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Research Article

Ethanol Production from Concentrated Food Waste Hydrolyzates Using Free and Immobilized Yeast *Saccharomyces cerevisiae* H058

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Abstract: The ethanol fermentation of concentrated food waste hydrolyzates by free and immobilized cells of *S. cerevisiae* H058 in a batch system was studied. The effect of initial reducing sugar and initial inoculum concentration on ethanol fermentation in both immobilized and free cell systems were studied. Parameters such as ethanol concentration, ethanol production rate, ethanol yield and sugar consumption rate were assessed and compared for both fermentation systems. In addition, repeated batch fermentations with the immobilized yeast cells were carried out for ethanol production. The result showed an optimal initial reducing sugar concentration of 180 g/L, inoculum concentration of 2% (v/v) and fermentation time of 60 h were determined for the free cell system. For the immobilized system, an optimal initial reducing sugar concentration of 200 g/L, inoculum concentration of 2% (w/v) and fermentation time of 48 h were selected. The immobilized cells were also proved to be reusable in 7 batches of fermentation. More than 98.5% reducing sugar was utilized during the 7 repeated batches by the same immobilized cells and overall ethanol concentration fluctuated around 94.24 g/L. The immobilized cell system was superior to the free cell system since lower substrate inhibition and less fermentation time and higher ethanol tolerance were realized.

Keywords: Enzymatic hydrolysis, ethanol, fermentation, food waste, immobilization

INTRODUCTION

The global demand for fuel ethanol has been increasing in recent years because of its wide use in chemical and motor-fuel industries and its important role in reduction of green house gas emissions. Ethanol has been produced mainly from corn in America and China and from sugarcane in Brazil. However, since corn is a major food source, its use as a fuel raw material has been criticized as it has led to a dramatic increase in the price of corn. Since 2006 the Chinese government has restricted the use of corn for ethanol production. Therefore, waste biomass such as corn stover, waste wood and waste food are much more attractive than corn as cheap raw material for ethanol production.

Food waste is a kind of organic solid waste discharged from restaurants, cafeterias, households and accounts for a considerable proportion of municipal solid waste in China (Cho *et al*., 1995). Food waste is also a major source of odor, vermin attraction, toxic gas emission and groundwater contamination during collection, transportation and disposal owing to their high organic concentration. This issue is particularly serious in China, as the generation of food waste is growing every year. Landfill was once the primary choice for handling these wastes but has now been banned because of the exhaustion of existing landfill sites, moreover, it is difficult to find new sites and the leachate generated by these materials requires secondary wastewater treatments (Choi *et al*., 2003). The incineration of food waste is unsuitable because of its high water content and the possibility of dioxin generation (Wang *et al*., 2005). The major conventional recycling method for food waste has been to employ it as animal feed and fertilizer, which has been practiced as ways of treating large amounts of the food wastes. However, large amounts of wastewater are generated when desalting the food wastes for fertilizer production and animal feeds produced from this material often creates hygiene problems for feeding animals (Moon *et al*., 2009). Therefore, it is imperative to overcome the technological and systematic dilemma of the conventional recycling method for food waste and

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simultaneously develop an environment friendly recycling method that can convert food waste to a high value product such as fuel ethanol.

Ethanol can be produced using either free or immobilized cells. Using immobilized cells is advantageous over free cells due to enhanced yield, ease to separate cell mass from the bulk liquid, feasibility of continuous processing, reduced risk of contamination, better operational stability and cell viability for several cycles of operations (Chandel *et al*., 2007; Nigam, 2000). A number of carrier materials (agar, calcium alginate, k-carrageenan, etc.) have been used for entrapping microbial cells for production of ethanol (Adinarayana *et al*., 2005; Kar *et al*., 2009). Among these, entrapment in calcium alginate beads is found most suitable in majority of studies as this matrix is cost effective, procedure is simple and easy to handle (Kar *et al*., 2009; Najafpour *et al*., 2004).

In this study, ethanol fermentation of concentrated food waste hydrolyzates by free and immobilized cells of yeast *S. cerevisiae* H058 was investigated in a batch system. The effect of initial reducing sugar and initial inoculum concentration on ethanol fermentation in both immobilized and free cell systems were studied. In addition, repeated batch fermentations with the immobilized yeast cells were carried out for ethanol production. This research can provide important information on the commercial utilization of food waste hydrolyzates for large-scale ethanol production.

MATERIALS AND METHODS

Microorganism and Inoculum preparation: *S. cerevisiae* H058 used in this study was obtained from Key Laboratory of Ion Beam Bio-engineering of Institute of Plasma Physics, Chinese Academy of Sciences. It was maintained on slants of the agar medium (w/v): 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar and kept at 4°C. The seed was grown in 5% YPD (5% glucose, 1% yeast extract, 2% peptone) medium. Further propagation, prior to immobilization and ethanol fermentation, was performed aerobically at 30°C for 24 h with mixing at 150 rpm using a rotary shaker, then the yeast suspension of a desired cell density was prepared by centrifugation of the cultured media.

Cell immobilization: *S. cerevisiae* H058 cells grown in a propagation medium were collected by centrifugation for 10 min at 4000 rpm, 4°C. The 2% (w/w) Na-alginate solution was prepared by dissolving 10 g of Na-alginate powder into 500 mL of distillated water. A separate solution of 60 g of calcium chloride was made in 1 L distilled water. Na-alginate and calcium chloride solution were autoclaved at 121°C for 15 min. The collected cells and the sterilized sodium alginate solution were thoroughly mixed. The mixed solution was then extruded through a pipette into a sterilized calcium chloride solution. Alginate drops solidified upon contact with calcium chloride solution, forming beads entrapping yeast cells. The beads were allowed to harden for 24 h at 4°C. In this way, the yeast cells were entrapped in the gel matrix of Ca-alginate. The initial mean density of the yeast cells in the Ca-alginate immobilized carrier reached 1.3×10^8 CFU/g of gel beads at the time of immobilization.

Enzymes: In the tests, two commercial enzyme solutions, fungal α-amylase and glucoamylase purchased from Shandong Longda Bio-Products Company Limited (China), were used for food waste saccharification. According to the information sheet, the optimum temperature for fungal α-amylase is in the range 50-60°C and for glucoamylase is in the range 55-60°C. Regarding optimum pH, the range for fungal α-amylase is from 4.0 to 6.5 and for glucoamylase is from 4.0 to 4.5. The specific activity of fungal α-amylase and glucoamylase is 5 000 u/mL and 150 000 u/mL, respectively. One fungal α-amylase unit is defined as the amount of enzyme that hydrolyzes 1 mg water soluble corn starch per minute under the assay conditions. One glucoamylase unit is defined as the amount of enzyme required to produce 1 mg of glucose in 1 h under the assay conditions.

Enzymatic hydrolysis of food waste: Food waste used in this study was collected from the dining room located in Institute of Plasma Physics, Chinese Academy of Sciences. Two kilogram of food waste (separating out bones and shells) was chopped into small pieces using a fruit mixer and transferred into a 5 L jar fermentor containing 1 kg tap water. Two kinds of enzymes, α-amylase and glucoamylase were then added to the mixture with the amount of 10 u/g and 140 u/g food waste, respectively. The enzymatic hydrolysis was performed at pH4.5, 55°C and 150 rpm for 2.5 h. Then saccharified liquid was separated by centrifugation at 8,000 rpm for 10 min and used for batch ethanol fermentation.

Ethanol fermentation: Food waste hydrolyzates containing initial reducing sugar concentration of 102.68 g/L (Table 1) was concentrated at 60°C by a vacuum evaporation. The total reducing sugar in the hydrolyzate was adjusted to approximately 160, 180, 200 and 220 g/L by concentrated process. Then each adjusted hydrolyzate was supplemented with 8 g/L YEP (3 g yeast extract and 5 g peptone) and used as ethanol production medium. All medium were adjusted to pH 5.0 with 3 N NaOH before use. It was considered that the pasteurization of the substrate achieved during the enzymatic hydrolysis (55°C, 2.5 h) and concentrated process (60°C) was sufficient thermal treatment and thus no additional sterilization prior to fermentation was carried out.

Table 1: Characteristics of food waste hydrolyzates

Parameters	Value
pH	4.65
Reducing sugar (g/L)	102.68±4.96
Fermentable nitrogen (mg/L)	146.72±5.34
Glucose (g/L)	90.71±2.69
Fructose (g/L)	4.31±0.53
Sucrose (g/L)	7.62±1.05
Sodium chloride (g/L)	15.31±1.24

The ethanol production medium containing various initial reducing sugar concentration were fermentated by free and immobilized yeast under anaerobic conditions (30°C, mixing rate 100 rpm, 72 h). The initial inoculum concentration for the free and the immobilized system was 2% (v/v) and 2% (w/v), respectively.

The effect of initial yeast concentrations on ethanol fermentation was also investigated by applying different inoculum concentration (2, 4 and 6% v/v in a free system and 2, 4 and 6% w/v in a immobilized system) at constant reducing sugar concentration. Initial viable cell number was ~1×10^8 CFU/mL when the *S.cerevisiae* H058 concentration was 2, 4 and 6% (v/v), respectively. In the case of immobilized yeast, the initial mean number of the yeast cells in the Ca-alginate immobilized carrier reached 1.3×10^8 CFU/g of gel beads. Furthermore, repeated batch fermentations with the immobilized yeast cells were carried out for ethanol production under optimized conditions.

Analytical methods: The reducing sugar and residual sugar were determined using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). Fermentable nitrogen or formol nitrogen in the fermentation broth was analyzed by formol titration method (Wu *et al*., 1984). Concentrations of sucrose, glucose, fructose in food waste hydrolyzates were determined by HPLC with a Agilent ZORBAX Bonus-RPUSP L60 (4.6 mm×250 mm, 5 μm) and a refractive index detector (Agilent Technologies, Inc., USA). The mobile phase was 1.0 mL/min of 85% CH_3CN (CH_3CN/H_2O = 85/15, v/v) (Chinese National Standard GB/T 22221, 2008). The ethanol concentration was measured by using Shimadzu GC-2050 gas chromatography with cbp-20 capillary column and a flame ionization detector. The chromatogram was run at 180°C oven temperature and 90°C injection temperature using N_2 as a carrier gas and H_2 as a flaming gas (Yu *et al*., 2009). Cell growth was measured using the dilution and plating method. After thorough dispersion, a 1-mL sample was serially diluted and plated (three plates per dilution) on YPD agar plates to obtain Colony Forming Units (CFU). The plates were incubated at 30°C for 48 h and the final colony count was calculated as the average of the CFU of the three plates for the dilution containing 30-300 colonies per plate. In the case of immobilized yeast cells, the first dilution was performed in 2% sodium citrate solution (pH 7.0) in order to dissolve alginate gel. The ethanol yield (g/g) was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized. The ethanol production rate (g/L/h) was calculated by ethanol concentration produced (g/L) divided by fermentation time (h).

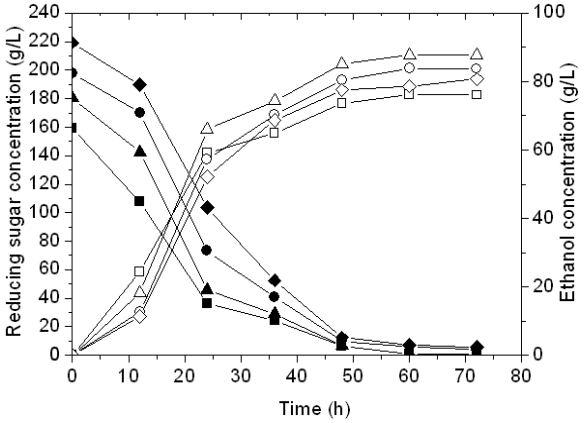


Fig. 1: Sugar consumption and ethanol production during batch ethanol fermentation by free cells of *S. cerevisiae* H058 from concentrated food waste hydrolyzates at various initial reducing sugar concentrations: 160 g/L (■, □), 180 g/L (▲, Δ), 200 g/L (●, ○) and 220 g/L (◆,◇), total reducing sugar (close symbol) and ethanol (open symbol)

Electron microscopic scanning: For electronic microscopic sanning (SEM) micrographs, samples were taken from fresh beads and 6 batches beads of repeated batch fermentations. The samples were dipped into liquid nitrogen for 10 min, then freeze-dried for 8 h into the Freeze Drier. Samples were then coated with gold particles, deposited on silicon plate and analyzed with SEM (USA FEI Company, Sirion200).

RESULTS AND DISCUSSION

Ethanol production with free yeast cells:

Effect of initial reducing sugar concentration: The ethanol fermentation depends on many factors, such as nitrogen sources, initial reducing sugar and inoculum concentrations required for efficient fermentation etc. Standard ethanol production medium contains 3 g/L of yeast extract and 5 g/L of peptone which total fermentable nitrogen equals to 1129 mg/L (Melzoch *et al*., 1994). Therefore in this study yeast extract and peptone at those concentrations were supplemented in each treatment as nitrogen sources. Table 1 shows the compositional data of the food waste hydrolyzate selected for this study.

The first set of experiments was conducted in order to determine an optimal initial reducing sugar concentration for the ethanol fermentation process. The ethanol production and reducing sugar consumption during the fermentation of concentrated food waste hydrolyzates with various initial reducing sugar concentrations (approximately 160, 180, 200 and 220 g/L) are presented in Fig. 1.

Table 2: Kinetic parameters of ethanol production from concentrated food waste hydrolyzates at various initial reducing sugar concentrations by free cells of *S. cerevisiae* H058

Initial reducing sugar (g/L)	Max. ethanol produced (g/L)	Sugar utilized (g/L)	Time (h)	Ethanolproduction rate (g/L/h)	Sugar consumption rate (g/L/h)	Ethanol yield (g/g)
159.12±1.06	76.24±0.56	158.20±1.48	60	1.27±0.01	2.64±0.02	0.48±0.04
181.23±1.29	87.81±0.63	179.24±1.36	60	1.46±0.01	2.99±0.02	0.49±0.03
199.48±1.97	83.82±0.75	193.85±1.44	60	1.40±0.01	3.23±0.02	0.43±0.02
220.06±2.04	80.72±0.91	213.65±1.55	72	1.34±0.02	2.97±0.03	0.38±0.01

As demonstrated by Fig. 1, the ethanol concentration gradually increased during the fermentation with initial reducing sugar concentrations of 160 and 180 g/L. However, lower ethanol concentrations were obtained at an initial reducing sugar concentration of 200 and 220 g/L, because the substrate and product inhibition took place at high sugar concentrations. The maximum values of ethanol concentration, ethanol production rate and ethanol yield during 72 h fermentation were achieved at the initial reducing sugar concentration of 180 g/L (Fig. 1, Table 2). Since, the highest values of ethanol concentration of 87.81±0.63 g/L, highest ethanol yield of 0.49±0.03 g/g were achieved after 60 h of fermentation with initial reducing sugar concentration of 181.23±1.29 g/L (Table 2), it is reasonable to reduce fermentation time to 60 h. Therefore, we concluded that the initial reducing sugar concentration of 180 g/L was selected as optimal.

Variations of sugar concentration with time for different initial reducing sugar concentrations are also shown in Fig. 1. At low reducing sugar concentrations (not exceed 180 g/L), sugar utilization was fast resulting in almost complete sugar utilization within 48 h. High initial reducing sugar concentrations (200 g/L, 220 g/L) caused a lag phase for sugar utilization probably due to high osmotic pressure. And a significant amount of residual sugars (approximately 2.58% of the original sugars) remained in the finished broth from concentrated food waste hydrolyzates at the initial reducing sugar of approximately 220 g/L. Therefore, initial reducing sugar concentration in concentrated food waste hydrolyzates should not exceed 180 g/L for fast sugar utilization, otherwise both the high sugar content and the resulting high ethanol concentration will exert inhibitory effects on yeast, which will result in incomplete fermentation of reducing sugars.

Viability of the yeast during ethanol fermentation from the concentrated food waste hydrolyzates under various conditions is shown in Fig. 2. As shown in Fig. 2, in all flasks, after a few hours of lag phase, the cells growth of *S. cerevisiae* H058 continued until the exponential phase and reached the stationary phase. After the stationary phase, a significant reduction in the number of viable cells was observed, indicating a product inhibition or nutrient depletion affect biomass yield.

Effect of initial inoculum concentration: In order to investigate the effect of initial inoculum concentration

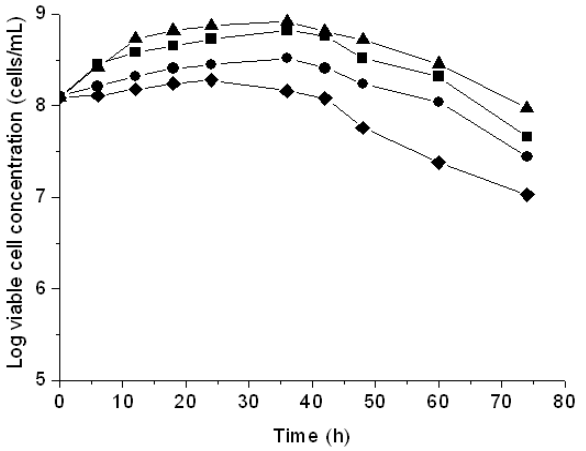


Fig. 2: Yeast viability of *S. cerevisiae* H058 during batch ethanol fermentation from concentrated food waste hydrolyzates at various initial reducing sugar concentrations: 160 g/L (■), 180 g/L (▲), 200 g/L (●) and 220 g/L (◆)

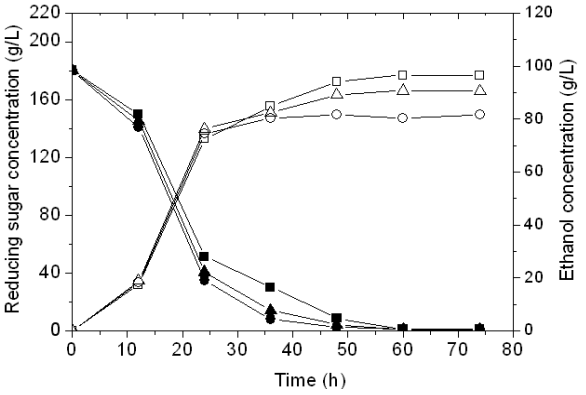


Fig. 3: Sugar consumption and ethanol production during batch ethanol fermentation from concentrated food waste hydrolyzates with various inoculum concentrations by free cells of *S. cerevisiae* H058: 2% (■, □), 4% (▲, Δ) and 6% (●, ○), total reducing sugar (close symbol) and ethanol (open symbol). Initial reducing sugar concentration was 180 g/L

on ethanol production from concentrated food waste hydrolyzates. Three different initial inoculum concentrations were tested: 2, 4 and 6% (v/v), at constant initial reducing sugar concentration of 180 g/L. The results are depiced in Fig. 3 and Table 3.

Figure 3 depicted variations of total reducing sugar and ethanol concentrations with time for different initial inoculum concentrations. Reducing sugar utilization was almost completed with 48 h when initial inoculum

Table 3: Kinetic parameters during batch ethanol fermentation from concentrated food waste hydrolyzates for various inoculum concentrations by free cells of *S. cerevisiae* H058. Initial reducing sugar concentration was 180 g/L

Fermentation time (h)	Inoculum concentration % (v/v)	Ethanol produced (g/L)	Sugar Utilized (g/L)	Ethanol production rate (g/L/h)	Sugar consumption rate (g/L/h)	Ethanol yield (g/g)
	2	84.12±1.13	171.55±1.79	1.75±0.02	3.57±0.04	0.49±0.02
48	4	83.16±1.75	175.96±1.43	1.73±0.04	3.67±0.03	0.47±0.03
	6	81.68±1.42	177.61±1.81	1.70±0.03	3.70±0.0	0.46±0.03
	2	87.75±1.22	178.16±0.98	1.46±0.02	2.97±0.02	0.49±0.02
60	4	84.65±1.87	179.08±1.23	1.41±0.03	2.98±0.02	0.47±0.02
	6	81.41±1.63	179.23±1.89	1.36±0.03	2.99±0.03	0.45±0.03
	2	87.46±1.31	179.26±1.26	1.21±0.02	2.49±0.02	0.49±0.01
72	4	85.55±1.42	179.43±1.48	1.19±0.02	2.49±0.02	0.48±0.03
	6	81.26±1.84	179.30±1.87	1.13±0.03	2.49±0.03	0.45±0.02

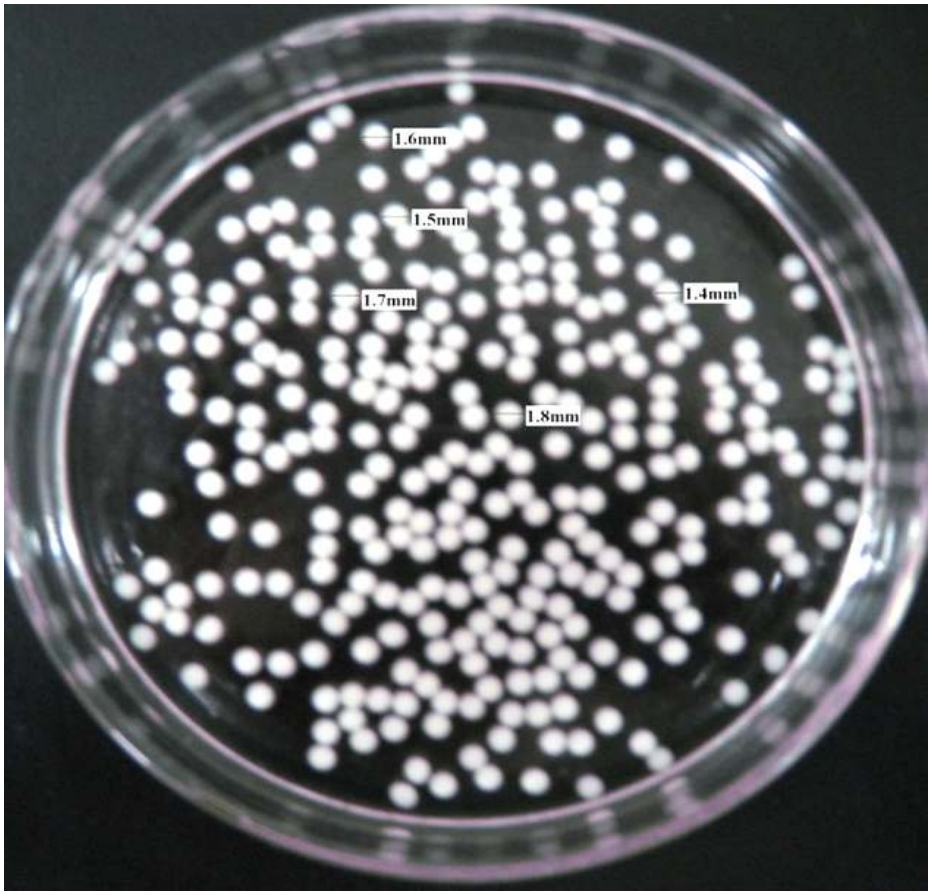


Fig. 4: Cells of *S. cerevisiae* H058 entrapped in the gel matrix of Ca-alginate

concentrations were above 4% (v/v). Reducing sugar utilization was relatively slow (60 h) for initial inoculum concentration was 2% (v/v) since the rate is directly proportional with the inoculum concentration. Table 3 presented the maximum ethanol produced, as well as ethanol yield, was achieved at an inoculum concentration 2% (v/v). The values of these parameters at higher inoculum concentrations of 4% and 6% (v/v) were lower than the values achieved at inoculum concentrations of 2% (v/v). This may result from overuse of substrate for growth and maintenance at high cell concentrations. However, it's obvious that higher initial cell concentrations resulted in shorter fermentation period and reduced the chance of contamination. Taking into account all these facts, inoculum concentration of 2% (v/v) was choosed as optimum initial inoculum concentration for free cell system.

In related study using Corn meal hydrolyzates as the main carbon source, has shown that maximal ethanol production by free cells of *S. cerevisiae* var. *ellipsoideus*, occurred with 2% inoculum size (Nikolić *et al*., 2009a) while Gibbons and Westby (1986) reported the effect of inoculum size on ethanol production, maximum yeast population and yield. A 3% of inoculum size was shown to be acceptable for ethanol fermentation. Narendranath and Power used corn meal as raw material and fermentation

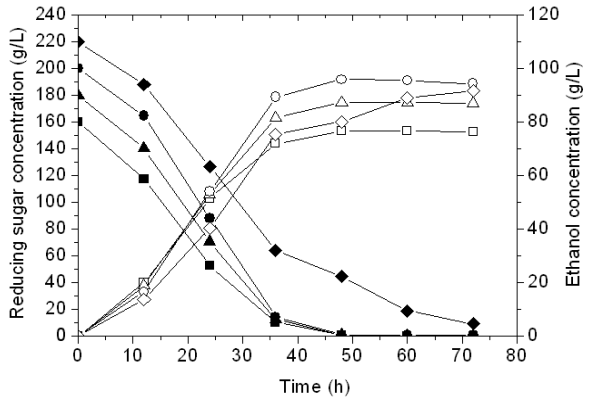


Fig. 5: Sugar consumption and ethanol production during batch ethanol fermentation by immobilized cells of *S. cerevisiae* H058 from concentrated food waste hydrolyzates at various initial reducing sugar concentrations: 160 g/L (■, □), 180 g/L (▲, Δ), 200 g/L (●, ○) and 220 g/L (◆, ◇), total reducing sugar (close symbol) and ethanol (open symbol)

was carried out for 72 h at 30°C in a batch system. They reported that no significant differences were observed in the final ethanol concentration produced by *S. cerevisiae* at any of the inoculum concentrations (1×10^6, 1×10^7, 2×10^7, 3×10^7 and 4×10^7 yeast cells/mL) tested (Sharma *et al*., 2004). These suggest that there was no need to use higher inoculum concentrations, because it did not contribute to higher ethanol concentration, which was also found in this study.

Ethanol production with immobilized yeast cells: In the experiments, the Ca-alginate beads with immobilized cells had an average diameter of 1.6 mm and a photograph of the immobilized yeast cells is presented in Fig. 4. Small diameter beads are generally preferred because of the more favorable mass transfer. The significance of this immobilization method is that the matrix is porous enough for substrate and products to traverse where a level of cell retention is maintained within the immobilization matrix (Nikolić *et al*., 2009b). Alginate as a suitable cell entrapment matrix is non-toxic, less expensive, reversible and has good mechanical properties (Vogelsang *et al*., 2000). According to the results, the yeast *S. cerevisiae* H058 cells entrapped in Ca-alginate showed good physical and chemical stability and no substrate and product diffusion restrictions were noticed.

Effect of initial reducing sugar concentration: Ethanol production and sugar consumption during batch fermentation of immobilized *S. cerevisiae* H058 cells from the concentrated food waste hydrolyzates at the initial reducing sugar of approximately 160, 180, 200 and 220 g/L are shown in Fig. 5.

As demonstrated by Fig. 5, at relatively low reducing sugar concentrations (160-200 g/L) sugar utilization was fast resulting in complete sugar utilization within 48 h. High reducing sugar concentrations of 220 g/L caused a lag phase for sugar utilization probably due to high osmotic pressure. And at initial reducing sugar concentration of 220 g/L, considerable sugar utilization was realized only after 72 h. Therefore, reducing sugar concentration should be kept below 200 g/L for fast sugar utilization.

Variations of ethanol concentration with time for different reducing sugar concentrations are also shown in Fig. 5. Ethanol concentration increased with time and reached the maximum level at the end of 48 h of incubation for reducing sugar concentrations below 200 g/L. Similar to sugar utilization, ethanol formation was slow for the first 48 h for reducing sugar concentration of 220 g/L, probably due to osmotic pressure caused by high reducing sugar concentrations. And only after the adaption period (48 h), ethanol formation was increased considerably. Final ethanol concentrations increased with the initial reducing sugar up to 200 g/L and then decreased with increasing reducing sugar above 200 g/L due to substrate inhibition (Fig. 5). The maximum final ethanol concentration of 95.98±0.78 g/L was obtained at the end of 48 h when initial reducing sugar was 200 g/L.

Behera *et al*. (2010) concluded that the concentrations of ethanol produced in both the free and immobilized batch systems (Ca-alginate) were relatively similar. However, our study demonstrated that immobilized cells exhibited an elevated tolerance to higher substrate and product concentrations compared with the free cells. Also, Nikolić *et al*. (2009a) investigated the batch fermentation of corn meal hydrolyzates by immobilized and free cells of *Saccharomyces cerevisiae* var. *ellipsoideus* with different initial glucose concentrations, they found during fermentation with immobilized cells, substrate inhibition occurred at an initial glucose concentration of 200 g/L, whereas free cells were inhibited with lower initial substrate concentration of 176 g/L (Nikolić *et al*., 2009a). Najafpour *et al*. (2004) reported that immobilization of the cells can eliminate inhibition caused by high concentrations of substrate and product, enhance the ethanol yield and productivity and increase the yeast stability. Immobilized cells are considered to be more tolerant to ethanol since the matrix provides a protective environment against ethanol toxicity as reported by Verbelen *et al*. (2006). Similar results were also observed by Ciesarová *et al*. (1998) and Wendhausen *et al*. (2001).

Table 4 also summarizes the important kinetic parameters of the ethanol fermentation under various initial reducing sugar concentrations. The results showed that initial reducing sugar concentration had significant effects on the main kinetic parameters. Ethanol production rate for the first 48 h period increased with reducing sugar concentration below 200 g/L due to substrate limitations, but decreased with increasing reducing sugar concentrations larger than

Table 4: Kinetic parameters of ethanol production from concentrated food waste hydrolyzates at various initial reducing sugar concentrations by immobilized cells of *S. cerevisiae* H058

Initial reducing sugar (g/L)	Max. ethanol produced (g/L)	Sugar Utilized (g/L)	Time (h)	Ethanol production rate (g/L/h)	Sugarconsumption rate (g/L/h)	Ethanol yield (g/g)
160.32±1.14	76.65±0.49	159.43±1.23	48	1.60±0.01	3.32±0.03	0.48±0.03
179.87±1.27	87.32±0.63	178.89±1.41	48	1.82±0.01	3.73±0.03	0.49±0.04
200.23±1.96	95.98±0.78	199.11±1.74	48	2.00±0.01	4.15±0.04	0.48±0.02
220.14±2.16	91.60±0.89	210.78±1.35	72	1.27±0.02	2.93±0.01	0.43±0.02

Table 5: Kinetic parameters during batch ethanol fermentation from concentrated food waste hydrolyzates for various inoculum concentrations by immobilized cells of *S. cerevisiae* H058. Initial reducing sugar concentration was 200 g/L

Fermentation time (h)	Inoculum concentration % (v/v)	Ethanol produced (g/L)	Sugar Utilized (g/L)	Ethanol production rate (g/L/h)	Sugar consumption rate (g/L/h)	Ethanol yield (g/g)
	2	89.45±1.37	184.81±1.64	2.48±0.04	5.13±0.05	0.48±0.02
36	4	89.35±1.85	190.21±1.52	2.48±0.05	5.28±0.04	0.46±0.03
	6	88.59±1.46	193.7±1.76	2.46±0.04	5.38±0.05	0.46±0.02
	2	95.97±1.18	198.53±1.39	2.00±0.04	4.14±0.03	0.48±0.03
48	4	93.15±1.84	199.15±1.82	1.94±0.04	4.15±0.04	0.47±0.02
	6	90.13±1.71	199.47±1.73	1.88±0.04	4.16±0.04	0.45±0.04
	2	95.94±1.56	198.73±1.39	1.60±0.03	3.31±0.02	0.48±0.02
60	4	93.08±1.67	199.41±1.55	1.55±0.03	3.32±0.03	0.47±0.02
	6	90.12±1.83	199.67±1.78	1.50±0.03	3.33±0.03	0.45±0.03

200 g/L due to substrate inhibition as a result of high osmotic pressure at high reducing sugar concenrations (Fig. 5, Table 4). The maximum values of ethanol concentration of 95.98 g/L, ethanol production rate of 2.00±0.01 g/L/h and sugar consumption rate of 4.15±0.04 g/L/h after 48 h were achieved when the initial reducing sugar concentration was 200 g/L (Table 4). Also, high final ethanol yield of 0.48±0.02 g/g was obtained in this case. Taking into account all these facts, one could select an initial reducing sugar concentration of 200 g/L as optimal.

Figure 6 shows the variations of viable cell counts as measured by the number of Colony-Forming Units (CFU) on the immobilized cells and in the fermentation broth, during the batch fermentations of concentrated food waste hydrolyzates. The time profiles of the number of viable cells at various reducing sugar concentrations were similar (Fig. 6). It was obvious that released cells were detected in the medium only after 18 h of cultivation, which approximately coincided with the start of intensive proliferation of immobilized cells. The free cell concentration probably represents both cells that grew outside the carriers as well as those leaked into the medium after growing inside the carriers. The maximum number of viable cells of 1.33×10^9 CFU/g of beads and 6.71×10^6 CFU/mL of broth were obtained in the immobilized system with initial reducing sugar concentration of 200 g/L during 48 h, justifying the highest ethanol concentration and sugar consumption rate obtained by immobilized cells at the same reducing sugar concentrations (Table 4).

Effect of initial inoculum concentration: Ethanol fermentation in the concentrated food waste hydrolyzates was carried out with different inoculums to determine the effect of inoculum concentration on ethanol fermentation. The inoculum concentration was increased from 2, 4, to 6% (w/v) at a reducing sugar

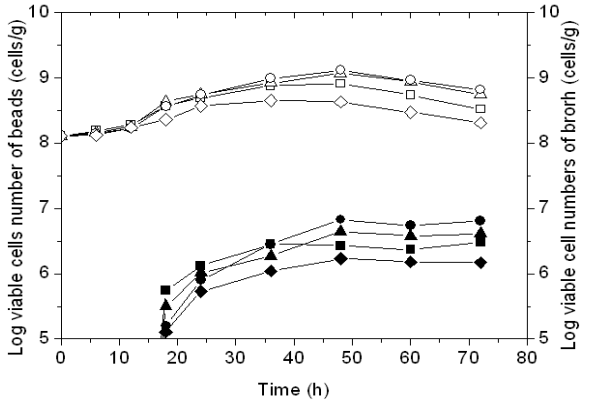


Fig. 6: Yeast viability of *S. cerevisiae* H058 during batch ethanol fermentation from immobilized system at various initial reducing sugar concentrations:160 g/L (■, □), 180 g/L (▲, Δ), 200 g/L (●, ○) and 220 g/L (◆, ◇), number of viable cells in immobilized beads (open symbol) and number of viable cells in fermentation broth (close symbol)

concentration of 200 g/L as optimal. The results of the ethanol and reducing sugar concentration achieved are presented in Fig. 7. The values of significant fermentation parameters achieved during the fermentation with various inoculum concentrations are presented in Table 5.

As shown in Table 5, the maximum ethanol concentration and ethanol yield were obtained at the time of 48 h with an initial inoculum of 2% (w/v). Rakin *et al*. (2009) investigated ethanol production from corn meal hydrolyzates in batch fermentations by immobilized *S. cerevisiae* cells. The optimum initial inoculum concentration and initial glucose concentration in the batch fermentation were 5% (w/v) and 176 g/L, respectively. At these conditions, the maximum ethanol concentration of 10.05% (w/w) was obtained. Also, Nikolić *et al*. (2010) investigated

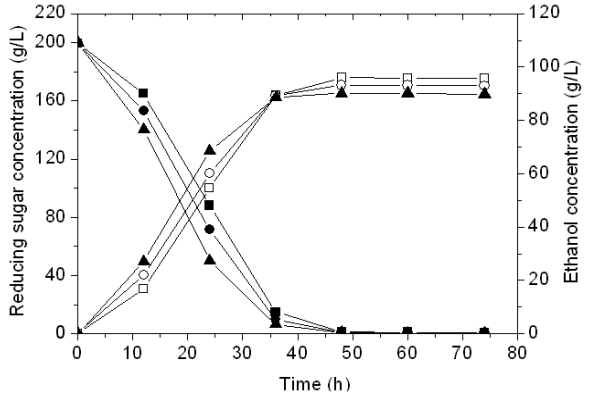


Fig. 7: Sugar consumption and ethanol production during batch ethanol fermentation from concentrated food waste hydrolyzates with various inoculum concentrations by immobilized cells of *S. cerevisiae* H058: 2% (■, □), 4% (▲, Δ) and 6% (●, ○), total reducing sugar (close symbol) and ethanol (open symbol). Initial reducing sugar concentration was 180 g/L

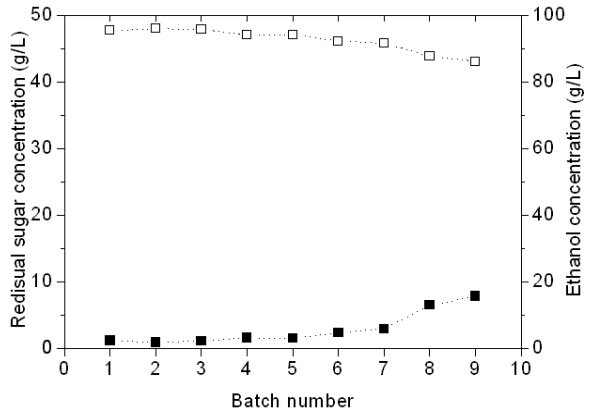


Fig. 8: Repeated batch kinetic profile of the immobilized yeast cells in concentrated food waste hydrolyzates. Reduisal sugar (close symbol) and ethanol (open symbol). Initial reducing sugar concentration was 200 g/L

ethanol production from corn meal hydrolyzates by immobilized *Saccharomyces cerevisiae* var. *ellipsoideus* and found that the maximum final ethanol concentration, as well as maximum ethanol yield, percentage of theoretical yield of ethanol and volumetric productivity was achieved at an inoculum concentration 2% (w/v). These results suggested that the inoculum concentration had a significant effect for ethanol production with *S. cerevisiae*. In the present study, there was a significant difference between an inoculum concentration of 2% and other inoculum concentrations evaluated especially in terms of ethanol concentration (Fig. 7). The optimum inoculum concentration for maximum ethanol production, ethanol productivity and ethanol yield was found to be 2% (w/v). Thus, we concluded that the optimum inoculum size was 2% (v/v).

REPEATED BATCH PRODUCTION OF ETHANOL BY IMMOBILIZED S. CEREVISIAE H058 CELLS

In repeated batch fermentation, sample was withdrawn every 6 h. The residual sugar concentration and ethanol concentration were determined immediately. After 48 h of incubation, all the media were withdrew and the immobilized *S. cerevisiae* H058 were then retrieved and transferred to a fresh batch juice. The process was repeated. Figure 8 shows the repeated batch kinetic profile of the immobilized yeast cells in concentrated food waste hydrolyzates. It can be seen in Fig. 8 that the overall ethanol concentration fluctuated around 94.24 g/L slightly during the first 7 repeated batches. And the utilization rate of reducing sugar could be maintained more than 98.5% after repeated use of immobilized cells for 7 batches. However, the ethanol concentration in the 8th and 9th cycle was rather lower (87.81, 86.23 g/L, respectively). This means that immobilized *S. cerevisiae* H058 cells in alginate gel retained its activity to produce ethanol for 7 batches, then the productivity began to decline.

In addition, the 8th batch of fermentation resulted in mostly destruction of Ca-alginate beads. The destruction of alginate beads after the 8th batches might due to intensive growth of cells and CO_2 evolution during the fermentation. It is assumed that the intensive proliferation of yeast cells inside the matrix caused instability of Ca-alginate in acidic condition during the fermentation. Najafpour *et al*. (2004) reported that the concentration of 2% (w/w) of alginate in beads was a suitable alginate concentration regarding the activity of beads for ethanol production in an immobilized cell reactor during 10 working days. Similar results were also observed by Rakin *et al*. (2009).

A series of electronic micrographs were taken from the fresh and 7th batch of immobilized beads (Fig. 9 and 10). It was apparent that after 7 batches, the yeast cells were accumulated close to the surface of the beads. Therefore the active sites were potentially available for ethanol production without diffusion problems. This phenomenon was comparable with other similar study (Laca *et al*., 2000; Wang *et al*., 1995). The inner surfaces of the beads before and after use were also compared (Fig. 10). Figure 10 showed the cells were initially trapped inside the beads, however, after 8 batches the cells gradually autolyzed or migrated from the inner side to the surfacer. This might due to the hindrance of substrate diffusion to the immobilized cells which limited the growth within the gel.

Comparison of the free and immobilized system: The maximum value of ethanol concentration, ethanol yield and ethanol production rate was achieved after 60 h fermentation using free yeast cells at initial inoculum concentration of 2% (v/v) and with initial reducing sugar concentration of 180 g/L.

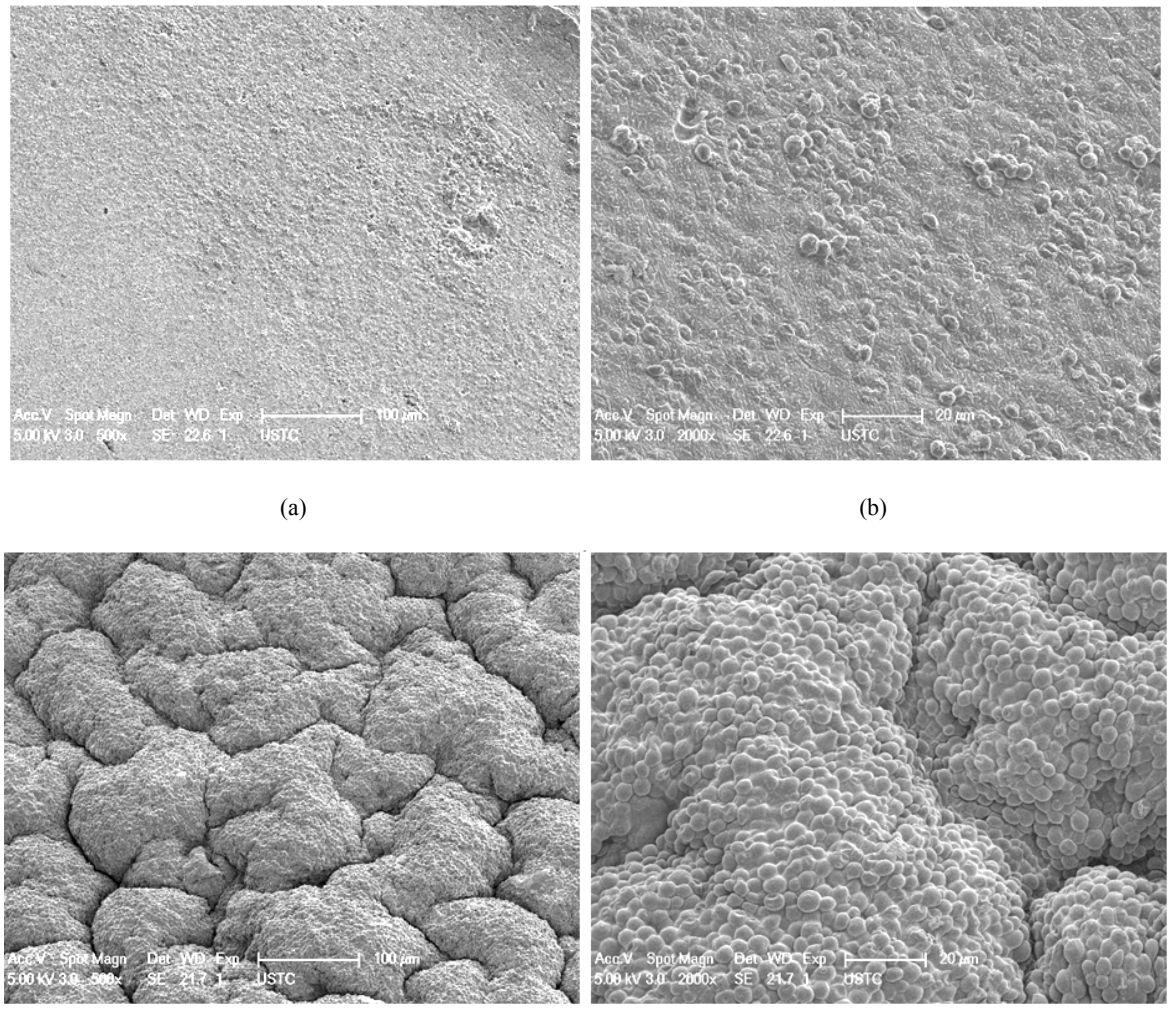
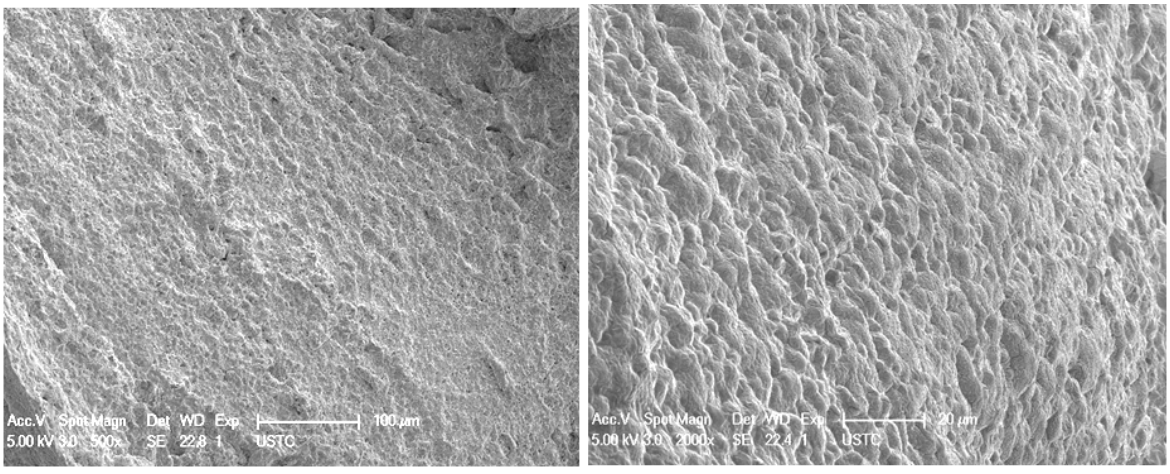
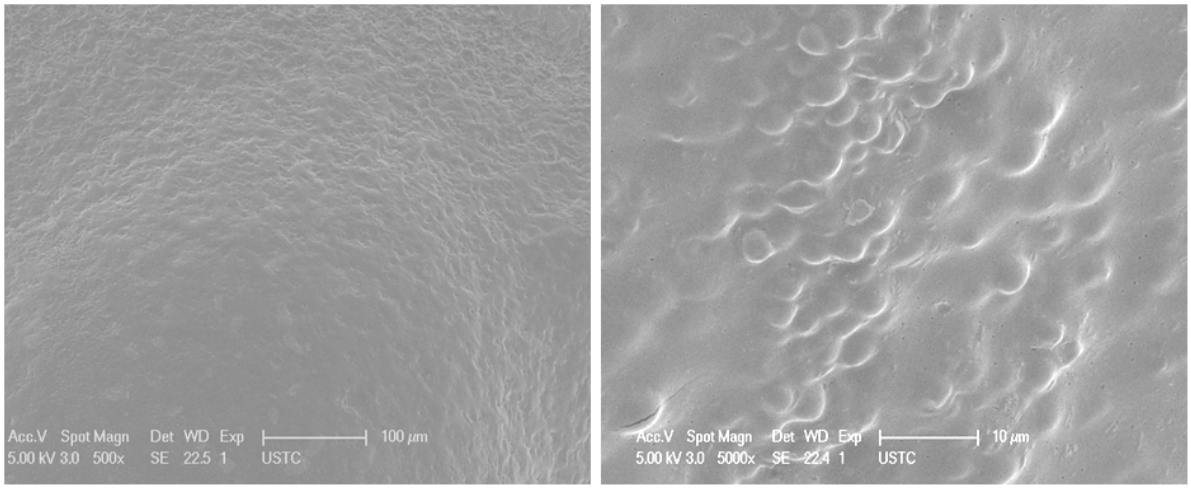


Fig. 9: Electronic photomicroscope of the outer surface of immobilized *S. cerevisiae* H058 beads; (a): Outer surface of the fresh beads with magnification of 500 µm; (b): Outer surface of the fresh beads with magnification of 2000 µm; (c): Outer surface of the used beads after 6 batches with magnification of 500 µm; (d): Outer surface of the used beads after 6 batches with magnification of 2000 µm





(c) (d)

Fig. 10: Electronic photomicroscope of the inner surface of immobilized *S. cerevisiae* H058 beads; (a): Inner surface of the fresh beads with magnification of 500 μm; (b): Inner surface of the fresh beads with magnification of 2000 μm; (c): Inner surface of the used beads after 6 batches with magnification of 500 μm; (d): Inner surface of the used beads after 6 batches with magnification of 5000 μm

However, in immobilized system the maximum values of all significant parameters were achieved at initial inoculum concentration of 2% (w/v) and with initial reducing sugar concentration of 200 g/L. Swain *et al*. (2007) investigated ethanol fermentation of mahula (Madhuca latifolia L.) flowers using free and immobilized yeast *S. cerevisiae*. They reported there was virtually insignificant difference on the ethanol yield whether free or immobilized cells were used. However, our study demonstrated that immobilized cells exhibited an elevated tolerance to higher substrate and product concentrations compared with the free cells. Also, immobilized cells showed maximum ethanol produced at 48 h of incubation, less than that of free system (60 h). The reason may be due to the microorganisms when entrapped with the spongy matrices led to decrease medium viscosity and enhance nutrient transfer which eventually showed more substrate consumption with faster rates yielding ethanol within less fermentation time (Angelova and Petricheva, 1997). Ganguly *et al*. (2007) also observed the lactic acid production reached its maximum level (80.75 g/L) after 48 h of incubation with immobilized cells while, the maximum production level (86.13 g/L) with free cells was obtained after 72 h of incubation. These systems, therefore, can be used for developing the repeated batch and continuous processes for economizing the production process (Ganguly *et al*., 2007).

Therefore, it can be generally concluded that under selected optimal process conditions the immobilized system was more productive. Further benefits of utilization of immobilized *S. cerevisiae* H058 could be expected in continuous fermentation system and our further research will also be focused on the improvement of the stability of alginate micro-beads.

CONCLUSION

Ethanol fermentation of concentrated food waste hydrolyzates by free and immobilized cells of *S. cerevisiae* H058 in a batch fermentation system was studied. The conditions of ethanol fermentation, such as initial reducing sugar concentration, inoculum concentration and the time required for the efficient ethanol production were optimized.

An optimal initial reducing sugar concentration of 180 g/L, inoculum concentration of 2% (v/v) and fermentation time of 60 h were determined for the free cell system. For the immobilized system, an optimal initial reducing sugar concentration of 200 g/L, inoculum concentration of 2% (w/v) and fermentation time of 48 h were selected. In addition, repeated batch fermentation of immobilized *S. cerevisiae* H058 cells was attempted for ethanol production for 7 batches. These results indicated immobilized cell system was superior to the free cell system since lower substrate inhibition, less fermentation time and higher ethanol tolerance were realized.

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