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Full Length Research Paper

Continuous ethanol production by *Kluyveromyces* sp. IIPE453 immobilized on bagasse chips in packed bed reactor

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Continuous ethanol production eliminates much of the unproductive down-time associated with batch process and increases the productivity. Many studies showed that the cell immobilization leads to improve fermentation rates by the high cell concentrations, option of reusability and protection of cells from toxic effects of low pH, temperature, inhibitors, etc. The thermotolerant yeast *Kluyveromyces* sp. IIPE453 was immobilized on sugarcane bagasse chips and packed in a column. The maximum volumetric productivity 21.87 ± 0.75 g l^{-1} h^{-1} was achieved with ethanol concentration of 17.5 ± 0.6 g/l and sugar utilization of 76 ± 2.4% at dilution rate of 1.25 h^{-1} by feeding 50 g/l glucose concentration. The maximum 18.65 ± 0.75 g l^{-1} h^{-1} volumetric productivity was achieved with ethanol concentration of 37.3 ± 1.5 g/l and sugar utilization of 54±6.5% at dilution rate of 0.5 h^{-1} by feeding 150 g/l glucose concentration.

Key words: *Kluyveromyces* sp., ethanol fermentation, continuous process, immobilization, sugarcane bagasse chips.

INTRODUCTION

The environment concern over the use and depletion of fossil fuels, the search for alternative fuel is desirable (Liang et al., 2008). Ethanol has attracted worldwide attention due to its potential use as a transportation fuel (Kumar et al., 2009a). Ethanol is traditionally produced in the batch fermentations by yeasts, mostly *Saccharomyces cerevisiae* and their interspecies hybrids, which provide the low productivity (Gunasekaran and Raj, 1999; Rebroš et al., 2005). High ethanol productivity from cheaper and renewable sources and minimum energy input are important aspects in the alcoholic fermentation research. Techniques such as continuous culture; cell immobilization and recycling of cells have been explored to achieve these objectives (Sheoran et al., 1998).

The continuous process can achieve substantial improvements in the efficiency of the process and product quality, subsequently higher productivities, lower operating costs, reduced product losses and environmental advantages (Bakoyianis et al., 1997; Verbelen et al., 2006). However, continuous process with free cells has disadvantages of higher cost of cell recycling, high contamination risk, susceptibility to environmental variations and the limitations of the dilution rate due to wash-out condition (de Vasconcelos et al., 2004). Cell immobilization facilitates the larger area of contact between cells and nutrient medium, potential for high fermentation rates offered by the high cell concentrations, option of reusability of cells, protection of cells from toxic effects of low pH, temperature, inhibitors, tolerance to high osmolalities etc. (Banat et al., 1998; Tata et al., 1999; Kocher et al., 2006). In many studies

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Abbreviations: D, Dilution rate, h^{-1} ; $\mathbf{P_f}$, ethanol concentration in outlet, g/l; $\mathbf{q_p}$, volumetric ethanol production rate; g l^{-1} h^{-1}; $\mathbf{q_{sp}}$, specific ethanol production rate, g g^{-1} h^{-1}; $\mathbf{q_s}$, specific sugar uptake rate, g g^{-1} h^{-1}; $\mathbf{S_o}$, glucose concentration in feed, g/l; $\mathbf{S_f}$, residual glucose concentration in outlet, g/l; $\bar{X}$, average biomass concentration, g/l; $\mathbf{Y_{P/S}}$, ethanol yield on glucose consumed, g/g; **η,** sugar utilization, %.

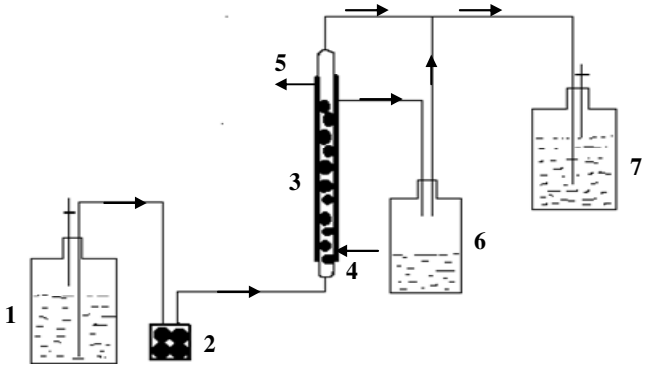

Figure 1. Schematic diagram of continuous ethanol fermentation process in a packed column; 1. Feed vessel; 2. Peristaltic pump; 3. Jacketed column; 4. Hot water in; 5. Hot water out; 6. Product vessel; 7. CO_2 absorber.

the immobilized microorganisms were found to be more stable than the free microorganisms, particularly when utilized over prolonged periods of time (Love et al., 1998; Göksungur and Zorlu, 2001).

The development of immobilized-cell processes, using low-cost support and low operational (immobilization) cost would be desirable for economical production process (de Vasconcelos et al., 2004). The selection of a suitable support for cell immobilization is difficult. A number of factors for example nature of support and its compatibility with the microorganisms, environmental conditions, etc. are known to influence the cell support matrix interactions (Bakoyianis et al., 1997). The four methods for immobilization are categorized as (Liang et al., 2008): (i) the methods involving binding of biocatalyst to a water insoluble support by using ionic or covalent bonding or adsorption; (ii) the methods involving multiple covalent bonding; (iii) the methods involving entrapment or encapsulation; and (iv) combinations of these methods.

A number of studies on ethanol production by immobilized microorganisms using *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Kluyveromyces marxianus* in organic or inorganic supports have been reported. The reported supports used in immobilization are calcium alginate (Bakoyianis et al., 1997; Sheoran et al., 1998; Love et al., 1998; Göksungur and Zorlu, 2001; Kocher et al., 2006; Valach et al., 2006), calcium pactate (Valach et al., 2006; Kesava and Panda, 1996), porous glass beads (Love et al., 1998; Tata et al., 1999), corn cobs (Kocher et al., 2006), sugarcane bagasse (de Vasconcelos et al., 1998,2004; Kocher et al., 2006), wood shavings (Kocher et al., 2006), kissiris (Bakoyianis et al., 1997; Nigam et al., 1997; Love et al., 1998), γ-alumina (Bakoyianis et al., 1997), delignified cellulosic material (Shindo et al., 2001), κ-carrageenan beads (Krishnan et al., 2000), sorghum bagasse (Yu et al., 2007) and zeolite (Kourkoutas et al., 2002). de Vasconcelos et al. (1998, 2004) and Liang et al. (2008) satisfactory demonstrated the use of sugar-cane stalks as a support for yeast cells in alcoholic fermentation.

The technical barriers, such as substrate and product inhibition, CO_2 hold up in the gel beads are generally encountered during the ethanol fermentation in immobilized yeast reactor. Therefore, reduction in mass transfer rate, floatation of beads and their accumulation near the exit of the reactor results in a considerable low productivity (Sheoran et al., 1998). However, CO_2 hold up could be over come by increasing height to diameter ratio and making high porosity of bed by using sugarcane stalks (de Vasconcelos et al., 2004). In the present study we have reported the ethanol fermentation at 50°C by thermotolerant yeast *Kluyveromyces* sp. IIPE453. The yeast was immobilized on sugarcane bagasse chips and packed in a column. The effect of different dilution rates and sugar concentrations in feed on volumetric productivity and sugar utilization rate were observed and compared with the reported literature.

MATERIALS AND METHODS

Microorganisms and culture conditions

A thermotolerant yeast, *Kluyveromyces* sp. IIPE453 (Kumar et al., 2009b; Kumar et al., 2010), was grown in Bioflow-110 bioreactor (ca. 5 L) on medium SM containing (g/l), di-sodium hydrogen orthophosphate, 0.15; potassium di-hydrogen orthophosphate, 0.15; ammonium sulphate, 2.0; yeast extract, 1.0; glucose 20. The temperature and pH were controlled at 50°C and 5.0, respectively, during the process. The dissolved oxygen was controlled by agitation and 1 vvm aeration rate at 40% of saturation to obtain maximum cell mass.

Cell immobilization

Cells of *Kluyveromyces* sp. IIPE453 were immobilized by adsorption on sugarcane bagasse chips of size 4 to 6 mm. The bagasse chips were washed with distilled water twice and dried before using for immobilization. 10 g dry bagasse chips were suspended in 500 ml salt medium (SM) with glucose concentration 5 g/l and cell concentration 4 g/l. The suspension was incubated overnight at 50°C in shaker at 150 rpm in two flasks of 2 L. The bagasse chips were separated from cell suspension and washed twice to remove free cells with sterile distilled water. The bagasse chips with immobilized cells were packed in a jacketed column of 50 cm height and 2.5 cm i.d. (Figure 1). The immobilized bagasse occupied approximately 160±3 cm^3 volume with void volume 50±5 cm^3 and void fraction 0.31±0.04. The height of the bed was 32.5±0.5 cm.

Fermentation conditions

The medium for fermentation was same as used for growth except ammonium sulphate, 1.0 g/l. The medium was passed through the packed bed column. The liquid was fed upward from bottom in the column. A high accuracy positive displacement peristaltic pump was used to vary the liquid feed flow rate. The dilution rates were varied from 0.5 to 1.25 h^{-1} with an increment of 0.25 h^{-1} and glucose concentrations were varied from 50 to 150 g/l with an increment of 50 g/l. The product stream was collected from the upper part of the

column. The temperature in the packed bed column was maintained at 50°C by passing the hot water through a jacket. Samples were taken at different time intervals and estimated for glucose and ethanol concentration in the broth.

Analytical methods

Glucose was analyzed by a HPLC using High Performance Carbohydrate Column (Waters) at 30°C and detected by a Waters 2414 refractive index detector. The acetonitrile and water mixture (75:25) as a mobile carrier at a flow rate of 1.4 ml min^{-1} was used. Ethanol was analyzed by gas chromatography using Ashco Neon II Gas Analyzer with a 2 m long x 1/8" dia Porapak-QS column with mesh range 80/100. The nitrogen gas was used as a carrier. The injector temperature, oven temperature and flame ionization detector temperature were kept at 220, 150 and 250°C, respectively.

The cell mass concentration in bagasse chips was analyzed on the basis of protein present in cell mass. 5 g dried immobilized bagasse chips were crushed in 50 ml 1 N sodium hydroxide and incubated at 80°C in water bath for 2 h. After incubation the suspension was centrifuged and protein concentration in supernatant was estimated by Folin-Lowery method. The same method was followed for estimation of protein in cell-free bagasse chips. The difference of protein concentration in bagasse chips immobilized with cells and cells free bagasse provides the loading of cells in bagasse. The protein concentration in the known quantity of yeast cells was also analyzed.

Mathematical modeling

The material balance on substrate concentration over a differential height of the column can be expressed as (Ozmihci and Kargi, 2008a):

$$-FdS = q_s \overline{X} dV = q_s \overline{X} A dz \quad (1)$$

Or,

$$-FdS = \frac{q_{sp} \overline{X}}{Y_{P/S}} dV = \frac{q_{sp} \overline{X}}{Y_{P/S}} A\, dz \quad (2)$$

where, F is the flow rate of the feed (l/h); dS is the difference between sugar concentration over the differential height (g/l); q_s is the specific rate of sugar uptake (g g^{-1} h^{-1}); q_{sp} is the specific rate of ethanol formation (g g^{-1} h^{-1}); $\overline{X}$ is the average biomass concentration (g/l); $Y_{P/S}$ is the product yield coefficient (g/g); dV is the differential volume (l); A is the cross-section area of the column (m^2); and dz is the differential column height (m). Assuming q_{sp}, $\overline{X}$ and $Y_{P/S}$ are constant. On integrating equation (Equation 2).

$$-F\int_{S_o}^{S} dS = \frac{q_{sp} \overline{X}}{Y_{P/S}} A \int_0^H dz \quad (3)$$

$$S_o - S = \frac{q_{sp} \overline{X}}{Y_{P/S}} \frac{A}{F} H \quad (4)$$

Where, S_o is the sugar concentration in feed (g/l); S is the sugar concentration at column height H (g/l); and H is the column height (m). Similarly, material balance on product concentration over a differential height can be expressed as:

$$FdP = q_{sp} \overline{X} dV = q_{sp} \overline{X} A dz \quad (5)$$

where, dP is the difference between ethanol concentration over the differential height (g/l). On integrating Equation (5):

$$F\int_{P_o}^{P} dP = q_{sp} \overline{X} A \int_0^H dz \quad (6)$$

$$P - P_o = q_{sp} \overline{X} \frac{A}{F} H \quad (7)$$

where, P_o is the ethanol concentration in feed (g/l); and P is the ethanol concentration at column height H (g/l). Rearranging equation (7) for calculating specific rate of ethanol formation (q_p):

$$q_{sp} = P \frac{D}{\overline{X}} \quad (8)$$

where, D is dilution rate, h^{-1}; $D = \frac{F}{A.H} = \frac{F}{V}$ and ethanol concentration in feed, P_o=0.

Rate of ethanol formation or volumetric ethanol productivity (q_p):

$$q_p = q_{sp} \overline{X} = PD \quad (9)$$

RESULTS AND DISCUSSION

The bagasse pieces were chosen for immobilization of thermotolerant yeast *Kluyveromyces* sp. IIPE453 for the production of ethanol because of its high porosity to adsorb the yeast cells, easy availability, natural source and stability at high temperature. The cells were adsorbed on bagasse chips with cell loading of 120±10 mg/g of bagasse on dry basis with 60% adsorption efficiency. de Vasconcelos et al. (2004) reported Fleischmann yeast cells loading of 477 mg/g on 2 cm long dry sugarcane stalks. The average cell mass concentration in column ($\overline{X}$) was 7.5±0.5 g/l of total packed volume. Liang et al. (2008) suggested that the immobilization of yeast cells on sugarcane pieces is a result of natural entrapment into the porous structure of the support and adsorption by electrostatic forces between cell membrane and support.

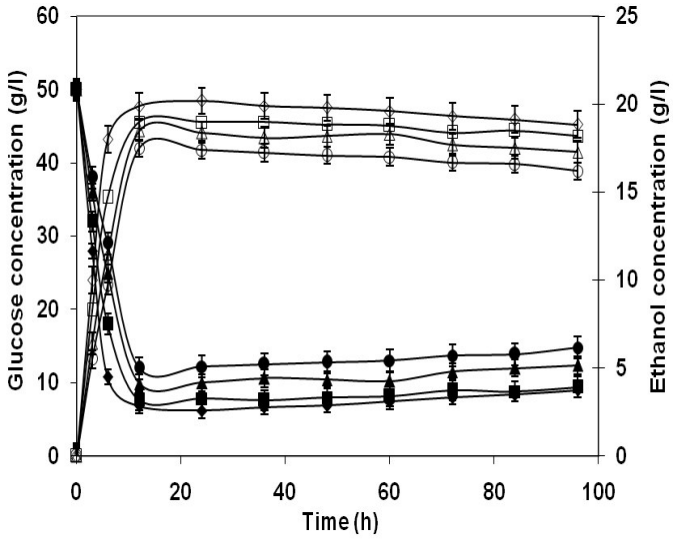

Figure 2. Ethanol fermentation by *Kluyveromyces* sp. IIPE453 immobilized in bagasse chips at different dilution rates with 50 g/l feed glucose concentration showing glucose concentrations: (♦) D=0.5 h^{-1}; (■) D=0.75 h^{-1}; (▲) D=1 h^{-1}; (●) D=1.25 h^{-1} and ethanol concentrations: (◊) D=0.5 h^{-1}; (□) D=0.75 h^{-1}; (Δ) D=1.0 h^{-1}; (○) D=1.25 h^{-1}.

Table 1. Effect of dilution rate on different parameters in ethanol fermentation by *Kluyveromyces* sp. IIPE453 immobilized in bagasse chips.

Kinetic parameters[a]	Dilution rate (h^{-1})			
	0.5	**0.75**	**1**	**1.25**
S_o (g/l)	50	50	50	50
S_f (g/l)	6.8	7.6	9.9	12
P_f (g/l)	20.2	19	18.5	17.5
$Y_{P/S}$ (g/g)	0.461	0.459	0.461	0.46
q_p (g l^{-1} h^{-1})	10.1	14.25	18.5	21.87
q_{sp} (g g^{-1} h^{-1})	1.35	1.9	2.46	2.91
η (%)	87.6	84.8	80.2	76

[a]S_o = Glucose concentration in feed; S_f = Residual glucose concentration in outlet; P_f = Ethanol concentration in outlet; $Y_{P/S}$ = Ethanol yield on glucose consumed; q_P = Volumetric ethanol productivity; q_{SP} =Specific ethanol productivity; η = Sugar utilization.

Effect of dilution rate

The medium containing glucose concentration of 50 g/l was passed through the column at different dilution rates varying from 0.5 to 1.25 h^{-1} (Figure 2). At a dilution rate of 0.5 h^{-1}, 20.2±0.7 g/l ethanol concentration was obtained in the outlet with an ethanol yield of 90.2±0.2% of its theoretical yield at steady state. At a dilution rate of 0.75 h^{-1}, 19±0.55 g/l ethanol concentration was obtained in the outlet with an ethanol yield of 89.8±0.16% of its theoretical yield at steady state. At a dilution rate of 1.0 h^{-1}, 18.5±0.6 g/l ethanol concentration was obtained in the outlet with an ethanol yield of 90.2±0.2% of its theoretical yield at steady state. At a dilution rate of 1.25 h^{-1}, 17.5±0.6 g/l ethanol concentration was obtained in the outlet with an ethanol yield of 90±0.1% of its theoretical yield at steady state.

The ethanol productivities (q_p) and specific productivities (q_{sp}) were calculated using equations 8 and 9 (Table 1). The ethanol productivity and specific ethanol productivity could be increased when the dilution rate was increased from 0.5 to 1.25 h^{-1} but the ethanol concentration and sugar utilization decreased significantly. The ethanol concentration of 20.2±0.7 g/l with 87.6±2.8% sugar utilization was achieved at a dilution rate of 0.5 h^{-1}. The ethanol concentration decreased up to 17.5±0.6 g/l with 76±2.4% sugar utilization when dilution rate was increased up to 1.25 h^{-1}. The volumetric productivity 21.87±0.75 g l^{-1} h^{-1} and specific productivity of 2.91±0.09 g g^{-1} h^{-1} was achieved at a dilution rate of 1.25 h^{-1}. The ethanol concentration was decreased due to less interaction of glucose molecule with the immobilized yeast or low hydraulic retention time. The higher ethanol productivity was achieved as compared to 1.71 and 3.7 g l^{-1} h^{-1} in a batch fermentation and continuous fermentation with cell recycle, respectively using free cells of the same strain (Kumar et al., 2009b). Thus, the ethanol fermentation using immobilized yeast is a better option.

Ozmihci and Kargi (2008a) reported the ethanol concentration of 10.5 g/l and volumetric productivity of 0.58 g l^{-1} h^{-1} with 63% sugar utilization at a dilution rate of 0.057 h^{-1} on feeding 50 g/l sugar concentration in cheese whey powder solution by *Kluyveromyces marxianus* (DSMZ 7239) in a packed column bioreactor. Yu et al. (2007) reported the volumetric productivity of 16.68 g l^{-1} h^{-1} with ~55% sugar utilization at a dilution rate of 0.3 h^{-1} on feeding 200 g/l sugar concentration using immobilized *S. cerevisiae* on sorghum bagasse. In the present study, we could achieve the highest ethanol concentration with the highest ethanol productivity and maximum sugar utilization at a dilution rate of 1.25 h^{-1} as compared to reported literature.

The ethanol yield at each dilution rate was almost same. Therefore, no effect was observed of dilution rate on ethanol yield. But Ozmihci and Kargi (2008a) reported that the ethanol yield was decreased by increasing dilution rate or increased by increasing hydraulic retention time on fermenting cheese whey powder by *Kluyveromyces marxianus* (DSMZ 7239) in a packed column bioreactor. As shown in Table 1, the volumetric productivity could be increased when dilution rate was increased from 0.5 to 1.25 h^{-1} whereas the ethanol concentration was declined consistently due to decrease in sugar utilization at high flow rate. de Vasconcelos et al. (2004) reported maximum 29.64 g l^{-1} h^{-1} volumetric

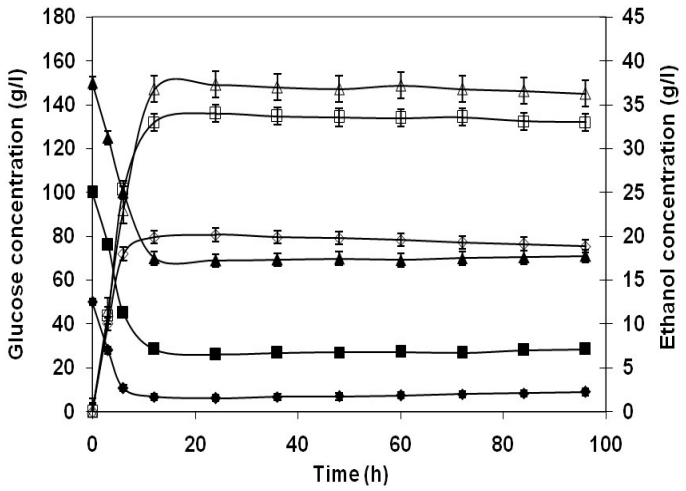

Figure 3. Ethanol fermentation by *Kluyveromyces* sp. IIPE453 immobilized in bagasse chips on varying glucose concentration at dilution rate 0.5 h^{-1} showing glucose concentrations: (♦) S_o=50 g/l; (■) S_o=100 g/l; (▲) S_o=150 g/l and ethanol concentrations: (◊) S_o=50 g/l; (□) S_o=50 g/l; (Δ) S_o=50 g/l.

Table 2. Effect of glucose concentration in feed on different parameters in ethanol fermentation by *Kluyveromyces* sp. IIPE453 immobilized in bagasse chips.

Kinetic parameters[a]	Glucose concentration in feed (g/l)		
	50	100	150
D (h^{-1})	0.5	0.5	0.5
S_f (g/l)	6.8	26	69
P_f (g/l)	20.2	34	37.3
$Y_{P/S}$ (g/g)	0.461	0.46	0.46
q_p (g l^{-1} h^{-1})	10.1	17	18.65
q_{sp} (g g^{-1} h^{-1})	1.35	2.27	2.5
η (%)	87.6	74	54

[a]S_o = Glucose concentration in feed; S_f = Residual glucose concentration in outlet; P_f = Ethanol concentration in outlet; $Y_{P/S}$ = Ethanol yield on glucose consumed; q_P = Volumetric ethanol productivity; q_{SP} = Specific ethanol productivity; η = Sugar utilization

productivity at a dilution rate of 0.83 h^{-1} with 74.61% sugar utilization using immobilized Fleischmann yeast cells in sugarcane stalks.

Effect of feed sugar concentration

The medium with varying glucose concentrations from 50 to 150 g/l was fed into the column at dilution rate 0.5 h^{-1} (Figure 3). At a feed glucose concentration of 100 g/l, 34±1.4 g/l ethanol concentration was obtained with an ethanol yield of 90±0.2% of its theoretical yield at steady state. At a feed glucose concentration of 150, 37.3±1.5 g/l ethanol concentration was obtained with an ethanol yield of 90±0.2% of its theoretical yield at steady state. The ethanol productivities (q_p) and specific productivities (q_{sp}) were calculated using equations 8 and 9 (Table 2). The ethanol concentration, ethanol productivity and specific ethanol productivity could be increased when glucose concentration in feed was increased but the sugar utilization decreased considerably. The ethanol concentration of 37.3±1.5 g/l with 54±6.5% sugar utilization was achieved on feeding glucose concentration of 150 g/l as compared to 20.2±0.7 g/l with 87.5±2.8% sugar utilization on feeding glucose concentration of 50 g/l.

The volumetric productivity of 18.65±0.75 g l^{-1} h^{-1} and specific ethanol productivity of 2.5±0.12 g g^{-1} h^{-1} was achieved on feeding glucose concentration of 150 g/l. The sugar utilization decreased on increasing glucose concentration in feed due to high ratio of glucose to cell mass concentration (S/X) or sugar uptake limit. Ozmihci and Kargi (2008b) reported that the ethanol concentration increased when sugar concentration was increased in feed from 50 to 100 g/l and decreased when sugar concentration was further increased due to lower sugar utilization. They obtained the ethanol concentration of 22.5 g/l with the specific productivity of 0.075 g g^{-1} h^{-1} at feed sugar concentration of 100 g/l in cheese whey powder solution by *Kluyveromyces marxianus* (DSMZ 7239) in a packed column bioreactor.

In the present study, the ethanol concentration further increased by increasing glucose concentration. Love et al. (1998) reported maximum ethanol concentration 46 to 48 g/l on feeding glucose concentration of 100 g/l with ethanol productivity of 4.8 g l^{-1} h^{-1} at a dilution rate of 1 h^{-1} by *Kluyveromyces marxianus* IMB3 immobilized on mixed alginate and kissiris whereas Gough and McHale (1998) reported ethanol concentration of 34 g/l on feeding glucose concentration of 120 g/l with ethanol productivity of 5.1 g l^{-1} h^{-1} at a dilution rate of 1.5 h^{-1} by the same strain immobilized on alginate. In the present study, the ethanol concentration is comparable whereas volumetric productivity is much higher than the reported literature at a glucose concentration of 150 g/l. Increase in specific ethanol productivity shows increase in sugar uptake rate by the yeast on increasing glucose concentration.

The ethanol yield on feeding glucose at any concentration was almost same. Therefore, no effect was observed of glucose concentration in feed on ethanol yield. But Ozmihci and Kargi (2008b) reported that the ethanol yield was decreased by increasing feed sugar on fermenting cheese whey powder by *Kluyveromyces marxianus* (DSMZ 7239) in a packed column bioreactor. As shown in Table 2, the ethanol concentration and volumetric productivity significantly increased when feed glucose concentration was increased from 50 to 100 g/l whereas at 150 g/l feed glucose concentration slightly

increase in ethanol concentration and volumetric productivity were observed. Thus, the immobilization on sugarcane bagasse chips is favorable for low feed sugar concentration.

Conclusion

Thus, ethanol fermentation at high temperature (50°C) with immobilized yeast *Kluyveromyces* sp. IIPE453 reveals that the high dilution rate is favorable to the feed with low sugar concentration. For a high sugar concentration feed, the maximum sugar utilization can be achieved either by increasing bed height or by increasing number of columns.

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