ISSN 0975-6299 Vol 3/Issue 2/April – June 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOTECHNOLOGY

STUDIES OF ALCOHOL DEHYDROGENASE AND ALDEHYDE DEHYDROGENASE ACTIVITIES DURING ACETIC ACID PRODUCTION BY AN ETHANOL RESISTANT IMMOBILIZED STRAIN OF *SACCHAROMYCES CEREVISIAE* AB_{100}

MODHURIMA CHAKRABORTI* AND AJIT KUMAR BANIK

Department of Chemical Engineering, University of Calcutta 92, A.P.C. Road, Kolkata - 700009, India

MODHURIMA CHAKRABORTI
Department of Chemical Engineering, University of Calcutta 92, A.P.C. Road, Kolkata - 700009, India

ABSTRACT

The wine yeast *Saccharomyces cerevisiae* was immobilized in sodium alginate beads for production of acetic acid. In order to optimize immobilization conditions, a study was conducted using various concentrations of alginate, $CaCl_2$, cell loading, bead diameter etc. The optimized parameters were alginate concentration 4% (w/v), $CaCl_2$ concentration 0.3 (M), cell: alginate ratio 5:4, storage period 24 hrs. and cell bead diameter of 3 mm. In comparison to free cells (1.0678 gm/100ml), the rate of fermentation by immobilized cell proved to be greater (1.395 gm/100ml), showing suitability for acetic acid production.

KEY WORDS

Acetic acid, alcohol dehydrogenase and aldehyde dehydrogenase, immobilization, *Saccharomyces cerevisiae*, calcium alginate.

INTRODUCTION

Acetic acid is an important feedstock for many chemicals, such as- sodium acetate is used as an acidulant and as a meat spray to inhibit microbial growth. Calcium magnesium acetate (CMA) has been identified by the US Federal Highway Administration as an environmentally safe and non corrosive deicer for use of roads in winter and potassium acetate was identified as a heat exchange fluid [1,2,3]. At present, these products are made from petroleum derived acetic acid at a cost of about $650 per ton. Fermentation is potentially a cost effective alternative for acetic acid production.

Efficient acetic acid production requires a rapid fermentation leading to high acetic acid concentration; therefore a yeast strain must have a good specific growth rate. During batch fermentation many parameters can cause the decrease of the specific rate of yeast growth and the inhibition can be caused either by product or substrate concentration. Immobilization of cells for fermentation has been developed to eliminate inhibition caused by high concentration of substrate and product, also to enhance the productivity [4, 5, 6, 7, 8, 9].

According to Groboillot et al (1994), the main advantages of the immobilization are the increase of acetic acid yield and cellular stability and a decrease of process expenses due to the ease for cell recovery and reutilization [10].

Prasad and Mishra (1995) reported other advantages of immobilization as-

1. greater volumetric productivity as a result of higher cell density,
2. tolerance to higher concentrations of substrate and products,
3. lacking of inhibition
4. relative easiness of down stream processing etc. [11].

Cell immobilization provides a means to improve solid-liquid separation, minimal clogging in continuous flow systems [12-15], protection of cells from toxic effects of low pH, temperature, inhibitors etc. (please specify where bracket ends) as ionic/hydrophobic interactions of the immobilization matrix induces increased stability and a buffered zone is provided by the immobilization material [16-18].

There are several procedures for immobilization, such as-

1. adsorption or adhesion of microorganisms to the surface of carriers (wood chips, porous ceramics, Raschig rings, plant fibres, silicates, titanium compounds, glass fibres etc.
2. covalent binding.
3. incorporation into a carrier gel (calcium alginate, carrageenan, agarose, chitosan, pectin, gelatin, polyacrylamide).
4. membrane retention of microorganisms (cellulose, diatomite, biphasic emulsions or membranes of various origin are used as carriers in this case) [19,20].

A good support material should be inexpensive, easily available, rigid, chemically inert, should bind cells firmly and should have high loading capacity. All these qualities are present in Na-alginate. So we have chosen it. Alginic acid is a heteropolysaccharide made of α-L-glucuramic acid and β-D-manuromic acid and is found in many algal species, especially in brown algae. The overall composition and the sequence of monomers in the alginated polysaccharide vary extensively depending on the origin. This

carboxylic polyelectrolyte is soluble from aqueous solutions and precipitates in the form of a coacervate in the presence of multivalent metal ions like- Ca^{2+} , Co^{2+} , Fe^{2+} , Fe^{3+} and Al^{3+} [21]. In this work, the ethanol resistant strain of *Saccharomyces cerevisiae* was entrapped using Na-alginate as the natural polymeric matrix and the effect of immobilization was studied on acetic acid production and on the activities of alcohol dehydrogenase and aldehyde dehydrogenase.

MATERIALS AND METHODS

Microorganism used:

Saccharomyces cerevisiae AB_{100} , a newly isolated ethanol resistant strain in our laboratory have been used in these studies [22].

Medium & cultural condition:

The maintenance media consisted of 1% D-glucose, 0.5% peptone, 0.5% yeast extract & 4% agar agar powder. pH was adjusted to 5.0. Organism was maintained at 30ºC for 48hours. Inoculums were harvested by washing the slant with sterile distilled water, the cell density to 2.05×10^5 per ml. The medium for fermentation consisted of 10% D-glucose, 0.125% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, $7H_2O$ and 10 µg/ml of each of $FeSO_4$, $7H_2O$, $MnSO_4$, H_2O and $ZnSO_4$, $7H_2O$. pH was adjusted to 4.5. Both medium were sterilized at 121ºC & 15lb/inch^2 pressure for 15 minutes. 250 ml. conical flask containing 85ml of the fermentation medium was inoculated with 5ml (1.025×10^5 cell / ml) of inoculums & incubated at 30ºC for 96 hours. After fermentation, the cell was separated by centrifugation & the supernatant was used for the analysis of acetic acid & determination of enzyme activity[22].

Estimation of acetic acid:

Concentration of acetic acid was determined by HPLC with a TPS Spectra System apparatus using a Biorad Aminex HPX-87H column heated to 40ºC & a Refraction Index Detector (Waters 640). The mobile phase was 0.005M sulfuric acid flowing at 0.4 ml / min [22].

Assay of Alcohol Dehydrogenase:

0.1ml of supernatant was added to the mixture of 15mM β- NAD^+, 50mM sodium pyrophosphate buffer (pH 8.8) & 95% ethanol & incubated for 6min at 25°C in a suitably thermostatted spectrophotometer. Absorbance was recorded at 340 nm. Enzyme activity was expressed as mu/ml. One unit of enzyme activity represents the amount of enzyme which can convert 1.0 µmole of ethanol to acetaldehyde per minute at pH 8.8 at 25°C [22].

Assay of Aldehyde Dehydrogenase:

0.1ml of supernatant was added to the mixture of 20mM β- NAD^+, 1 (M) Tris-HCl buffer (pH 8.0), 100mM acetaldehyde solution, 3 (M) KCl & 1(M) 2-mercaptoethanol & incubated for 5min at 25°C in a suitably thermostatted spectrophotometer. Absorbance was recorded at 340 nm. Enzyme activity was expressed as mu/ml. One unit of enzyme activity represents the amount of enzyme which can oxidize 1.0 µmole of acetaldehyde to acetic acid per minute at pH 8.0 at 25°C in presence of β- NAD^+, potassium & thiols [22].

Preparation of Ca-alginate beads:

Yeast cell suspension was formed by adding 5ml of thick yeast suspension in 10ml distilled water at room temperature. The 4% (w/v) Na-alginate solution was prepared by dissolving 0.6gm of Na-alginate powder (Sigma, medium viscosity) in this 15ml of yeast cell suspension. 0.1 (M) $CaCl_2$ solution was prepared of 50ml. Rigid near spherical micro beads were formed by drop wise addition of sterile Na-alginate-yeast cell suspension to 0.1 (M) $CaCl_2$ solution by a blunt 5ml pipette by ionotropic gelation. After formation, the beads were placed in double distilled water for removal of unreacted material and low molecular weight byproducts

and also for stabilization. Finally, the distilled water was removed and fermentation medium was added to start the production of acetic acid (23).

Statistical Analysis:

Data was presented as the mean of at least six independent experiments along with SEM [(mean±SEM), where n=6]. Statistical analysis of data was done by Student's *t* test, by using MS Excel.

RESULTS AND DISCUSSION

i)Effect of $CaCl_2$ in synthetic media for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae:

$CaCl_2$ is required for yeast growth and flocculation. To study the effect of $CaCl_2$ in our synthetic media, we made 2 sets of fermentation media, one with 0.01% $CaCl_2$ and other without $CaCl_2$. We observed that, the previous one produced greater amount of acetic acid and showed higher activities of alcohol dehydrogenase and aldehyde dehydrogenase than the later one (fig.1). So we can conclude that, $CaCl_2$ is must for our synthetic media, otherwise beads will be dissolved in medium.

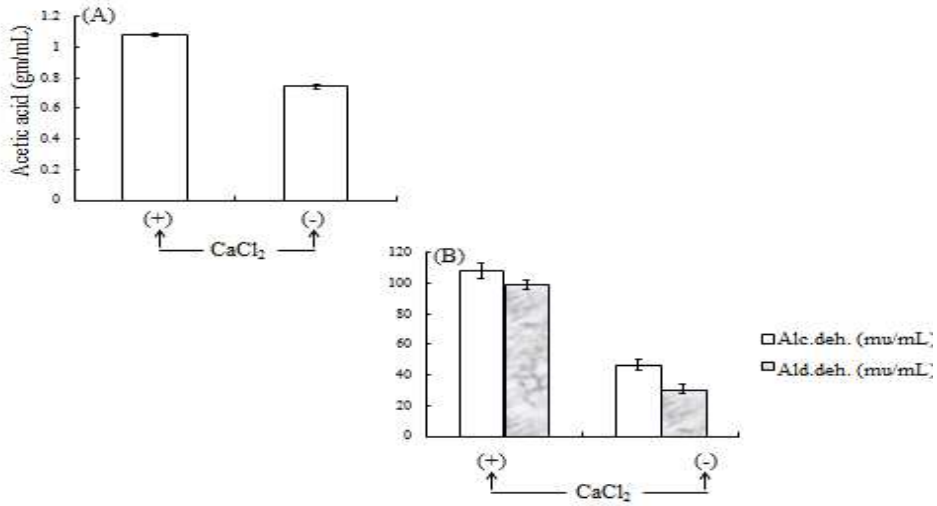

Figure 1.
Effect of $CaCl_2$ in synthetic media for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

ii) Optimization of $CaCl_2$ concentration in synthetic media for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae :

Different concentrations of $CaCl_2$ were added in the synthetic media, such as- 0.005%, 0.01%, 0.02% to find out the optimum concentration of $CaCl_2$. Fig.2 indicates that, 0.01% of $CaCl_2$ produces maximum acetic acid. Higher and lower concentrations produces lower amount of acetic acid and cell growth due to osmotic stress.

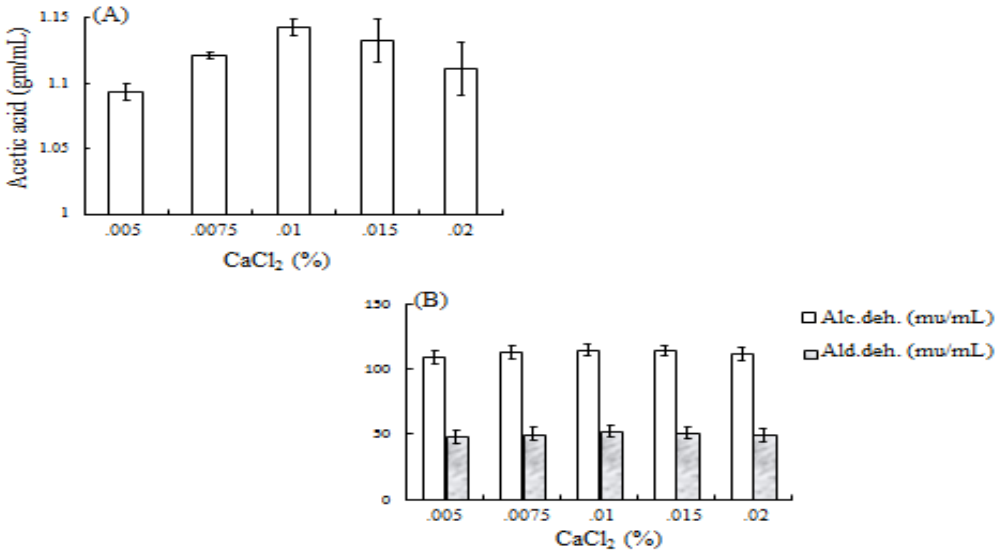

Figure . 2.

Optimization of $CaCl_2$ concentration in synthetic media for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

iii) Effect of $CaCl_2$ on Ca-alginate bead formation for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae :

During bead formation $CaCl_2$ is used to ensure the saturation of alginate molecules with Ca^{2+} ions and to prevent drying and also to give proper shape and porosity [24]. Different concentrations of $CaCl_2$ were produced, such as- 0.1(M), 0.2(M), 0.3(M), 0.4(M) and 0.5(M) to study their effects on bead formation as well as on acetic acid production. Fig.3 indicates that 0.3(M) concentration of $CaCl_2$ shows maximum effect on acetic acid production as well as on enzyme activities.

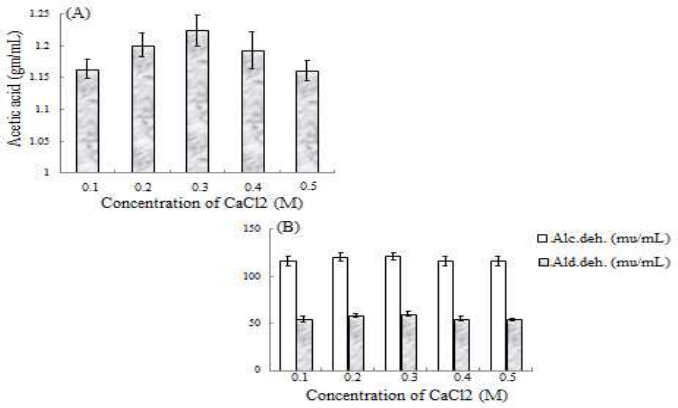

Figure .3

Effect of $CaCl_2$ on Ca-alginate bead formation for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

iv) Effect of Na-alginate on bead formation for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae :

To study the effect of Na-alginate concentrations on acetic acid production, alginate solutions of 1-6% were prepared. Fig.4 indicates that, in 4% Na-alginate concentration, there was maximum production of acetic acid. Lower alginate concentrations have problems of cell leakage. Beads with higher alginate concentrations have good mechanical strength, but, it hinders the transport of the solute and thus reduces the diffusion coefficient. Higher alginate concentration decreases the pore size in the beads. It results in more retardation of substrate molecule i.e. increase in internal mass transfer resistance and decrease in effective diffusivity of the substrate molecule and thus resulting in lower rates of fermentation [25].

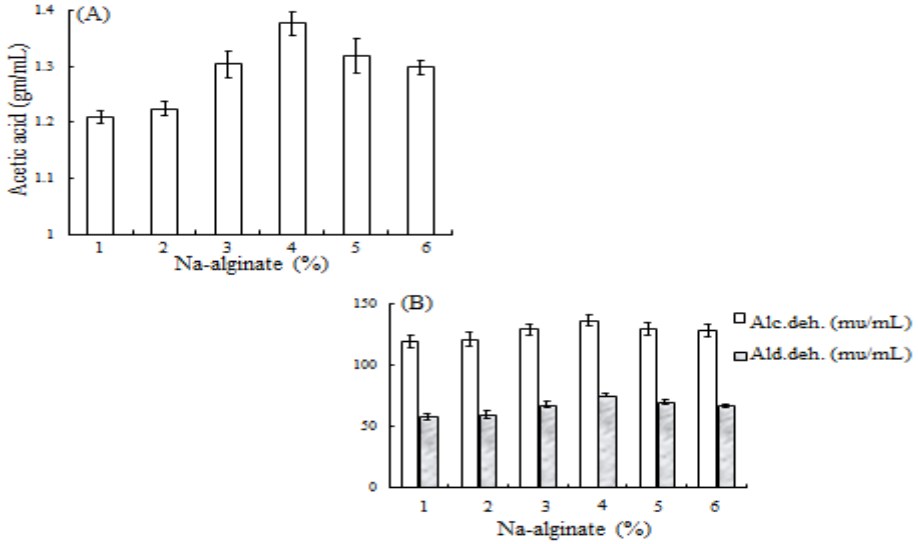

Figure . 4

Effect of Na-alginate on bead formation for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

v) Effect of storage period on Ca-alginate bead formation for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae :

The storage periods of the Ca-alginate beads on acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase were investigated using different ages of beads, such as- 24, 30, 48, 54, 72 and 96 hours. Fig.5 indicates that, 24 hr. aged beads showed maximum production and activities. The cells entrapped in properly solidified beads had better storage stabilities than the free cells [26, 27]. Beads without storage deform quickly and produce minimal acetic acid.

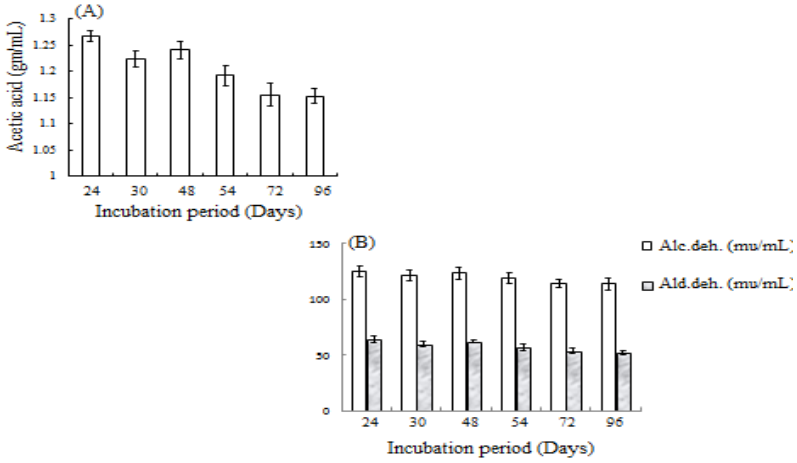

Figure . 5

Effect of storage period on Ca-alginate bead formation for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

vi) Effect of cell: alginate ratio for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae :

Different volume of yeast cell inoculums was added during bead formation with 4% Na-alginate and 0.3 (M) $CaCl_2$ making the cell: alginate ratio, such as- 2:4, 3:4, 4:4, 5:4, 6:4, 7:4 and 8:4. Fig.6 indicates that, the cell: alginate ratio 5:4 shows maximum production of acetic acid with maximum activities of alcohol dehydrogenase and aldehyde dehydrogenase. Low cell volume with 4% alginate decreases the production due to lower cell density and higher alginate impairs glucose transport. In case of higher cell volume, the cell density is so high that a marked reduction of the cell surface available for enzyme reaction occurred [28-30].

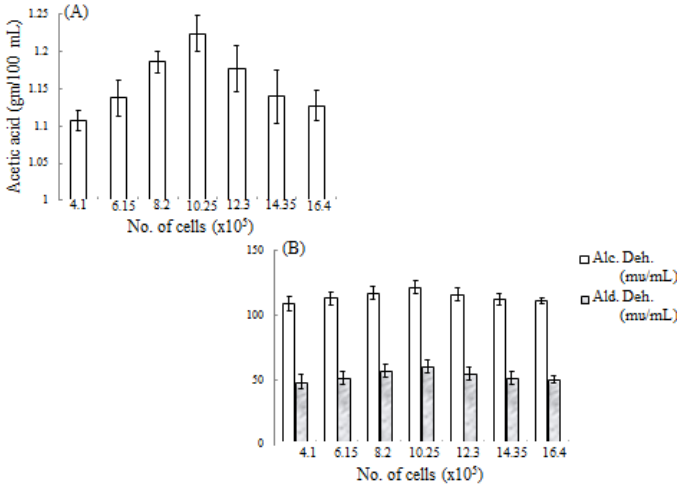

Figure 6.

Effect of cell: alginate ratio for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

vii) Effect of bead volume for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae :

Beads with various sizes Such as- 2, 3, 4, 6, 7 mm were prepared in 4% alginate solution. These beads were added to production media and subjected to fermentation. Fig. 7 represents the effect of bead volume on acetic acid production with immobilized cells. There was gradual decrease in acetic acid production with increasing bead diameter. Lower bead diameter results in higher acetic acid production, because- as the diameter of the bead increases, the substrate molecule has to travel more to reach the centre of the bead [25].

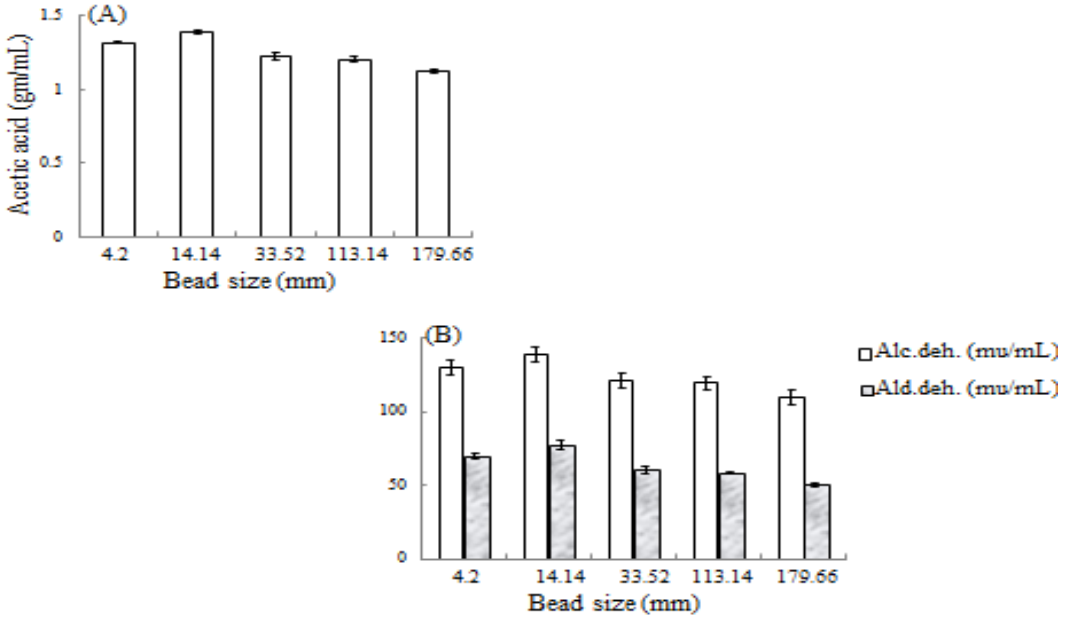

Figure 7

Effect of bead volume for acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase by an Na-alginate entrapped ethanol resistant Saccharomyces cerevisiae [S.D.=mean±0.05]

CONCLUSION

From the above studies, it is very clear, that, the acetic acid production and activities of alcohol dehydrogenase and aldehyde dehydrogenase increase severely in case of Ca-alginate entrapped ethanol resistant *Saccharomyces cerevisiae* than the free cells. The immobilized cells are highly stable in 4% Na-alginate, 0.3 (M) $CaCl_2$, 5:4 cell: alginate ratio and in 24 hr storage. So, we can conclude that, cellular activities of immobilized cells increase or remain same than that of the free cells.

ACKNOWLEDGEMENT

We show our sincere gratitude to the Dept. of Food Processing Industries and Horticulture, Govt. of West Bengal, for their financial assistance.

REFERENCES

1. Shah M.M. and Cheryan M., Acetate production by *C. thermoaceticum* in corn steep liquor media. Journal of Industrial Microbiology, 15: 424-428, (1995).
2. Dunn S. and Schenk R., Alternative Highway Deicing Chemicals. Federal Highway Administration Report FHWA-RD, 78-108, (1980).
3. Johnson, K.I., Cryotech Deicing Technologies, Ford Madison (1994).
4. Rakin M., Mojovic L., Nikolic S., Vukasinovic M. and Nedovic V., Bioethanol production by immobilized *Saccharomyces cerevisiae* var *ellipsoideus* cells. African Journal of Biotechnology, 8(3): 464-471, (4th Feb. 2009).
5. Baptista C.M.S.G., Coias J.M.A., Oliveira N.M.C., Roche J.M.S., Dempsey M.J., Lannigan K.C. and Benson P.S., Natural immobilization of microorganisms for continuous ethanol production. Enzyme Microbial Technology, 40:127-131, (2006).
6. Kourkoutas Y., Bekatrou A., Banat IM., Marchant R. and Koutinas AA., Immobilization techniques and support materials suitable in alcohol beverages production: A review. Food Microbiology, 21: 277-397, (2004).
7. Sakurai A., Nishida Y., Saito H. and Sakakibara M, Ethanol production by repeated batch culture using yeast cells immobilized within porous cellulose carriers. J. Biosci. Bioengg., 90: 526-529, (2000).
8. Strehaiano P., Portugal FR. and Tailandier P., Yeast as biocatalyst. In Querol. A, Fleet GH (ed.) Yeasts in Food & Beverages, Springer-Verlag, Berlin, Germany, 243-285, (2006).
9. Williams D. and Munnecke DM., The production of ethanol by immobilized yeast cells. Biotech. Bioengg., 23: 1813-1825, (1981).
10. Groboillot A., Boadi DK., Poncelet D. and Neufeld RJ, Immobilization of cells for application in the food industry. Crit. Rev. Biotechnol., 14: 75-107, (1994).
11. Prasad B. and Mishra IM., On the kinetics and effectiveness of immobilzed whole cell batch cultures. Biores. Technol., 53: 269-275, (1995).
12. Kacar Y., Arpa C., Tan S., Denizli A., Genc O. and Arica MY., Biosorption of Hg(II) and Cd(II) from aqueous solutions: comparison of biosorptive capacity of alginate and immobilized live and heat inactivated *Phanerochaete chrysosporium.* Process Biochem., 37: 601-610, (2002).
13. Mittar D., Khanna PK., Marwaha SS. And Kennedy JF., Bioleaching of pulp and paper mill effluents by *Phanerochaete chrysosporium*. J. Chem. Tech. Biotechnol., 53: 81-92, (1992).
14. Ting YP. And Sun G., Use of polyvinyl alcohol as a cell immobilization matrix for copper biosorption by yeast cells. J. Chem. Tech. Biotechnol., 75: 541-546, (2000).
15. Arica MY., Sharif FA., Alaeddinoglu NG., Hasirci N. and Hasirci V., Covalent immobilization of *Aspergillus niger* on pHEMA membrane:application to continuous flow reactor. J. Chem. Tech. Biotechnol., 58: 281-285, (1993).
16. Kocher GS., Kalra KL. And Phutela RP., Comparative production of sugarcane vinegar by different immobilization techniques. J. Inst. Brew., 112(3): 264-266, (2006).
17. Brodelius P. and Vandamne EJ., Immobilized cell systems: In: Biotechnology, vol. 7A, HJ. Rehm and G. Reed, Eds. Verlag-Chemie: Germany, 405-464, (1987).
18. Durham DR., Marshall LC., Miller JG. and Chmurny AB., New composite biocarriers engineered to contain adsorptive and ion

exchange properties improve immobilized cell bioreactor process dependability. Appl. Environ. Microbiol. 60: 4178-4181, (1994).
19. Martynenko NN. and Gracheva IM., Physiological and Biochemical characteristics of immobilized Champagne yeasts and their participation in champagnizing process: A review. Appl. Biochem. Microbiol. 39(5): 439-445, (2003).
20. Sarishvili NG. and Reitblat BB., Mikrobiologicheskie osnovy tekhnologii shampanizatsii vina (Microbiological basic principles of technology of wine champagnazing), Moscow: Pishchevaya prom-nost', (2000).
21. Yalcinkaya Y., Arica MY., Soysal L., Denizli A., Genc O. and Bektas S., Cadmium and Mercury uptake by immobilized *Pleurotus sapidus*. Turk. J. Chem. 26: 441-452, (2002).
22. Chakraborti M. and Banik AK., Effect of complex nutrients, vitamins and amino acids on alcohol dehydrogenase and aldehyde dehydrogenase activity during acetic acid production by an ethanol resistant strain of *Saccharomyces cerevisiae* AB_{100}. J. Indian Chem. Soc.88:1005-1009, (2011).
23. Beshay U., Production of alkaline protease by *Terredinobacter tumiae* cell immobilized in calcium alginate beads. African J. Biotech. 2: 60-65, (2003).
24. James M. and Johansen F & A., A novel method for immobilization of yeast cells in alginate gels of various shapes by internal liberation of Ca-ions. Biotech. Lett. 7(10): 765-768, (1985).
25. Sevda SB. and Rodriguez L., The making of pomegranate wine using yeast immobilized on Na-alginate. African J. of Food Sci. 5(5): 299-304, (2011).
26. Gosman B. and Rehm HJ., Oxygen uptake of microorganisms entrapped in calcium alginate. Appl. Microbiol. Biotechnol. 23: 163-267, (1986).
27. Johnsen A. and Flink JM., Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells. Enz. Microb. Technol. 8: 737-748, (1986).
28. Gianfreda L., Parascandola P. and Scardi V., A new method of whole microbial cell immobilization. European J. Appl. Microbiol. Biotechnol. 11: 6-7, (1980).
29. Colagrande O., Silva A. and Fumi MD., recent applications of biotechnology in wine production. Rev. Biotechnol. Progr. 10: 2-18, (1994).
30. Nigam JN., Gogoi BK. and Bezbaruah RL., Alcoholic fermentation by agar-immobilized yeast cells. World J. Microb. Biotech. 14: 457-459, (1998).