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Effect of Zeolite NaY and Ca-Montmorillonite on Ethanol Production Using Synthetic Molasses

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Abstract The influence of Ca-Montmorillonite (Ca-MNT) and zeolite NaY addition on ethanol production from synthetic molasses by *S. cerevisiae* 251 TP(3-2) was studied by the measurement of biomass concentrations and metal ion concentration with respect to fermentation time. Addition of 5 g/L Ca-MNT and 10 g/L zeolite NaY resulted in an increase in both ethanol concentration and ethanol production rate. This increase was 24 and 40% for ethanol concentration and 65 and 87% for production rate, respectively. From the ion analyses, it was observed that the NaY added to the medium decreased the toxic concentration of zinc, manganese, and iron cations and acted also as a pH regulator. Ca-MNT added to the medium decreased the concentration of Na^+ ions, which is known to have a toxic effect on glycolysis and cell concentration. These effects caused improvement in the ethanol production rate.

Keywords *S. cerevisiae* · Zeolite · Clay mineral · Sucrose · Ethanol · Fermentation

Introduction

Ethanol production is dependent on different factors. The presence of metal cations can change the rate of glycolysis and subsequently the conversion of pyruvate to ethanol. The lowering of pH during the progress of fermentation causes cell damage leading to decrease in productivity. The metal cations zinc, manganese, sodium, and iron are known to directly influence fermentative metabolism in yeast. These metal cations have a positive effect on the respiratory activity and the growth rate of *S. cerevisiae*. On the other hand, a high concentration of these metal cations in the substrate may produce significant problems during fermentation [1–6]. From the view point of industrial ethanol production, the

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sucrose-based substrates such as sugarcane molasses and sugar beet juices present many advantages including their relative abundance and renewable nature. Molasses, which have been used as the main substrate for yeast and ethanol production, contain most of the necessary microelements but not in the optimal concentrations. One way of controlling the concentration of metal ions is to add ion exchange agents to the medium to decrease the concentration of free metal ions to the desired values. Several additives (metal-chelating agents, surfactans, vitamins, zeolites) have been reported to improve the ethanol production [8–15].

Zeolites and clay minerals are crystalline aluminosilicates whose primary structural elements form a framework with cavities and channels of different size and of molecular dimensions. These substances have ionic exchange, adsorption, and catalytic properties, and the use of them is simple, cheap, and reproducible. These properties underlie the use of zeolites in a wide range of industrial and agricultural applications and biotechnological application. Additionally, after fermentation, zeolite could be regenerated by heating at a high temperature for further use. The catalytic effect on the acceleration of yeast fermentation by the presence of natural zeolite (clinoptilolite) was tested, and it was observed that the addition zeolite to the fermentation media increases the alcoholic fermentation rate both in laboratory and large-scale processes [10]. Synthetic zeolites such as silicalite have been shown to act as accelerators of sugarcane molasses to ethanol by *S. cerevisiae* [9]. In the literature, it was reported that zeolite NaY acted as a pH regulator and the addition of NaY improved ethanol production from a high concentration of glucose by *S. bayanus* [14].

From the review of literature, it was observed that several authors have reported the positive effect of zeolite on ethanol fermentation, without investigating the change in metal ion concentration and pH variations with respect to time during fermentation [8–10, 14]. The aim of this study was to investigate the effect of the addition of Ca-montmorillonite (MNT) and NaY on the production of ethanol from synthetic molasses by following biomass concentration, metal ion concentration, and pH with respect to time during fermentation.

Materials and Methods

Microorganism

S. cerevisiae 251 TP (3-2) was obtained from the Hıfzıssıhha Center in Turkey. It was maintained by transferring to fresh agar–malt slants each month and storing at 4 °C. The agar–malt extract contained (g/L): malt extract (3.0), yeast extract (3.0), peptone (5.0), glucose (10), and agar (20), pH 4.5. The fermentation system was inoculated 1.0 g cell/L.

Ca-Montmorillonite

Ca-MNT was from Edirne city in Turkey. Its composition was (wt%); SiO_2 (59.40), Al_2O_3 (16.94), Na_2O (0.08), Fe_2O_3 (4.54), and CaO (2.51). Ca-MNT was activated by heating to 100 °C for 1 h and cooling to room temperature before use. The amount of Ca-MNT added to the medium was 1, 5, 10, and 15 g/L. These values were selected on the basis of ion exchange capacity (1.34 meq/g) of Ca-MNT.

NaY

Zeolite NaY was from Aldrich. This synthetic zeolite was of the following elemental composition (wt%): SiO_2 (63.8), Al_2O_3 (22.9), Fe_2O_3 (0.13), CaO (0.38), and Na_2O (13.0). NaY was activated before use by heating to 100 °C for 1 h and cooling to room temperature. Varying amounts of NaY namely, 1, 5, 10, and 15 g/L, were added to the fermentation medium.

Fermentation Medium

The synthetic medium was prepared with pure sucrose and chemical substances for nutritional requirements of the microorganism. Synthetic molasses composition was determined by the use of a composition of molasses taken from Ankara Sugar Company in Turkey, which consisted of (g): sucrose (100), $(NH_4)_2SO_4$ (5), Na_2SO_4 (4.13), KH_2PO_4 (25), $CaCl_2$ (2.8), $MgSO_4.7H_2O$ (0.709), $FeSO_4.H_2O$ (0.040), $MnSO_4.H_2O$ (0.017), $ZnSO_4.H_2O$ (0.021), $CuSO_4.5H_2O$ (0.00152), peptone (2.67), H_3BO_3 (0.01), pantothenate (0.0010), inosite (0.0005), biotin (0,000125), thiamine (0,005), pyridoxine (0.006), and pure water to 1,000 mL. All the chemical substances used were of analytical grade.

Operating Conditions

The yeast was subcultured on agar–malt extract slants at the start of each experiment. This starter culture was grown in an incubator at 30 °C for 24 h. For the seed culture, the inoculum from the fresh slant culture were transferred aseptically to previously sterilized 100 mL growth medium, which consisted of (g): glucose (50), KH_2PO_4 (12), $(NH_4)_2SO_4$ (5), $MgSO_4.7H_2O$ (1), yeast extract (5), $CaCl_2$ (0.5), and pure water to 1,000 mL. The liquid culture was agitated using a magnetic stirrer at 440 rpm to ensure homogeneity. After approximately 19 h, corresponding to the midexponential growth phase, 5 mL of seed culture was added to 50 mL fermentation medium. Repetition of this procedure in all cases ensured a relative constancy in the initial cell concentrations (1 g cell/L) of the fermentation flasks.

Batch cultures were grown in 250-mL erlenmeyer flasks. The initial pH value was adjusted to 4.5 with 1 mol/L H_2SO_4. All the experiments were carried out at 30 °C in a shaking water bath. The growth and fermentation media were sterilized by autoclaving at 121 °C for 15 min to avoid contamination.

The concentration of ethanol, metal ions, and pH were monitored with respect to time. All experiments were performed at least twice. In most of the experiments, the data have good reproducibility. When two experiments values deviate from each other, an additional experiment was carried out, and the average of the nearest two values among them were taken as a data point in this research.

Analytical Techniques

Ethanol in the supernatant fluids was measured using gas–liquid chromatography (Unicam 610 model), equipped with a capillary column (BP20) and flame ionization detector after centrifugation and microfiltration. The temperature of the injector, detector, and oven were kept at 200, 150, and 180 °C, respectively. Hydrogen, nitrogen, and air were used as the carrier gas at a flow rate of 30, 30, 330 mL/min, respectively. Zinc, manganese, and iron

were analyzed using atomic absorption spectrophotometry (Unicam 929 model) with external standardization. Sodium and calcium were determined by flame photometry (Jenway PFP 7). Microbial growth was measured spectrophotometrically at the 620-nm wavelength using a Baush and Lomb spectrophotometer. Measurements were done in cells with 1 cm path length. Culture samples were diluted with distilled water to confine the absorbance readings to the range 0.1–0.35 optical density as required by the Lambert–Beer law.

Result and Discussion

Zeolite NaY

Figure 1a shows the concentration of ethanol in cultures with different concentrations of zeolite NaY. The highest ethanol concentration (3.5%v/v) and microorganism concentration (18 g/L) were reached in the case of addition of 10 g/L NaY into the fermentation medium

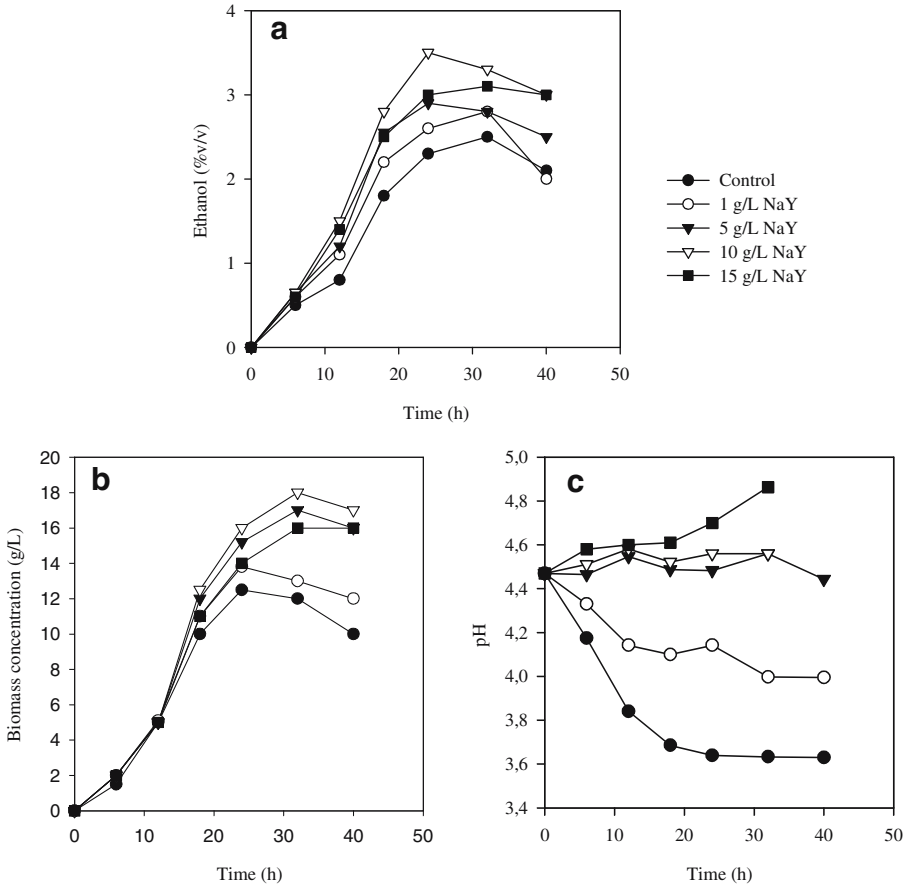

Fig. 1 The effect of zeolite NaY on ethanol concentration (**a**), on biomass concentration (**b**), and on variation of pH (**c**)

(Fig. 1b). The period required to reach this concentration was 24 h with 10 g/L NaY. The corresponding time to obtain maximum concentration of ethanol was 32 h in the absence of the additive. Additional improvement was not observed at zeolite concentrations higher than 10 g/L. Increase in maximum ethanol concentration and together with a decrease in time to reach this maximum value caused the production rate to increase by 87% in comparison with the values of fermentation medium without the additive. This represents an important improvement for process development. Other authors have previously reported the improvement of ethanol productivity by yeast *Candida pseudotropicalis* and *S. cerevisiae* in the presence of zeoliteX, clinoptilolite, and silicalite [7–9]. This positive effect of zeolite NaY on ethanol fermentation was demonstrated also in this study.

Variation of pH with time is given in Fig. 1c. As it is seen from Fig. 1c, pH decreases from 4.5 to 3.6 in 20 h for the control culture. The lowering of pH during the progress of fermentation causes the damage of cell concentration leading to decreased productivity. However, pH remains approximately constant for the fermentation media, which contain zeolite NaY in between 5 and 10 g/L. NaY acted here as a pH regulator. As a result of this effect, cell concentration and ethanol productivity were increased. Although ethanol fermentation by *S. cerevisiae* could also be improved with the pH control, the use of zeolite for this purpose was simple, cheap, and reproducible, and it permitted the pH control system to be suppressed [14].

Zinc and manganese are essential micronutrients in yeast metabolism. They function as a cofactor of essential enzymes such as alcohol and aldehyde dehydrogenases that are directly involved in ethanol production. However, the presence of metal ions in toxic concentrations can be significant problem during fermentation [6]. From our previous research [15], it was observed that when zinc and manganese concentration increase above 2.4 and 5 mg/L, respectively, yeast concentration decreased. The ion analyses were carried out at the time at which ethanol concentration reached its maximum value and presented in Fig. 2. From this figure, it was observed that the addition of 10 g/L NaY to the fermentation medium caused the concentration of zinc and manganese to decrease to the values below the toxic ones given above. This effect is most probably due to the ion exchange property of NaY zeolite. NaY of 15 g/L added to the medium decreased ethanol production. It is known from literature that zinc deficiency is a potential risk factor for disease in humans because it leads to increased oxidative stress and deoxyribonucleic acid damage. This fact was observed for also *S. cerevisiae* [16]. Therefore, the decrease in the production of ethanol with the addition of 15 g/L NaY may be explained with zinc ion deficiency, which results in a decrease in the growth rate of yeast.

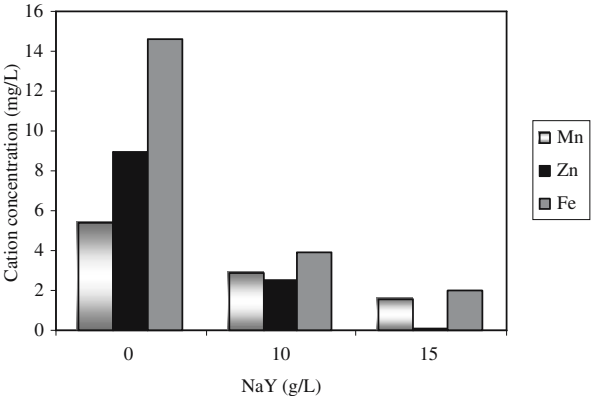

Fig. 2 Variation of cation concentrations in fermentation in the presence of NaY zeolite

Clay Mineral Ca-MNT

Figure 3a shows the concentration of ethanol in the fermentation medium containing different concentrations of Ca-MNT. Because the results were similar for Ca-MNT concentration of 5 and 10 g/L considering 24-h fermentation, it is more economical to us 5 g/L Ca-MNT to reach the highest ethanol concentration in the case of Ca-MNT addition. This means that addition of 5 g/L of Ca-MNT contributes to the reduction in respective operation time values by an amount equal to 24%. Addition of 5 g/L Ca-MNT resulted in an increase in ethanol production rate by 65%. This represents an important improvement for process development.

Biomass concentration profiles with and without Ca-MNT are reported in Fig. 3b. At an initial fermentation phase, in the first 12 h, a similar growth pattern was obtained with and without Ca-MNT. However, at fermentation times greater than 12 h, more cellular growth

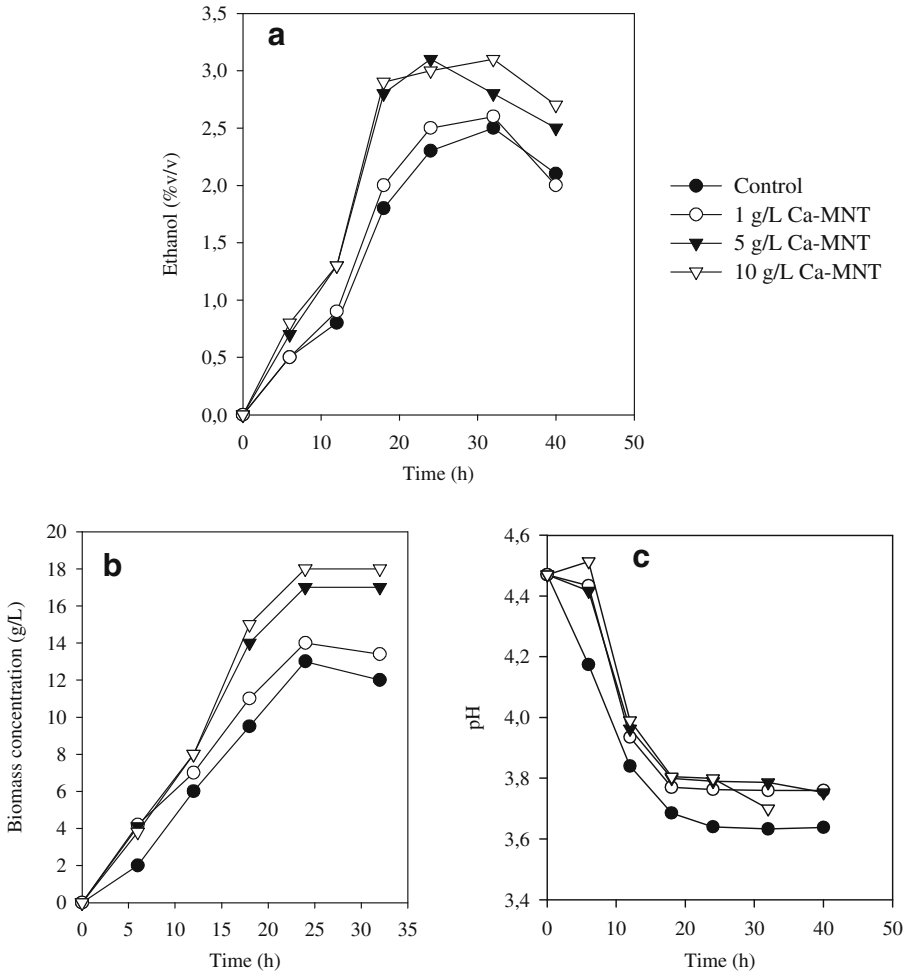

Fig. 3 The effect of Ca-MNT on ethanol concentration (**a**), on biomass concentration (**b**), and on variation of pH (**c**)

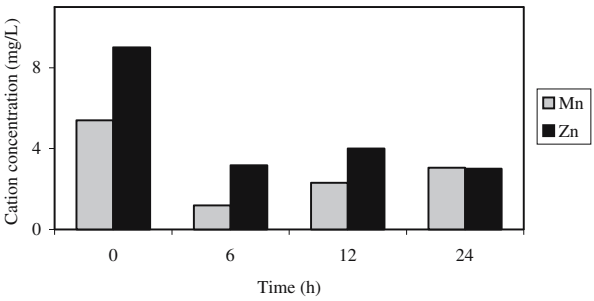

Fig. 4 The effect of the presence of 5g/L Ca-MNT on zinc and manganese concentration

was observed in the presence of clay mineral. Therefore, ethanol concentration was increased.

Figure 4 represents the variation of manganese and zinc ions with the fermentation time for 5-g/L Ca-MNT-containing medium. As it is seen from this figure, manganese ion concentration decreases first at the fermentation time of 6 h, and the ion concentration increases during the remaining period of fermentation. It seems that Ca-MNT releases ions as they are utilized by growing microorganism, so it controls the ion concentration on the behalf of microorganism growth. Investigation of reversibility of adsorption in aqueous solution is suggested to support this argument.

Sodium ion has a negative effect on glycolysis and ethanol production. High sodium ion concentration is toxic to yeast cells. It was observed that when sodium concentration increase above 930mg/L, ethanol concentration decreased [15]. One mechanism that has been proposed to explain sodium toxicity in *S. cerevisiae* is the competitive inhibition of potassium uptake, leading to potassium depletion in the cell and an increased level of sodium. Sodium becomes toxic for the sodium potassium cellular ratio greater than 0.7 [4]. From our previous research [15], it was observed that the intracellular sodium–potassium ratio was 2.87 for *S. cerevisiae*. From the sodium ion analyses (Fig. 5), it was observed that the 5 g/L Ca-MNT added to the medium decreased sodium ion concentration. The lowering of sodium concentration in the fermentation medium through ion exchange with the calcium ion contained in clay mineral under consideration could in part explain the increase of ethanol concentration and biomass concentration in the presence of the additive. Controlled zinc, manganese, and sodium ion concentrations by the clay mineral increased biomass and ethanol concentrations.

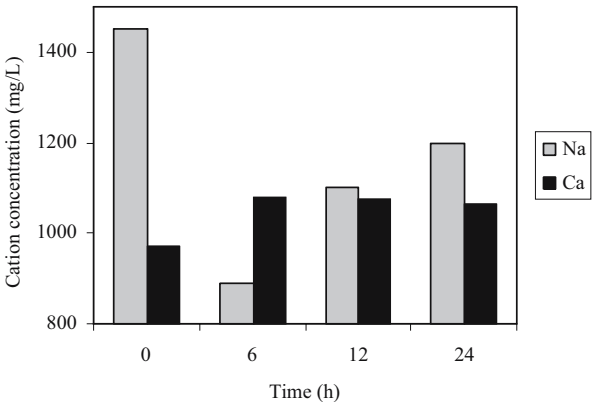

Fig. 5 The effect of the presence of 5g/L Ca-MNT on sodium and calcium concentration

As it is seen from Fig. 3c, pH decreases to 3.6 in 20 h for the control culture, and the addition clay mineral did not affect pH lowering. Therefore, the positive effect of Ca-MNT is due to only its ion exchange property.

Conclusion

Results presented in this article reveal that the addition of NaY and Ca-MNT to the fermentation of *S. cerevisae* resulted in an improvement in ethanol and biomass concentration and ethanol production rate. The effect of the addition was found to be dependent on the concentration of the additives employed and was explained on the basis of the ion exchange property of these substances. This work is a contribution to the study and improvement of ethanol fermentation and to the use of clay mineral and zeolite in biotechnological processes.

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