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Continuous Bioethanol Production Using *Saccharomyces cerevisiae* Cells Immobilized In Nata De Coco (Biocellulose)

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Abstract. The performance of Nata de coco (NDC) and Calcium alginate (CA) as an immobilization medium for *Saccharomyces cerevisiae* cells are compared in terms of production rate and conversion. *S. cerevisiae* cells are immobilized in NDC and CA beads using a cell suspension with an average approximate live cell density of 232.1288 ± 1.5387 cells/mL. The biocatalysts NDC and CA are charged into horizontal fermentation reactors. A centrifugal pump and manifold is used to control the flow rate to a desired flow rate of 9 mL/hr. Samples are collected every 12 hours and tested for ethanol by gas chromatography and glucose concentration by colorimetry. The average steady state effluent ethanol concentration, productivity and conversion in NDC are 5.093 % by volume, 52.329 mL/hr and 0.7779, respectively. One-way ANOVA showed that the immobilization medium has a significant effect on the parameters under consideration. T-test is further performed between NDC and CA biocatalysts which showed that effluent ethanol concentration, productivity and conversion of NDC and CA are statistically equal. The study showed that the NDC biocatalyst performs equally well in the conditions optimized for CA biocatalyst. The structural strength and cost effectiveness of Nata de Coco makes it a very promising immobilization medium for continuous bioethanol production.

Keywords: Immobilization, Biocellulose, Calcium Alginate, Continuous Fermentation, Horizontal Reactor, Baker's Yeast

1. Introduction

Continuous fermentation systems offer important economic advantages and significantly improves production rate. In freely suspended cell systems, continuous operation is limited by flow rate as cells are carried in the effluent resulting to a decrease or complete loss of cells. Cell immobilization supports the cells promoting operation at higher flow rates. Immobilization is achieved by various mechanisms. One of which is surface adsorption where cells naturally adhere to the surface of the material through electrostatic force. Yeast cells are adsorbed in the surface of NDC which is a very hydrophilic and strong material with a young's modulus comparable to aluminum (Titech, 2001). Studies on optimization of the immobilization process are reported in the literature (Nguyen, Ton, & Le, 2009). In another mechanism, matrix encapsulation, cells are trapped in a polymer matrix of materials such as alginate and carrageenan. Entrapment in CA is commonly used in studies with ethanol fermentation due to "the requirement for mild conditions and the simplicity of the used procedure" (Ramakrishna & Prakasham). Several disadvantages in immobilization with CA include damage to gel particles due to carbon dioxide, "diffusion limitations of nutrients, metabolites and oxygen due to the gel matrix and the high cell densities in the gel beads, the chemical and physical instability of the gel and the non-regenerability of the beads, making this immobilization type rather expensive" (Verbelen, De Schutter, Delvaux, Vestrepen, & Delvaux, 2006). Carbon dioxide production decreases the working volume of the reactor thereby reducing its fermentative

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capacity. Several studies showed that a horizontal reactor decreased gas hold up and that ethanol production rate is 1.5 times higher relative to a vertical configuration (Shiotani & Yamane, 1981).

In this study, the performance of NDC and CA immobilized *S. cerevisiae* cells are compared during continuous fermentation in a horizontal bioreactor in terms of effluent ethanol concentration, ethanol production rate and conversion.

2. Materials and Methodology

2.1. Yeast Cell Suspension

Commercially available Eagle Brand Instant dry yeast was used as the source of *S. cerevisiae* cells. The cell density in the suspension was determined by dissolving dry yeast and counting by hemocytometry. A linear correlation was observed in the cell density-mass plot and the mass of dry yeast corresponding to the desired cell density was determined. A factor of 80% was used to consider viability of cells as suggested in literature (Taeymans, Roelans, & Lenges, 1986).

The cell suspension was prepared by combining 10 g of glucose, 5 g peptone, 3 g dextrose, 3 g yeast extract, 10 mL of pH4 acetic acid-acetate buffer and enough distilled water. The mixture was autoclaved at 121°C for 15 minutes, afterwards it was cooled to approximately 40°C before adding 4.1597 g dry yeast and dilution to 1 Liter.

2.2. Fermentation Broth Composition

One liter of the fermentation broth was prepared by mixing 100 g glucose, 5 g yeast extract, 5 g $(NH_4)_2SO_4$, 0.125 g K_2HPO_4, 0.875 g KH_2PO_4, 0.1 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl2 \cdot 2H_2O$ and 10 mL of pH4 acetic acid-acetate buffer 500 mL of distilled water was added prior to autoclaving. After autoclaving the broth was diluted to 1L using distilled water.

2.3. Immobilization Media (Nata de Coco & Calcium Alginate)

Unsweetened NDC procured from local market was cut into 1x1x1 cm cubes, washed and soaked for one week. The initial cell density in the cell suspension was $200x10^6$ cells/mL. 200 grams of NDC was mixed with 1 liter of cell suspension at 200 rpm for 5 hrs. The detailed immobilization method is described in literature (Nguyen, Ton, & Le, 2009), however the incubation step is skipped due to contamination issues.

Analytical Reagent grade Sodium Alginate (2.5 g) was dissolved in 125 mL of distilled water and mixed with 125 mL of cell suspension. A 10 mL glass pipette is used to drop the Alginate into 500 mL of calcium chloride solution containing 30 g of calcium chloride. CA beads were soaked in calcium chloride solution for 22hrs as suggested in literature (Ogbonna, Amano, & Nakamura, 1989).

After immobilization, the biocatalysts NDC and CA were washed with distilled water, weighed and charged in the reactors until a void fraction of approximately 0.7 is reached.

2.4. Continuous Fermentation

A horizontal bioreactor with a total dimension of 320 x 30 x 50 mm was constructed from plexi-glass and was packed with the biocatalysts. An over flow weir maintained liquid height to approximately 25 mm with a working dimension of 300 x 30 x 25 mm. Fermentation broth was fed into the reactor at a rate of 9 mL/hr to give a residence time of 25 hrs. A constant temperature bath was used to maintain the temperature at 30°C.

Four fermentation set-ups (with NDC, CA, a control without biocatalyst and a control batch run) were run simultaneously in replicate over a 72 hour period. Batch fermentation was performed for the first 12 hours to reach a constant outlet concentration faster. This was followed by continuous fermentation until the 72nd hour except for the control in batch run. Samples were collected every 12 hours, stored, sealed and frozen in vials prior to testing.

2.5. Glucose and Ethanol Measurement

Residual glucose in the samples were measured using colorimetry with 3,5-dinitrosalicylic acid and a glucose internal standard at 575 nm. Details of the procedure are described elsewhere (Wang).

Ethanol concentration in the samples was measured using an HP 5890 series II Gas Chromatograph equipped with flame ionization detector and a HP-5 Crosslinked 5% PhMe Silicone column. Helium was used as carrier gas with isopropanol as internal standard and an oven temperature of 40°C (Templeton, 1994).

3. Results and Discussion

3.1. Results and Discussion

The ethanol concentration profiles for two reactors are summarized in figure 1.

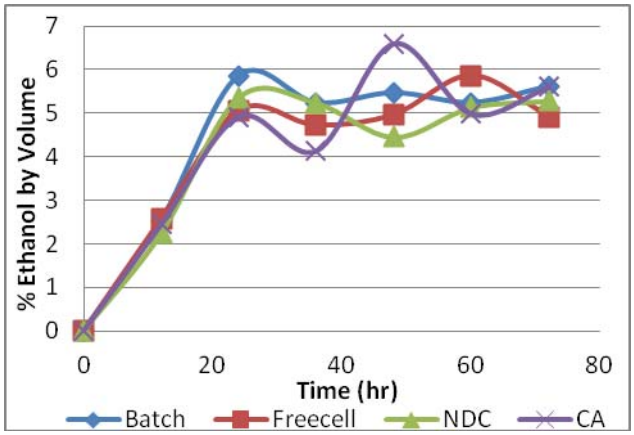

Fig. 1: Average Effluent Ethanol Concentration

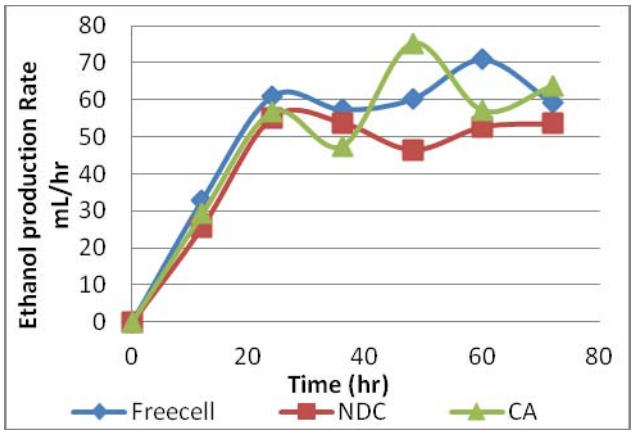

Fig. 2: Average Ethanol Production Rate

Ethanol concentration generally increased until a steady value is reached. This trend is observed across all immobilization media and in the batch process. Ethanol production rate is calculated by multiplying the fermentation broth flow rate and the ethanol concentration.

$$Prodution\ Rate\mid_t = Q_{average} * [C_2H_5OH]_t$$

Figure 2 shows the plot of production rate versus time showing a trend similar to the change in ethanol concentration. Fluctuation is present but the plots generally approach a final value. Glucose concentration relative to time is plotted and the average for the two trials is shown in figure 3.

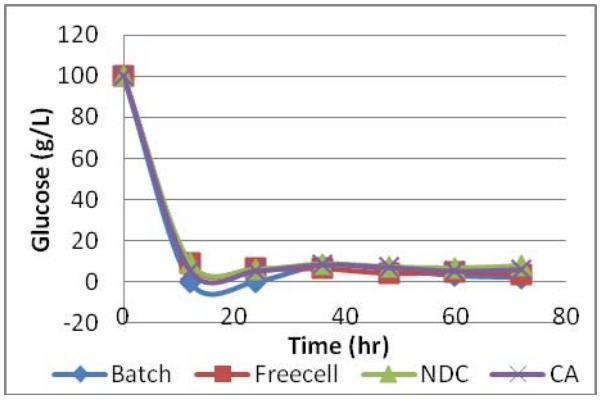

Fig. 3: Average Residual Glucose Concentration

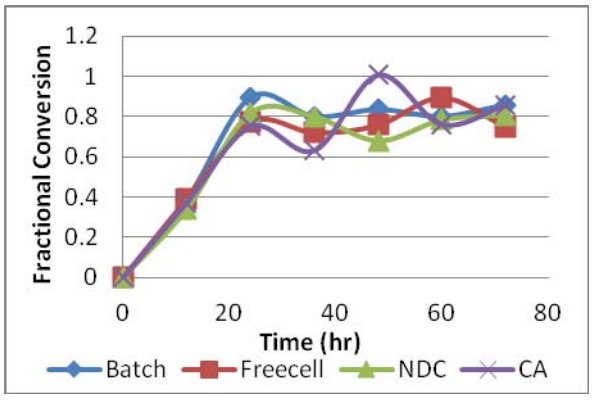

Fig. 4: Average Conversion

As expected, glucose concentration is decreasing. For the continuous runs (Freecell, NDC and CA) there are instances where glucose concentration increased. This could be the consequence of variation in the fermentation broth flow rate, the interference of an invading microorganism thus decreasing the activity of *S. cerevisiae* or a combination of both. Fractional conversion is calculated based on outlet ethanol concentration using stoichiometry. The variation of fractional conversion with time is illustrated in figure 4. It is expected that the ethanol concentration would increase and gradually reach a steady state value. The steady state value is not well defined due to the fluctuating ethanol concentration but the expected trend is achieved. In many

studies in ethanol fermentation, the outlet concentration does fluctuate thus other parameters also fluctuate. The steady state outlet ethanol concentration, production rate and conversion are summarized in table 1.

Table 1: Summary of Performance Parameters

	Freecell	NDC	CA	NDC-CA %difference
Average Ethanol Concentration	5.1034	5.093	5.2499	**2.9898**
Average Ethanol production rate mL/hr	61.788	52.329	60.029	**12.827**
Average Fractional Conversion	0.7795	0.7779	0.8019	**2.9898**

Immobilization with NDC generally resulted in the lowest ethanol concentration, production rate and fractional conversion. Percent difference is smaller for ethanol concentration and fractional conversion but the production rate difference is significant at 12.82%. For all samples, fractional conversion is high with an average of 0.7976 across all immobilization media. Yield considers the residual glucose unaccounted by conversion. Yield is calculated and a plot of the average yield for trials 1 and 2 is presented in figure 5.

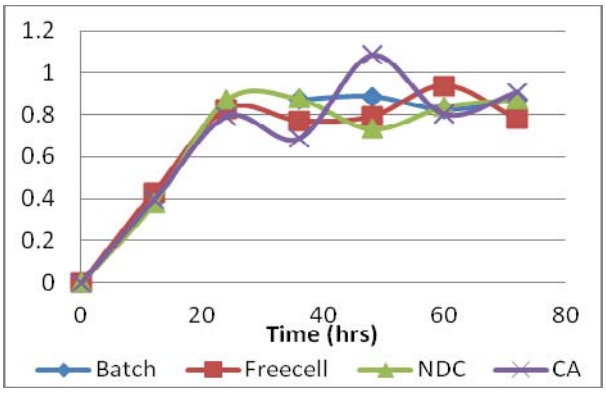

Fig. 4: Average Fractional Yield

A disparity is observed in alginate at t = 48 hrs since the yield cannot be greater than 1. A yield of 95% is the highest attainable yield using a chemostat (Shiotani & Yamane, 1981). Generally the yield is slightly below this value. The lower yield may be an effect of the fermentation setup since it is not completely anaerobic. Dissolved oxygen in the fermentation broth is not accounted and may act as the initial oxygen acceptor until it is exhausted by *S. cerevisiae* cells for growth.

3.2. Statistical Analysis

The relationship between the immobilization medium and three factors: ethanol concentration, ethanol production rate, and fractional conversion are investigated. These factors are independently analyzed and the significance of the immobilization media is determined at four levels of immobilization medium, NDC in batch run, free cell, NDC and CA. The results of one-way ANOVA and the proportion of variability (R^2) are summarized in table 2.

Table 2: Summary of ANOVA Results and T-Test Analysis (Ho No significant difference)

Factor	Trial	F_0	F	R^2	Conclusion	t0	T	Conclusion
Ethanol Concentration	1	118.3969	3.238872	0.956895	Reject	0.461953	1.859548	Accept Ho
	2	11.31415	3.490295	0.738804	Reject Ho	-1.75942	1.94318	Accept Ho
	Average	53.9243	3.238872	0.909998	Reject Ho	-0.3543	1.859548	Accept Ho
Ethanol Production rate	1	31.24345	3.885294	0.838898	Reject Ho	-0.64272	1.859548	Accept Ho
	2	9.271864	3.885294	0.607121	Reject Ho	-2.77996	1.859548	Reject Ho
	Average	13.76714	3.885294	0.696466	Reject Ho	-1.59158	1.859548	Accept Ho
Fractional Conversion	1	118.3969	3.238872	0.956895	Reject Ho	0.461953	1.859548	Accept Ho
	2	11.31415	3.490295	0.738804	Reject Ho	-1.75942	1.94318	Accept Ho
	Average	53.9243	3.238872	0.909998	Reject Ho	-0.3543	1.859548	Accept Ho

For all trials of all the factors $F^0 > F$ and the null hypothesis is rejected. ANOVA analysis shows that the treatment means differ i.e. the immobilization medium significantly affects the ethanol concentration, the ethanol production rate and the fractional conversion. The proportion of variability ranges from moderately strong to strong for all factors and levels as shown by the R^2 values greater than 0.60.

The Two-Sample t-test analysis for the continuous fermentation processes is shown in the table 3. For eight trials, the null hypothesis is accepted. Two-Sample t-test analysis shows that the difference between the mean values of the steady state effluent ethanol concentration, ethanol production rate and fractional conversion in NDC and CA are statistically insignificant. Although table 1 showed that generally, the CA biocatalyst is better than NDC, t-test does not verify that claim.

One noticeable advantage of Nata de coco immobilization is the structural integrity of the biocatalyst. Calcium alginate beads become soft and mushy after just 72 hours which is a disadvantage observed in several studies on continuous fermentation.

4. Conclusion

One-way ANOVA showed that the immobilization medium significantly affects the performance of a continuous fermentation process. Fermentation using the CA biocatalyst resulted to a higher average effluent ethanol concentration, production rate and conversion than NDC but the difference is statistically insignificant.

Nata de Coco is a promising immobilization medium as its performance was statistically similar to CA in conditions optimized for CA. The advantage of NDC in terms of material strength and reusability should also be credited. The economic advantage of NDC may also be considered as the cost of immobilization with CA (based on alginate alone) is 22.5 times that of NDC at the time of the experiment.

5. References

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