residue was dispersed in the remaining volume of ethanol and centrifuged again at 3780 rpm for 5 min;

Result

The collect the upernatants and filtere through a pre weighed Whattman s filter paper (47 mm) and further diluted with 95% (v/v) ethanol to a final volumetric dilution factor of 20.

1. Observe the absorption spectrum between 300 600 nm using spectrophotometer.

2. Yield is calculated by: E= A/CLr

3. Fourier Transform Infrared spectroscopy analysis for pigment.

Experiment 4

ISOLATION OF LIPASE PRODUCING BACTERIA FROM OIL CONTAMINATED SOIL

Aim : To screen the lipolytic fungi from oil contaminated soil

Introduction

Lipases are the enzymes capable of catalysing the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids (Sharma et al., 2001; Svendsen et al., 2000). Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Only about 2% of world's microorganisms have been tested as enzyme sources. Microbial lipases are produced mostly by submerged culture (Ito et al., 2001), but solid-state fermentation methods can be also used (Chisti, 1999). In general, solid-state fermentation is a well-adapted and cheaper process than submerged fermentation for the production of a wide spectrum of bioproducts (animal feed, enzymes, organic acids, biopulp, aroma compounds, antibiotics, compost, biopesticide, biofertilizer etc). Solid state fermentation is a high recovery method for the production of industrial enzymes (Pandey et al., 1999).

Materials and methods

Sample collection for isolation of lipase producing organism, soil sample was collected from 4-5 cm depth with help of sterile spatula in a sterile plastic bag from the vicinity of Vellore petrol bunks in Tamilnadu. Screening of microorganism After collection, sample was brought to the laboratory and 1 g of

sample was suspended in 100 ml of sterile distilled water, agitated for 30 min on a shaker at 500 C and 0.2 ml was spread on nutrient agar plates and incubated at 300 C for 24-48 hours. Enriched sample was used for plating to get only lipolytic isolates thus enriched samples was plated containing (gm/lit): beef extract 3.0, peptone 5.0, sodium chloride 5.0, agar 15.0, calcium chloride 0.05, and glycerol tributyrate 0.2ml, after incubation for 24 h a total of 10 colonies showing clear zone were picked. One isolate OCR-02 which showed maximum activity was selected and maintained on tributyrin agar slant at 40 C. The culture was examined for various morphological and biochemical characteristics as per Bergey' s Manual of determinative Bacteriology.

Results and Discussion

Isolation of bacteria from soil sample Microbiological analysis of soil samples revealed that contaminated sites with organic waste contain high bacterial count. For Total Viable Count, the soil samples were diluted up to 10-7 in Ringer's Solution and plated in Nutrient agar. After incubation the colonies were counted and the results were tabulated. The bacterial count varied from 1.0 x 108 CFU/g to 5.2 x 108 CFU/g of the soil sample. Isolation of lipolytic strains The serially diluted soil samples were plated on Tributyrin agar and lipolytic count varied from 1.1 x 108 CFU/g to 4.9 x 108 CFU/g of the soil sample. It was found that the soil samples collected from oil refineries waste contaminated sites showed high bacterial count. Isolation of Lipase Producing Bacteria from Oil Contaminated Soil 589 Screening of potential bacterial strain for lipase production On the basis of larger clear zone formation on Tributyrin agar, 08 potential isolates were selected in the study. The isolates were grown in Tributyrin broth at pH 8 and the supernatant were assayed for lipase activity after incubating for 48 hours. It was found that the isolate OCR – 02 was found to have highest enzyme production as compared to other isolates.

Experiment 5

STUDY OF ALGAL SINGLE CELL PROTEINS

INTRODUCTION

The algae are a diverse collection of chlorophyll-a-containing organisms that includes many divisions of the plant kingdom, including seaweeds, and a number of single-celled and multicellular 1microscopic forms. Broad assemblages of microalgae are grouped into major categories together with macroalgae (seaweeds) on the basis of pigmentation, cell wall composition, chemical constitution of food reserves, presence and kind of flagellation and features unique to different groups. Microalgae are important constituents of many ecosystems ranging from marine and fresh water environments to desert sands and from hot springs to snow and ice. They account for more than half total primary production at the base of the food chain worldwide. Comprehensive analysis and nutritional studies have demonstrated that the algal proteins are of high quality and comparable to conventional vegetable proteins. However, due to high production costs as well as technical difficulties to incorporate the algal material into palatable food preparations, the propagation of algal proteins is still in its infancy. To date, the majority of microalgal preparations are marketed as health food, cosmetics or animal feed. Nutritional supplements produced from microalgae have been the primary focus of microalgal biotechnology for many years. Dried biomass or cell extracts produced from Chlorella, Dunaliella and Spirulina have dominated the commercial opportunities. These products are directed mainly at the nutraceutical or health food market and collectively are like worth many hundred of million dollars. *(PDF) Algae for the production of SCP*.

MICROALGAE AS HUMAN FOOD OR ANIMAL FEED

Microalgae for human nutrition are nowadays marketed in different forms such as tablets, capsules and liquids. They can also be incorporated into pastas, snack foods, candy bars or gums, and beverages. Owing to their diverse chemical properties, they can act as a nutritional supplement or represent a source of natural food colorants. The commercial applications are dominated by four strains: Spirulina, Chlorella, D. salina and Aphanizomenon flos-aquae. In addition to its use in human nutrition, microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals. In fact, 30% of the current world algal production is sold for animal feed applications. Many nutritional and toxicological evaluations have proved the suitability of algal biomass as feed supplement. Spirulina is largely used in this domain and concerns many types of animal: cats, dogs, aquarium fish, ornamental birds, horses, cows and breeding bulls. Algae positively affect the physiology (by providing a large profile of natural vitamins, minerals, and essential fatty acids; improved immune response and fertility; and better weight control) and their external appearance (resulting in healthy skin and a lustrous coat) of animals

Fat, Oil and Hydrocarbon Content of Microalgae

Fatty acids are primarily metabolites of acetyl CoA pathway which is generally determined, evolutionary very old, and therefore conservative [32]. Microalgae may contain significant quantities of fats and oils (lipids) with compositions similar to those of vegetable oils. Under certain conditions, microalgae have

been reported to contain up to 85% of the dry weight as lipids. This exceeds the lipid content of most terrestrial plants. The range of potential applications for these algal fats and oils is very wide. The algal oils resemble fish and vegetable oils, and could therefore be considered as potential substitutes for petroleum products. Direct extraction and refinement of the microalgal oils also would seem to be a more efficient way of obtaining fuels from microalgae, compared to the alternative of fermenting the algal biomass to produce either methane or ethanol.

Protein/Amino Acids Content of Microalgae

The high protein content of various microalgal species is one of the main reasons to consider them as an unconventional source of protein [31]. Most of the figures published in the literature on concentration of algal proteins, dominantly enzymatic proteins, are based on estimates of so called crude proteins, commonly used in evaluating food and feed. These figures are the result of hydrolysis of the algal biomass and estimation of the total nitrogen [4]. Proteins are composed of different amino acids and hence the nutritional quality of a protein is determined basically by the content, proportion and availability of its amino acids [3]. As the cells are capable of synthesizing all amino acids, they can provide the essential ones to humans and animals [31]. Selected data on the amino acid profile of various algae are compiled in Table 3 and compared with some basic conventional food items and a reference pattern of a well-balanced protein recommended by WHO/FAO (1973). It can be seen that the amino acid pattern of almost all algae compares favorably with that of the reference and the other food proteins [3]

Vitamin Content of Microalgae

Microalgae also represent a valuable source of nearly all essential vitamins (e. g., A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid and pantothenic acid. Vitamins improve the nutritional value of algal cell

but their quantity fluctuates with environmental factors, the harvesting treatment and the method of drying the cells

CONCLUSION

Microalgae, are a good and unstudied candidates to be used as SCP as human food or animal feed, because of their high content of protein, fatty acids and minerals. We can easily cultivate them, their growth rate is high, their productivity is high, there is no risk for pathogenicity, their culture media is simple and inexpensive, and finally there are different resources for finding and screening other strains of naturally isolated microalgae for SCP.