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OPTIMIZATION OF BIOETHANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE* MICROENCAPSULATED ON ALGINATE-DELIGNIFIED CELLULOSE MATERIAL

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ABSTRACT

The aim of this research was to enhance bioethanol production via immobilization and microencapsulation of *Saccharomyces cerevisiae* CICC 1001 into delignified cellulosic material (DCM), prepared from sawdust and Na alginate. It has been demonstrated that microencapsulation process using Alginate: DCM ratio of (1:1.5) and alginate solution of 2.5% by wt, attained the highest microencapsulation efficiency. In addition, microwave alkali pretreated sugarcane bagasse was used as a substrate for second-generation ethanol production. A maximum ethanol yield of 0.37g/g theoretical yield was obtained after 96h of incubation from DCM-Alginate microcapsule, which was 42.3% higher than that of alginate alone. The microcapsule was used up to nine cycles in order to study about its stability and the strength of yeast cell for ethanol production. Optimization of ethanol production was examined using the Box–Behnken design (BBD) of response surface methodology. The pH (4-5.5), fermentation time (24-72 h), and solid content loading (15-20%w/v) were tested to maximize bioethanol yield in the SSF process. The predicted optimum conditions were pH 4.5, 56.42h fermentation time, and 15.16%w/v solid content loading. Overall, this work will be meaningful in the conversion of lignocellulosic agro-waste to generate bioethanol.

KEYWORDS: Bioethanol; Immobilized cell; Microencapsulation; Delignified cellulosic material; Response surface methodology

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1. INTRODUCTION

The concept of cell immobilization was the physical confinement of intact cells to a particular region of space without changing of desired catalytic activity [1]. Cell immobilization considered as a way of increasing bioreactor productivity, especially in fermentation processes. This method allowed better substrate utilization, sensitive external substances protection, and greater productivities among various fermentation processes. There are four main immobilization techniques for yeast cells: containment behind barriers, attachment to a supports surface, entrapment within a porous matrix and cell aggregation (flocculation) [2]. Containment behind a barrier can be achieved by two main methods: entrapment of the cells in microcapsules or hollow fiber matrix [1,3]. Microencapsulation defined as the technology of packaging active ingredients in small capsules that release their content at controlled rates over time [4]. It can be achieved either by chemical or physical methods [4]. Microencapsulation in alginate is the most common system of immobilization, because of its simplicity, nontoxicity, biocompatibility and low cost [5]. The microencapsulation of yeasts proposed as a suitable vehicle for the source material, for a target controlled release. They could prevent the loss of viability and protect the yeast against unfavorable circumstances [6,7]. In addition, Wandrey et al. [8] reported that the microencapsulation could increase the shelf life of the encapsulated yeast and achieve a controlled and targeted release.In this study, we use containment behind barriers technique using DCM and alginate supports. Alginate beads and delignified cellulosic materials (DCM) carriers of *S. cerevisiae* were fabricated via microencapsulation technique to carry yeast cells for ethanol fermentation. This research investigated the optimal ratio of DCM to alginate and the suitable concentration of alginate. The ethanol productions by batch and repeated batch fermentation using DCM-immobilized microcapsule were compared with those of single DCM as well as alginate beads while studying its stability in a long-term operation. Overall, this paper highlights the need for a possible application of alginate beads and delignified cellulosic material as carriers for *S. cerevisiae* for ethanol production under batch and repeated batch fermentation. The present paper was divided into different steps as follows: (i) studying DCM-Alginate microcapsule, in terms of encapsulation yield, viability of loaded *S. cerevisiae*, (ii) applying the microcapsule as carriers for the yeasts (iii) using microcapsule to start the fermentation of lignocellulosic biomass, (iv) evaluating the bioethanol production through response surface methodology approach.

2. MATERIALS AND METHODS

2.1 *Microorganism and Inoculum preparation*

The hyper ethanol production yeast strain used in this paper, *Saccharomyces cerevisiae* CICC 1001, was purchased from China Center of Industrial Culture Collection. The yeast was inoculated in a 1000mL Erlenmeyer flask containing 500mL cultivation medium. The cultivation medium (YEPD medium) consisted of 20 g/l yeast extract, 20 g/l peptone and 10 g/l glucose and was sterilized at 121^{o}C for 15 min prior to use. Yeast inoculum was prepared based on the method proposed by Ofori-Boateng [9] and was further incubated at 30^{o}C for 16 h in an incubator shaker (150 rpm).

2.2 *Feedstock preparation and pretreatment procedures*

In this study, an air-dried and grounded lignocellulosic biomass was utilized as substrate for ethanol production. Sugarcane bagasse (SCB) was collected from the local market in Nanjing city, China. The biomass used for further treatment were dried 65^{o}C overnight after ground to 1 mm size.

2.3 Microwave-alkali assisted pretreatment

SCB was subjected to NaOH - microwave pretreatment. The lignocellulosic biomass used was soaked in 2.5% NaOH solution, then exposed to 540 W microwave powers

pretreatment for 15 minutes. Liquid-to-solid ratio was maintained until 10:1. Biomass were washed several times in tap water until reached neutral pH, and then followed by a final rinse in the distilled water. The pretreated biomass were dried at 65°C overnight later they were cooled down at room temperature and stored in the airtight containers until further use.

2.4 Yeast immobilization

In this present paper, delignified cellulosic material (sawdust), Ca-alginate and mixed DCM-alginate were used as support for immobilization of yeast. The sawdust was prepared using the protocol as described previously by Bardi and Koutinas [10]. After that, the material was washed extensively until achieved neutral pH. The sawdust biomaterial was dried at 65°C overnight. In addition, the DCM was cooled down to room temperature and kept in a desiccator for subsequent use. For DCM preparation, the pretreated DCM mixed with 1L of minimal salt (MS) medium. The MS medium consists of 30 g/l glucose, 5g/l yeast extract, 10g/l $(NH_4)_2SO_4$, 4.5g/l KH_2PO_4 and 1g/l $MgSO_4.7H_2O$ as described in [11]. Yeast cells then inoculated into the support medium and left to ferment for 48h at 35°C. In addition, the liquid was then decanted, and the immobilized cells were washed with sterile water and used for fermentation runs. For alginate preparation, 2.5% (w/v) of Na-alginate and the yeast cells were added to a (5% w/v) $CaCl_2$ solution. Mean diameter of immobilized cell was around 3 to 4 mm. For DCM-alginate preparation, the treatment was followed the above protocol for the alginate and sawdust preparation. The alginate was mixed with immobilized yeast-DCM at different dry weight ratios (w/w). Xanthan gum was added to the $CaCl_2$ solution to form spherical capsules. The homogenized mixture of immobilized cell was dropped by a syringe dispenser (Hamilton, MA, USA) into sterile 0.1 M $CaCl_2$ stirred by a magnetic bar. The formed microcapsules were left for solidification for 1h.

2.5. Microencapsulation efficiency

In order to determine the microencapsulation efficiency of *S.cerevisiae*, 1 g of microcapsules was dissolved by placing them in 50 mL sodium citrate solution (10 g/L) using a stirrer until disintegration. The cell viability was determined by colony forming units (CFU/g) counting via a pour-plate method using MRS agar and then incubating at 37°C for 48 h. Microencapsulation efficiency (ME) was calculated using Eq. (1):

$$ME\ (\%) = \frac{N}{N0} \times 100$$

where N is the number of microencapsulated cells released from the microcapsules (log CFU/g microcapsule) and N_0 is the number of free cells added to the polymer mixture during the production of the microcapsules (log CFU/g).

2.6 Microcapsule size

Diameter of ten wet beads were evaluated using caliper (Mitutoyo, Japan) and the average diameter were measured and recorded. The measurement was replicated three times.

2.7 Simultaneous Saccharification and Fermentation (SSF)

The fermentation medium of on-site crude cellulase enzyme using microwave-alkali pretreated single and mixed materials was examined for ethanol production. SSF experiments for ethanol production were conducted in 500 ml flasks with a working volume of 250 ml. The flasks were incubated in 150 rpm at 37°C for 96 h. All of the experiments performed in triplicate. In addition, the untreated substrate without yeast cell was used as a control. Subsequently repeated batch fermentations were carried out for ethanol production followed the protocol in Chandel et al. [12].

2.8 Experimental design for ethanol production optimization

A statistical approach was used to screen out the factors affecting bioethanol production.

Response surface methodology is an effective tool for optimizing different non-homogenous parameters and conditions. Box–Behnken factorial design with three factors and levels was used to ascertain the best combination of parameters for maximizing the bioethanol yield. The pH, solid content (%, w.v-1) and fermentation time (h) were selected as independent variables for optimizing ethanol production. The second-order polynomial response function to consider the effects of the three variables can be approximated by the Eq.1 below:

$$Y = a_0 + a_1A + a_2B + a_3C + a_{11}A^2 + a_{22}B^2 + a_{33}C^2 + a_{12}AB + a_{13}AC + a_{23}BC$$

where Y is the predicted response; a_0 is the intercept; a_1,a_2,a_3 the linear coefficients; a_{11},a_{22},a_{33} the squared coefficients; and a_{12}, a_{13}, a_{23} the interaction coefficients. All of the results of the experimental designs were analyzed and interpreted using Design Expert 9 statistical software (Stat ease trial versions).

2.9 Validation of the experimental models

In order to examine the optimum conditions in bioethanol production, additional confirmation experiments were conducted. Optimization of bioethanol production condition was obtained by keeping all values of parameters “in range” and setting the response goal to “maximize” [13]. The predicted optimum condition experiments produced by the software were carried out to verify the validity of the statistical experimental strategies [11]. Analysis on the actual responses obtained and the predicted values will indicate the validity of the model.

3. RESULTS AND DISCUSSION

3.1 Microencapsulation establishment efficiency

In order to examine the ability of *S.cerevisiae* to form microencapsulation, cultures were inoculated into alginate beads and DCM containing YPED medium. Unattached biomass was removed, while we measured the remaining attached cells. The microcapsules were analyzed via cell viability after the microencapsulation process and calculated as microencapsulation efficiency. High efficiency of immobilization and encapsulation was imperative as the resultant microcapsules will be subjected to bioethanol production.

Table 1. Microencapsulation efficiency and diameter of microcapsules synthesized under different condition

NaA concentration (%)	NaA: DCM ratio	Microencapsulation efficiency (%)	Microcapsule diameter (µm)
1	1:0	90.54 ± 1.9	299.80 ± 58.32
	1:1	91.23 ± 0.71	350.69 ± 72.30
	1:1.5	93.60 ± 0.49	452.18 ± 26.88
2	1:0	91.10 ± 0.85	351.60 ± 34.40
	1:1	92.27 ± 1.51	438.26 ± 80.1
	1:1.5	94.88 ± 0.50	502.73 + 62.8
2.5	1:0	94.30 ± 0.63	385.9 ± 69.57
	1:1	95.71 ± 1.60	427.7 ± 70.11
	1:1.5	97.24 ± .0.85	499.6 ± 51.42
3	1:0	92.40 ± 0.77	390.10 ± 47.78
	1:1	93.51 ± 1.19	480.29 ± 53.12
	1:1.5	95.36 ± 0.20	520.18 ± 44.30

Table 1
Microencapsulation efficiency and diameter of microcapsules synthesized under different condition

Table 1 showed the effects of the alginate concentration on the microencapsulation of DCM-NaA at the various NaA: DCM blending ratios after 48 h fermentation. It has been

demonstrated that a high alginate concentrations results in a low value of the mass transfer diffusivity of the gel matrix [14]. However, a matrix formulated with a low concentration of alginate gel (≤1.0%) is brittle and vulnerable when it is used in a shaking incubator or a packed-bed reactor. On the other hand, the DCM microencapsulated with an alginate solution at a higher concentration (more than 3.0%) showed extensive declined during the cross-linking step. The microcapsule also formed a multilayer structure instead of an interconnected porous structure (data not shown). As can be seen from the table (above), there was a high retainability or small loss of cell viability. Microencapsulation process using NaA : DCM ratio of (1:1.5) and (1:1) attained the highest efficiency of 97.24 ± 0.85 and 95.71 ± 1.60, respectively. No significant differences were found between conditions when preparing the microcapsules. Overall, these results emphasized the benefit and importance of immobilization stage before microencapsulation. Surprisingly, results showed incorporation of DCM enhanced the retainability of yeast cell after microencapsulation process in comparison to the single alginate carrier. These results indicated that DCM-alginate incorporation provides larger surface area for the attachment of yeast before encapsulation. Moreover, the dense structure of Alginate-DCM inside the microcapsule could enable the immobilized yeast to remain entrapped inside the matrix.

3.2 Microcapsule size analysis

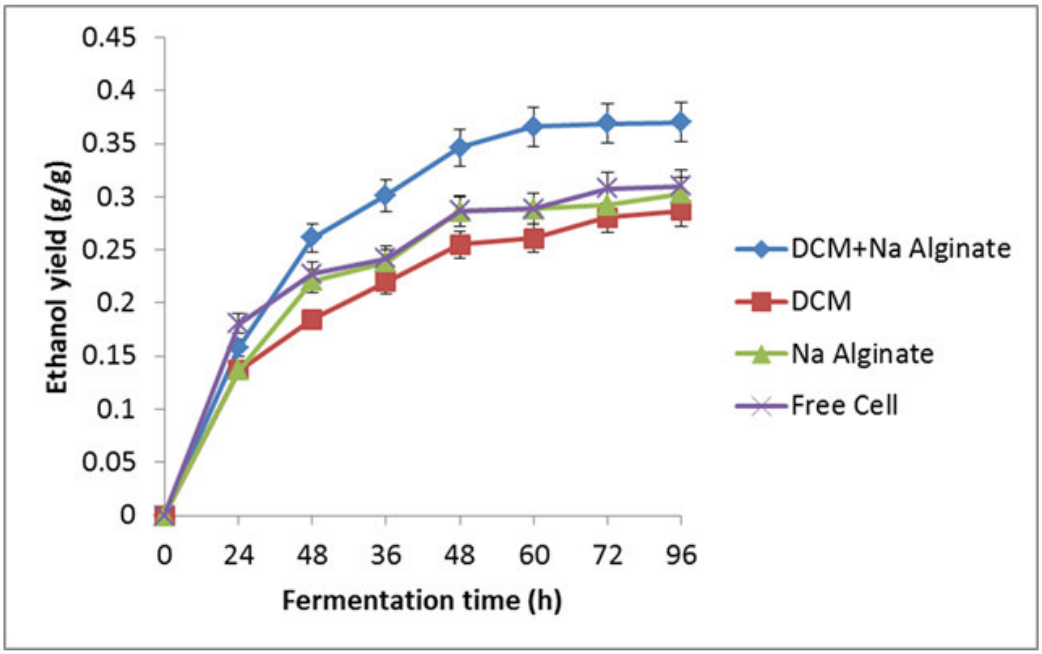
It has been suggested that the size of the microcapsule affects the homogeneity and enables better substrate utilization in the fermentation production [15]. Smaller microcapsule size may provide better distribution subsequently improving the homogeneity of the additive. It is suggested that the adequate and recommended size range of microcapsule is 50–500 μm [15]. Table 1 showed the microcapsule size synthesized from the nine different combinations of microencapsulation performed in this study. From this data, we can see the microcapsules ranged in size from 292.80 to 557.20 μm. There was an increase of the microcapsule diameter associated with the increase of the concentration of NaA and ratio of NaA: DCM. It is apparent from this table that the average sizes of microcapsules with pre-immobilized cells were significantly increased in comparison to that without immobilization process. This finding is due to the presence of DCM component in the formation of microcapsule. However, the increase in microcapsule diameter was not significant when the NaA:DCM ratio increased from 1:1 to 1:1.5. This phenomenon could be attributed to the fast flowing of mixing solution during extrusion that limited the space of immobilized yeast on DCM during encapsulation [15]. In addition, acceptable microcapsule diameter was also archived due to the size reduction of DCM after autoclave process [15].

3.3. Bioethanol production by immobilized yeast in a batch system

Batch fermentation using sugarcane bagasse as the primary carbon source was carried out at 37^{o}C and 150 rpm for 96 h. It was found that native biomass is extremely recalcitrant to enzyme saccharification [16]. Therefore, numbers of pretreatment methods have been designed to improve enzyme amenability. In this study, we use the microwave-alkali assisted pretreatment method to enhance susceptibility of sugarcane bagasse. According to the previous result in Table.1, alginate solution of 2.5% by wt was applied to formulate the DCM-alginate microcapsule since it has the highest efficiency compare to another concentration.

Fig.1 showed a comparison between the ethanol yields produced by DCM, alginate, and DCM-alginate carriers at different fermentation time. All of the immobilized yeast cell carriers resulted in a slightly higher ethanol yield than the free yeast cell. Moreover, a maximum ethanol yield of 0.37 g/g theoretical yield was obtained after 96h of incubation from DCM-Alginate microcapsule, which was 42.3% higher than that of alginate alone. Thus, this result indicates that DCM-alginate is a superior carrier of immobilized cell for ethanol production.

Figure 1
Comparison of immobilized yeast and free yeast cell fermentation state



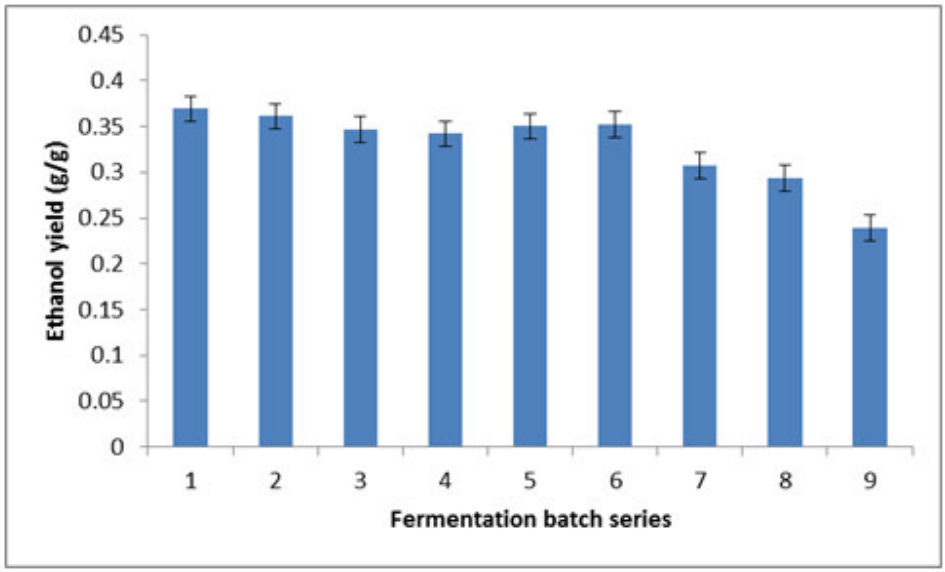


Figure 2
Repeated Batch Fermentation of DCM Alginate carrier microencapsulation

Fig. 2 showed a comparison of the ethanol yield between each batch systems in the DCM-NaA carrier. A 9 cycle repeated batch system was used to investigate the long-term stability of the DCM-alginate microcapsule and the strength of yeast cell for ethanol production. The microencapsulation formed by the 1.5:1 ratio of DCM/NaA (using a 2.5% by wt. alginate solution) was used as a cell carrier. Repeated batch fermentation was carried out under similar conditions; with each batch lasting 60 h. The final ethanol concentrations of each batch were fluctuated between 0.25 and 0.35 g/g theoretical yield during the repeated batch fermentation. The leakage of the microcapsule was observed after the sixth batch.

Consequences of degradation and breakage of the capsule undergoing long-term operations are the leakage of cells and a reduction of ethanol production. However, our results showed that the DCM-alginate was more stable as it retained its overall carrier size and ethanol yield production at about 0.24-0.35 g/g theoretical yield for the entire course of the examination. What is interesting in this data is that the incorporation of DCM enhanced the mechanical strength of the microcapsule. These results provide further support for the hypothesis that the existence of substrate or product inhibition in long-term repeated batch fermentation may hinder the yeast cells; therefore, it lowered the ethanol production efficiency. In this case, the protection of the cells by immobilization in a suitable support can lead to significantly improved tolerance to inhibitors and inadequate conditions compare to the free cell system [17].

3.5 Optimization of Bioethanol Production

Evaluation of the bioethanol production from the sugarcane bagasse was examined using response surface methodology approach. Delignification cellulosic material used in this paper, sawdust, was encapsulated with alginate to be used as the immobilized carrier. Table.2 below showed the levels of selected variables for the BBD and summarized the response values (bioethanol yield) along with the predicted values in the design matrix. Based on the preliminary selection of the process parameters, three independent variables, pH, fermentation time (h) and solid content (% w/v-1) were chosen. 17 design experiments with various combinations of selected variables were performed for statistical analysis.

Table 2

Three level Box-Behnken design and the experimental responses of dependent variable

Three level Box-Behnken design and the experimental responses of dependent variable (bioethanol yield (g/g)					
Run	pH	Fermentation time (h)	solid content (% w/v)	Bioethanol yield (g/g)	
				Observed	Predicted
1	5.5	24	17.5	0.2995	0.28986
2	5	24	17.5	0.3120	0.32297
3	5.5	72	17.5	0.2839	0.28317
4	5	72	17.5	0.3523	0.34554
5	5.5	48	20	0.2189	0.21952
6	5	48	20	0.2894	0.29642
7	5.5	48	15	0.3992	0.40898
8	5	48	15	0.4387	0.42756
9	4.5	24	20	0.2835	0.29556
10	4.5	72	20	0.3316	0.32961
11	4.5	24	15	0.3463	0.35059
12	4.5	72	15	0.4122	0.42019
13	4.5	48	17.5	0.4118	0.41897
14	4.5	48	15	0.4451	0.43425
15	4.5	72	17.5	0.3945	0.39602
16	4.5	48	17.5	0.4214	0.41897
17	4.5	24	20	0.3133	0.29556

From examine the findings, the bioethanol yield varied from 0.2188g/g to 0.4451g/g. The highest bioethanol yield of 0.4451g/g theoretical yield was observed at pH 4.5, 48h fermentation time and 15% w/v solid content. This result could reflect the significance of the various factors on bioethanol production. Therefore, it is imperative to study interactions of each parameter through response surface methodology.

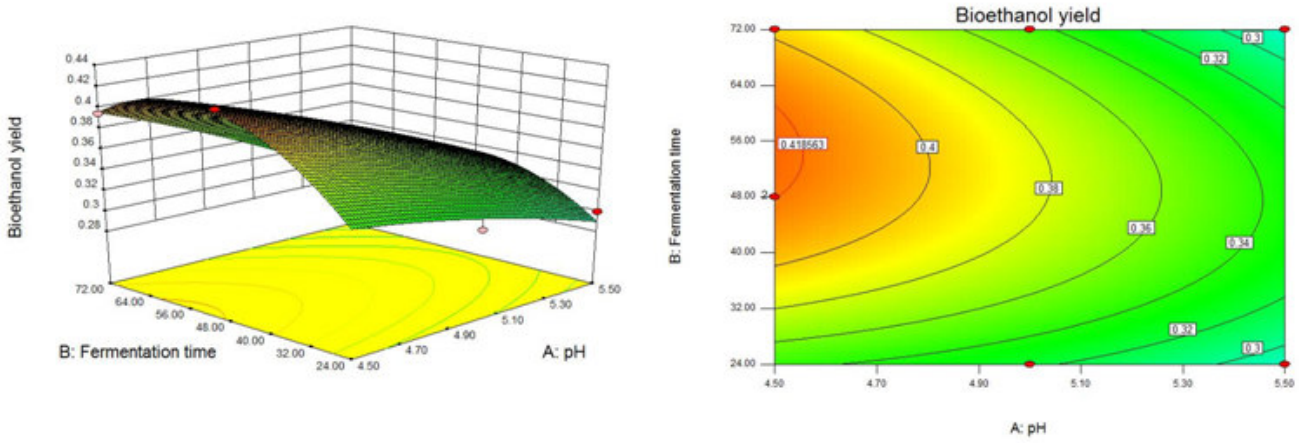


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Figure 3
3-D response surface and contour plots of the interactive effects of fermentation time and pH on bioethanol production, solid content loading was held constant at 17.5% w/v

Based on the response surface and contour plots of the developed quadratic model, the interactive effects of pH, fermentation time and solid content loading can be seen in Fig 3-5, respectively. Fig.3 showed the response surface plots of the effects of pH and fermentation time on bioethanol yield. It could be observed from Fig.3 that the slight curvature plots were obtained when the lower pH and longer time were applied. Bioethanol yields increased gradually with decreasing pH value and prolong fermentation time (40-60h). Nonetheless, decreasing the pH value would not make any increase in bioethanol yields if fermentation time were less than 40h.

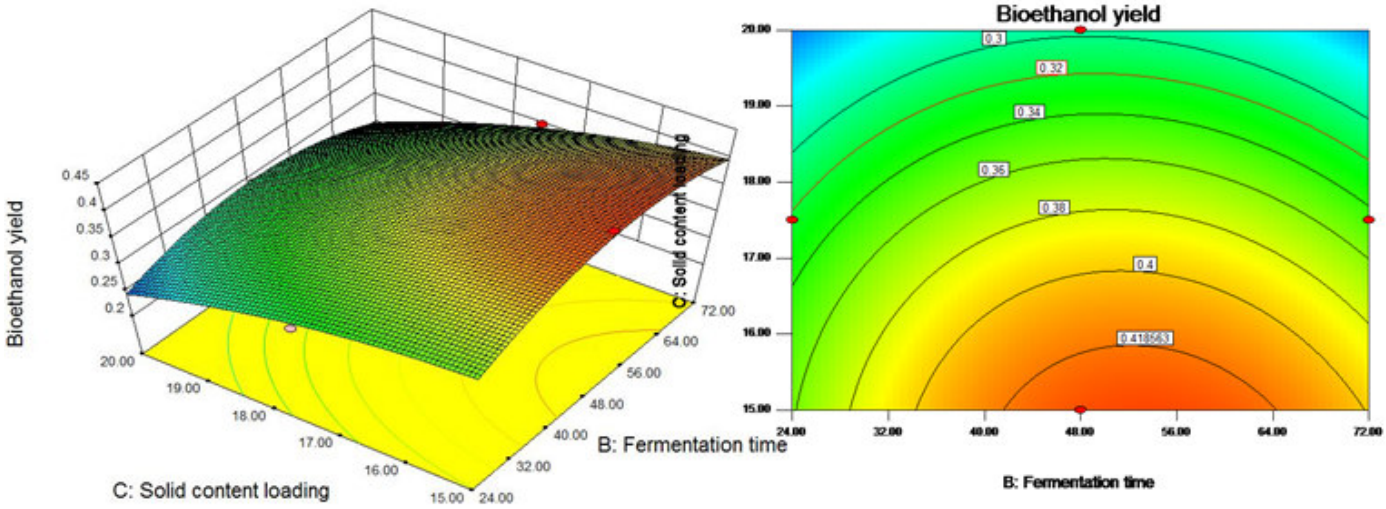

Figure 4
3-D response surface and contour plots of the interactive effects of solid content loading and fermentation time on the bioethanol production, pH was held constant at 5

Fig.4 depicted the effects of solid content and fermentation time on bioethanol production when the fermentation time was held constant at 48h. It is apparent from this table that there was a non-linear effect of the solid content loading and the fermentation time. The longer the fermentation time to some extent will increase the yield of bioethanol; while the same effect could be obtained if solid content kept at lower level. However, prolong the fermentation time would not make any increase in bioethanol yield if high density of solid content used (more than 17%w/v). m

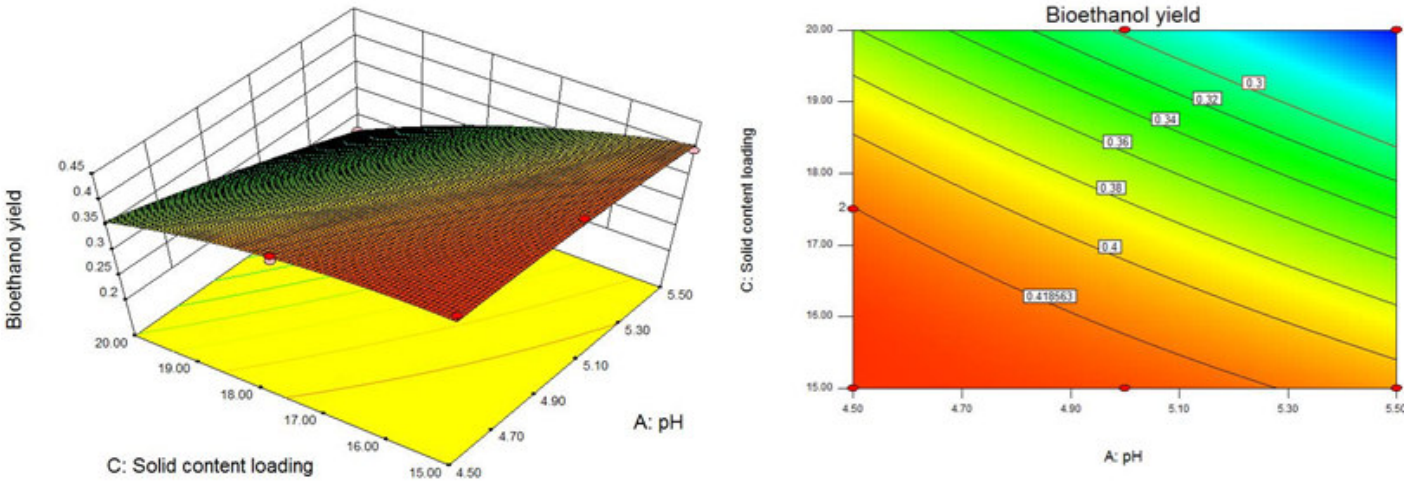

Figure 5

3-D response surface and contour plots of the interactive effects of pH and solid content loading (%w/v) on bioethanol production, fermentation time was held constant at 48h.

In Fig.5, the bioethanol yield showed a non-linear effect with an increase of pH from 4.5 to 5.5 under the conditions of fermentation time was held constant at 48h. There was a linear increase in the bioethanol yield with decrease in solid content loading and pH value. The high bioethanol yield could be obtained from higher solid content only if pH were maintained at lower level (4.5-5). It could be inferred that acidic condition was important in bioethanol production. It also could be inferred that the increase of pH and solid content would gave the negative effect in bioethanol production. This finding was corroborated with the finding by W Zhao et al [18]; the optimum pH level suggested to obtain maximum bioethanol production was 4.6 on wheat straw raw material. This view is supported by Shuler [19] who suggested that the variations of the pH medium resulted in changes in the activity of the enzyme and hence change the reaction rate. Nonetheless, this interpretation contrast with Turhan et al [20] on carob pods; they obtained maximum bioethanol production at pH 5.5 in carob raw material. Overall, according to the predicted optimal conditions, we can infer that the pH, fermentation time and solid content loading were 4.5, 56.42h and 15.16%w/v, respectively. It would then result in 0.4405 g/g theoretical bioethanol yield. Thus, this finding agrees well with the observed optimal condition. In bioethanol production, the data analyzed by regression analysis was represented by quadratic model equation. Equation of the optimization of bioethanol production terms of actual parameters is shown in Eq.(4):

$$Y = 46.50 + 3.75A - 0.511B + 9.382C + 2.977AB - 0.210AC + 2.236BC - 5.99\,A^2 - 7.93B^2 - 0.873\,C^2$$

where, Y= Percent reducing sugar yield, A, B, C are pH, fermentation time, and solid content loading, respectively.

Source	Sum of square	Degree of freedom	Mean square	F-ratio	Prob>F
Model	0.068	9	0.0076	42.37	< 0.0001
A-pH	0.018	1	0.018	102.19	< 0.0001
B-Fermentation time	0.001	1	0.001	5.59	0.0499
C-solid content loading	0.034	1	0.034	188.83	< 0.0001
AB	0.0013	1	0.0013	7.41	0.0296
AC	0.0053	1	0.0053	29.47	0.001
BC	0.0003	1	0.0003	2.03	0.1975
A^2	0.0001	1	0.0001	0.57	0.4744
B^2	0.0085	1	0.0085	47.75	0.0002
C^2	0.0016	1	0.0016	8.92	0.0203
Residual	0.0013	7	0.0002		
Lack of Fit	0.0008	5	0.0001	0.62	0.7115
Pure Error	0.0005	2	0.0002		
Cor Total	0.069	16			

Table 3
Analysis of variance (ANOVA) for the fit of experimental data to response surface models

R^2 0.9820; Adj-R^2 0.9588
Pred-R^2 0.8630
p value below 0.05 are significant and above are not significant
To test statistical significance of experimental data, ANOVA (analysis of variance) were conducted. The coefficient values and the analysis of variance (ANOVA) are presented in Table.3, which indicate that the predictability of the model is at 95% confidence level. Analysis of variance (ANOVA) in this experiment indicated that the model is highly reliable with its coefficient of determination (R^2) value of 0.9897 and 'prob>F' value was<0.0001. The statistically insignificant lack of fit value 10.12 ($p> 0.05$) also prove that the model fitted well to the experimental data. Therefore, the model is found to be adequate for prediction within the range of variables employed [21].

4. CONCLUSION

This research proposed a new support for the immobilization of microbial cells. DCM sawdust was microencapsulated with Na alginate. DCM-Alginate was found useful as a yeast cell carrier in ethanol fermentation due to its advantageous porous structure, mechanical strengths, stability and hydrophilic character. From the present study, it can be concluded that repeated-batch ethanol productions using DCM-Alginate microencapsulation were more stable than those using free yeast cell and Na-alginate carrier. An examination using nine cycles of repeated batch fermentation demonstrated that DCM-Alginate is capable reused as a support for ethanol production. In addition, it is found to be an appropriate yet stable long-term use support for *S. cerevisiae* immobilization to carry out the bioethanol fermentation in agro wastes. The DCM-Alginate microcapsule also showed an excellent mechanical property, withstanding the shear force in the mixing process during ethanol fermentation. Good correlations between the observed and predicted bioethanol yield were found in bioethanol production. The predicted optimum pH, fermentation time and solid content loading were 4.5, 56.42h and 15.16%w/v, respectively. It would then result in 0.4405g/g theoretical bioethanol yield. However, further research should be carried out on this issue for a scale-up of the system for the fermentation of large volumes of agro wastes.

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