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CONTINUOUS ETHANOL FERMENTATION FROM CAROB POD EXTRACT MEDIUM AT DIFFERENT HYDRAULIC RESIDENCE TIME (HRT)

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ABSTRACT
Production of bioethanol is one of the important bioprocesses for the energy industry to provide inexpensive renewable resources all over the world. In this context, this research was organized for continuous ethanol fermentation from carob pod extract which is an inexpensive carbon source by free or immobilized *S. cerevisiae* cells. Continuous ethanol fermentations were performed with different HRT (from 4 to 20 h) and optimal HRT were 8 h for the free cell, and 6.67 h for immobilized cell, respectively. The highest volumetric ethanol productivities for free cell and immobilized cell fermentations were 3.12 g/L/h and 3.37 g/L/h at HRT of 5.71 h, respectively. All kinetic parameters clearly showed that both cell types can be used for ethanol fermentation, and immobilized *S. cerevisiae* ethanol fermentation can be operated at higher dilution rates independent of biomass than a free cell.
Keywords: Continuous fermentation, free and immobilized cells, stirred tank bioreactor

FARKLI HİDROLİK ALIKONMA SÜRELERİNDE KEÇİBOYNUZU EKSTRAKTI BESİYERİNDE SÜREKLİ ETANOL FERMANTASYONU

ÖZ
Enerji endüstrisinin tüm dünyada ucuz yenilenebilir kaynaklar sağlaması için önemli biyoproseslerden birisi biyoetanol üretimidir. Bu çalışmada serbest veya immobilize edilmiş *S. cerevisiae* hücreleri ile ucuz bir karbon kaynağı olan keçiboynuzu ekstraktından sürekli etanol fermantasyonları amaçlanmıştır. Sürekli etanol fermantasyonları farklı hidrolik alıkonma sürelerinde (4-20 saat) gerçekleştirilmiştir. Optimum hidrolik alıkonma süreleri, serbest haldeki hücreler için 8 sa ve immobilize edilmiş hücreler için 6.67 sa olarak belirlenmiştir. Serbest ve immobilize hücre fermantasyonları için en yüksek etanol üretim oranları sırasıyla 3.12 g/L/sa ve 3.37 g/L/sa olarak hidrolik alıkonma süresi 5.71 saatte elde edilmiştir. Tüm kinetik parametreler, her iki hücre tipinin etanol fermantasyonu için kullanılabileceğini ve immobilize edilmiş *S. cerevisiae* hücreleri ile gerçekleştirilen etanol fermantasyonunun, süspansiyon haldeki hücrelere kıyasla biyokütleden bağımsız olarak daha yüksek seyreltme oranlarında gerçekleştirilebileceğini açıkça göstermiştir.
Anahtar kelimeler: Sürekli fermantasyon, serbest ve immobilize hücreler, karıştırmalı tank tipi biyoreaktör

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INTRODUCTION

The carob tree (*Ceratonia siliqua* L.), which is mostly grown in Mediterranean countries (Yousif and Alghzawi, 2000), could be grown in mild and dry places with poor soils. It could be used as an alternative tree for diversification and revitalization in dryland areas of forests in Mediterranean-climate countries (Sánchez et al., 2010). Although the tree is grown in poor land areas, the carob pods have enough amount and type of sugar for biotechnological applications with its high total soluble content (62–67%) (Ayaz et al., 2007). These soluble solids consist of macro elements such as sucrose, glucose, and fructose; and microelements such as amino acids, minerals, and phenolic compounds (Ayaz et al., 2007). Higher carbohydrate contents make the carob pod and its extract valuable for fermentation processes. So, carob pod extract was used to produce lots of microbial value-added products such as citric acid (Roukas, 1998; Alani et al., 2007), succinic acid (Carvalho et al., 2014), lactic acid (Turhan et al., 2010a), β-mannanase (Yatmaz et al., 2016a; Yatmaz et al., 2016b), and ethanol (Roukas, 1993; Turhan et al., 2010b; Yatmaz et al., 2013; Germec et al., 2015) etc.

Renewable energy sources such as wind turbine, solar panels, bioethanol, biodiesel, their combinations etc. have been started to use in the last decade because of the high carbon emissions and ever decreasing fossil fuels. As a result of the decisions taken by some developed countries, the use of vehicles that work with petroleum products will be limited in the future. Using renewable sources to produce bioethanol, that is an environmental and simple bioprocess, is one of the most important process for the energy industry in the last 30 years (Yatmaz et al., 2013). *Pichia stipitis, Zymomonas mobilis,* and *Saccharomyces cerevisiae* are the most commonly used microorganisms for industrial bioethanol production from starch, sugar or cellulose (Brethauer and Wyman, 2010). Using industrial crops and food wastes or cheap materials for fermentation is very important to decrease cost values of the products. Worldwide, corn and sugar cane extracts are generally used as carbon sources for bioethanol production because of high sugar content and availability of these industrial crops (Cardona and Sánchez, 2007). World total fuel ethanol production in 2009, 2012, and 2015 were 20303, 21812, and 25682 million gallons, respectively (AFDC, 2017). And also, 85.27% of the total fuel ethanol production was done by the two major bioethanol producers; USA and Brazil in 2015 (AFDC, 2017). These statistical values clearly showed that the bioethanol production increases every year. Additionally, it is clearly seen that the bioethanol is one of the most important resources for fossil fuel substitution.

Previous fermentation studies about carob pod extract showed that different microorganisms could be used as free or immobilized for bioethanol production. Some of the used microorganisms by researchers are *Saccharomyces cerevisiae* (Roukas, 1994; 1996; Sánchez et al., 2010; Sánchez-Segado et al., 2012; Saharkhiz et al., 2013) , *Saccharomyces cerevisiae* (ATCC 36858) (Turhan et al., 2010b; Yatmaz et al., 2013; Germec et al., 2015; Germec et al., 2016) , *Saccharomyces cerevisiae* (F13A) (Lima-Costa et al., 2012; Raposo et al., 2017) , *Saccharomyces cerevisiae* (ATCC 7754) (Bahry et al., 2017) , and *Zymomonas mobilis* (PTCC 1718) (Vaheed et al., 2011; Mazaheri et al., 2012; Saharkhiz et al., 2013) .

The first work is about the global process of ethanol production from carob pod. They used different stages to produce ethanol such as aqueous extraction of sugars, acid or alkaline hydrolysis and fermentation of the hydrolysate. The results showed that 95 g/L of ethanol was acquired after 24h at 30 °C, 125 rpm, 200 g/L of initial sugar concentration by *Saccharomycess cerevisiae* (Sánchez et al., 2010).

Different microorganisms were used for batch and fed-batch ethanol fermentation from carob pod extract. *Saccharomycess cerevisiae* (F13A) was used for ethanol fermentation; final ethanol concentrations were 100-110 g/L in all the batch runs and 130 g/L for fed-batch strategy (Lima-Costa et al., 2012). Researchers used *Saccharomycess cerevisiae* (F13A) for evaluating a cost-effective ethanol production with different organic and

inorganic nitrogen sources in a stirred tank bioreactor system by batch fermentation strategy. They carried out that urea can be used as a nitrogen source for ethanol fermentation with 44% ethanol yield and 115 g/L ethanol concentration (Raposo et al., 2017). Ethanol fermentation from carob pod extract by *Zymomonas mobilis* was performed to optimize medium composition and fermentation conditions, and maximum ethanol production was obtained to be 0.34 g ethanol/g initial sugar (Vaheed et al., 2011).

Saccharomyces cerevisiae (ATCC 36858) was also studied for ethanol fermentation with different fermentation techniques. Firstly, free cells were used for ethanol fermentation in a stirred tank bioreactor system. Maximum production rate was 3.48 g/L/h with a meat-bone meal (Turhan et al., 2010b). Immobilized cells in Ca-alginate beads were used for batch ethanol fermentation in a stirred tank bioreactor and the validation results for ethanol concentration, yield, production rate and sugar utilization rate were 40.10 g/L, 46.32%, 3.19 g/L/h and 90.66%, respectively (Yatmaz et al., 2013).

Biofilm reactor was performed for ethanol fermentation. Ethanol concentration and production rate were found to be 24.51 g/L, and 2.14 g/L/h (Germec et al., 2015). Packed bed reactor system had been also investigated for continuous ethanol fermentation by immobilized *Saccharomyces cerevisiae*. Maximum ethanol productivity was obtained at 0.4 h^{-1} dilution rate with 150 g/L substrate concentration (Roukas, 1994). The continuous ethanol fermentation from non-sterilized carob pod extract by immobilized *Saccharomyces cerevisiae* was studied by one and two reactor systems. A maximum volumetric ethanol productivities were 9.6 g/L/h for one-reactor system, and 11.4 g/L/h for two-reactor system at 0.4 h^{-1} dilution rate and 200 g/L initial sugar concentration (Roukas, 1996).

These studies have been carried out under different fermentation conditions and strategies with different strains. Batch, fed-batch, and continuous fermentations were performed with free or immobilized cells. The aims of this research are to study the continuous ethanol producing capability of free and immobilized *S. cerevisiae* (ATCC 36858) cells in carob pod extract medium in a modified stirred tank bioreactor system and to determine the best dilution rates (or hydraulic residence time: HRT) for free and immobilized cells.

MATERIALS AND METHODS

Microorganism

S. cerevisiae (ATCC 36858) was grown in glucose medium at 30ºC for 24 h (Turhan et al., 2010b; Yatmaz et al., 2013). 50 g of glucose, 6 g of yeast extract, 0.3 g of $CaCl_2.2H_2O$, 4 g of $(NH_4)_2SO_4$, 1 g of $MgSO_4.7H_2O$, and 1.5 g of KH_2PO_4 were added per liter of deionized water to form glucose medium. Stock culture was stored at 4ºC for short-term storage, and -80ºC in 20% glycerol for long-term storage, respectively. The culture was renewed monthly to provide high cell viability.

Carob pod extraction and fermentation medium

The chopped carob pods (without seed) were supplied from a local manufacturer (Yenigun Food Inc., Antalya, Turkey). Carob pods were mixed with water (1:4 ratio), incubated for 2 h at 80ºC, and filtrated to obtain particle-free carob pod extract (Turhan et al., 2010b). Then, carob pod extract enriched with 6 g/L of yeast extract, 0.3 g/L of $CaCl_2.2H_2O$, 4 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4.7H_2O$, and 1.5 g/L of KH_2PO_4 to obtain Carob Pod Extract Fermentation Medium (CPEM).

Immobilization

Immobilized beads were prepared with 2% alginate solution by mixing 5% pre-culture of total working volume (Yatmaz et al., 2013). For this purpose, pre-culture (12.51±0.13 g dry biomass/L) was centrifuged in 50 ml sterile centrifuge tubes at 1582 g and 4ºC for 20 min. The supernatant was removed. The cells were mixed with 20 ml 2% alginate solution and mixed carefully (Yatmaz et al., 2013). The mixture was dropped into a sterile 0.1 M $CaCl_2$ solution with a syringe (3P21G 0.80×38 mm) while the solution was stirred continuously. After beads formation,

the solution was replaced with 0.05 M sterile $CaCl_2$ solution to harden the beads for overnight. Finally, the beads were washed with 0.85% NaCl sterile solution to remove $CaCl_2$ ions and non-adherent cells used for inoculation (Lee et al., 2011; Razmovski and Vučurović, 2011).

Continuous ethanol fermentation in a 2L fermenter

CPEM was used for the initial batch phase and the continuous phase of the ethanol fermentation. All fermentations were performed in a reorganized continuous stirred tank bioreactor system (Sartorius Biostat A, Germany) with a 2L vessel (working volume of 1.5L) (Fig 1).

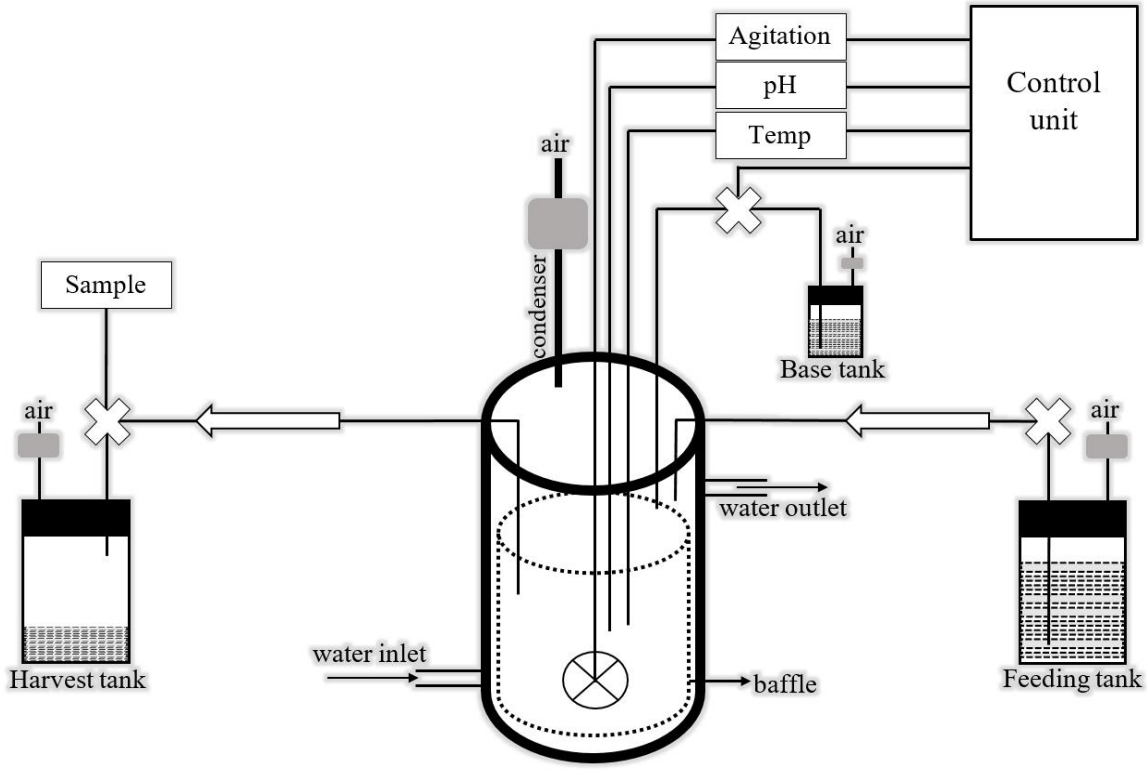

Fig 1. Continuous ethanol fermentation system for free or immobilized cells

The temperature, pH, and agitation were adjusted to 30°C, 5.5, and 150 rpm, respectively (Turhan et al., 2010b; Yatmaz et al., 2013). pH was controlled using fermenter automatic control unit by addition of 2N NaOH. Inoculum size was chosen to be 3% for submerged free cell fermentation (Turhan et al., 2010b) and 5% for preparing immobilized beads with 2% alginate solution (Yatmaz et al., 2013). Fermentations were started as a batch for fermentation till late log phase by sugar utilization for immobilized cell fermentation and biomass concentration for free cell fermentation. Then, the systems were switched to continuous fermentation by switching on inlet and outlet pumps at the different HRT values for each dilution rate were 20 h, 13.33 h, 10 h, 8 h, 6.67 h, 5.71 h, 5 h, 4.44 h, and 4h which calculated from specified dilution rates (D) (0.05 h^{-1}, 0.075 h^{-1}, 0.10 h^{-1}, 0.125 h^{-1}, 0.15 h^{-1}, 0.175 h^{-1}, 0.20 h^{-1}, 0.225 h^{-1}, and 0.25 h^{-1}) respectively ($D=1/HRT$).5 L autoclavable bottles were used to feed the sterile fresh medium to the fermenter and the fermented broth was collected into 5 L bottles.

Analysis
All samples for continuous fermentation were taken at steady-state conditions and analyzed in duplicate.

Biomass
Biomass analyses were done by measuring optical cell density at 620 nm (Thermo Scientific Evolution 201, Waltham, MA, USA). Samples were diluted with deionized water. Absorbance values were converted to biomass concentration (g dry biomass/L) by a standard curve (Turhan et al., 2010b; Yatmaz et al., 2013).

Residual sugar and ethanol
Residual sugar and ethanol concentrations were determined by using a Dionex Ultimate 3000 Ultra High-Pressure Liquid Chromatography (Thermo Scientific Corp., Germering, Germany) equipped with a RefractoMax520 refractive index detector (ERC, Germering, Germany). An ICSep ICE-ORH-801 column (300 × 6.5 mm) (Transgenomic Inc., Omaha, Nebraska, USA) was used to analyze sugars and ethanol from samples by using 0.01 N H_2SO_4 as the mobile phase. The flow rate was adjusted as 0.5 ml/min with a 20 µL injection volume at 70°C column oven temperature. The samples were diluted with HPLC grade water and filtered through 0.20 µm filters to remove all solid particles.

Kinetic parameters
The kinetic parameters were calculated by the following equations:

$\Delta S = S_o - S_e$ (1.1)

Where; ΔS is the total amount of the sugar utilized (g/L), S_o is the feed sugar concentration (g/L), S_e is the effluent sugar concentration (g/L).

$\Delta P = P_e - P_o$ (1.2)

Where; ΔP is the total amount of the ethanol produced (g/L), P_o is the feed ethanol concentration (g/L), P_e is the effluent ethanol concentration (g/L).

$\Delta X = X_e - X_o$ (1.3)

Where; ΔX is the total amount of the biomass (g/L), X_o is the feed biomass concentration (g/L), X_e is the effluent biomass concentration (g/L).

$$\frac{Y_P}{S} = \frac{\Delta P}{\Delta S} \quad (1.4)$$

Where; $\frac{Y_P}{S}$ (g ethanol/g sugar) is the yield coefficient.

$HRT = 1 / D$ (1.5)

Where; *HRT* is hydraulic residence time (h), and *D* is dilution rate (h^{-1}).

Productivity (g / L / h) = DX (1.6)

Ethanol productivity (g / L / h) = DP (1.7)

Statistical analysis
Statistical Analyses System (SAS University Edition, Online Version) was used for analysis of variance using the General Linear Models. All analyses and fermentations were performed in duplicate. The significance was given at $p<0.05$ (Data was given as mean ± std deviation).

RESULTS AND DISCUSSION

This study was designed with two goals in mind. First, effects of free or immobilized cells on the continuous ethanol fermentation from CPEM. In addition, fermentations were performed at nine different HRT levels between 4 and 20 h at 1.5 L constant vessel volume to evaluate the effects of HRT on ethanol production from CPEM.

Sugar consumption, ethanol production and ethanol productivity for free cells
Free cell continuous ethanol fermentation from CPEM were performed at seven different HRT (from 5 h to 20 h). Figure 2 shows the percent sugar utilization, effluent sugar concentration and biomass content with the HRT for a constant feed sugar content (S_{feed}=69.75±2.42 g/L). Percent sugar utilization increased from 61.24% to 87.23% and the effluent sugar decreased from 27.39 g/L to 9.18 g/L when the HRT increased from 5 to 20 h. The highest percent sugar utilization and the lowest effluent sugar content were calculated to be 87.70% and 8.99 g/L respectively at HRT of 13.33 h. Biomass content increased from 6.46 to 12.39 g/L when the HRT increased from 5 to 20 h. Effluent sugar concentration was dramatically increased in lower HRT because of the reduction of biomass concentration (Fig 2). And also, HRT of 8 h or over was not statistically important for effluent sugar content, percent sugar utilization and

biomass concentration for free cell fermentation ($p<0.05$). Effluent sugar concentration increases not only because of the reduction of biomass concentration, but also (and more importantly) due to the low retention time of substrate in the bioreactor.

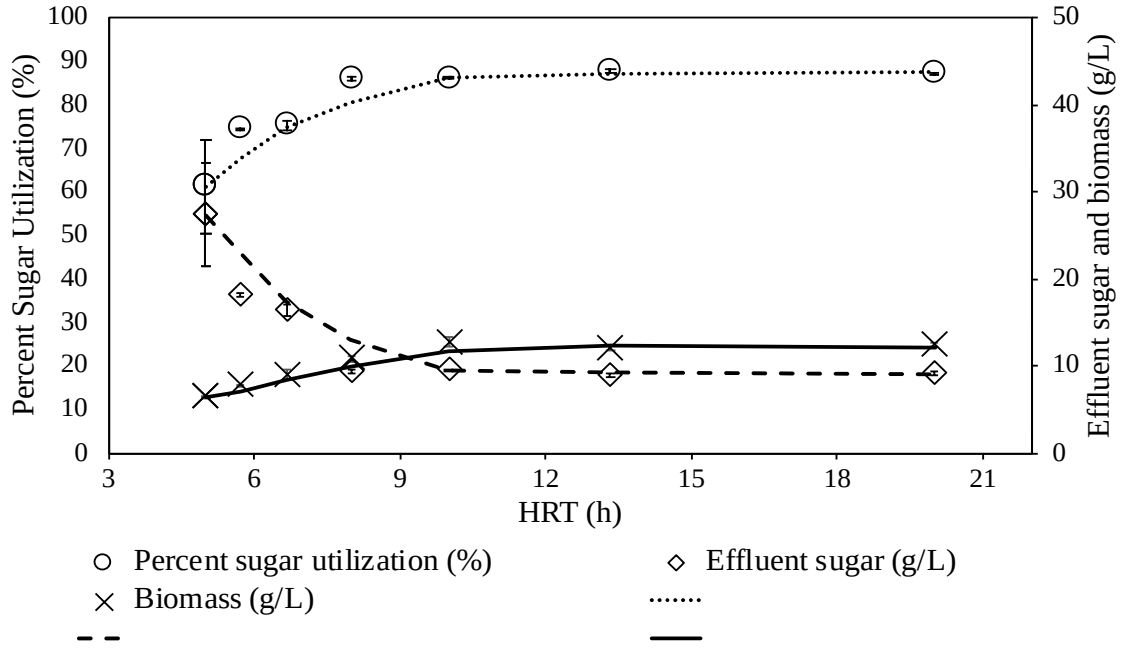

Fig 2. Variation of percent sugar utilization, biomass and effluent sugar concentration for free cell fermentations

Productivity, ethanol productivity and ethanol concentration versus HRT is given in Fig 3. Although ethanol concentration increased at higher HRT levels due to higher sugar consumption values, ethanol productivity values decreased. The highest ethanol productivity value was calculated to be 3.12 g/L/h at HRT of 5.71 h. HRT was statistically important for ethanol productivity, but there was no significant difference in ethanol productivity lower than HRT of 8 h ($p<0.05$). HRT was also not statistically important for ethanol concentration at higher than HRT of 8 h ($p<0.05$). Ethanol concentration remained in the same range from 19.31 g/L to 22.60 g/L, respectively, when HRT changed from 8 to 20 h in free cell submerged continuous fermentation (Fig 3). For a free cell continuous ethanol fermentation from CPEM, the highest feasible or applicable ethanol productivity was 2.41 g/L/h at HRT of 8 h, because the wash out started to appear in lower HRT (DX values are nearly same between HRT of 5 to 10 h because biomass values also decreased from 10.82 to 6.46 g/L when HRT changed from 8 to 5 h).

Sugar consumption, ethanol production and ethanol productivity for immobilized cells

Nine different HRT (from 4 to 20 h) were used for immobilized cell continuous ethanol fermentation from CPEM. Effluent sugar concentration and percent sugar utilization are given in Fig 4. Percent sugar utilization and effluent sugar varied in inverse proportion with increased HRT (constant feed sugar content, S_{feed}=64.69±3.17 g/L). Lower values than HRT of 5.71 h was statistically important for effluent sugar and percent sugar utilization ($p<0.05$). Percent sugar utilization increased from 41.15% to 83.71% and the effluent sugar decreased from 37.92 g/L to 10.18 g/L when the HRT increased from 4 to 20 h. The highest feasible dilution rate or lower HRT value for immobilized cell ethanol fermentation from CPEM was 0.175 h^{-1} or 5.71 h.

Because over values than HRT of 5.71 h caused the effluent sugar concentration increased rapidly which mean that the productivity or sugar consumption rate started to decrease.

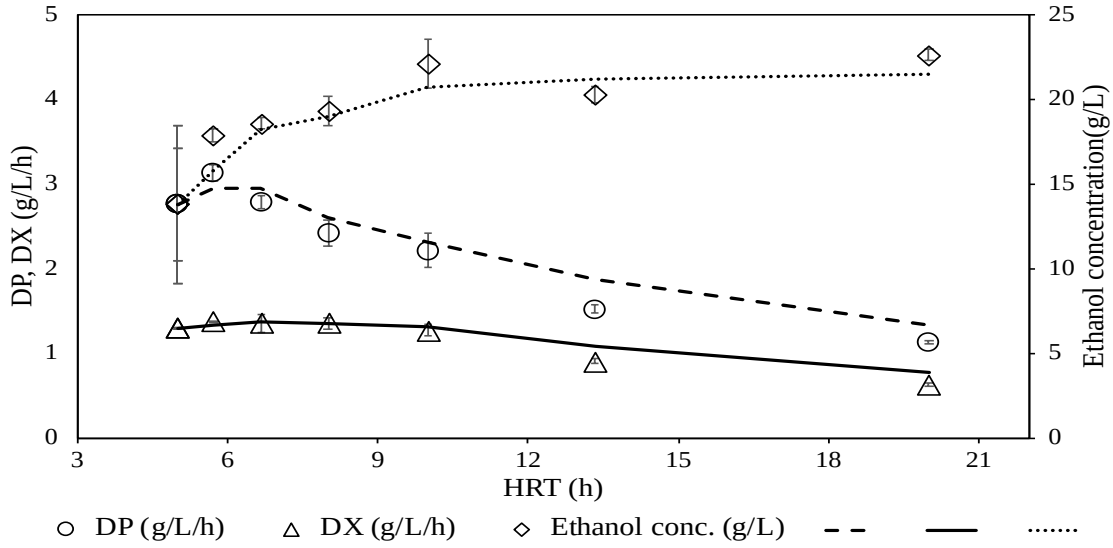

Fig 3. Variation of ethanol productivity (DP), productivity (DX) and ethanol concentration for free cell fermentation

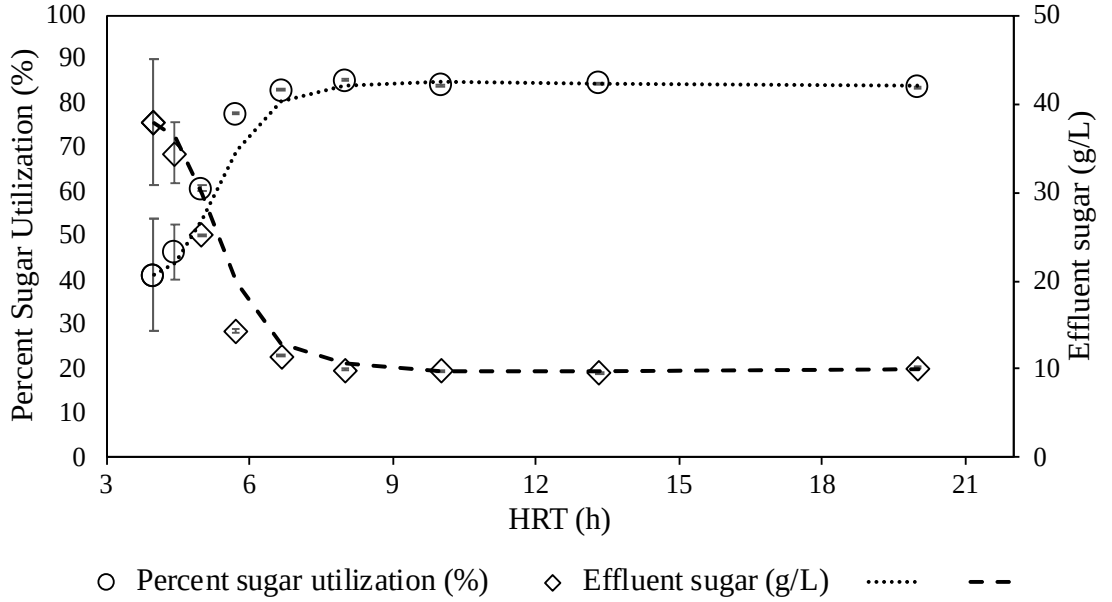

Fig 4. Variation of percent sugar utilization and effluent sugar concentration for immobilized cell fermentations

Ethanol productivity and ethanol concentration are given in Fig 5 to evaluate the effect of HRT. HRT was not statistically important for ethanol concentration which varied from 21.69 to 22.29 g/L, when HRT changed from 6.67 to 20 h ($p<0.05$). But lower HRT values had a significant effect on ethanol concentration because of high dilution rate ($p<0.05$). The highest ethanol

productivity was calculated as 3.37 g/L/h at HRT of 5.71 with 19.27 g/L ethanol concentration. 6.67 h was the lowest feasible HRT value for continuous ethanol fermentation from CPEM with ethanol productivity of 3.25 g/L/h and ethanol concentration of 21.69 g/L (It could be seen from the effluent sugar concentration in Fig 4 as well). It meant that immobilized cell could be used lower HRT than free cell for continuous ethanol fermentation from CPEM in a modified stirred tank bioreactor.

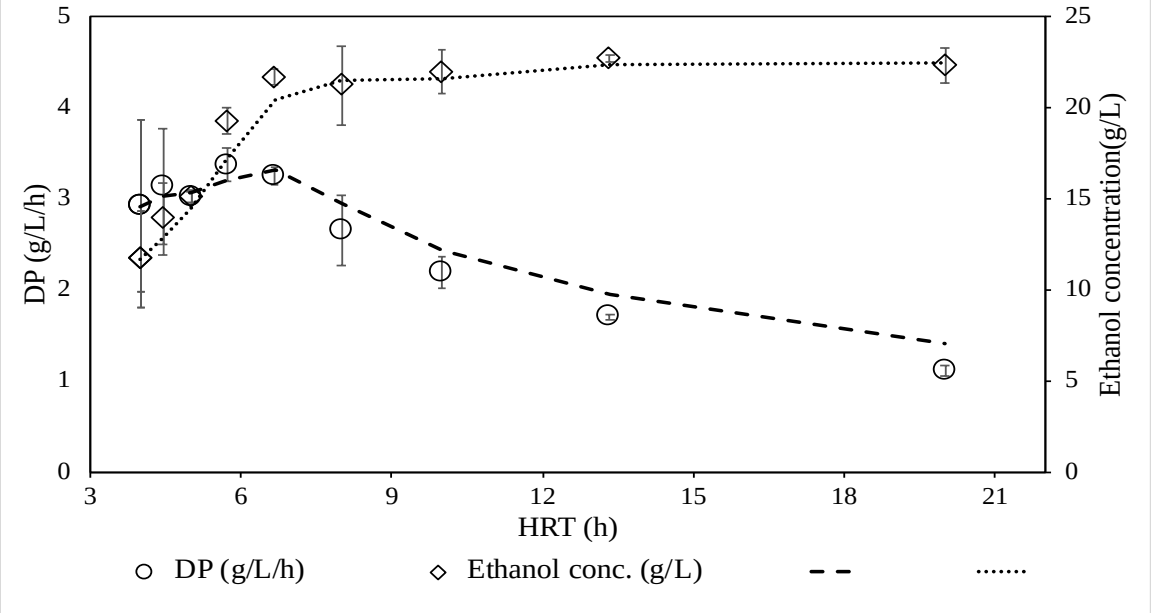

Fig 5. Variation of ethanol productivity (DP) and ethanol concentration for immobilized cell fermentation

Continuous ethanol fermentation from CPEM

Both free and immobilized cell ethanol fermentations were accomplished successfully. Kinetic parameters of percent sugar utilization and ethanol productivity results are given in Fig 6, and ethanol productivity values of immobilized cell fermentation were higher than free cell fermentation for all HRT levels. It meant that immobilized cells could be used more effectively for continuous ethanol production than free cells. Immobilized cell continuous ethanol fermentation could be operated until HRT of 6.67 h which was lower than the free cell (HRT of 8 h). The highest ethanol productivity values were 3.12 g/L/h for the free cell and 3.37 g/L/h for the immobilized cell at HRT of 5.71 h.

The researches about ethanol production from CPEM by using free *S. cerevisiae* cells showed that maximum production rates were 3.70 g/L/h for batch fermentation in a stirred tank bioreactor (STB) (Turhan et al., 2010b) , 2.04 g/L/h for batch fermentation in a STB (Lima-Costa et al., 2012) , 3.64 g/L/h for fed-batch fermentation in a STB (Lima-Costa et al., 2012), 1.604 g/L/h for batch fermentation (Raposo et al., 2017) when it was 3.12 g/L/h in this research. Some of the results obtained by the researchers were lower than ours, though this outcome is expected as continuous fermentation with free cells are used for carob pod extract ethanol fermentation.

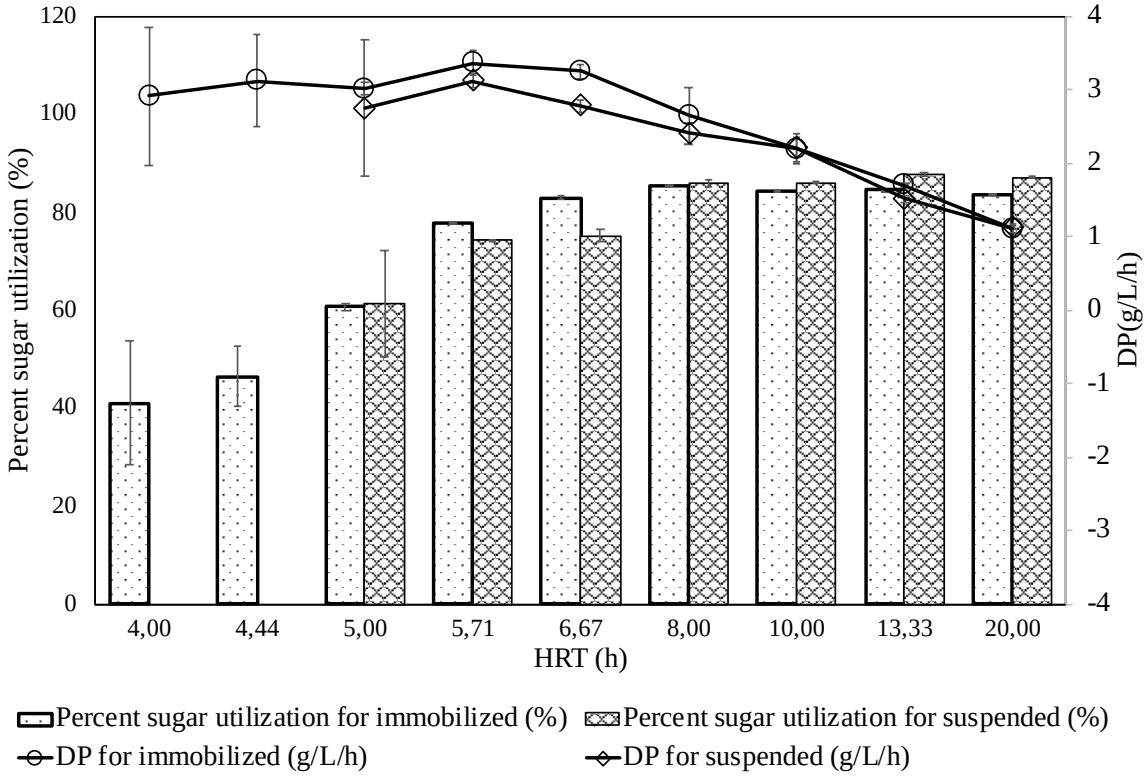

Fig 6. Comparison of immobilized and free cell continuous ethanol fermentations from carob pod extract

Roukas (1994) used immobilized *S. cerevisiae* in a packed bed reactor and the maximum ethanol productivity was 24.5 g/L/h at HRT of 20 h and 200 g/L initial sugar concentration. They also used immobilized yeast in a two-reactor system, and maximum ethanol productivity of 11.4 g/L/h was obtained at HRT of 2.5 h and 200 g/L initial sugar concentration (Roukas, 1996). Yatmaz et al. (2013) also conducted a study with immobilized cells in a STB, and maximum ethanol productivity was determined to be 3.19 g/L/h for batch fermentation. STB biofilm system was used by *S. cerevisiae*, and maximum ethanol productivity of 2.14 g/L/h obtained at 7.71 ºBx, pH 5.18, and 120 rpm (Germec et al., 2015). The maximum production rate of immobilized cells in this research was higher than what was obtained by Yatmaz et al. (2013), and Germec et al. (2015). So, these results also show that immobilized cell continuous ethanol fermentation from CPEM is compatible for ethanol production.

CONCLUSION

The results showed that CPEM could be used successfully for continuous ethanol fermentation with free or immobilized *S. cerevisiae* cells. The lowest non-wash out HRT (6.67 h) and maximum volumetric ethanol productivity (3.37 g/L/h) were obtained from immobilized cell fermentation. 6.67 h was the feasible HRT value for continuous ethanol fermentation from CPEM with ethanol productivity of 3.25 g/L/h. As a result, all kinetic parameters clearly showed that immobilized *S. cerevisiae* cell could be operated at lower HRT independent of biomass than a free cell ethanol fermentation from CPEM in a modified stirred tank bioreactor system.

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