

Fermentation of Starch By *Klebsiella oxytoca* P2, Containing Plasmids with α -Amylase and Pullulanase Genes

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Abstract: *Klebsiella oxytoca* P2(pC46), an ethanol-producing recombinant, has been evaluated in fermentation of maltose and starch. The maximum ethanol produced by P2(pC46) was 0.34 g ethanol/g maltose and 0.38, 0.40, or 0.36 g ethanol/g starch in fermentation of 1, 2, or 4% starch, representing 68, 71, and 64% the theoretical yield. The pC46 plasmid transformed to cells of *K. oxytoca* P2 reduced the ethanol production from maltose and starch. In fermentation of starch after its digestion at 60°C for 24 h, in two-step fermentation, the time for maximum ethanol production was reduced to 12–24 h and the theoretical yield was around 90%. The increase in starch concentration resulted in lower α -amylase activity but in higher pullulanase activity. The high activity and thermostability of the amylolytic enzymes from this transformant suggest that it has a potential for amylolytic enzymes source. © 1999 John Wiley & Sons, Inc. *Bio-technol Bioeng* 65: 673–676, 1999.

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INTRODUCTION

Different strategies have been tested for ethanol production from starch, such as its hydrolysis before fermentation (Kim et al., 1988); simultaneous saccharification and fermentation with mixed culture using amylolytic and fermenting microorganism (Tanaka et al. 1986; Han and Steinberg, 1987; Kurosawa et al., 1989); and use of amylolytic enzymes and fermenting microorganism or direct fermentation of starch by a microorganism that produces amylolytic enzymes and performs alcohol fermentation (Park et al., 1996; Nakamura et al., 1997). The commercial enzymes used for starch digestion represent a significant cost in the fermentation process.

Ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca* carrying genes for pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase II (*adhB*) from *Zymomonas mobilis* have been developed (Beall et al., 1991; Ohta et al.,

1991a,b; Burchhardt and Ingram, 1992; Wood and Ingram, 1992). *K. oxytoca* P2 is able to transport and metabolize xylo-oligosaccharides (Burchhardt and Ingram, 1992) and gluco-oligosaccharides, products of starch hydrolysis (Al-Zaag, 1989; Wood and Ingram, 1992) and to produce ethanol from starch (Santos et al., 1998). It is expected that *K. oxytoca* transformed with plasmids carrying genes for thermostable amylolytic enzymes can promote efficient hydrolysis and fermentation of starch.

The objective of the present study was to evaluate the fermentation of maltose and starch by *K. oxytoca* P2 transformed with plasmid carrying thermostable α -amylase and pullulanase genes. The thermostability of these enzymes is important, considering that the industrial starch saccharification is usually done at temperatures higher than 60°C.

MATERIAL AND METHODS

Bacterial Strain

K. oxytoca P2 (Wood and Ingram, 1992) transformed with pC46 plasmid was used in this study. The pC46 (17.8 kbp) was constructed by cloning a 2.5-kbp *EcoRI* fragment from pCOS2EMBL (Poustka et al., 1984) containing the tetracycline gene into pLOI568, after partial digestion with *EcoRI*, to permit the plasmid selection in strain P2. The pLOI568 contains the ampicillin gene and the α -amylase gene from *Bacillus stearothermophilus* (Mielenz and Mickel, 1985) and the pullulanase gene from *Thermoanaerobium brockii* (Coleman and McAlister, 1986; Coleman et al., 1987). The pC46 was quite stable in strain P2, with 92% stability/96 h incubation. The stock culture was grown at 30°C/18–24 h, in Luria broth medium (LBM), containing per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, supplemented with 2% maltose plus 40 mg/L chloramphenicol and 12.5 mg/L tetracycline. The cells were maintained at –20°C in LBM + 40% glycerol.

The inoculum was prepared by transferring cells from

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stock-culture to LBM agar and incubated at 30°C/12–18 h. Isolated ethanologenic colonies from the agar medium (Santos et al., 1998) were inoculated in LBM broth and incubated at 30°C/18–24 h. The cells were centrifuged at 7000g/10 min, and the cell mass was added to the fermentation medium to make 0.1 OD (550 nm) as final cell concentration.

One- Step Fermentation

Fermentation was carried out in 500-mL Fleakers with 200 mL of LB broth with 5% maltose or 1, 2, or 4% starch, 40 mg/L chloramphenicol, and 12.5 mg/L tetracycline, and the mixtures were incubated at 30°C/96 h, pH 6.0, and 100 rpm in pH-controlled fermenter (Beall et al., 1991). Maltose solution was sterilized separately by filtration. The medium with starch was cooked in a water-bath before autoclaving. During fermentation, samples of 5.0 mL were collected at 12-h intervals for determination of ethanol concentration and α -amylase and pullulanase activities.

Two-Step Fermentation

Cells harvested from a previous fermentation in LB broth + 5% maltose at 30°C for 72 h were added to 200 mL of LB medium with 1, 2, or 4% starch, heated for 10 min at 70°C, and then kept at 60°C for 24 h. The digested mixture was cooled to 30°C, and tetracycline (12.5 mg/L), chloramphenicol (40 mg/L), and inoculum were added to make 0.1 OD (550 nm). Fermentation was conducted at 30°C for 96 h, pH 6.0, and stirring at 100 rpm. Samples were removed at 12-h intervals for ethanol determination.

Analyses

Ethanol concentration was determined by gas chromatography (Hewlett Packard 5890, Series II Plus) equipped with a flame ionization detector and a HP Wax (polyethylene glycol) column. The dilution caused by the addition of 2 N KOH was considered for ethanol determination. The theoretical yield was determined based on the total sugar present in the medium. Maximum yield was considered to be 0.56 g of ethanol/g of starch and 0.53 g of ethanol/g of maltose. The results were the average of three or more fermentations with two replications.

The concentration of the residual sugars, glucose and maltose, produced during fermentation was determined with an HPLC chromatograph equipped with a refraction index detector and an Aminex HPX-87H (7.8 × 300 mm) column (Bio-Rad), using 5 mM sulfuric acid as the mobile phase at a flow of 0.7 mL/min at 60°C.

The α -amylase and pullulanase activities were determined in the cells of *K. oxytoca* P2(pC46) and in the supernatant obtained after centrifugation of 4 mL of fermented medium at 10,000g for 10 min and washed with 2 mL of 10 mM Tris-HCl, pH 6.0. The pellet was resuspended in 2 mL of 0.5-M mannitol, 1 mM EDTA, and lysozyme (1 mg/mL)

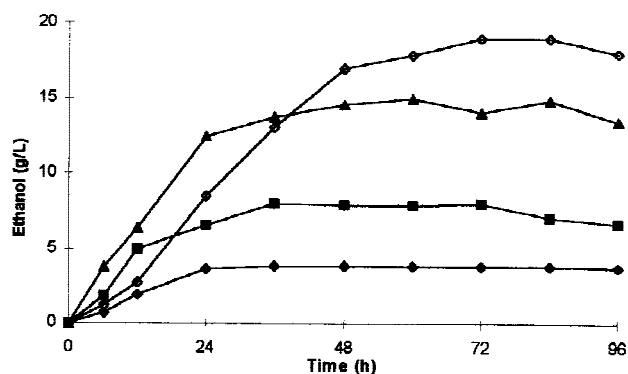


Figure 1. Ethanol production by *K. oxytoca* P2(pC46). Fermentation in LBM with 1% (◆), 2% (■), or 4% (▲) of starch or 5% maltose (open tilted squares) in pH controlled at 6.0, incubation at 30°C, and stirring at 100 rpm.

and incubated at room temperature for 1 h. The cell suspension was maintained in an ice-bath, and the cells were disrupted in a sonicator (ultrasonic homogenizer Cole-Parmer, Series 36260), with two pulses of 15 s with 20 s interval. The α -amylase activity was assayed by measuring the reducing sugars liberated from the soluble starch when 200 μ L of the enzyme was mixed with 200 μ L of 2% soluble starch in 40 mM potassium phosphate buffer, pH 6.0/1 mM CaCl₂, and incubated at 70°C for 30 min. The reaction mixture for pullulanase activity contained 200 μ L of 1% pullulan in 40 mM sodium phosphate-citrate buffer, pH 6.0, and 200 μ L of the diluted enzyme and was incubated at 60°C for 30 min. One unit of α -amylase or pullulanase activity was defined as the amount of enzyme that liberated 1 μ mol of reducing sugar/mL/min, using maltose as a standard. Reducing sugars were estimated by the dinitrosalicylic acid (DNS) method (Miller, 1959).

RESULTS AND DISCUSSION

The fermentation capacity of *K. oxytoca* P2(pC46) was analyzed in LB medium with 5% maltose or 1, 2, or 4% starch

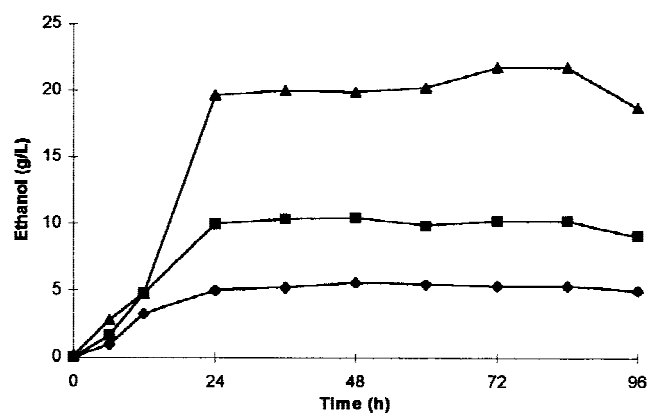


Figure 2. Ethanol production by *Klebsiella oxytoca* P2(pC46). Two-step fermentation in LBM with 1% (◆), 2% (■), or 4% (▲) starch in pH controlled at 6.0, incubation at 30°C, and stirring at 100 rpm.

Table I. Maximum ethanol production and maximum extracellular (extra) and intracellular (intra) α -amylase and pullulanase activities of *K. oxytoca* P2 (pC46) in one-step starch fermentation.

% starch	Time (h)	Cellular mass (g/L) ^a	Ethanol		VP (g/L/h) ^b	α -Amylase (U)		Pullulanase (U)	
			g/L	g/g starch		Intra	Extra	Intra	Extra
1	36	0.75	3.80	0.38	0.20	86.67	15.00	0.50	0.08
2	36	1.12	8.00	0.40	0.54	75.10	17.00	0.79	0.11
4	48	1.41	14.54	0.36	0.63	56.79	17.00	0.80	0.12

^aCellular mass was calculated from the OD at 550 nm (approximately 0.33 g dry weight per liter/OD unit).

^bVP, volumetric productivity.

(Fig. 1). The fermentation of 5% maltose resulted in 19 g of ethanol/L as maximum yield observed at 72 h. The presence of pC46 reduced the ethanol production of *K. oxytoca* P2 from 25 to 33% as compared to the original *K. oxytoca* P2 (Santos et al., 1998). Several authors reported that the secretion of enzymes at high levels frequently affects the normal cell processes (Coleman et al., 1987; Burchhardt and Ingram, 1992, Kurland and Dong, 1996; Viaplana et al., 1997). According to Wood and Ingram (1992), the presence of plasmids, encoding endoglucanases from *Clostridium thermocellum*, reduced the final cell density and ethanol yield in glucose fermentations of *Klebsiella oxytoca*. The increase in starch concentration resulted in increase of ethanol yield (Table 1) and the maximum ethanol concentration was observed between 36 and 48 h of incubation, representing 68, 71, and 64% of the theoretical yield (Fig. 1) for 1, 2, or 4% starch, respectively.

K. oxytoca P2 has a higher ethanol yield at 30°C but higher growth at 37°C (Burchhardt and Ingram, 1992); however, the pullulanase and α -amylase activities of P2(pC46) are higher at 60 and 70°C, respectively (Santos, 1997). Considering the difference in temperature for growth and for enzyme activity, experiments were conducted by digesting the starch at 60°C prior to fermentation (two-step fermentation). The maximum ethanol production was observed at 24 h of fermentation (Fig. 2) as compared to 36–48 h for one-step fermentation (Fig. 1) representing 96.0, 89.2, and 89.0% of the theoretical ethanol yield for 1, 2, or 4% of starch, respectively. The hydrolysis of starch prior to fermentation resulted in higher volumetric productivity (0.28, 0.53, and 1.27 for 1, 2, or 4% of starch) as compared to the one-step fermentation process (Table I). A high level of reducing sugars liberated during the high temperature digestion resulted in faster fermentation. The lysate of P2(pC46) after its growth on 4% starch resulted in 20.5 mM of glucose and 36.8 mM of maltose. High levels of hydrolytic enzymes would be advantageous at the beginning of fermentation to increase reducing sugars for fermentation. Sills et al. (1984) and Wilson et al. (1982) reported that insufficient glucoamylase activity was the main limiting factor for direct starch fermentation by *Schwanniomyces* spp.

The ethanol yield for maltose and starch fermentation observed by Guimarães et al. (1992) using *E. coli* KO11, harboring plasmids with genes for starch hydrolysis was

lower than the ones observed with P2(pC46) in this study. The better ethanol production of *K. oxytoca* could be due to its higher capacity of metabolizing gluco-oligosaccharides.

The intra- and extracellular α -amylase activity of P2(pC46) at the maximum ethanol yield in different starch concentration is indicated in Table I. The increase in starch concentration resulted in increase of cell mass, in the ethanol concentration, and in the extracellular enzyme activity, but the intracellular enzyme activity was decreased.

A relationship between production of amylolytic enzymes and starch concentration in the medium has been reported by several authors (Srivastava and Baruah, 1986; Wind et al., 1994; Nakamura et al., 1997), with contradictory results. Srivastava and Baruah (1986) observed that the optimum starch concentration for α -amylase production by *B. stearothersophilus* was 30 g/L, and that higher and lower concentrations resulted in lower α -amylase production. According to Wind et al. (1994), the highest production of α -amylase by *B. stearothersophilus* was observed with the lowest starch concentration. These investigators suggested that the inverse relationship between starch concentration and enzyme activity could result from the repression of α -amylase synthesis by products of starch hydrolysis or from differences in available O₂, which is affected by the change in medium viscosity in the presence of high starch concentrations.

The pullulanase activity at the maximum ethanol yield increased with starch concentration (Table I); however, the maximum activity was observed during the stationary growth phase of the culture. About 80% the amylolytic enzymes activity observed in this study were associated with the cells, suggesting limitation in the secretion system of the cells.

The high amylolytic activity of P2(pC46) observed in fermentation of starch represents a potential for the production of active and thermostable amylolytic enzymes for industrial use or for ethanol production.

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