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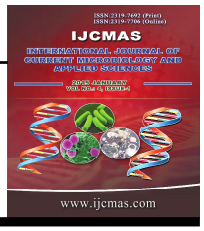

Original Research Article

Statistical Optimization of Alginate Immobilization Process of *Candida stauntonica* strain MY1 for Bioethanol Production

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Keywords

Isolation, Identification, Yeast, Cell immobilization, Optimization, Calcium alginate, Bioethanol

A B S T R A C T

In this study a new yeast strain was isolated from Egyptian sugarcane molasses for its high capability of bioethanol fermentation, under anaerobic conditions. It was identified on the basis of its 18S rDNA to be *Candida stauntonica* MY1 (Accession No. KM657091). The central composite face centered design CCFD matrix and response surface methodology were applied in designing and optimizing the process of Calcium-alginate immobilization of MY1 yeast cells to maximize its bioethanol productivity from glucose and evaluate the influence and interactive effect of three critical immobilization parameters; bead size (diameter, mm), initial inoculum size (g/L) and alginate concentration (g/L) on the bioethanol yield. Three quadratic model equations have been predicted finding out how statistically significant the effects of these variables (factors) and their interactions are in practice. The validity of the predicted models was confirmed. The optimum conditions for cell immobilization were found to be 2.5 mm, 2.5 g/L and 5.5 g/L, respectively. That produced 4.4 g/L bioethanol, with actual yield of 41.9% i.e. $Y_{P/S}$0.42 g ethanol/g glucose, which was about 2.3 fold higher than that produced with free cells batch fermentation operated under the same conditions;48 h, pH 5.5, 30^{o}C and 100 rpm. The immobilized cells showed good stability, with long storage time 21 d and can be used for four successive batches with maximum bioethanol productivity.

Introduction

Microbial immobilization in bioethanol production process has been recently received great attention, due to its technical, environmental and economic advantages (de Vasconcelos et al., 2004; Kopsahelis et al., 2007; Singh et al., 2009; Winkelhausen et al., 2010; Inal and Yigitoglu, 2011). Immobilization step usually results in a

better reaction rate due to the increased cell density, which would consequently increase the product yield. It also improves the cell stability and its resistance against shear forces, and reduces the high contamination risks, susceptibility of the microorganism to environmental variations, costs associated with the recycling and downstream processing. Immobilized cells can be applied in continuous processes at high dilution rate without washout. These factors help in reducing cost associated with fermenter design and control, as compared with traditional fermentation techniques with free cells. The immobilizing support must be available at an affordable cost, should provide good immobilization, high cell loading, low mass transfer limitations, stability, and rigidity, can be sterilized and has flexibility in preparation. Many methods namely adsorption, covalent bonding, cross linking, entrapment and encapsulation are widely used for immobilization. These categories are commonly used in immobilized enzyme technology. However, due to 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. Among the different cell immobilization techniques, entrapment in calcium alginate gel has been one of the most used matrices for whole cell entrapment due to its simplicity and nontoxic character (Ghorbani et al., 2011).

Many studies have been performed on bioethanol fermentation using calcium-alginate (Ca-alginate) immobilized *Saccharomyces cerevisiae and Zymomonas mobilis* as they are suitable candidates to produce ethanol (Gunasekaran andKamini, 1991; Sangryeol and Lee, 1997; Najafpour et al., 2004; Ghorbani et al., 2011; Inal and Yigitoglu, 2011; Behera et al., 2012).

The growing market of bioethanol fuel is driven by its advantages as a clean burning fuel. This necessitates for a high ethanol yielding strain and hence this study has been carried out to isolate and identify a yeast strain with high ethanol productivity. Response surface methodology RSM based on statistical central composite face centered design CCFD of experiments will be applied to optimize the process of Ca-alginate immobilization of the new yeast strain to maximize its bioethanol production from glucose and evaluate the influence of different immobilization variables like bead size (diameter, mm), initial inoculum size (g/L), alginate concentration (g/L) and their interactive effect on the bioethanol yield. The stability, storage and reusability of the immobilized cells will be also investigated.

Materials and Methods

Chemicals

D-(+)-Glucose (99.5%) is product of Sigma, Germany. Alginic acid sodium salt (SA) was purchased from Aldrich, United Kingdom. Calcium chloride (99%) was purchased from Honeywell, Germany. All other chemicals were of analytical grade, commercially available and used without further purification.

Isolation and identification of the yeast strain used in this study

The yeast strain was isolated from a sugarcane molasses sample purchased from Sugars and Integrated Industries Egyptian Distillation Plants in Hawamdeia City, Giza, Egypt. Identification of yeast isolate was done using 18S ribosomal DNA (rDNA) amplification and sequencing, which was

determined by direct sequencing of PCR-amplified 18S rDNA. Blast program (http:www.ncbi.nlm.nih.gov/blast) was used to assay the DNA similarities. Evolutionary history was inferred using the Neighbor-joining method (Patil et al., 2008). The tree was drawn to the scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree (Dhanve et al., 2009).The sequence generated in this study was deposited in the GenBank and the accession number was presented.

Preparation of inoculum

The yeast isolate was grown in Wickerham broth medium (Wickerham, 1951), pH5.5; at 30°C in shaking incubator 150 rpm for 48 h. Cells were harvested by centrifugation at 10,000 rpm at 4°C for 10 min and washed twice with sterile saline (8.5 g/L NaCl). The concentration of desired cells density was adjusted according to the experimental conditions as wet cells weight per unit volume, WCW g/L (Table 1) in 10 mL saline and then vortexed to produce a homogenous cell suspension.

Preparation of beads

In order to investigate the optimum sodium alginate concentration for immobilization, sodium alginate with varying concentrations according to the experimental conditions (Table 1) was used to prepare the beads. Cells were prepared and harvested as mentioned before. When the temperature of SA cooled to 45°C after sterilization, 10 mL of cell suspensions (with different initial inoculum size i.e. biomass concentrations g/L) was added to the solution under aseptic condition and stirred (100 rpm) for 10 min to get a uniform mixture. The effect of alginate beads size was also studied (Table 1). The slurry was filled in a sterile hypodermic syringe and an injection needle was fixed over its end. The bead size was varied by using hypodermic syringe needles of different gauge sizes. The alginate solution was dropped into a 0.2 M ice cold solution of calcium chloride and the formed beads were incubated overnight for curing. The cured beads were washed with sterile saline (8.5 g/L NaCl) four times to remove the residual calcium ions and then kept in sterile saline at about 4°C overnight and used to inoculate fermentation media.

For morphology studies of the immobilized yeast cells in the alginate beads, scanning electron microscopy (SEM, JEOL-model JSM-53000, Japan) was used, under different magnification powers. The alginate beads were fixed with 2% glutaraldehyde and then dehydrated in ethanol. Finally, the samples were dried, coated with gold and observed.

Batch fermentation

Batch fermentation experiments of 48 h, were carried out in 100 mL Erlenmeyer flasks containing 50 mL, pH 5.5 minimal salts medium of 0.24 g/L KH_2PO_4, 1 g/L $MgSO_4.7H_2O$ and 11 g/L glucose solution as sole carbon source, in shaking incubator set at 30^oC and 100 rpm. In order to evaluate the performance of the immobilized cells, parallel batch experiments using free MY1 cells for the fermentation was carried out, by the inoculation of the same mass of cells (WCW g/L) as that have been used in the immobilized beads.

Determination of glucose concentration

Glucose concentrations before and after fermentation were determined by 3,5-dinitro salicylic acid DNS method (Miller, 1959), using UV/VIS/NIR spectrophotometer (Model Jasco V- 570) at λ_{540nm}. Calibration

curve was done using glucose as a standard (0.25 - 4 g/L), as above that, does not obeying Beer's law.

Quantification of ethanol

Ethanol yield was measured by Gas chromatography (model 6890 (G1530A), Agilent, USA), equipped with flame ionization detector and nominal capillary column (HP-5, 5% phenyl- 95% Methylsiloxane 30 m x 250 µm I.D., 5.00 µm film, USA). Helium was the carrier gas, flow rate was 25 mL/min. Oven and detector temperature was 300°C. Calibration curve was done for ethanol concentration (1 - 60 g/L). The actual bioethanol yield was calculated by the modified formula (1) proposed by Gunasekaran and Kamini (1991).

$$Actual\ bioethanol\ yield\ \% = \frac{Produced\ bioethanol\ (g/L)}{Utilized\ TRS\ (g/L)} \times 100 \quad (1)$$

Experimental Design and Process optimization

Response surface methodology (RSM) was used to optimize the preparation of the immobilization matrix entrapping the new yeast isolate to maximize the bioethanol production process from glucose and investigate the influence of different immobilization variables and their interactive effect on the bioethanol yield. The central composite face centered design CCFD was applied to study the process variables. The experimental runs were carried out according to a 2^3 full factorial design for the three identified design independent variables, namely; bead size (diameter) mm (A), initial inoculum size g/L (B) and alginate concentration g/L (C), with low (-1) and high (+1) levels. The total number of experiments (runs) was given by the simple formula $[20 = 2^k + 2k + 6]$. Where; k is the number of independent variables ($k = 3$), this includes; 8 factorial points from 6 full factorial CCFD were augmented with 6 replicates at the center point to assess the pure error. Responses selected were bioethanol production g/L, utilized glucose g/L and actual bioethanol yield %. The levels were selected based on preliminary study results (data not shown). The design factors (variables) with low -1 and high +1 levels, are namely A [2 and 3.5 mm], B [2.5 and 10g/L], and C [2 and 8 g/L]. The central values (zero levels) chosen for experimental design were; bead size 2.75 mm, inoculum size 6.25 g/L and alginate concentration 5 g/L (Table1).

Statistical analysis

Once the experiments were performed, the next step was to perform a response surface experiment to produce a prediction model to determine curvature, detect interactions among the design factors (independent variables) and optimize the process, i.e. determine the local optimum independent variables with maximum utilization of TRS and bioethanol yield. The model used in this study, to estimate the response surface is the quadratic polynomial represented by the following equation:

$$Y = \beta_o + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j + \sum_{i=1}^{4} \beta_{ii} x_i^2 \quad (2)$$

where, Y is the response, β_o is the value of the fixed response at the center point of the design, β_i, β_{ij} and β_{ii} are the linear, interactive and quadratic coefficients, respectively, x_i and x_j are the independent variables (factors) under study.

The statistical software Design Expert® 6.0.7., (State-Ease Inc., Minneapolis, USA) was used for design of experiments,

regression and graphical analysis of the data obtained and statistical analysis of the model to evaluate the analysis of variance (ANOVA).

Stability and reusability of immobilized cells

In order to investigate the stability of the immobilized cell beads, a batch fermentation process of 72 h, was performed as mentioned above, using the obtained optimum conditions for the beads preparation.

To investigate the storage stability of the yeast cells upon fermentation activity, the free and immobilized cells, were stored in 100 mL sterile saline (8 g/L NaCl) at 4°C and after the storage of the biocatalysts for 0, 7, 14, 21, and 28 d and then the batch fermentation process was performed as mentioned above.

For the investigation of the reusability; free and immobilized cells were used repeatedly for fermentation cycles. Each batch fermentation cycle was proceeded for 48 h. At the end of each batch, the immobilized and free cells were collected by centrifugation at 5000 rpm for 15 min. The collected immobilized and free cells after each batch were washed twice with sterile saline (8 g/L NaCl) and then re-inoculated into a new fresh fermentation batch. All the experiments were done in triplicates and the average values of the obtained data are listed.

Results and Discussion

Yeast identification

The yeast isolate was identified to be *Candida stauntonica* MY1, with accession No. KM657091 and percent similarity of 99.47% to *Candida stauntonica* strain ATCC MYA 4699 (phylogenetic tree, Figure1).

Statistical analysis and validation of the elucidated regression models

The objective of the data analysis is to fit the regression model equations specify regression coefficients and recognizing significant model terms, and finally determine the factors optimum levels which would lead to a maximum response. Based on CCFD and the obtained experimental results; the following three second-order quadratic model equations were obtained;

$$Y_1 = 3.37 - 0.24A - 0.4B + 0.38C - 1.7A^2 + 0.29B^2 - 1.22C^2 + 0.056AB - 0.099AC - 0.28BC \quad (3)$$

$$Y_2 = 10.50 + 0.012A + 0.027B + 0.015C - 0.027A^2 - 0.012B^2 - 0.16C^2 + 0.0025AB - 0.083AC - 0.14BC \quad (4)$$

$$Y_3 = 32.02 - 2.12A - 3.84B + 3.65C - 16.81A^2 + 2.95B^2 - 11.10C^2 + 0.52AB - 0.90AC - 2.6BC \quad (5)$$

Where Y_1, Y_2 and Y_3 are the bioethanol production g/L, utilized glucose g/L and actual bioethanol yield %, respectively.

The validity of the fitted models was evaluated, and their statistical significance was controlled by F-test. The analysis of variance (ANOVA) for the response surface full quadratic models is given in Table 2. It can be indicated that the three elucidated models are highly statistically significant at 95% confidence level, with F-values of 311.57, 49.81 and 346.90, respectively and very low p-values <0.0001. The determination coefficients R^2, R^2_{adj} and

predicted R^2 which measure the model fitting reliability were calculated to be ≥ 0.9964, 0.9932 and 0.9372 for the model equations, respectively. R^2 indicates the ratio between sum of the squares (SSR) with total sum of the square (SST) and it describes up to what extent perfectly the model estimated experimental data points. Based on R^2, it can be suggested that approximately more than 99.64% of the variance is attributed to the studied variables and indicated the high significance of the estimated models. This was also confirmed by the good agreement between experimental and predicted values of the response variables (Table 1).

The "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 50.115, 24.792 and 53.114 for model equations 3, 4 and 5, respectively, indicated the adequate signal. These models are reliable and can be used to navigate the design space. Determination of coefficient of variance CV value is essential, as it indicates the ratio between standard error of estimate with the mean value of the observed response as percentage. It measures the reproducibility of the model. If the value is less than 10% then the model can be considered reasonably reproducible. The standard deviation SD and the CV were low, recording; 0.11 and 5.38 for model eq.3, 0.03 and 0.28 for model eq.4 and 0.99 and 5.09 for model eq.5, respectively, showing the reproducibility of the predicted model equations.

Analysis of variance (ANOVA) was applied to establish the statistical significance of the models parameters at 95% confidence level. The significance of each parameter (coefficient) was determined by F and p values (Table 2). The larger the magnitude of the F-values and the smaller the p values, the more significant is the corresponding coefficient. It is obvious that within the studied range of variables, bioethanol production (g/L) and actual yield (%) are highly statistically significantly affected. Where, they decreased by increase of bead size (diameter mm), initial biomass concentration (g/L) and alginate concentration (g/L) recording its maximum 4.26 g/L and 40.69% at 2.75 mm, 6.25 g/L and 5g/L, respectively. But the utilized glucose (g/L) is not statistically significantly affected. Similar observations were also recorded with free cells (Figure 2), where; the consumed amount of glucose did not significantly changed with the increase of initial inoculum size, i.e. the initial concentration of the free yeast cells, recording glucose consumption ≥ 91%, but the produced ethanol increased with the increase of initial cell concentration, from 1.85 g/L with actual yield of 18.48% at initial free cell concentration of 2.5 g/L to 1.94 g/L and 19.21% at initial free cell concentration of 6.25 g/L, respectively, then the ethanol production decreased with further increment of the initial free cells concentration, recoding ≈ 1.3 g/L with actual yield of ≈ 13% at initial free cell concentration of 10 g/L.

Karapatsia et al (2014) reported that low initial inoculum size of *Saccharomyces cerevisiae* equivalent to optical density of 0.5 produced higher ethanol concentration than that of high initial inoculum size equivalent to optical density of 4, in a batch fermentation of *Phalaris aquatica* L. hydrolysate containing 20 g/L glucose.

The performance of the model can be observed by the plots of the predicted versus experimental results (Figure 3) and the plot of standardized residual versus run order was tested (Figure 4) and the residuals were scattered randomly around ±3.00. This means that the data fit well with the models

and give a convincingly good estimate of response for the system in the studied experimental range.

The perturbation plots (Figure 5) show the comparative effects of all the studied independent variables on the three responses. It is obvious from the negative curvature of the independent variable B, the negative impact of the increase of the initial biomass concentration (B, g/L) on the bioethanol production g/L (Figure 5a) and the actual bioethanol yield % (Figure 5c). But from the shape of curvatures of the variables A and C, the positive impact of the low bead size (A, diameter mm) and the low alginate concentration (C, g/L) on the bioethanol production g/L (Figure 5a) and the actual bioethanol yield % (Figure 5c) can be indicated and the occurrence of negative impact at higher levels of these independent variables was also observed. The sensitivity of the bioethanol production g/L and the actual bioethanol yield % towards the studied variables can be ranked in the following decreasing order; initial biomass concentration (B, g/L) ≥ alginate concentration (C, g/L) > bead size (A, diameter mm). The perturbation plot for the glucose consumption (Figure 5b) confirms that it is not statistically significantly affected by the change in the studied range of the immobilization factors, as compared to bioethanol production and its sensitivity towards the studied variables can be ranked in the following decreasing order B > C ≥ A.

Statistical significance of the models' parameters

Analysis of variance (ANOVA) of the regression models were carried out to find the statistical significance of the main and interactive effects of the three studied parameters on the fermentation process of glucose at 95% confidence level. The significance of each coefficient was determined by F-values and p-values (Table 2). The larger the magnitude of the F-value and the smaller the p-values, the more significant is the corresponding coefficient, where, the value of Prob> F less than 0.05 would also indicate that the model terms are significant. This implies that within the studied range (levels) of the three independent variables (parameters); the main effect of bead size, i.e., its diameter (mm) and inoculum size, i.e. the yeast concentration (g/L) have high statistical negative significant effect on the production of bioethanol g/L and its actual yield % ($p < 0.0001$). But these two independent factors expressed a statistical positive; non-significant ($p = 0.228$) and possible-significant ($p = 0.0161$) effects on the consumption of glucose. i.e., the increase of the bead size or inoculum concentration would slightly increase the consumption of glucose as there would be larger amount of microbial cells needing carbon source for its survival. But within the studied range of these independent variables, their increase decreased the ethanol production and consequently its actual yield %. But within the studied range of alginate concentration, it expressed a high statistical positive significant effect on the production of bioethanol g/L and its actual yield % ($p < 0.0001$), with a statistical positive non-significant effect ($p = 0.1395$) on the glucose consumption. The interactive effect of bead diameter and yeast concentration (AB) expressed a statistical non-significant positive impact on the produced amount of bioethanol g/L ($p = 0.1796$), the consumed amount of glucose g/L ($p = 0.8157$) and consequently the actual bioethanol yield % ($p = 0.1661$). While, the interactive effect of the bead diameter with the alginate concentration (AC) showed a statistical possible-significant negative impact on the produced amount of bioethanol g/L and its

actual yield % ($p = 0.0297$ and 0.028, respectively) with a high statistical negative significant effect on the consumed amount of glucose g/L ($p < 0.0001$). Also, the interactive effect of the utilized yeast and alginate concentrations (g/L) in the preparation procedure of the beads, expressed a high statistical negative significant effect on the consumed amount of glucose and ethanol production ($p < 0.0001$), i.e. with the increase of yeast and alginate concentrations, the consumption of glucose would decrease which consequently would lower the production of bioethanol and its actual yield. The quadratic effect of the bead size, i.e. doubling the diameter of the prepared beads, expressed a high statistical negative significant effect on the ethanol production g/L and its actual yield % ($p < 0.0001$), but it expressed a statistical non- significant negative effect on the consumed amount of glucose g/L ($p = 0.1632$).

While, The quadratic effect of the inoculum size, i.e., doubling the amount of yeast concentration g/L used in the preparation of the beads, showed a statistical positive significant effect on the ethanol production g/L and its actual yield % ($p = 0.0015$ and 0.0006, respectively), with a statistical non-significant negative impact on the consumed amount of glucose g/L ($p = 0.5222$). But, the quadratic effect of the alginate concentration, i.e. doubling alginate concentration in the preparation of the beads had a high statistical negative significant effect on the consumed amount of glucose and ethanol production ($p < 0.0001$).

Response surface methodology and variables interactive effects

The highly statistically significantly negative interactive effect of the initial inoculum size, i.e., biomass concentration (B, g/L) and alginate concentration (C, g/L) is very obvious in RSM and contour plots (Figure 6). At low initial biomass concentration, the bioethanol production (g/L, Figure 6a), glucose utilization (g/L, Figure 6b) and actual bioethanol yield (%, Figure 6c) increased with increase of alginate concentration reaching its maximum ≈ 4.3 g/L, 10.5 and 41%, respectively, within the range of alginate concentration of 5.5 – 6.5 g/L, then decreased again with further increment of the alginate concentration. It is also obvious that the increment of initial biomass concentration has a negative impact on ethanol production.

The decrease of bioethanol production and its actual yield by the increase of alginate concentration might be due to the diffusion and mass transfer problems (Najafpour et al., 2004).

Optimization of the fermentation process

The optimization process was carried out to determine the optimum values of the studied immobilization process variables to maximize the bioethanol production (g/L), glucose utilization (g/l) and actual bioethanol yield (%). This was done using Design Expert® 6.0.7., (State-Ease Inc., Minneapolis, USA). According to the software optimization step, the desired goal for each immobilization variable (A bead size mm, B initial biomass concentration g/L and C alginate concentration g/L) was defined within the studied levels range to achieve the highest performance. The program combines the individual desirability into a single number and then searches to optimize this function based on the response goal. Accordingly, the optimum alginate-immobilization conditions was found to be 2.5 mm bead size (i.e. diameter), 2.5 g/L initial inoculum size (i.e. initial biomass

concentration) and 5.5 g/L alginate concentration (i.e. carrier concentration 0.55% w:v) with predicted 4.15 g/L ethanol production, 10.48 g/L glucose consumption and 39.74 % actual bioethanol yield, with desirability function of 0.946. Additional experiments were then performed to confirm the optimum results and the experimental respective responses recorded; 4.4 g/L, 10.5 g/L and 41.9 %, respectively, which agreed well with the aforementioned predicted responses values. The maximum bioethanol yield in this study was achieved at lower (0.55% w:v) alginate concentration than that reported in previous published researches, which would have a positive impact on the economy of the fermentation process. Carvalho et al (2002) reported the maximum xylitol production from sugarcane bagasse was achieved by Ca-alginate immobilized *Candida guilliermondii*, using beads prepared by 2% sodium alginate and 0.1 M calcium chloride concentration. Najafpour et al (2004) reported that, 2% alginate is the most appropriate concentration for maximum stability of the beads and production of bioethanol. Milessi et al (2013) reported the maximum ethanol production from xylose was achieved by Ca-alginate immobilized *Scheffersomyces stipitis*, using beads prepared by 2% sodium alginate and 0.1 M calcium chloride concentration.

The obtained results in this study showed that, not all the consumed amount of glucose would be converted to ethanol, either in case of free or immobilized cells. Similar observation was reported by Sangryeol and Lee (1997) where the maximum ethanol productivity by Ca-alginate immobilized *Zymomonas mobilis*, was increased from 77.5 g/L/h with 10% glucose to 87 g/L/h with 15% glucose. However, only 48% of the utilized glucose in the 15% glucose medium was converted to ethanol, while 82% of the utilized glucose in the 10% glucose medium was converted to ethanol.
In all the experimental runs, the glucose consumption was not affected by the change in experimental conditions or the presence of carrier, where in all trials the assimilation of glucose was $\geq$ 91.09%. Similar observation was reported by Milessi et al (2013) for the consumption of xylose by Ca-alginate immobilized *Scheffersomyces stipitis* cells for production of bioethanol.

The increase of bioethanol yield through immobilization of yeast cells in calcium-alginate gel proved that, it is an efficient matrix for the entrapment of *Candida stauntonica* MY1 cells. Similar observation was reported for continuous and static fermentation of glucose by Ca-alginate immobilized *Saccharomyces cerevisiae* cells (McGhee et al., 1982).

Scanning morphology of calcium-alginate cell immobilized beads

The operability of bead preparation under the aforementioned optimum conditions was easy, its shape is symmetric and smooth and its average size is $\approx$ 2.67 mm (Figure 7a, under magnification power of x100, 30 kv). A few yeast cells can be observed on the surface of beads, and also a highly macro-porous structure was found in the beads which would favor the diffusion of substrates and products (Figure 7b, under magnification power of x3500, 30 kv). From the cross-section (Figure 7C, under magnification power of x3000, 30 kv), it can indicated that large numbers of cells were evident within the internal structures of the beads, and *Candida stauntonica* MY1 cells appeared to be entrapped and maintained their structural integrity within the alginate matrix.

Stability and reusability of immobilized cells

In reality, the storage, stability and reusability of the cell-entrapped alginate beads constitute important parameters for the successful application of these immobilized cells for the bioethanol fermentation.

Rate of ethanol production by free and immobilized cells

The rate of ethanol production by free and immobilized *Candida stauntonica* MY1, using optimum conditions for immobilization, was investigated. The time course profiles are shown in Figure 8. The glucose consumption resulted in biomass growth and ethanol production. The cell concentration in free cells batch fermentation (Figure 8a) increased with time reaching its maximum 18.85 g/L, within 48 h, incubation period and then slightly decreased with longer incubation period, recording 14.41 g/L after 72 h of incubation, with a continuous consumption of glucose. The decline in growth might be due to the production of some inhibitory byproducts (Najafpour et al., 2004). In the free cell fermentation batch (Figure 8a), there was a regular increase in the ethanol production up to 48h, with maximum ethanol yield of ≈ 18.5%, but further increase in the fermentation time decreased the ethanol production. Similar observation was reported by Kalhorinia et al (2014) in the fermentation batch of xylose using *Candida intermedia* MTCC-1404. In case of immobilized cells batch fermentation (Figure 8b), there was a sharp glucose consumption ≈ 78.66% with a low bioethanol yield of ≈ 3.6%, within the first 12 h of incubation, then the glucose consumption was continued to reach ≈ 99% after 72 h. The cell leakage was not so significant, recording ≈ 3 g/L after 72 h of incubation. The total beads weight increased from 140 g/L to reach ≈ 145 g/L within 36 h of incubation, indicating the viability and growth of the entrapped yeast cells. But the total beads weight was then slightly decreased to reach 142 g/L within 48 h and then further decreased recording ≈ 137 g/L at the end of incubation period 72 h, due to some cell leakage. There was a continuous recorded increase of bioethanol yield with time, recording 41.29% within 48 h. That was 2.24 fold higher than of free cells, then it was slightly increased to reach its maximum 43.33% within 60 h, which was 4.66 fold higher than that in free cells batch. The sustainable production of bioethanol within 48 – 60 h incubation period would indicate that, the alginate matrix protected the encapsulated yeast cells from the inhibitory byproducts, maintaining a high concentration of cells in the capsules with improved activity, and a long-time operational stability with higher ethanol production and yield relevant to the fermentation with the free yeast cells. However, the bioethanol yield was sharply decreased to 20.48% with longer incubation period 72 h, but it was still higher than that of free cells batch by 7.02 fold. There was no observed breakage or disruption of Ca-alginate beads within the whole batch. Therefore, cells entrapped in Ca-alginate matrix with an average diameter of 2.5 – 2.67 mm showed high stability under the fermentation conditions which consequently showed high ethanol production. In addition, there was no need to use antibiotics and contamination was not observed.

Storage stability and reusability of free and immobilized cells

It can be concluded from results illustrated in Figure 9, that the immobilized cells can be stored for 21 days in sterile saline at 4^{o}C

without compromising the fermentation capacity, recording ≈ 41 and 39% bioethanol yield, using freshly prepared and 21 days-old immobilized cells beads, respectively. That decreased to 32.36% in batch fermentation inoculated with 28 days-old immobilized cells beads (Figure 9a). In case of free cells, the storage stability was low, recording ≈ 19 and 9.87% bioethanol yield, in batch fermentation inoculated with fresh and 28 days-old free cells, respectively (Figure 9b).That indicated, the bioethanol yield in batch fermentation inoculated with 28 days-old immobilized cells beads was 3.3 fold higher than that inoculated with 28 days-old free cells, assuring the storage stability of the immobilized cell beads.

Additionally, the immobilized and free cells were used in consecutive five batches. The results of these batches are illustrated in Figure 10. The immobilized beads was found to be effectively reused over a long period of time; 192 h for four fermentation batches, without a high significant change in bioethanol yield, recording ≈ 41, 40, 37 and 36% in the first, second, third and fourth fermentation cycles, compared to ≈ 20, 13, 10 and 9.9% bioethanol yield with the free cells cultures, respectively. But the ethanol yield was sharply decreased to ≈ 29% within the fifth fermentation batch cycle, relevant to 9.88% bioethanol yield in the free cells cultures, indicating that, the bioethanol yield with immobilized cells was ≈ 3 fold higher than that with free cells.

The ethanol productivity was decreased with the extent of cell leakage (Figure 9b and 10b), where the beads become fragile and deformed in shape. However, the observed stability of the immobilized cells beads with high production of ethanol would indicate the biological compatibility and good mechanical strength of the low-cost Ca-alginate matrix. These are important parameters in repeated fermentation cycles, which would consequently improve the process efficiency, productivity and save inoculum preparation time and other associated processing steps.

Conclusions and recommendations

Calcium-alginate beads did not show negative interaction with the fermentation medium and cultivation conditions; otherwise, it increased the bioethanol yield. The maximum bioethanol yield was 0.43 and 0.19 g ethanol /g glucose, in immobilized and free cells fermentation batches, respectively, with a net 2.26 fold higher yield. The immobilized cells were found to be stable for four successive fermentation batches and stably stored for 21 days. The most significant feature of the immobilized cells is their long-term-life-time. All of these would add to the advantage of ethanol production, reducing the tine of inoculum preparation. The support matrix is rather cheap and easy to prepare.

Since immobilization is the restriction of cell mobility within a define space, so the obtained results in this study, would provide; cell reusability, eliminates washout problems at high dilution rates and the cost process of cell recovery and cell recycle, reduce the possibility of contamination, it would improve genetic stability and tolerance of high ethanol concentration, high volumetric productivities can be also obtained, which could decrease the overall cost of bioethanol fermentation. It is difficult to compare the obtained results with other published individual reports due to the difference in experimental conditions, substrate and microbial strains. However, it is apparent that the formed beads are quite good in terms of stability, storage, reusability, and bioethanol production.

Table.1 Experimental design matrix with experimental and predicted values of the studied responses

Run number	Bead Size mm A		Inoculum Size g/L B		Alginate Concentration g/L C		Response 1 Bioethanol Production g/L		Response 2 Utilized Glucose g/L		Response 3 Actual Bioethanol Yield %	
	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Experimental value	Predicted value	Experimental value	Predicted value	Experimental value	Predicted value
1	+1	3.50	+1	10.00	-1	2.00	0.14	0.15	10.55	10.54	1.33	1.47
2	0	2.75	0	6.25	0	5.00	3.29	3.37	10.51	10.50	31.30	32.02
3	0	2.75	-1	2.50	0	5.00	4.26	4.05	10.47	10.46	40.69	38.81
4	0	2.75	0	6.25	0	5.00	3.30	3.37	10.49	10.50	31.45	32.02
5	-1	2.00	0	6.25	0	5.00	2.00	1.90	10.49	10.46	17.45	17.34
6	-1	2.00	-1	2.50	-1	2.00	0.59	0.67	10.02	10.02	5.89	6.39
7	0	2.75	0	6.25	0	5.00	3.32	3.37	10.47	10.50	31.70	32.02
8	+1	3.50	-1	2.50	-1	2.00	0.28	0.28	10.21	10.21	2.74	2.90
9	0	2.75	0	6.25	0	5.00	3.29	3.37	10.43	10.50	31.54	32.02
10	+1	3.50	-1	2.50	+1	8.00	1.30	1.40	10.35	10.35	12.56	13.60
11	+1	3.50	0	6.25	0	5.00	1.49	1.43	10.47	10.48	14.23	13.09
12	+1	3.50	+1	10.00	+1	8.00	0.20	0.16	10.13	10.13	1.97	1.78
13	0	2.75	0	6.25	0	5.00	3.34	3.37	10.50	10.50	31.81	32.02
14	0	2.75	0	6.25	+1	8.00	2.67	2.53	10.37	10.35	25.75	24.57
15	0	2.75	+1	10.00	0	5.00	3.21	3.26	10.52	10.51	30.51	31.13
16	0	2.75	0	6.25	0	5.00	3.35	3.37	10.53	10.50	31.81	32.02
17	-1	2.00	+1	10.00	+1	8.00	0.68	0.72	10.26	10.27	6.63	6.78
18	-1	2.00	-1	2.50	+1	8.00	2.15	2.18	10.48	10.49	20.52	20.70
19	-1	2.00	+1	10.00	-1	2.00	0.37	0.31	10.34	10.35	3.58	2.86
20	0	2.75	0	6.25	-1	2.00	1.79	1.77	10.32	10.54	17.34	17.26

Table.2 Analysis of variance of the fitted quadratic regression model equations

	Bioethanol production g/L, model equation 3					
Source	**SS***	**df***	**MS***	**F-value**	**p-value**	**Remarks**
Model	34.09	9	3.79	311.57	< 0.0001	Highly significant
A	0.57	1	0.57	46.60	< 0.0001	Highly significant
B	1.58	1	1.58	130.31	< 0.0001	Highly significant
C	1.47	1	1.47	120.67	< 0.0001	Highly significant
A^2	7.98	1	7.98	656.22	< 0.0001	Highly significant
B^2	0.23	1	0.23	18.61	0.0015	Significant
C^2	4.08	1	4.08	335.70	< 0.0001	Highly significant
AB	0.025	1	0.025	2.08	0.1796	Non-significant
AC	0.078	1	0.078	6.42	0.0297	Possibly significant
BC	0.61	1	0.61	50.22	< 0.0001	Highly significant
Residual	0.12	10	0.012			
Corrected total	34.21	19				
Utilized glucose g/L, model equation 4						
Source	**SS***	**df***	**MS***	**F-value**	**p-value**	**Remarks**
Model	0.39	9	0.043	49.81	< 0.0001	Highly significant
A	0.0014	1	0.0014	1.65	0.2280	Non-significant
B	0.0073	1	0.0073	8.35	0.0161	Possibly significant
C	0.0023	1	0.0023	2.58	0.1395	Non-significant
A^2	0.0020	1	0.0020	2.27	0.1632	Non-significant
B^2	0.0004	1	0.0004	0.44	0.5222	Non-significant
C^2	0.072	1	0.072	82.48	< 0.0001	Highly significant
AB	0.00005	1	0.00005	0.057	0.8157	Non-significant
AC	0.054	1	0.054	62.36	< 0.0001	Highly significant
BC	0.15	1	0.15	173.24	< 0.0001	Highly significant
Residual	0.009	10	0.0009			
Corrected total	0.40	19				
Actual bioethanol yield %, model equation 5						
Source	**SS***	**df***	**MS***	**F-value**	**p-value**	**Remarks**
Model	3086.80	9	342.98	346.90	< 0.0001	Highly significant
A	45.11	1	45.11	45.63	< 0.0001	Highly significant
B	147.30	1	147.30	148.99	< 0.0001	Highly significant
C	133.59	1	133.59	135.12	< 0.0001	Highly significant
A^2	776.96	1	776.96	785.83	< 0.0001	Highly significant
B^2	23.95	1	23.95	24.23	0.0006	Significant
C^2	339.05	1	339.05	342.92	< 0.0001	Highly significant
AB	2.20	1	2.20	2.23	0.1662	Non-significant
AC	6.52	1	6.52	6.59	0.0280	Possibly significant
BC	53.87	1	53.87	54.49	< 0.0001	Highly significant
Residual	9.89	10	0.99			
Corrected total	3096.69	19				

*SS: sum of squares df: degree of freedom MS: mean square

Figure.1 Phylogenetic tree constructed by neighbor joining method of 18S rDNA gene for MY1 and closely related yeast

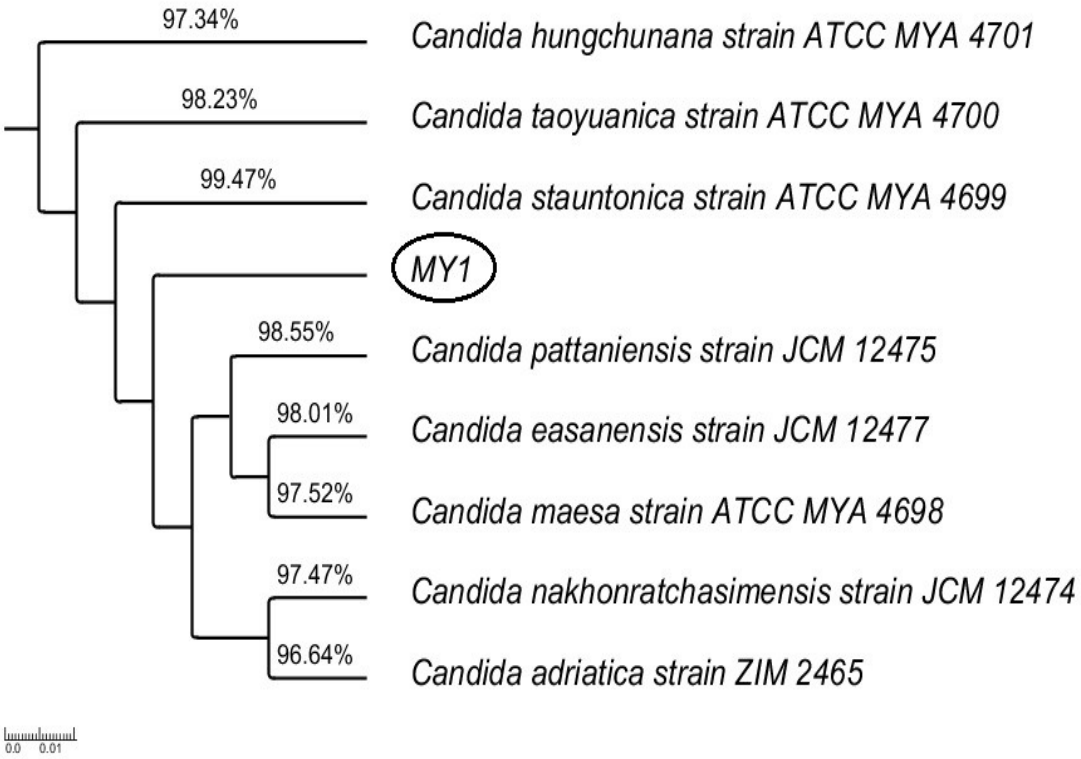


Figure.2 Effect of initial concentration of free cells on fermentation process

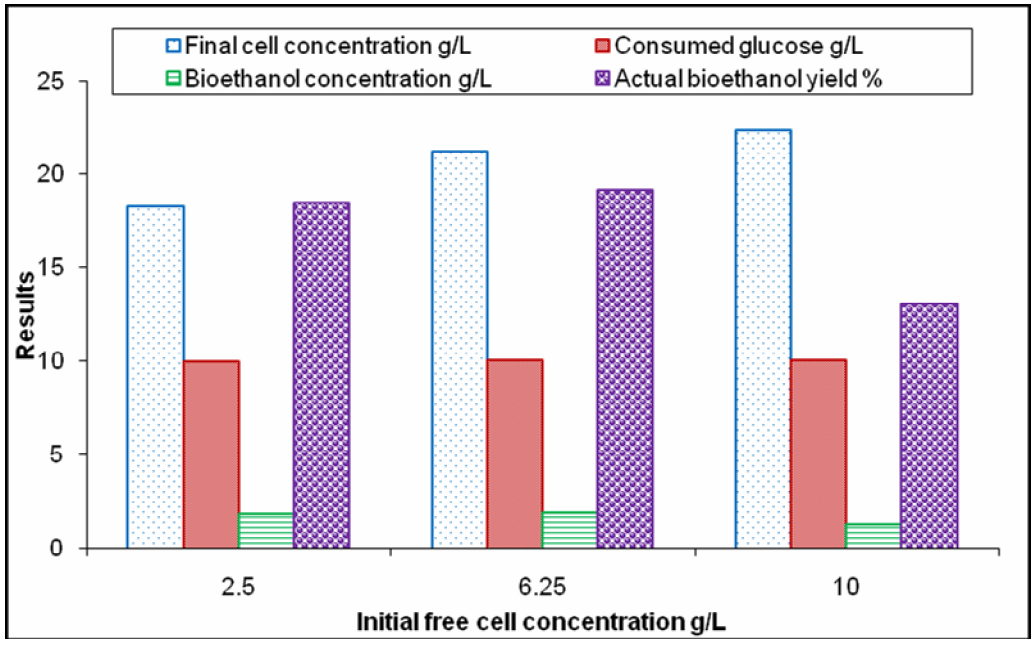


Figure.3 Plots of actual and predicted responses (a) bioethanol production g/L, (b) utilized glucose g/L and (c) actual bioethanol yield %

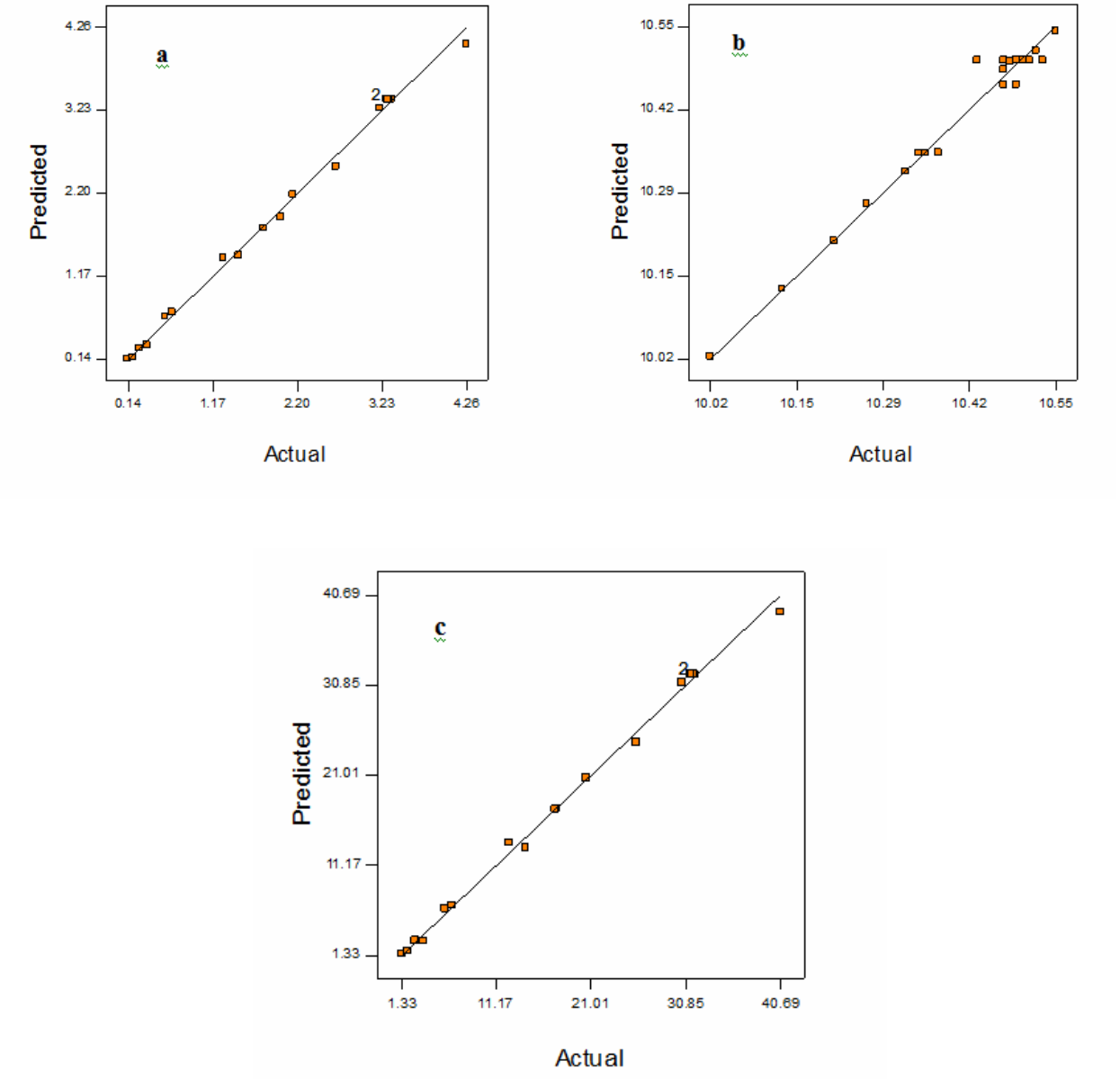


Figure.4 Diagnostic plots for residuals versus run number (a) bioethanol production g/L, (b) utilized glucose g/L and (c) actual bioethanol yield

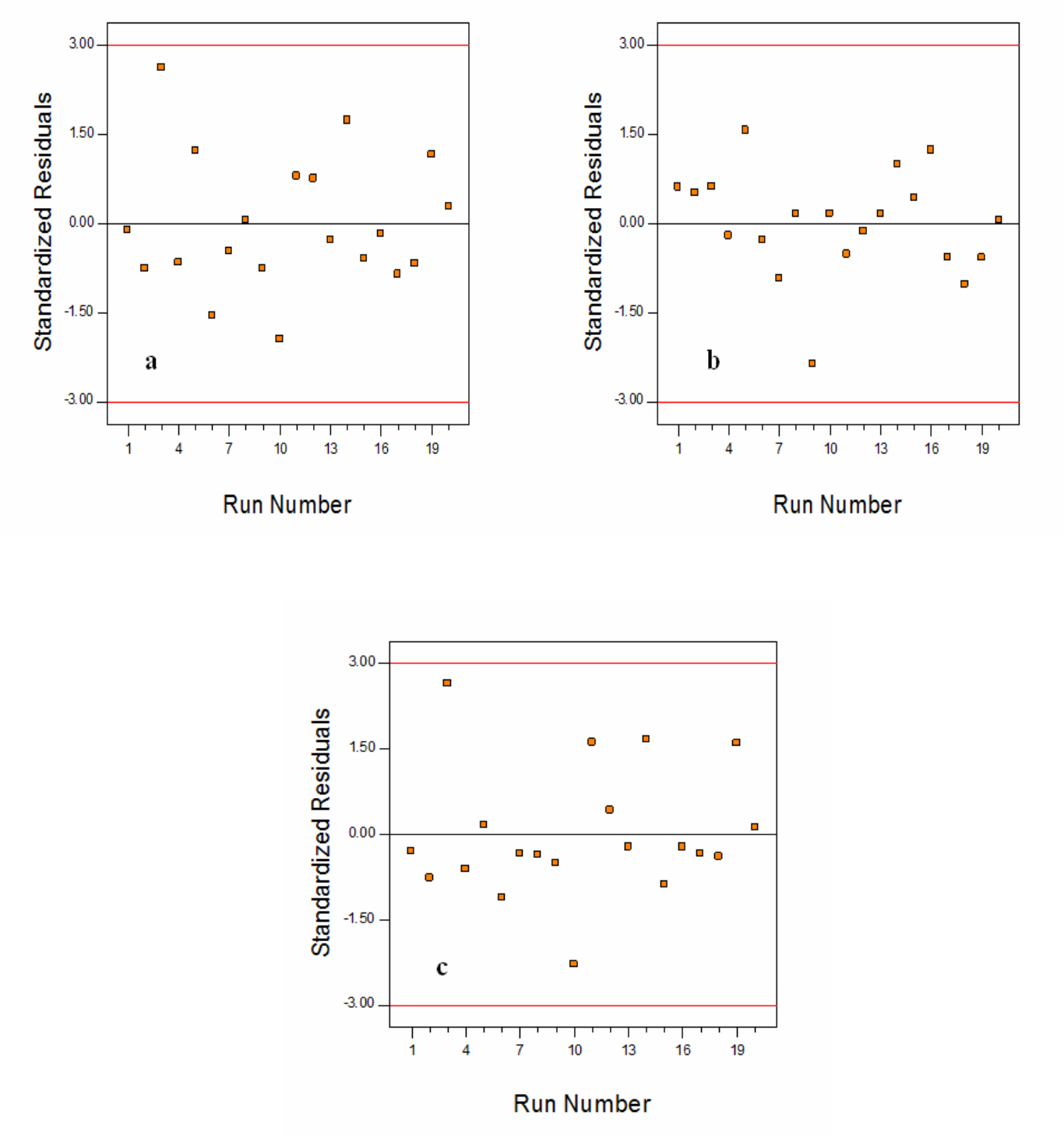
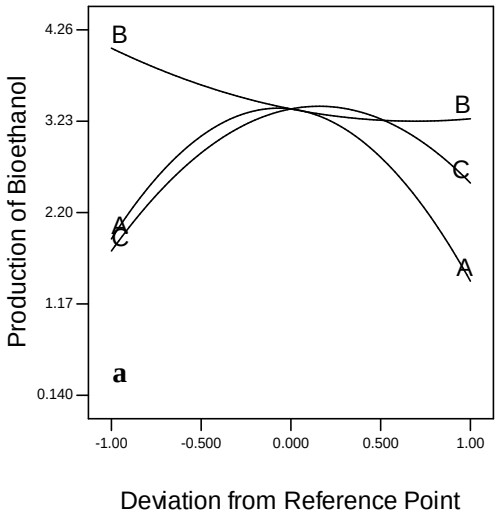
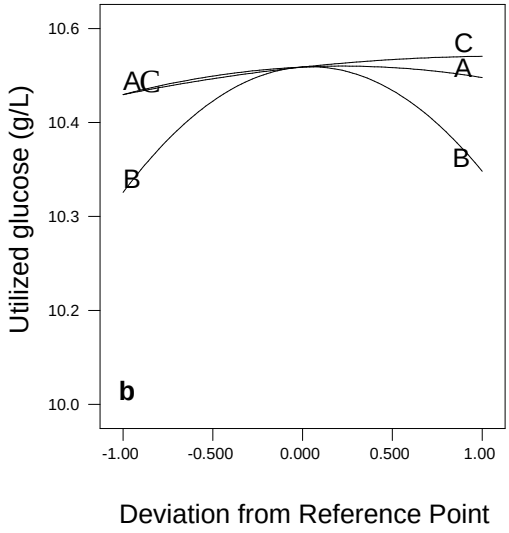


Figure.5 Perturbation plots (a) bioethanol production g/L, (b) utilized glucose g/L and (c) actual bioethanol yield %





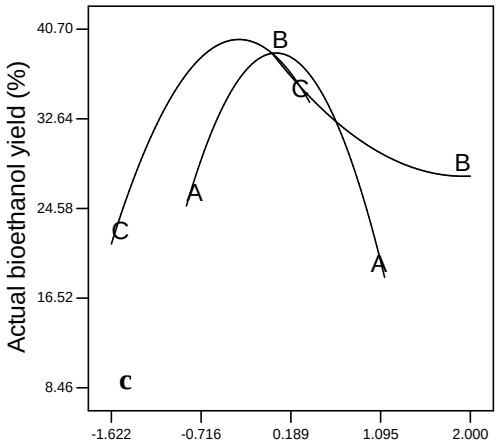


Figure.6 RSM and contour plots (a) bioethanol production g/L, (b) utilized glucose g/L and (c) actual bioethanol yield %

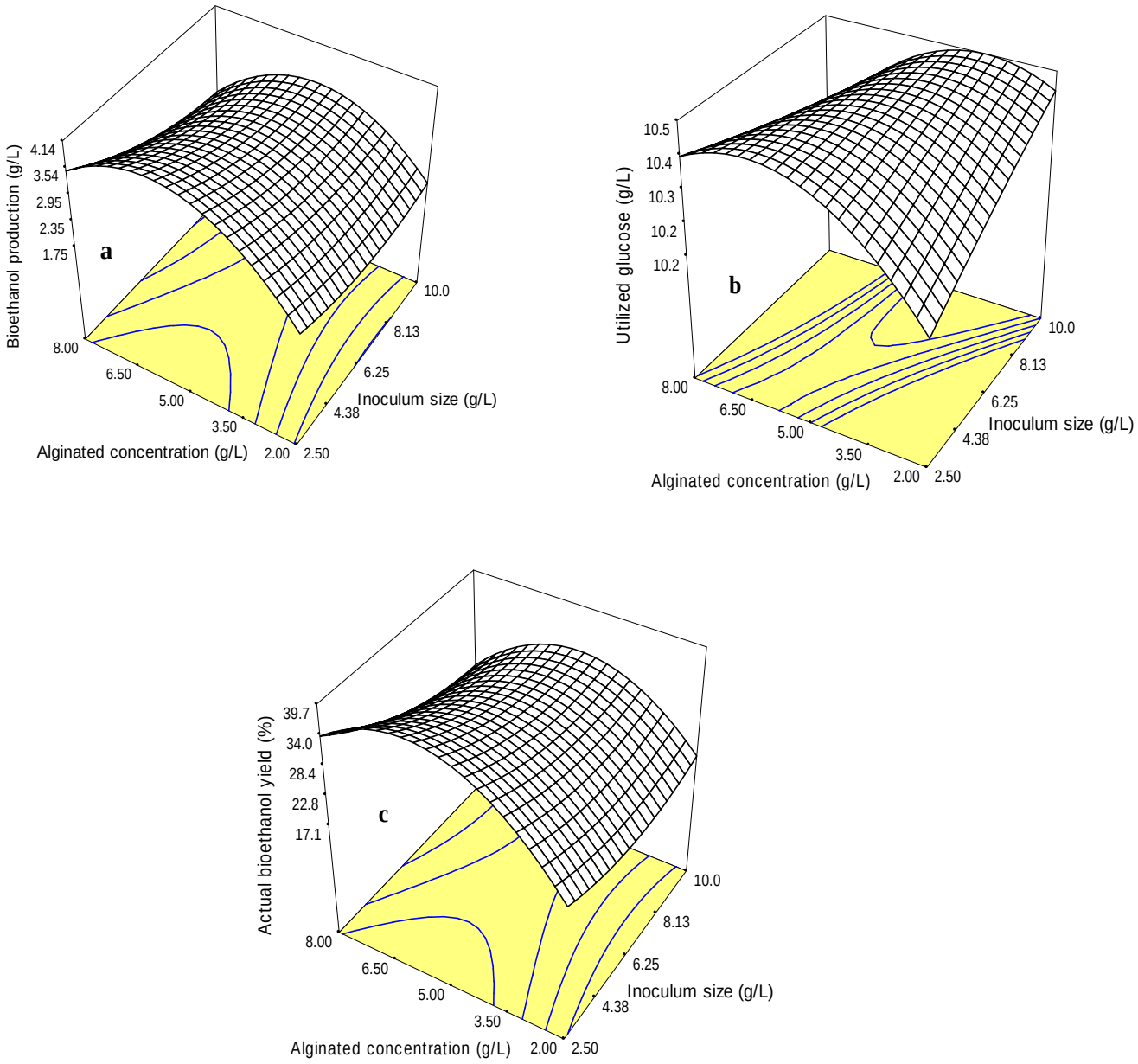
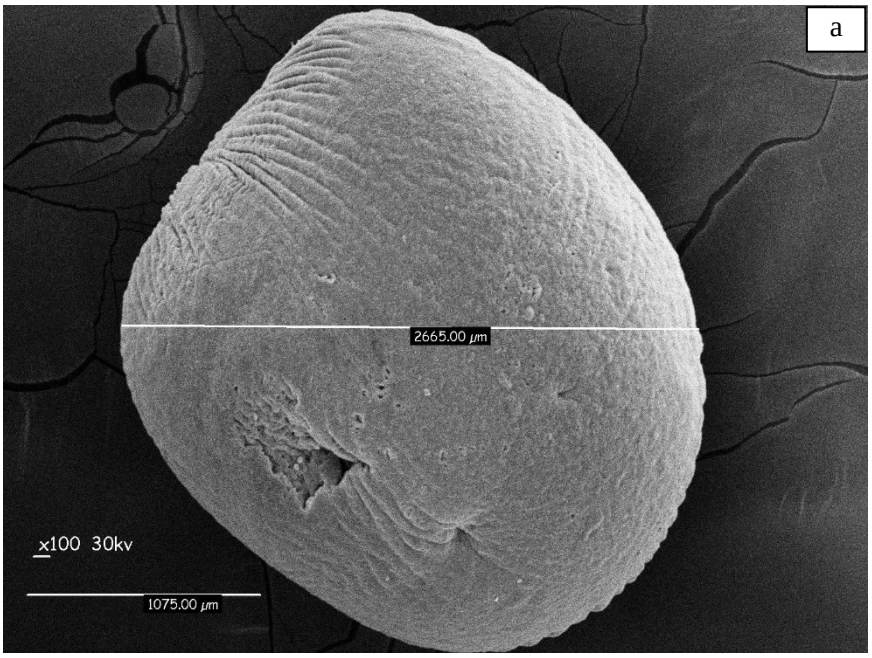
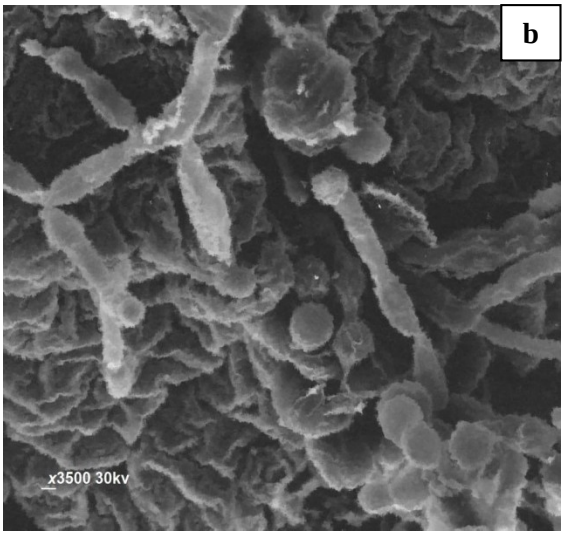


Figure.7 SEM pictures (a and b) the immobilized bead under different magnification powers and (c) cross-section of the alginate





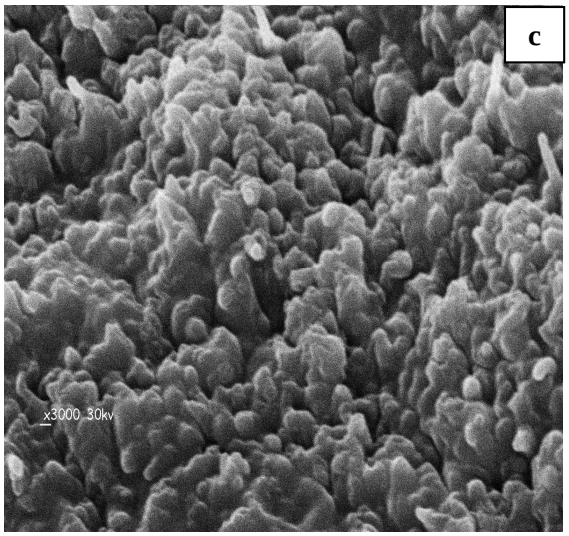


Figure.8 Time profile of bioethanol batch fermentation by (a) free cells (b) immobilized cells

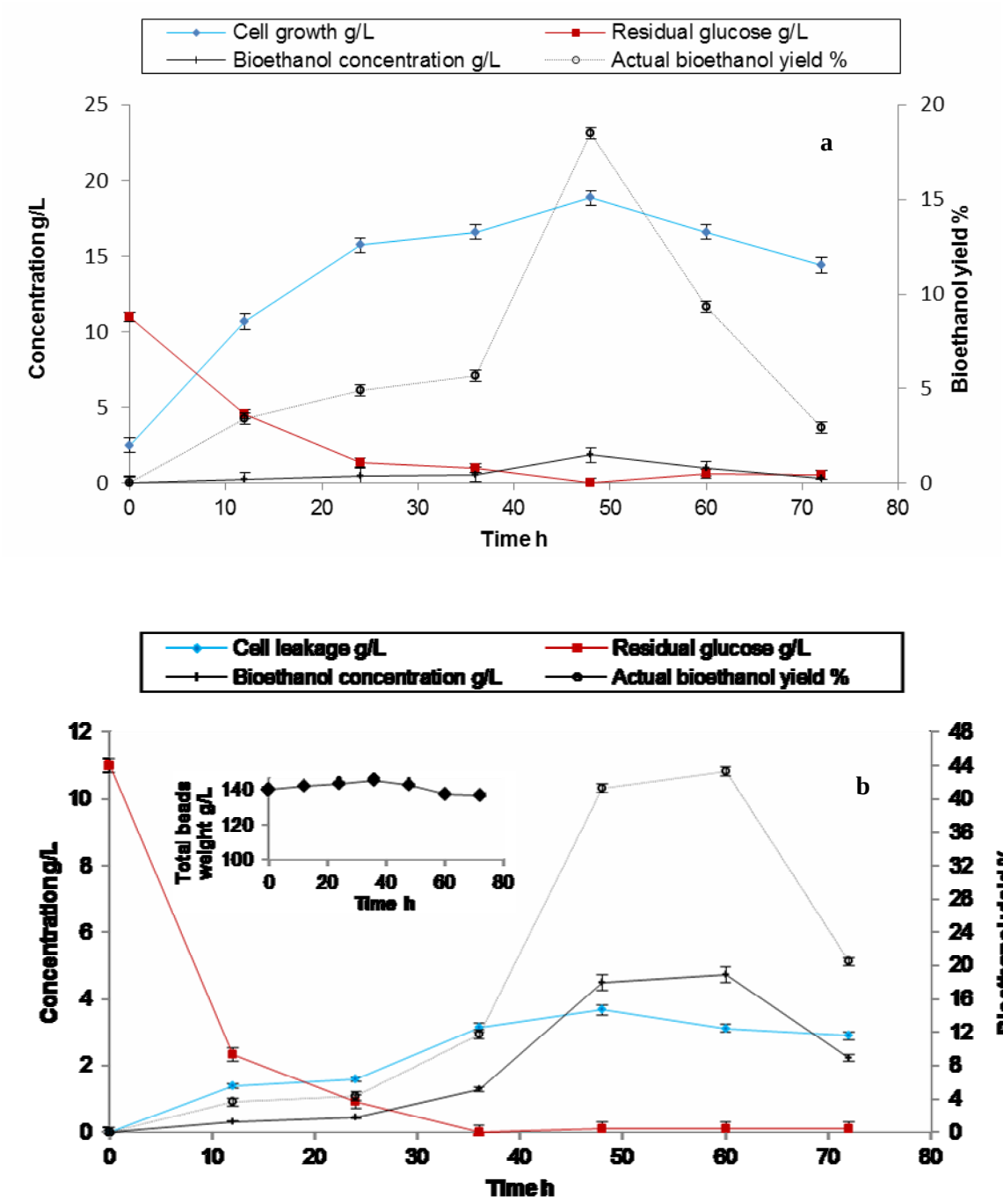
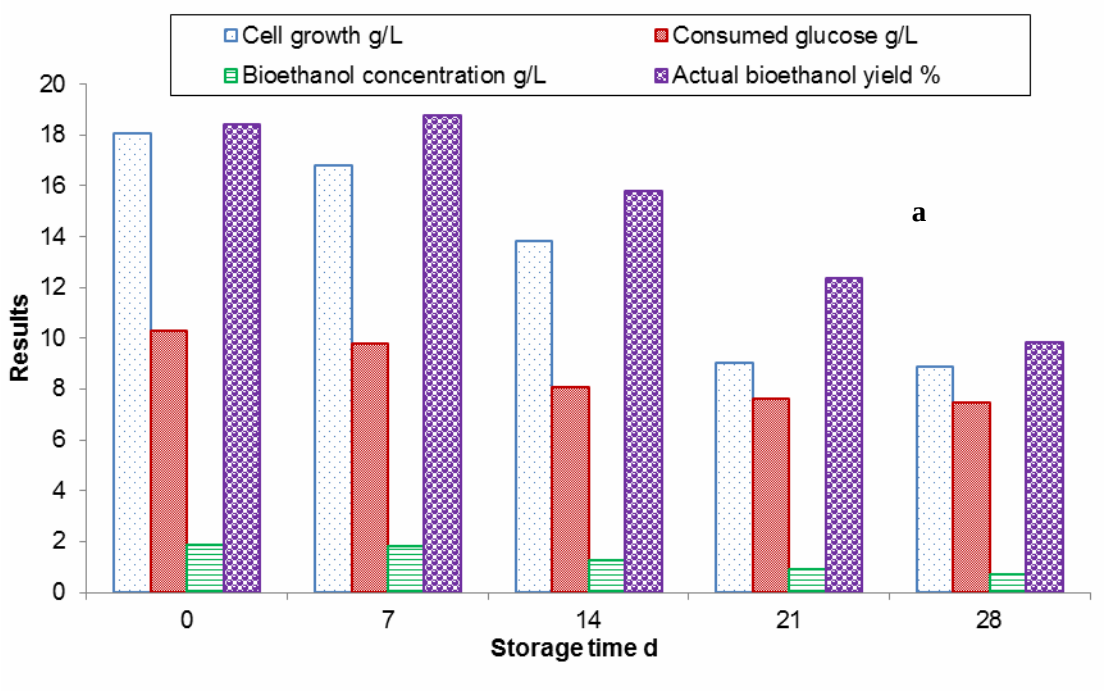


Figure.9 Storage stability (a) free cells (b) immobilized cells



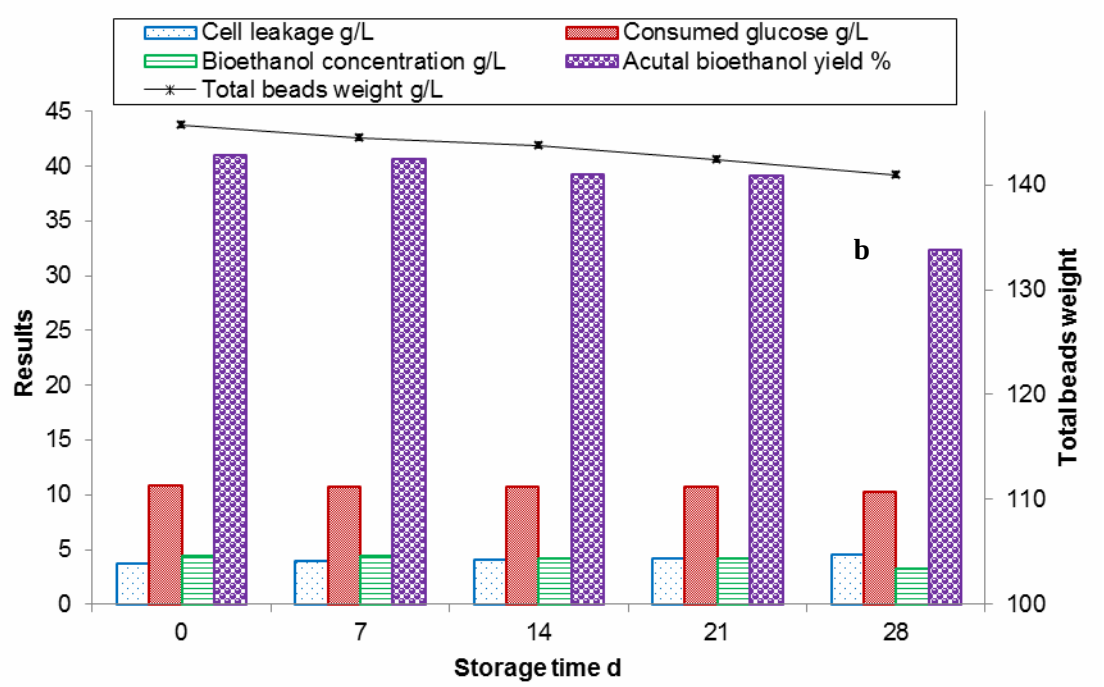
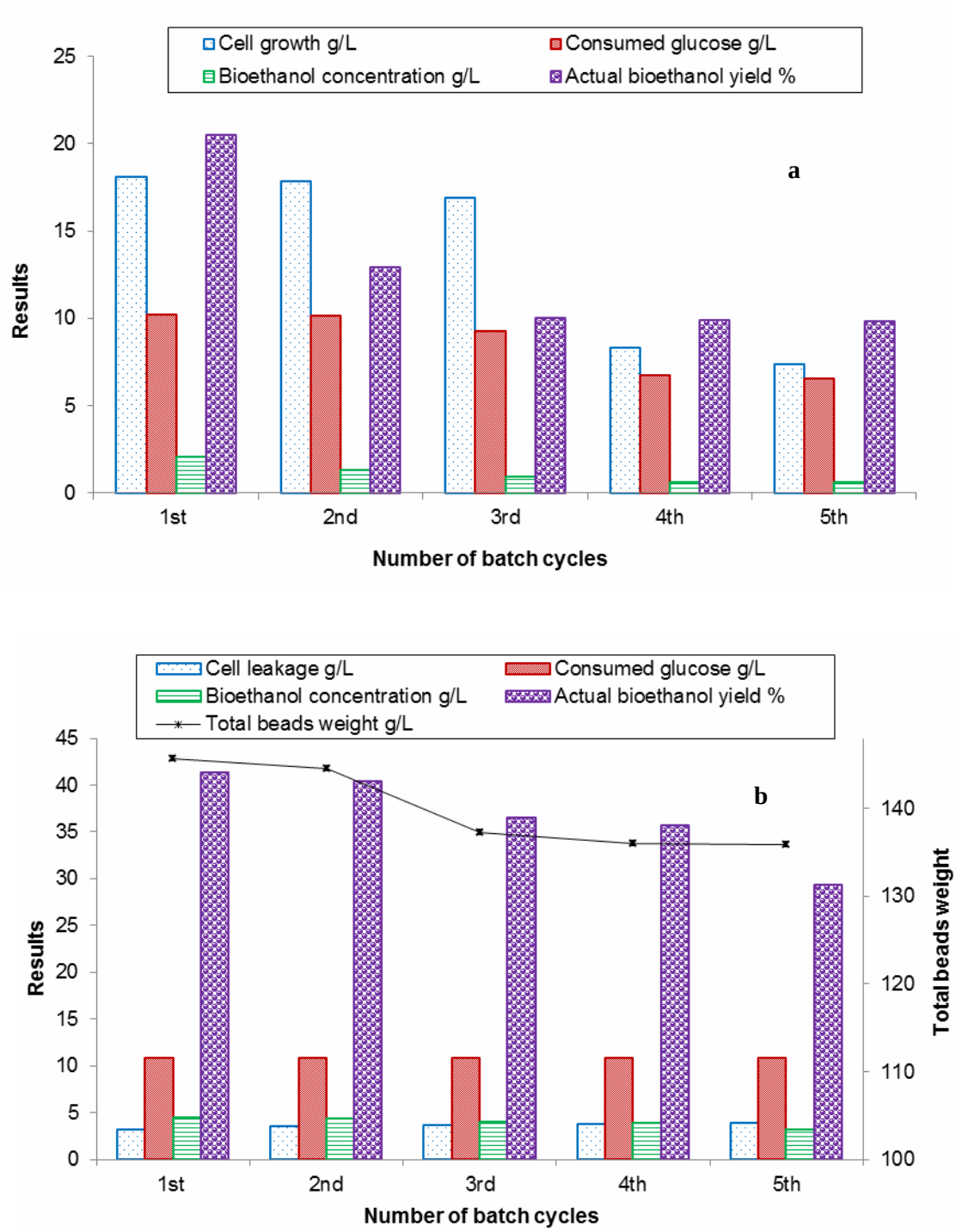


Figure.10 Reusability of (a) free cells (b) immobilized cells



It could be employed in a continuous process in a tubular reactor and for large scale industrial applications.

Further studies are undertaken in EPRI biotechnology Lab to study the effect of cell leakage, age of the alginate-entrapped yeast cells and initial glucose concentration. Since the substrate cost is about 60-70% of ethanol production cost (Williams and Munnecke, 1981) and the age of the entrapped cells is one of the critical factors that influence efficient fermentation of glucose to ethanol (McGhee et al., 1982).Sugar concentration is also critical in the fermentation process, influencing the physiological growth of the yeast, rate of ethanol production and its final yield (Ghorbani et al., 2011). Also, further work is still needed on the recovery of ethanol from the fermentation process and improvement of the substrate consumption rate by the organism at higher substrate levels, with higher bioethanol yield.

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