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Comparative Studies Of Ethanol Production And Cell Viability: Free Cells Versus Immobilized Cells.

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ABSTRACT

Use of yeast cells in fermentation industry on economic grounds proposes entrapment of yeast cell and their utilization. Immobilization of yeast cells offers many advantages as compared to free cells like easy recovery of product, its cost effectiveness, stability, reusability in further fermentation process, etc. In this study, three yeast strains *Saccharomyces cerevisiae* NCIM 3570, *Candida shehatae* NCIM 3500 and *Dekkera naardenensis* NCIM 3575 were immobilized using 3% sodium alginate and were used as inoculums for ethanol production independently. Effect of immobilization on ethanol production was compared between both free cell and encapsulated cell. Also, viability of immobilized yeast cell stored in different conditions like 4^{o}C, room temperature, and used bead at 4°C for 45 days was checked. It was revealed that ethanol production using immobilized cells was comparable to that of production by free cells. The cell viability check studies also gave significant positive results suggesting utilization of these parameters for further development in fermentation technology.

Keywords: Immobilization, fermentation, cell viability, ethanol production

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INTRODUCTION

Immobilization is the imprisonment of all types of biocatalysts including enzymes, cellular organelles, and animal and plant cells in a distinct phase that allows exchange with but is separated from the bulk phase or the external environment. Immobilization is the technique used for the physical or chemical fixation of cells, organelles, enzymes, proteins onto or into a solid support, or retained by a membrane, so that their stability is increased with their continuous use. Immobilization has a wide range of application in many industries like biotechnology, pharmaceutical, environmental, food and biosensor industries (Zhou et al. 2010). This technique possesses many benefits over free cells. Encapsulation is the most extensively studied method due to its simplicity and operationally convenient workup. Encapsulation refers to a physicochemical or mechanical process to entrap a substance in a material thus the produced particles are with diameters of a few nanometers to a few millimeters. So here, the active agents are entrapped within the polymer matrix or the capsules. In such case, cells are restricted by the membrane walls that is in a capsule, but free-floating within the core space. This technique creates a protective barrier around the entrapped microbes which ensures their prolonged viability during processing and storage in polymers (Martins and Santaella 2013). Alginate is the most suitable biomaterials for encapsulation technique due to their abundance, excellent biocompatibility and biodegradability properties (Margaritis and Kilonzo 2005). Large volume of calcium alginate beads can be produced without any sophisticated instrument and these gels are also thermostable at a temperature range of 0-100°C (Gacesa 1988). Calcium alginate bead is one of the most commonly used supports for the immobilization of cells and it is not only easy to carry out but also provides extremely mild conditions, so that it has a higher potential for industrial applications. Fermentation industry is a growing industry and has immense potential in growth of Indian economy in near future. Necessary research and advancement in the area of cell and enzyme immobilization can revolutionize the fermentation industry. Alcohol can be produced by utilizing free cells of yeast or by immobilized yeast cell with in calcium alginate beads. There are various method of ethanol production such as continuous fermentation and vacuum distillation with recycling of cells, and immobilization of yeast cells. Bioreactor productivity, improved cell stability, better substrate utilization are the main objectives of immobilization (Ghorbani et al. 2011). Fermentation by immobilized cell has many technical and economic advantages as compared to free cell system or traditional method, for example high fermentation rate, simple manner of preparation and handling, better substrate utilization, longer working life time, ease of separation to facilitate their reuse and easy harvest from the product, increased bioreactor productivity, reduction in cost of bioprocessing by eliminating long and expensive processes of cell recovery and cell recycle, maintenance of high cell density per volume, permeable to reactant and product, less inhibition by product, no reduction in the desired biocatalytic activity of cell, protection against high shear damage, provide favorable microenvironment to cell, less chances for contamination, high tolerance to alcohol, etc. (Wendhausen 1998; Tata et al. 1999; Kourkoutas et al. 2004). Immobilized yeast made by calcium alginate produces more ethanol by consuming more sugar as compared to free yeast cell under the same condition (30°C during fermentation, pH 5, 10 % glucose concentration and 2% sodium alginate concentration). The fermentation time in case of free cell was 36 hour that was more than the fresh (24 hours) and reused bead (10-14 hour). Immobilized yeast produced 100% ethanol as compare to free cell that produced 88% ethanol (Inloes et al. 1983; Najafpour et al. 2004; Lin and Tanaka 2006; Bai et al. 2008; Lee et al. 2011; Puligundla et al. 2011; Yao et al. 2011; Ylitervo et al. 2011; Yu et al. 2010). Considering the merits of immobilization of cells, the present work was planned to study the effect of immobilization on ethanol production and cell viability in calcium alginate beads stored under different temperature.

MATERIALS AND METHODS

Culture Maintenance

Three yeast strains i.e. *Saccharomyces cerevisiae* NCIM 3570, *Candida shehatae* NCIM 3500 and *Dekkera naardenensis* NCIM 3575 was purchased from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune. These cultures were maintained on MGYP (Malt extract 1%, glucose 1%, yeast extract 0.3%, peptone 1%) medium at pH 6.0.

Immobilization of Yeast Cells Using Calcium Alginate Beads

Yeast cells were cultured on MGYP broth at pH 6.0, for 48 h at 30°C and 120 rpm. Three percent sodium alginate and 0.05 M calcium chloride solution was prepared using deionised water and both the

solutions were autoclaved at 121°C (15 psi) for 15 minutes. To the autoclaved sodium alginate solution, 48 h old yeast culture was added in 1:1 (v/v) and the solution was homogenized using vortex. This solution was added drop wise using a syringe with needle diameter of 1 mm to the calcium chloride solution. The capsules/beads formed were allowed to harden for 10 minutes, washed in deionized water twice and stored in aseptic condition for future studies (Manasouripour et al. 2013).

Utilization of the Immobilized Yeast Cells in Ethanol Production

Immobilized yeast cells obtained in the form of beads were utilized for fermentation of sugar solution. Fermentation media was prepared using 10% initial glucose concentration for *S. cerevisiae* and *D. naardenensis* at pH 6.0-6.5 and 5% glucose to 5% xylose concentration for *C. shehatae* at pH 5.5. Ten percent of inoculum (immobilized and free cells) was added to fermentation media. Fermentation media was incubated for 120 h at 30°C and 120 rpm. After incubation, the free and immobilized cells were filtered and crude broth was analyzed for ethanol content.

Estimation of Ethanol Production by HPLC Analysis

Amount of ethanol in the crude broth was estimated by HPLC analysis using Hiplex-H Agilent column having column and detector temperature of 57°C and 50°C, respectively. 1 mM H2SO4 was used as mobile phase with 0.7 ml/min as flow rate. Refractive index detector was used to detect ethanol.

Storage of Immobilized Yeast Cells Under Variable Conditions

Immobilized yeast cells were aseptically stored in different conditions like at 4°C, at room temperature and in dried form. Immobilized yeast cells used in fermentation were also harvested, washed with autoclaved distilled water and stored in aseptic condition at 4°C to check the viability and culture load.

Viability of Immobilized Yeast Cells Stored Under Different Storage Conditions

The entrapped bacterial cells were released from the capsules using 0.2 M/l phosphate buffer. To count the encapsulated bacteria 1 g sample was aseptically re-suspended in 9 ml of 0.2 mol/l phosphate buffer. It was vortexed for 20 min to allow complete release of the yeast cells from alginate capsules. Sample so obtained was serially diluted in autoclaved distilled water and plated on MGYP agar plates. All samples were analyzed in triplicates (Godward & Kailasapathy 2003).

Field Emission Scanning Electron Microscopy Images of Yeast Cells

Three yeast strains i.e. *Saccharomyces cerevisiae NCIM 3570*, *Candida shehatae NCIM 3500* and *Dekkera naardenensis NCIM 3575* were subjected to FESEM (NOVA NANOSEM 450) imaging to study the morphology. All the three strains were cultured for 24 h at 30°C at120 rpm. Culture samples were centrifuged at 5000 rpm for 15 min. and washed twice with PBS. The final pellets were suspended in PBS and vortexed. Slides were fixed using 2.5% glutaraldehyde for 16 h and then with 1% tannic acid for 5 min. Slides were then washed with distilled water and dehydrated by using 30%, 50%, 70%, 90% and absolute ethanol in a series.

RESULTS AND DISCUSSION

Immobilization and Utilization of the Immobilized Yeast Cells in Ethanol Production

Three percent sodium alginate and 0.5 M $CaCl_2$ were used to produce stable, active beads of three yeast strains, *S. cerevisiae, C. shehatae and D. naardenensis* using a 0.5 mm diameter syringe. The beads were of same size, same diameter and are spherical shaped. Immobilized cells in ethanol production media retained their shape and did not degrade during the fermentation process. Fermentation media in case of *C. shehatae* was showing dark colour than *S. cerevisiae* and *D. naardenensis* due to the presence of both glucose and xylose in the media. Bubble formation in the sealed flask during the fermentation process confirms the production of CO_2 and ethanol.

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Estimation of Ethanol Production by HPLC Analysis

Ethanol production was estimated in High Performance Liquid Chromatography with refractive index detector at National Institute of Renewable Energy, Kapurthala, Punjab. The HPLC column used here was HIPlex-H Agilent having column temperature and detector temperature 57 °C and 50 °C respectively, with 1 mM H2SO4 as mobile phase with flow rate of 0.7 ml/min. The produced ethanol shows peak at retention time of 18.85 min (Fig. 1).

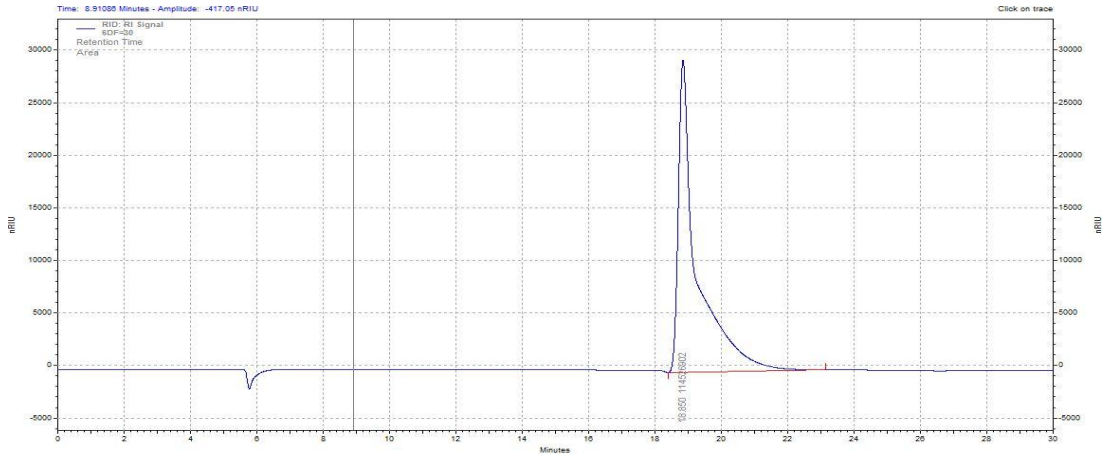

Figure 1: HPLC Chromatogram for ethanol

Fermentation of glucose by *S.cerevisiae* for 72 hrs. in immobilized form resulted in 10.19 g/L and incase of free cell it is 9.65 g/L (Table 1). In case of *C. shehatae* 13.98 g/L and 6.43 g/L ethanol production was recorded for immobilized and free cells respectively. Subject to *D. naardenensis,* failed in fermenting the substrates into desirable amount of ethanol, resulting in very low concentration of ethanol i.e. 1.74 g/L and 0.68 g/L in encapsulated and free cell mass respectively. Encapsulation provides favorable microenvironment to the yeast cells for their growth and protects them from wear and tear thereby increasing their potential and activity. Ethanol conversion both by encapsulated and free cells were carried out under similar temperature and incubation time. All results obtained were from the crude fermented broth containing 10% of initial sugar concentration.

Table 1: Amount of ethanol estimated using Hiplex-H Agilent column

Sl.No.	Samples	Ethanol Concentration (g/L)
1	Immobilized *S. cerevisiae*	10.19±0.1414
2	Free *S. cerevisiae*	9.65±0.1923
3	Immobilized *C. shehatae*	13.98±0.1144
4	Free *C. shehatae*	6.43±0.2097
5	Immobilized *D. naardenensis*	1.74±0.0768
6	Free *D. naardenensis*	0.68±0..282

Storage of Immobilized Yeast Cells Under Variable Conditions

Beads did not show any contamination or degradation after 45 days of storage in either 4°C or room temperature condition. Used beads, which were stored at 4°C, were swollen after their use in first cycle of fermentation. It signifies that nutrient media components as well as substrate were imbibed well into the beads and products like ethanol, CO_2 have come out. The colors of the used beads were different from the colour of their respective fresh beads, which may be as a result of increase in cells density inside the beads due to growth of yeast cells. Storage of beads is easier as compared to free cells and doesn't even require

utilization of antibiotics which increases their reusability in consecutive cycles eliminating serious economic and technical issues faced in fermentation industries.

Viability of Immobilized Yeast Cells Stored Under Different Storage Conditions

After 45 days of storage period, viable yeast colonies were evidenced on MGYP agar plates. Used beads were also showing good but lesser number of colonies than 4°C and room temperature stored beads, no contamination was recorded (Fig. 2). It suggested that the cells within the beads are viable and they can be used and reused in the fermentation process. Beads stored at 4°C indicate maximum viability of cells i.e. more than 75% in all the three strains of yeast. For *S.cerevisiae* the cell viability percentage at 4°C was found to be 99.33%, for *C. shehatae* it was recorded as 78.33% and *D. naardenensis* as 75.66%. Beads stored at room temperature signify decrease in cell number, reason being lack of nutrition and appropriate growth conditions. For *S.cerevisiae* the cell viability percentage at room temperature was found to be 59.73%, for *C. shehatae* as 45% and *D. naardenensis* as 71%. Used beads denote lesser cell number due to prolonged usage in fermentation process, stationary cell phase or may be due to production of toxic secondary metabolites. For used *S. cerevisiae* beads the cell viability percentage was found to be 56.04%, for *C. shehatae* it was 45% and *D. naardenensis* was 35.33%. Percentage of survivability was calculated by comparing the number of cells present in freshly synthesized beads of same dimension and in same quantity (Table 2). FESEM images of the used beads were taken to check cell morphology and images suggests that the cells are healthy even after their use in consecutive fermentation cycles (Fig. 3).

Table 2: Cell viability of immobilized yeast cells under different storage conditions

S. No.	Yeast Strains	Storage Conditions (45 days)	CFU /mg	Percentage Survivability
1	*S. cerevisiae*	4 ºC	298±1.9146	99.33
2	*S. cerevisiae*	30 ºC	178±6.5574	59.73
3	*S. cerevisiae*	Used	167± 8.1034	56.04
4	*C. shehatae*	4 ºC	238±13.735	78.33
5	*C. shehatae*	30 ºC	135±5.354	45
6	*C. shehatae*	Used	85±10.198	28.33
7	*D. naardenensis*	4 ºC	227±11.343	75.66
8	*D. naardenensis*	30 ºC	213±13.589	71
9	*D. naardenensis*	Used	106±8.602	35.33

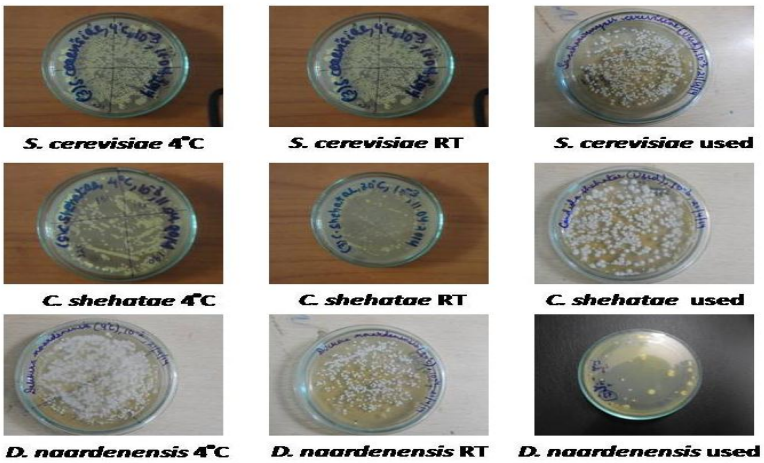

Figure 2: MGYP agar plates showing viable yeast colonies subcultured from the immobilized cells stored at 4°C, room temperature and from reused alginate beads

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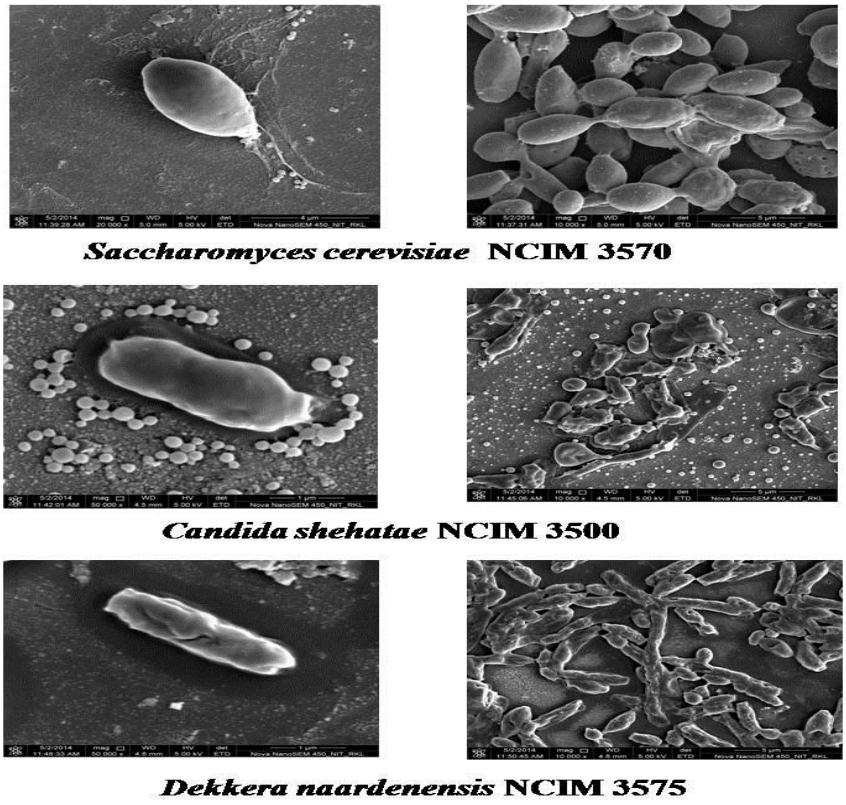

Figure 3: Field Emission Scanning Electron Microscopy (FESEM) images of three yeast cells

CONCLUSION

The encapsulation of three yeast cells *Saccharomyces cerevisiae, Candida shehatae and Dekkera naardenensis* in calcium alginate beads is the most suitable and efficient process of immobilization of yeast cells. From the present study it was concluded that *C. shehatae* demonstrated best results in terms of ethanol production under immobilized condition as compared to *S.cerevisiae*. Whereas for *D. naardenensis* extensive studies needs to be carried out in order to monitor ethanol production in both free and immobilized conditions. For *S.cerevisiae* both immobilized and free cells resulted in almost similar ethanol yield whereas for *C. shehatae* it was noticed that ethanol production was doubled in fermentation process utilizing immobilized cells as compared to that of free cells. In context with the cell viability studies after 45 days of storage under different conditions, *S.cerevisiae* displays best survivability results under all conditions followed by better results from *D. naardenensis* and *C. shehatae.* Beads stored at 4 ºC exhibits better survivability as compared to those stored at room temperature or once used in fermentation process.

This immobilized yeast cells have greater application in the industrial production of ethanol and many more by products. If these immobilized yeast cells are used in industries like baking, brewing, food etc., the production rate will surely increase as well as it will be cost effective and favor reuse. Immobilized yeast cells produce higher ethanol as compared to free cells and having various benefits over free cells like rapid fermentation time, fewer by products and no residual glucose content that is present on initial fermentation broth. For the economical ethanol production utilization of these yeast cells in the fermentation broth is the best method. Raw biological substrates like lignocelluloses, agricultural wastes can be utilized in place of pure substrates like glucose and xylose. Thus bioethanol produced from this have greater role in biofuel industries which will meet the demand of energy of human beings. In the present study, they are showing good storage capabilities, so they can be stored for many days and reused repeatedly.

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