

Enhanced ethanol fermentation of brewery wastewater using the genetically modified strain *E. coli* KO11

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Abstract We have used liquid waste obtained from a beer brewery process to produce ethanol. To increase the productivity, genetically modified organism, *Escherichia coli* KO11, was used for ethanol fermentation. Yeast was also used to produce ethanol from the same feed stock, and the ethanol production rates and resulting concentrations of sugars and ethanol were compared with those of KO11. In the experiments, first the raw wastewater was directly fermented using two strains with no saccharification enzymes added. Then, commercial enzymes, α -amylase, pectinase, or a combination of both, were used for simultaneous saccharification and fermentation, and the results were compared with those of the no-enzyme experiments for KO11 and yeast. Under the given conditions with or without the enzymes, yeast produced ethanol more rapidly than *E. coli* KO11, but the final ethanol concentrations were almost the same. For both yeast and KO11, the enzymes were observed to enhance the ethanol yields by 61–84% as compared to the fermentation without enzymes. The combination of the two enzymes increased ethanol production the most for the both strains. The advantages of using KO11 were not demonstrated clearly as compared to the yeast fermentation results.

Keywords Ethanol fermentation · *E. coli* KO11 · Simultaneous saccharification and fermentation · Brewery wastes · Yeast · Pectinase

Introduction

Ethanol is a major fuel additive and promising energy alternative in the future. Although the energy content of ethanol is lower than that of gasoline, ethanol has many advantages as an alternative energy: Its use reduces pollutant emissions and it can be produced from renewable wastes, which is attractive to economy and environmental sustainability. Although diverse alternatives in biofuel production are available, ethanol constitutes 99% of total biofuel consumption in the US.

Annual ethanol production in North America was more than 3.41 billion gallons at the end of 2004, which grew by 29% by the end of 2005, to 4.4 billion gallons (RFA 2005). Almost all the ethanol was produced from 35 million tons of corn (12% of the US corn crop; RFA 2005). The greater quantities of ethanol fuel are expected to be used as a motor fuel because of changing federal policies. According to the new Energy Policy Act, 7.5 billion gallons of renewable fuel is to be used by 2012 and taxpayers will get \$0.51 credit per gallon of ethanol used in their fuel (Urbanchuk 2006; Farrell et al. 2006). The average gasoline cost is more than \$2.20 per gallon now, whereas ethanol cost is around \$1.20 a gallon. Thus, if a consumer uses 10% ethanol-blended gasoline instead of pure gasoline, he can save as much as 8 cents per gallon (Cooper 2005).

However, as one of the major sources of food, corn, as a feedstock, is not only the most significant cost of ethanol fermentation, but also the use of it as a fuel source is criticized as an inefficient use of food (Doty 2005). Instead of corn, biomass wastes such as corn fiber, waste wood, and food processing wastes are recommended as a cheap feedstock for ethanol production (Zaldivar et al. 2001).

For more efficient ethanol fermentation of biomass, improved microbial strains are sometimes required to

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ferment mixed sugars and to be tolerant to by-products (Bothast et al. 1999). Conventional ethanol fermentation uses wild type microorganisms such as *Saccharomyces* or *Zymomonas*, and these commonly used wild-type microorganisms metabolize just a few types of sugars such as glucose, fructose, and sucrose (Amutha and Gunasekaran 2001), being unable to ferment pentose sugars such as arabinose and xylose. Also, usually their ethanol production is inhibited by the fermentation by-products such as organic acids and ethanol (Alfenore et al. 2004).

To overcome these problems, several promising recombinant strains of Gram-negative bacteria, *Zymomonas mobilis* (Zhang et al. 1995) and *Escherichia coli* (Ingram et al. 1987), and *Klebsiella oxytoca* (Ohta et al. 1991; Wood et al. 2005), were developed. These strains are known to ferment xylose and other pentose sugars as well as hexose sugars to ethanol. Also ethanol yields were improved by eliminating competing reactions. In this study, the *E. coli* strain KO11 developed in the laboratory of Dr. Ingram (Ingram et al. 1987; Lindsay et al. 1995) was chosen as the fermenting organism because it is able to ferment a wide range of sugars, including hexose and pentose, which are possibly found in wastewater. Moreover, it is known to be ethanol-tolerant (i.e., less product inhibition) and tolerant to process errors (Moniruzzaman et al. 1998a).

In the processes employed for current industrial-scale ethanol production, starch is first hydrolyzed by adding a liquefying enzyme to avoid gelatinization, namely α -amylase (EC 3.2.1.1, 1,4- α -D-glucan-4-glucohydrolase), and then cooked at a high temperature around 45°C. Next, the liquefied starch is hydrolyzed to glucose with a saccharifying enzyme, namely glucoamylase (EC 3.2.1.3, 1,4- α -D-glucan glucohydrolase). Finally, the glucose is converted to ethanol by yeast cells.

In this study, we have used brewery wastewater collected from a local brewery company as a feedstock. This feedstock is readily available and is of no competitive value to any other processes. Also brewery wastes are relatively easier to hydrolyze than raw biomass wastes and they have high biodegradable organics. Wastewater management in any brewing industry is very important from both economic and ecological standpoint (Fillaudeau et al. 2006).

The freshly collected wastewater was characterized first. After characterization, the wastewater was either directly fermented without hydrolyzing enzymes or simultaneously hydrolyzed and fermented, i.e., simultaneous saccharification and fermentation (SSF), using yeast or *E. coli* KO11 with (1) α -amylase, (2) pectinase, or (3) a combination of α -amylase and pectinase. Sugar concentrations and ethanol concentration were measured before, during, and after SSF. This approach is thought to be novel that we successfully used a genetically modified strain with the unique combi-

nation of commercially available enzymes, which are much less expensive than the specialty enzymes commonly used in SSF systems.

The SSF process is generally done at higher temperature ranges of 45–70°C (Wood and Ingram 2001). According to Kádár et al. (2004), the 0.30–0.34 g/g (~40 vol%) of ethanol was obtained from lignocellulosic sources using SSF at 40°C. We also ran a SSF process at 40°C in this study. Though the study was done on specific substrate, the process used was general and can be applied to any fermentable medium with complex sugars.

Materials and methods

Origin of wastewater

Brewery wastes were obtained from a local brewery company in Toledo, OH. In the brewing process, beer alternately goes through three chemical and biochemical reactions (mashing, boiling, fermentation and maturation) and three solid–liquid separations (wort separation, wort clarification, and rough beer clarification; Urbanchuk 2006). Each step in this process generates wastewater. The wastewater used in our study was the one obtained when water was used to extract the desired sugars from crushed malt in the brewing process. This liquid extract called wort is separated from the spent grains (the barley grains after saccharification; Fig. 1).

Microorganisms and enzymes

Recombinant *E. coli* KO11, which was kindly provided by Dr. Ingram at University of Florida, was used for the fermentation experiments. This strain was cloned with chloramphenicol acyl transferase (*cat*) and the *Z. mobilis* genes for ethanol production (*pd*, *adhB*), which were integrated into the *E. coli* chromosome.

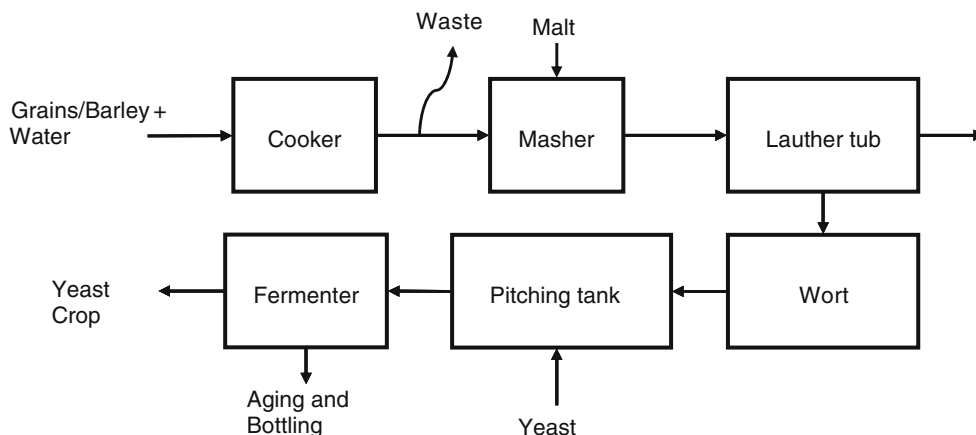
Saccharomyces cerevisiae, which is commonly known as baker's yeast, was obtained from Sigma-Aldrich and was stored at 8°C.

α -Amylase and pectinase were obtained from L.D. Carlson, which provide many home brewing enzymes and supplies.

Growth media and trace mineral solution

E. coli KO11 was grown on agar plates containing solid LB agar medium, containing 5.0 g/l NaCl, 5.0 g/l yeast extract, 10.0 g/l tryptone, 15.0 g/l agar, supplemented with 20.0 g/l glucose and chloramphenicol, and incubated at 30°C for 24 h. The stock culture was stored at –70°C in LB broth with 5% (w/v) glucose and 80% (w/v) glycerol as

Fig. 1 The brewing process and waste generated



recommended. Isolated colonies were transferred daily to new plates containing alternating high (600 mg/ml) and low (40 mg/ml) concentrations of chloramphenicol. This was done to retain the viability of *E. coli* KO11 to consume both pentose and hexose sugars.

The KO11 inoculum was prepared using a mixture of LB broth and 5% (w/v) glucose solution in 1:1 ratio. The LB broth stock solution was initially prepared by dissolving 10 g/l of tryptone, 5 g/l of yeast extract, and 5 g/l NaCl in deionized water. After 12–16 h of culture, the inoculum's optical density became 2–3 at 550 nm wavelength. Inoculum of 25 ml (0.5 g dry cell weight) was transferred to the fermentor. No separate culture media were prepared for baker's yeast because baker's yeast can be directly inoculated and cultured in a fermentor at the beginning of fermentation. Yeast (2.5 g) was added into the fermentor.

Trace mineral solution (1 l) contained 5.0 g disodium EDTA, 0.22 mg zinc sulfate ($\cdot 7\text{H}_2\text{O}$), 0.5 g calcium chloride, 0.5 g manganese chloride, 0.5 g ferrous sulfate, 0.1 g ammonium molybdate ($\cdot 4\text{H}_2\text{O}$), 0.16 g cupric chloride, and 0.16 g cobalt chloride (Leite et al. 2000). This trace metal mixture was reported to increase the productivity of the recombinant *E. coli* KO11.

Fermentation

Four types of fermentation reactions were carried out for both strains, viz.:

1. Fermentation without saccharification enzymes. Trace minerals, Zn^{2+} , Fe^{2+} , Cu^{2+} , Co^{2+} , were added to the raw wastewater for cellular metabolism.
2. SSF with pectinase.
3. SSF with α -amylase (fungal).
4. SSF with a combination of α -amylase and pectinase.

Pectinase and α -amylase were chosen because both of them were readily available in the local brewing companies at a cheap price. Known as a strong versatile hydrolytic enzyme, the effects of pectinase on the brewery waste were

focused. All the experiments were performed in 500-ml closed conical flasks with 250 ml of working volume. The flasks were provided with the openings for sampling and gas escape. The wastewater obtained from the brewery was autoclaved at 110°C for 15 min. After cooling, the substrate was supplemented with 0.05 to 0.5% yeast extract or LB broth for *S. cerevisiae* and *E. coli* KO11, respectively, following the procedure in Leite et al. (2000). The pH of the solution was adjusted and maintained above 6 using 0.1 M phosphate buffer. The buffer solution was directly made in the wastewater culture solution to make a 250-ml fermentation medium. This is the pH for the optimal activity of the genetically modified *E. coli* KO11.

The phosphate buffer was made by mixing 400 ml of 0.1 M sodium phosphate dibasic (Na_2HPO_4), and 100 ml of 0.1 M sodium phosphate monobasic (NaH_2PO_4). Finally, enzymes (4 g/l) were added to the fermentor. The reaction was carried out in an incubator shaker at 120 rpm. Samples were taken at every 2 h and analyzed using HPLC.

For the first set of experiments, the fermentation was run on the raw wastewater without the saccharification enzymes. However, to maintain proper cellular metabolism, the fermentation was supplemented by the addition of 5 ml/l of the trace mineral solution and 1 μg of thiamin.

Analytical methods

The fermentation samples were analyzed with Shimadzu HPLC equipped with a refractive index detector, using an Aminex 87H column (Biorad) maintained at 65°C. The mobile phase used was 0.5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The standard components used for calibration were maltotetraose, maltotriose, maltose, glucose, xylose, and galactose. These were purchased from Sigma (St. Louis, MO). The chromatograms obtained had the results in terms of micro-refractive index units.

Before the fermentation, the raw wastewater samples were first analyzed as was (i.e., as a control) for the sugar

content using HPLC. Then the samples were spiked with the purchased standards to accurately verify each peak in the blank chromatograms. The presence of maltotriose, maltose, glucose, and galactose were determined using the spiking method by observing the changes in the peak areas as shown in Fig. 2.

The wastewater samples were also analyzed using electrospray ionization mass spectrometry (ESI-MS). The instrument used was Mass-Spec Esquire LC 00139 ion trap LC/MS with ESI/APCI. Nitrogen was used as nebulizing gas at the inlet pressure of 3 psi and temperature of 320°C. The sampling rate was 1 scan/s. The samples were directly infused into the ESI-MS instrument by means of a syringe pump (Cole Palmer, 74900 Series). The mass spectrometer was operated on the scan mode from m/z 200 to 1,100. This was done to check the presence of any other saccharides (Mauri et al. 2002). The standards were first analyzed by the addition of 3 mM of KCl. The samples were then spiked with 3 mM of KCl. The peaks were identified as the potassium adducts $(M+K)^+$. Hence, maltose would show a peak at $(320+39)$, which corresponds to 381 (m/z). Similarly maltotriose and maltotetraose would be seen at 543 and 705 (m/z). Hence, the presence of glucose, maltose, maltotriose, and maltotetraose was confirmed by the presence of the particular peaks as shown in Fig. 3.

Results

Sugar composition of the wastewater

The initial composition and the parameters of the wastewater are shown in Table 1. The chemical oxygen demand and the total dissolved solids of the water samples were

Fig. 2 The characterization of wastewater samples using HPLC

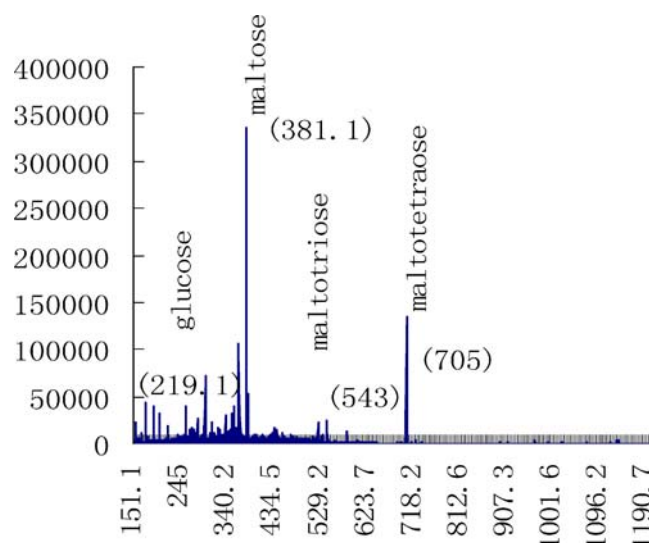
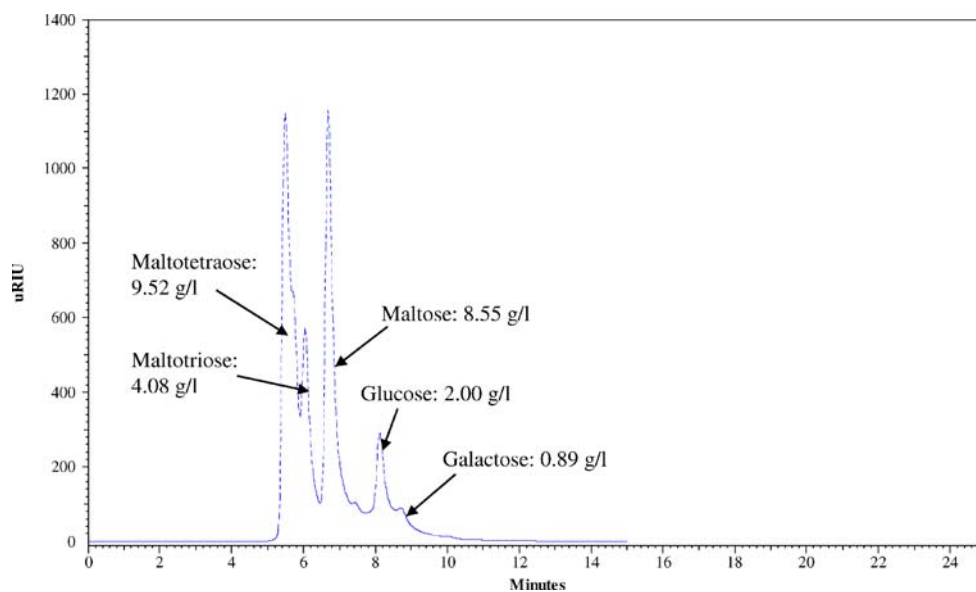


Fig. 3 The ESI-MS analysis for various sugars in the wastewater

initially analyzed by John and Henry Laboratories, Northwood, OH. Approximately 2,880 mg/l of starch were estimated in the total solid contents in the wastewater. It was found that the total dissolved sugar concentration was 24.2 g/l (Fig. 2), which is in agreement to literature values (Roukas 1999).

Figures 2 and 3 show the analytical results of HPLC and ESI-MS for the wastewater. The HPLC chromatogram of the control wastewater (Fig. 2) shows that this particular wastewater contained 9.52 g/l of maltotetraose, 4.08 g/l of maltotriose, 8.55 g/l maltose, which are tetramer, trimer, and dimer of hexose sugar, respectively, as major components. The wastewater was also observed to have some starchy components suspended in it. The peaks in Fig. 3 represent the different types of sugars and their concentrations. The peaks at 5.48, 6.017, 6.683, 8.233, and

Table 1 Initial analysis of the wastewater

| Test parameter | Result (mg/l) |
|--------------------------------------|---------------|
| Chemical oxygen demand (COD) | 15,600 |
| Solids, total 104°C | 13,400 |
| Solids, total, volume 550°C | 13,400 |
| Solids, suspended, 104°C | 120 |
| Solids, vol. suspended, 550°C | 120 |
| Solids, dissolved 180°C | 10,400 |
| Total dissolved sugar content (HPLC) | 24,150 |
| Total starch content | 2,880 |

8.75 min, represent maltotetraose, maltotriose, maltose, glucose, and galactose, respectively.

Fermentation without saccharification enzymes

The wastewater samples were directly fermented without the saccharification enzymes (i.e., no hydrolysis step). Both of the fermentations by *E. coli* KO11 and yeast resulted in low yields of ethanol at around 8 g/l (0.92 vol%) after 6 (yeast) and 30 h (KO11) of fermentation as shown in Fig. 4. Maximum ethanol yields obtained were 8.42 g/l (0.97 vol%) by *E. coli* KO11 and 8.48 g/l (0.98 vol%) by yeast. The ethanol production rate of yeast was higher than *E. coli* KO11.

Figure 5 shows the concentrations of sugars and ethanol before and after fermentation. For both yeast and KO11, maltotetraose was not consumed much and the final ethanol seemed to come from the other sugars. Although there was a considerable difference in the ethanol production rates between yeast and KO11, the final concentrations of

ethanol were almost the same, 8.42 g/l for yeast and 8.48 g/l for KO11.

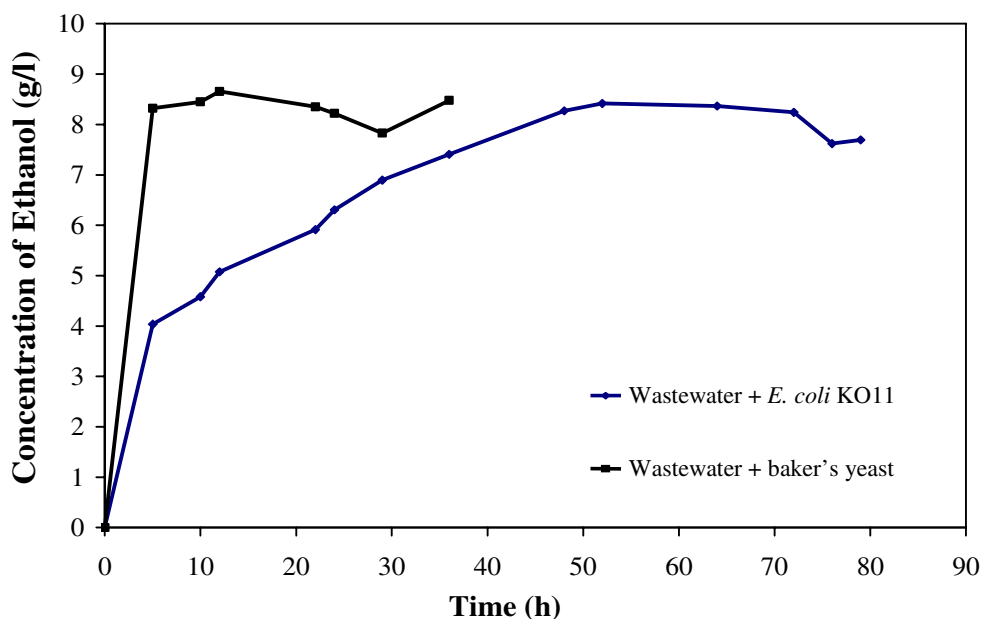
Yeast seemed to be able to use a small portion of maltotetraose as shown in Fig. 5b. However, it is not clear whether it converted maltotetraose directly to ethanol or to some other sugars. A small decrease of maltotetraose in the yeast fermentation was observed persistently in the repetitions of the same experiment. Despite the maltotetraose decrease, which was not observed in the KO11 fermentation, the final ethanol concentrations were not noticeably different between yeast and KO11.

Fermentation with saccharification enzymes

The wastewater was hazy in texture and the large amount of suspended solids reading indicates the presence of oligo-saccharides in this wastewater. Enzymes like pectinase and amylase can break specific bonds of sugar oligomers to give fermentable sugars (Gummadi and Panda 2003). In our experiments we used commercially available enzymes, which are readily available at any local brewing store. As shown in Figs. 6 and 7, the use of pectinase enzymes gave enhanced yields of ethanol from the brewery waste. Maximum concentrations of 12.18 g/l (1.40 vol%) and 12.45 g/l (1.44 vol%) of ethanol were obtained from *E. coli* KO11 and yeast, respectively, which were higher than those of the no-enzyme cases in Figs. 4 and 5.

Figure 6 compares the ethanol production rates for the four cases, fermentation by yeast with and without pectinase and by KO11 with and without pectinase. Yeast with pectinase produced the highest ethanol concentration, 12.45 g/l within 15 h. Yeast without pectinase produced ethanol at a similar rate, but there was about a 7-h delay and

Fig. 4 Ethanol production rate in the fermentation of the raw brewery wastewater for *E. coli* KO11 and baker's yeast



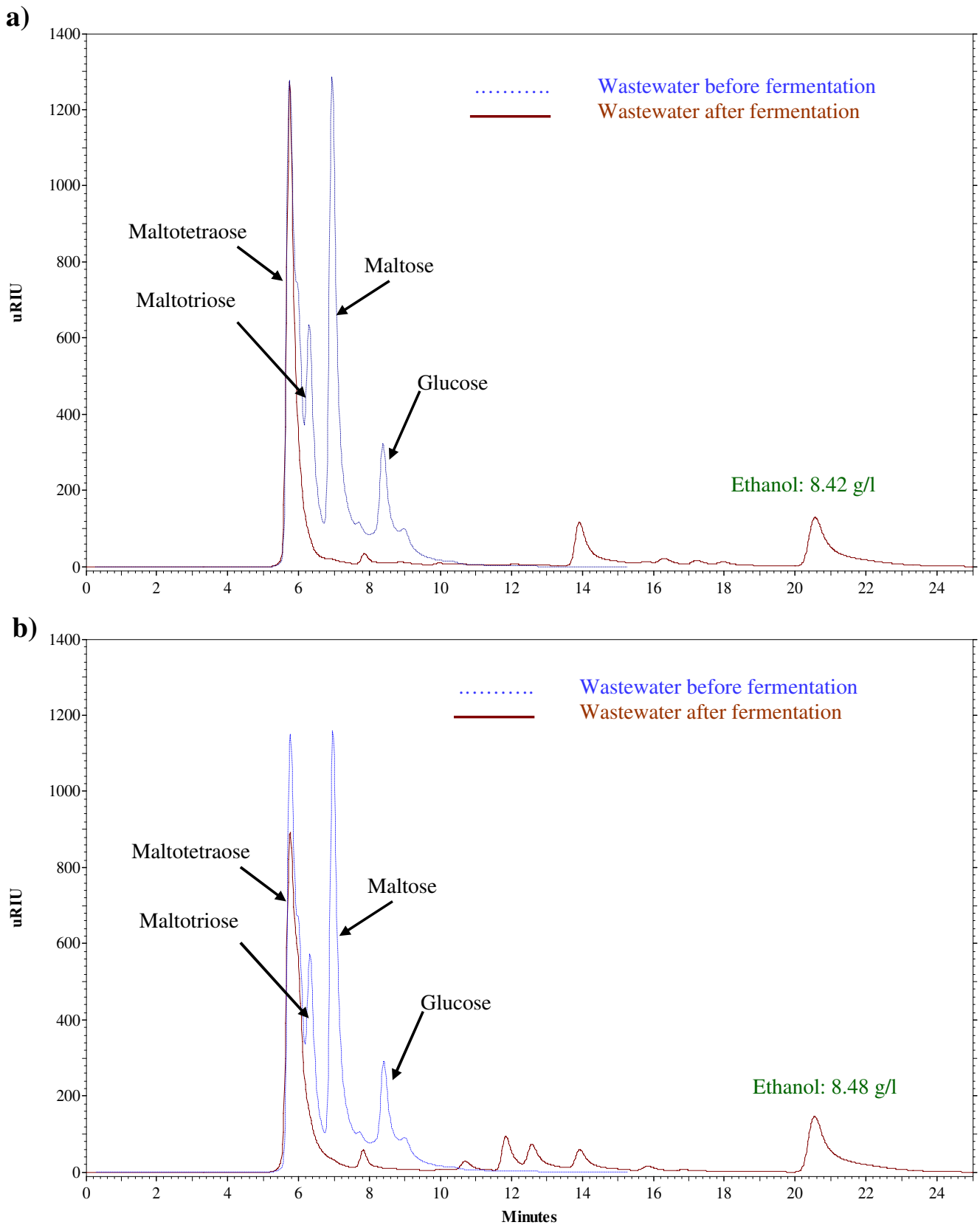
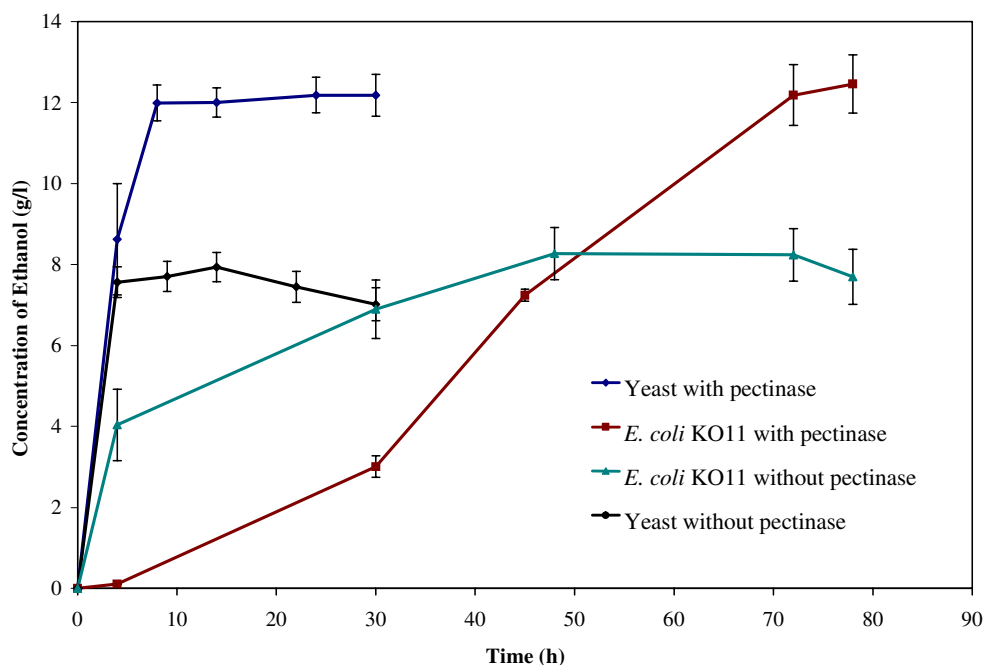


Fig. 5 Concentrations of sugars and ethanol in wastewater before and after fermentation without enzymes. **a** *E. coli* KO11 **b** yeast

Fig. 6 Ethanol production by *E. coli* KO11 and yeast with and without pectinase



the final ethanol concentration was 8.48 g/l, about two-thirds of the highest ethanol concentration.

Likewise, *E. coli* KO11 with pectinase produced more ethanol (12.18 g/l) than without pectinase (8.42 g/l).

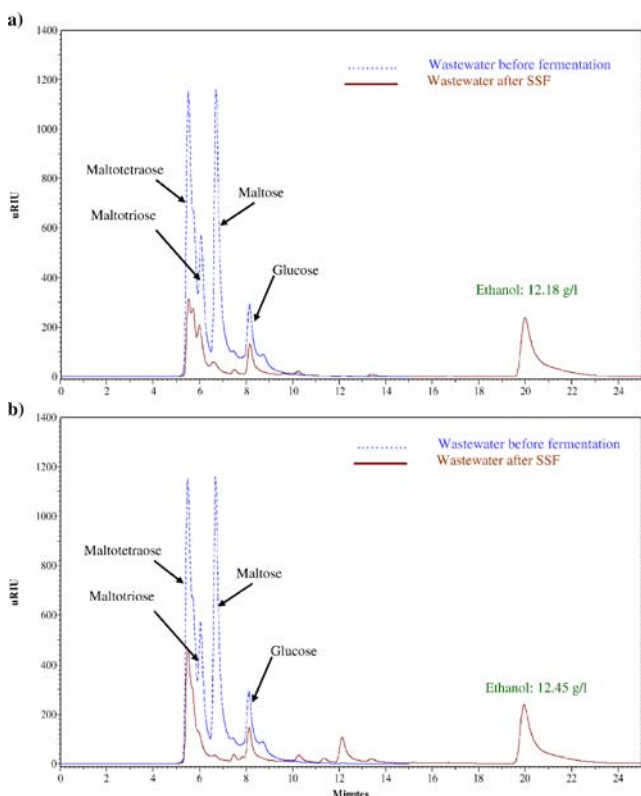


Fig. 7 Concentrations of sugars and ethanol in wastewater before and after fermentation without pectinase. **a** *E. coli* KO11 **b** yeast

However, the ethanol production rate of KO11 with pectinase was even slower than the without pectinase case.

Figure 7 compares the initial and final concentrations of the sugars and ethanol before and after fermentation in the presence of pectinase. Compared to Fig. 5 where no enzymes were used, maltotetraose decreased significantly in Fig. 7. For both yeast and KO11, maltotriose and maltose were almost completely consumed. However, interestingly the glucose peak was not reduced as much for the two strains. The difference of the final ethanol concentration was marginal between the two strains.

The similar results were obtained in the chromatogram for the fermentation with α -amylase (Fig. 8) and the combination of pectinase and α -amylase (Fig. 9). Similar to the pectinase experiments, use of α -amylase enzyme resulted in up to 13.66 g/l (1.57 vol%) of ethanol (Fig. 8). Interestingly Fig. 8 (with α -amylase) shows an increase in the maltotetraose peak instead of a decrease after the fermentation. Despite this increase of maltotetraose, however, the final ethanol concentration was not much different from that of the fermentation with pectinase in Fig. 7, where a decrease of maltotetraose was observed.

Figure 9 shows the effect of the combined enzymes on the ethanol fermentation. The combination of amylase and pectinase enzymes gave the highest yields of ethanol, which were up to 15.52 g/l (1.79 vol%) and 14.60 g/l (1.68 vol%) in the *E. coli* KO11 and yeast fermentations, respectively. Unlike the pectinase and α -amylase cases in Figs. 7 and 8, all the sugars presented were consumed for both strains. Also, KO11 noticeably produced about 6.3% more ethanol (15.52 g/l) than yeast (14.6 g/l), compared with the results

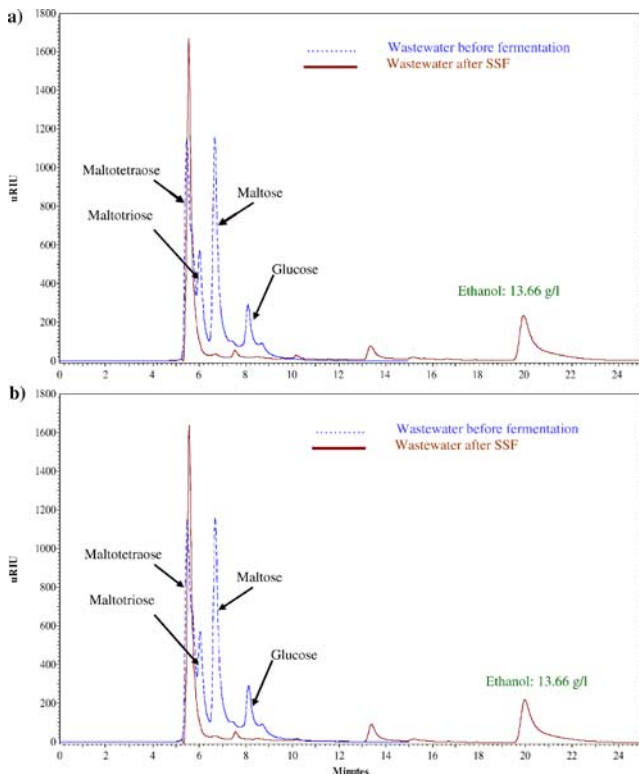


Fig. 8 Concentrations of sugars and ethanol in wastewater before and after fermentation with α -amylase. **a** *E. coli* KO11 **b** yeast

of the pectinase and α -amylase fermentation where both strains produced almost the same amount of ethanol.

Discussion

Comparison of the ethanol production rates of yeast and KO11 without the enzymes in Fig. 4 shows a clear difference between the two strains. Yeast produced ethanol roughly three times more rapidly than KO11. For both strains, however, the final ethanol concentrations were not significantly different within the experimental error range. The stationary phase was reached within 6 h for yeast and 30 h for KO11. The termination of ethanol production is thought to be due to the depletion of the nutrients, not due to the product inhibition, because more than 99% conversion of sugars were achieved in almost all the fermentation experiments performed in this study. Also the concentration of ethanol is not thought to be high enough to measurably show the product inhibition effect.

With regards to product inhibition, we did not observe any advantage of using the recombinant *E. coli* over yeast in the experimental results shown in Fig. 4 and also in the other experimental results. As mentioned above, it is thought that the advantage of ethanol tolerance of KO11

was not well-demonstrated because the concentrations of produced ethanol were not high enough to induce inhibition of cell growth to begin with. In other words, in the absence of significant inhibition effects of by-products, i.e., ethanol, yeast and KO11 showed little difference in ethanol production. However, it appears that without the effect of product inhibition, yeast produced marginally more ethanol by presumably using maltotetraose more than KO11, as shown in Fig. 5. However, as stated earlier in the Results section regarding ‘Fermentation without saccharification enzymes’, at this point it is not clear how maltotetraose was used.

According to Fig. 6, the ethanol production rates of yeast with and without pectinase were almost the same except that the ethanol production began earlier and the rate slowed down rapidly in the presence of pectinase. This earlier onset of ethanol production demonstrates the effect of pectinase that helped produce low molecular weight sugars, and with a higher concentration of consumable sugars, the cells started growing earlier. However, *E. coli* KO11 showed the opposite trend, i.e., the ethanol production rate in the presence of pectinase was slower than the no-pectinase case. It is not clear how pectinase affects the KO11s ethanol production rate.

Although a further ethanol kinetics study is needed, we speculated the possible reasons for the unconsumed glucose

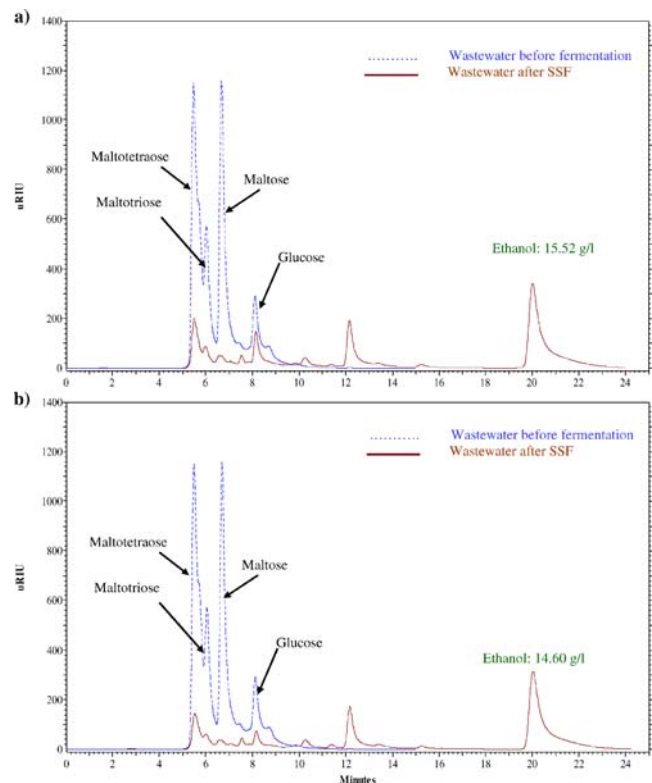


Fig. 9 Concentrations of sugars and ethanol in wastewater before and after fermentation with the combination of amylase and pectinase. **a** *E. coli* KO11 **b** yeast

at the end of fermentation and slower ethanol production in the presence of pectinase for the two strains. According to the chromatograms shown in Fig. 7, the high oligosaccharide peak found at a retention time of 5.5 min (maltotetraose) was considerably used up by the pectinase enzyme. Note that the maltotetraose peak was not reduced much when there was no enzyme present in the fermentation shown in Fig. 5. For the glucose peak, with pectinase, it did not decrease as much as in the no-enzyme cases. Also, the extra dimer and monomer sugars resulted from the degradation of maltotetraose by pectinase did not help the strains to produce more ethanol. Similar results were observed in the fermentation of hydrolyzate of rice hulls by *E. coli* KO11 (Moniruzzaman et al. 1998b). These observations indicate that the leftover glucose in Fig. 7 was possibly due to the effects of pectinase activity on the cell growth: being a strong hydrolytic enzyme, pectinase might have damaged the cell wall membranes by disintegrating it as reported in Liu and Li (1988). As a result, the cells could not fully utilize the benefits of extra glucose degraded from maltotetraose by pectinase.

Furthermore, the cytotoxic effect of pectinase was observed in the rate data in Figure 6 as it slowed down the fermentation of KO11 considerably due to the enhanced cell death rate. Yeast growth was also affected by pectinase leaving some glucose unreacted. It appears that the yeast cell walls are less susceptible to pectinase than the KO11s. According to Fig. 6, in the beginning of fermentation, yeast could take advantage of the excess low molecular sugars derived by pectinase from oligomers and produced ethanol about 5 h earlier than the no-enzyme case. However, the ethanol production rate slowed down rapidly after 4 h and stopped eventually leaving some available glucose unreacted due to cell death.

In addition, Gummadi and Panda (2003) have observed that even though pectinase was very effective in saccharification and solubilization of the polysaccharides, the resultant hydrolyzates showed the presence of some inhibitory substances that affected the *S. cerevisiae* growth. For the result shown in Fig. 7, we concurred in the similar effects of pectinase on *E. coli* KO11 and yeast. Due to these two reasons, the cell growth was inhibited and cells died early, and as a result they could not consume the glucose appreciably in the pectinase case, leaving the extra glucose unconsumed at the end of the fermentation.

Compared to pectinase, α -amylase helped the two strains produce more ethanol. The noticeable differences between Figs. 7 and 8 were the maltotetraose and glucose concentrations. Unlike the pectinase case in Fig. 7, maltotetraose increased and the glucose concentration was almost zero after the fermentation as shown in Fig. 8. Despite this increase of maltotetraose, the final ethanol concentration was not much different from that of the fermentation with

pectinase in Fig. 7. This increase of the maltotetraose peak with almost the same ethanol yields as the experimental results of the pectinase cases (Fig. 7) indicates that the suspended starch materials were broken down to maltotetraose, maltotriose, and maltose by α -amylase. α -Amylase is also known to convert tetramers and trimers into dimers, maltose in this case, helping both strains to produce more ethanol than the no-enzyme cases. The additional maltose, resulting from the maltotetraose degradation by α -amylase, helped the cells produce more ethanol.

Here, all the sugars except maltotetraose were consumed fully; hence, we obtained enhanced ethanol yields. The consumption of the originally present glucose and maltose in the raw feed and the additional maltose from the α -amylase was stopped until all the sugar was consumed and no additional maltose was produced from the degradation of starch due to the deactivation of amylase. It is known that α -amylase action on starch is inhibited by the hydrolysis products and proteins present in the fermentation mixture (Robyt 1984). α -Amylase decomposed starch into maltotetraose and maltose, and the two strains took advantage of these extra sugars from starch, producing more ethanol than the no-enzyme case. However, in this case also, the ethanol production stopped at 13.66 g/l because all the sugars were depleted and no more extra maltose was produced by amylase due to the possible enzyme.

Consequently, the simultaneous use of a mixture of enzymes in fermentation would help more consumption of unfermentable (or hard to ferment) sugars, resulting in high ethanol yields. It is thought that α -amylase converted starch into more fermentable maltotetraose and then pectinase converted it to even more fermentable trimers and dimers. The experimental results are summarized in Table 2. The percentage of yield was calculated based on the theoretical ethanol production. The last column shows how much ethanol production increased for each case compared to the no-enzyme case.

Unfortunately, the versatility of KO11 in utilizing different types of sugar was not demonstrated in this study. It was because the sugars resulting from saccharification were all hexose sugars, mostly maltotetraose, maltotriose, maltose, and glucose. Likewise, the advantage of KO11s ethanol tolerance was not well-demonstrated due to the low concentration of the resulted ethanol. However, it is clearly showed that the combination of enzymes enhanced the ethanol production compared to single enzyme. Also, when growing on low substrate concentration, KO11 produced ethanol slower than yeast.

It is thought that when the substrate consists of mostly hexose and its concentration is low, yeast may work better than *E. coli* KO11 for ethanol production because of the more rapid production rate of yeast.

Table 2 Comparison of the experimental results

| | Fermentation type | Fermentation time (h) | Ethanol production (g/l, vol%) | Increase as compared with the no-enzyme case |
|---------------------|--------------------------------------|-----------------------|--------------------------------|--|
| <i>E. coli</i> KO11 | No enzymes | 84 | 8.42, 0.971 | – |
| | With α -amylase | 84 | 13.66, 1.575 | 62.2% |
| | With pectinase | 84 | 12.18, 1.405 | 44.6% |
| | With α -amylase and pectinase | 84 | 15.52, 1.790 | 84.3% |
| Yeast | No enzymes | 48 | 8.48, 0.978 | – |
| | With α -amylase | 30 | 13.66, 1.575 | 61.1% |
| | With pectinase | 30 | 12.45, 1.436 | 46.8% |
| | With α -amylase and pectinase | 30 | 14.60, 1.684 | 72.2% |

However, it still needs more work to investigate the effect of product inhibition when the ethanol yield is high.

In summary, the use of a mixture of the enzymes pectinase and amylase enhanced the ethanol yields up to 15.5 g/l (84.3% increase) and 14.6 g/l (72.2% increase) for *E. coli* KO11 and yeast, respectively, compared to the results without the enzymes. When one enzyme was used, the fermentation resulted in an increase of ethanol production yield by an average of 2 g/l (~15%) regardless of the strains.

Pectinase is traditionally used to reduce the size of the pectin molecule, which helps clearing the haze in the beer brewing process. In our experiments, pectinase was used to clear the haze due to starch particles and oligosaccharides suspended in the brewery wastewater. Pectinase was observed to help hydrolyze maltotetraose into the smaller sugars, i.e., maltotriose, maltose, and glucose, whereas α -amylase hydrolyzed starch to maltotetraose, which resulted in an increase of maltotetraose concentration after fermentation. Therefore, the combination of the two enzymes appears to enhance the consumption of both starch and maltotetraose, resulting in an increase of the ethanol yield by an average of 84%, compared to the no enzyme case, and an increase by an average of 54% compared to the single enzyme cases. The method can be easily applied to different substrates that contain complex sugars. The process is economically attractive, as it does not use expensive enzymes.

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