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Influence of fermentation conditions on levan production by *Zymomonas mobilis* CT2

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Zymomonas mobilis produces two extracellular sucrases namely levansucrase (*sac* B) and sucrase (*sac* C). A mutant strain of *Z. mobilis* CT2 defective in sucrase *sac* C, constructed earlier by gene disruption[1], produced higher levels of levan (27.2 g L^{-1}) than the parent strain B14023 (15.4 g L^{-1}) from 200 g L^{-1} of sucrose at 25°C and *p*H 5.0. Increasing fermentation temperature from 25 to 35°C enhanced ethanol concentration from 17.8 to 46.4 g L^{-1} due to increased rate of sucrose hydrolysis but decreased the transfructosylation activity from 165 to 56 U mL^{-1}. Addition of glucose or fructose to the fermentation medium considerably reduced the levan production due to the inhibition of levansucrase activity.

Keywords: *Zymomonas mobilis*, levansucrase, levan, sucrase, fermentation

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Introduction

Zymomonas mobilis, an ethanologenic bacterium produces levan, a polymer of fructose, by the transfructosylation reaction of levansucrase. Levan is commercially used as an emulsifier, formulation aid, stabilizing thickener, surface-finishing agent, encapsulating agent and a carrier for colour or flavours in the food industry[2]. Levan, with varying of molecular weight, exhibits antitumour activity against Sarcoma 180[3] and as cholesterol lowering agent in blood[4]. Several bacteria, viz. *Bacillus subtilis*, *B. stearothermophilus, Rahnella aquatilis, Aerobactor levanicum*, *Erwinia herbicola*, *Streptococcus salivarius, Pseudomonas syringae, P. aurantiaca* and *Zymomonas mobilis* produce levan of high molecular weight when grown in sucrose media. The enzymatic production of levan from a recombinant *Escherichia coli* overexpressing cloned levansucrase gene of *Z. mobilis* has been reported[5]. Several authors have critically emphasized the importance of biochemical and medium optimization strategies for levan production[6,7]. In the present investigation, the authors report the influence of fermentation conditions on levan production by the mutant strain of *Z. mobilis* CT2 defective in *sac* C.

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Materials and Methods

Organism and Culture Conditions

Z. mobilis CT2, defective in extracellular *sac* C developed earlier by gene disruption[1] was used in this study. Culture of *Z. mobilis* was grown in RM medium consisting of (g L^{-1}) glucose, 20; yeast extract, 10; KH_2PO_4, 2; and tetracycline (2 µg ml^{-1}) at 30°C (*p*H 6.0) without agitation. For levan production, sucrose (5 to 25 g L^{-1}) was used.

Batch Fermentation

Batch fermentation was carried out at 30°C with 100 mL fermentation medium in 250 mL Erlenmeyer flask, inoculated with 10% (v/v) of 12 h old seed culture. Samples were periodically withdrawn at regular intervals and analyzed for levan, biomass, ethanol, residual sucrose, sucrase and levan forming activity.

Analytical Methods

Biomass was estimated by suspending the cells in 0.85% (w/v) sodium chloride solution and measuring the absorbance at 550 nm. The corresponding dry weight of the cells was obtained from the established standard curve of absorbance against dry weight of cells. Residual sucrose was evaluated by phenol sulphuric acid method[8] while reducing sugar was determined by Somogyi method[9]. Levan was separated by ethanol precipitation, hydrolyzed in 0.1N HCl at 100°C for 30 min and equivalent to fructose

units[10]. Ethanol was analyzed by the method of Caputi *et al*[11].

Enzyme Assay

Cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and the supernatant was used as enzyme source. Sucrase activity was assayed by analyzing the reducing sugar liberated during sucrose hydrolysis. The reaction mixture [250 µL of enzyme extract and 250 µL of 1 *M* sucrose in acetate buffer (50 m*M*, *p*H 5.0)] was incubated at 30°C for 30 min. Reducing sugar released was determined as per the method of Somogyi[9]. One unit of levansucrase activity was expressed as the amount of enzyme required to liberate 1 µmol of reducing sugar from sucrose in 1 min under experimental conditions. Levan forming activity was estimated by incubating the reaction mixture at 30°C for 2 h. Turbidity was measured at 540 nm and the amount of levan was calculated from standard curve of absorbance against the concentration of purified levan (Sigma, USA). One unit of levan forming activity was expressed as the amount of the enzyme required to form 1 µg of levan in 1 min under experimental condition.

Results and Discussion

All the *Z. mobilis* strains have inherent capability to produce levan in sucrose containing fermentation medium. A levan hyperproducing mutant strain capable of levan production at 7°C was isolated[12]. Attempts were made to increase the levan production through cloning and expression of levansucrase gene (*sac* B) from *Z. mobilis*. *Z. mobilis* CT2 (*sac* C$^-$) mutant strain developed by gene disruption has shown higher levels of levan production compared vis-à-vis wild type[1].

The effect of fermentation conditions on levan and fructo oligosaccharide production by *Z. mobilis* 113S was determined and the fructo-oligosaccharide forming activity was high at 25°C in 150 gL^{-1} sucrose from the biomass and cell-free culture supernatant of fermentation[4]. The sucrose concentration and temperature also affected the levan production by *Z. mobilis*[12]. Therefore, the effect of these fermentation conditions on levan production by *Z. mobilis* CT2 was studied here.

Effect of Sucrose Concentration

The effect of sucrose concentration (50-250 gL^{-1}) on levan production by *Z. mobilis* CT2 in fermentation medium at *p*H 6 and 30°C was studied. The production of biomass, ethanol and levan is shown in Fig. 1. Increase in sucrose concentration from 50 to 200 gL^{-1} enhanced levan production from 15.5 to 22.8 gL^{-1} with concomitant increase in biomass from 1.1 to 2.4 gL^{-1} at 30°C. However, levan concentration decreased after 24 h due to degradation of levan by levanase type of activity exhibited by the levansucrase. This activity was prominent in the culture grown in the fermentation medium containing 50 and 100 gL^{-1} sucrose[3]. At higher concentration of sucrose (above 150 gL^{-1}), the decrease in levan concentration after 24 h was not prominent due to the inhibition of levanase activity by available reducing sugar in the fermentation medium. Further increase in sucrose concentration above 200 gL^{-1} strongly affected levan and biomass production. However, the ethanol production continued to increase (46-53 gL^{-1}) in the fermentation from 200-250 gL^{-1} of sucrose. These results suggested that higher concentration of sucrose affected levan formation by levansucrase.

Effect of *p*H

A maximum concentration of levan 22.8 gL^{-1} was produced from the fermentation at *p*H 6 with the initial sucrose concentration of 200 gL^{-1} at 30°C. No levan production occurred at *p*H 4, which could be attributed to the poor growth of the cells (Fig. 2). Further, at *p*H 4 the levan forming activity of levansucrase was lesser than the sucrose hydrolyzing activity (Table 1). Crittenden and Doelle[14] also found higher sucrose hydrolyzing activity of levansucrase at *p*H 4, than levan forming activity. Although the biomass production in the fermentation with an initial *p*H 6-7 was maximum (2.3-2.4 gL^{-1}), the levan production was low at initial *p*H 7. These results indicated that the optimum *p*H for levan forming activity of the levansucrase lies within 5 and 6. Lyness and Doelle[13] also reported that *p*H 5 was optimum for levan production, while higher *p*H favours oligosaccharide formation. The ethanol production was more (25-36 gL^{-1}) in the fermentation with the initial *p*H 6-7 due to the enhanced sucrose hydrolyzing activity of levansucrase resulting in the formation of reducing sugars.

Effect of Temperature

Maximum concentration of levan (27.2 gL^{1}) was produced at 25°C with initial *p*H 6, while low concentration of levan was produced (7.1 and 4.1 gL^{-1}) at 35 and 40°C (Fig. 3). On the contrary, ethanol production was low (16.5 gL^{1}) at 25°C, but more

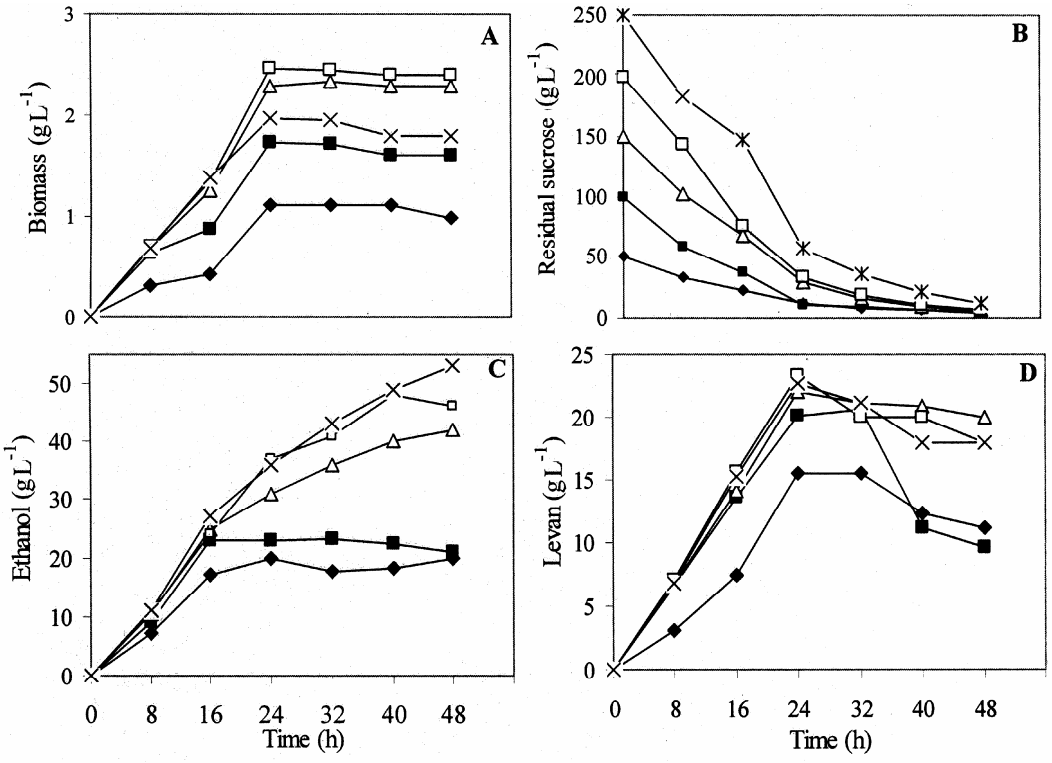

Fig. 1—Sucrose concentration on fermentation profile of *Z. mobilis* CT2 as a function of time [Sucrose (♦) 50 gL^{-1} (■) 100 gL^{-1} (Δ) 150 gL^{-1} (□) 20 gLl^{-1} (×) 25 gL^{-1})]

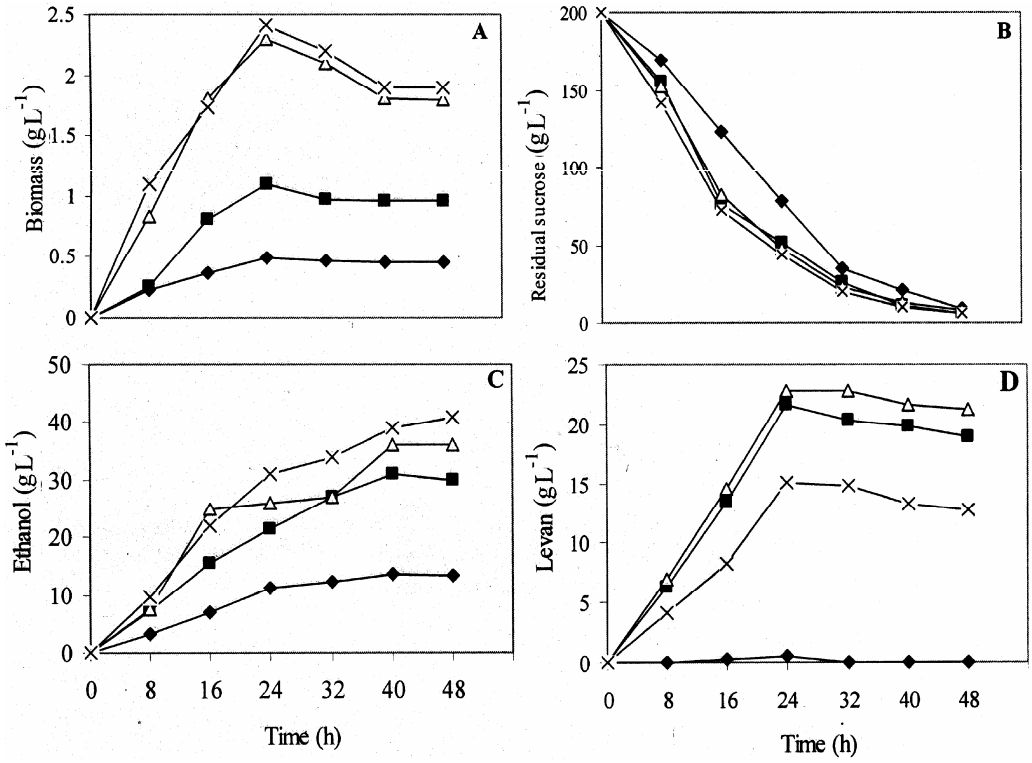

Fig. 2—Effect of pH on fermentation profile of *Z. mobilis* CT2 as a function of time [*p*H (◊) 4 (■) 5 (Δ) 6 (×) 7]

ethanol was produced (54 gL^{-1}) at temperature above 30°C. The effect of temperature on levan forming and sucrose hydrolyzing activities in the fermentation is shown in Fig. 4. Levan forming activity was high at 25°C (156 U mL^{-1}), but the enzyme activity was significantly decreased at 40°C (56 U mL^{-1}). However, the sucrose hydrolyzing activity was increased from 1.3 to 1.9 U mL^{-1} as the fermentation temperature increased from 25 to 35°C, but considerably decreased to 1.2 U mL^{-1} at 40°C. These results suggested that optimum temperature for the levan forming activity was 25°C while for the sucrose hydrolyzing activity was 35 to 40°C (Fig. 4). The levan production was more at low temperature due to the transfructosylation reaction[10]. The levansucrase has been reported to lose irreversibly the levan forming ability at 35°C while retaining the sucrose hydrolyzing activity[10]. Recently, it was shown that more levan production occurred at 7°C by levan hyperproducing mutant of *Z. mobilis*. The levansucrase of this strain retained its activity for 29 days of incubation at 7°C[12].

In order to determine the stability of levanforming and sucrose hydrolyzing activity of levansucrase, the enzyme extract was pre-incubated for 12 h at different temperatures (25 to 40°C) and assayed for sucrose hydrolysis, levanforming activities at 30°C (Fig. 5A). The levan forming and sucrose hydrolyzing activities were also assayed at different temperatures ranging from 25 to 40°C (Fig. 5B). Levan forming activity decreased from 137.1 to 87.3 U mL^{-1} as the temperature increased from 25 to 40°C while sucrose hydrolyzing activity increased from 1.25 to 1.87 U mL^{-1} as the temperature increased from 25 to 35°C.

Table 1–Effect of *p*H on sucrose hydrolyzing and levan forming activity of levansucrase in the fermentation medium (200 gL^{-1}) at 30°C after 24 h

Initial *p*H of the fermentation medium	Sucrose hydrolyzing activity (U mL^{-1})	Levan forming activity (U mL^{-1})
4	2.3	15
5	2.0	54.1
6	2.0	106.6
7	1.8	85.4

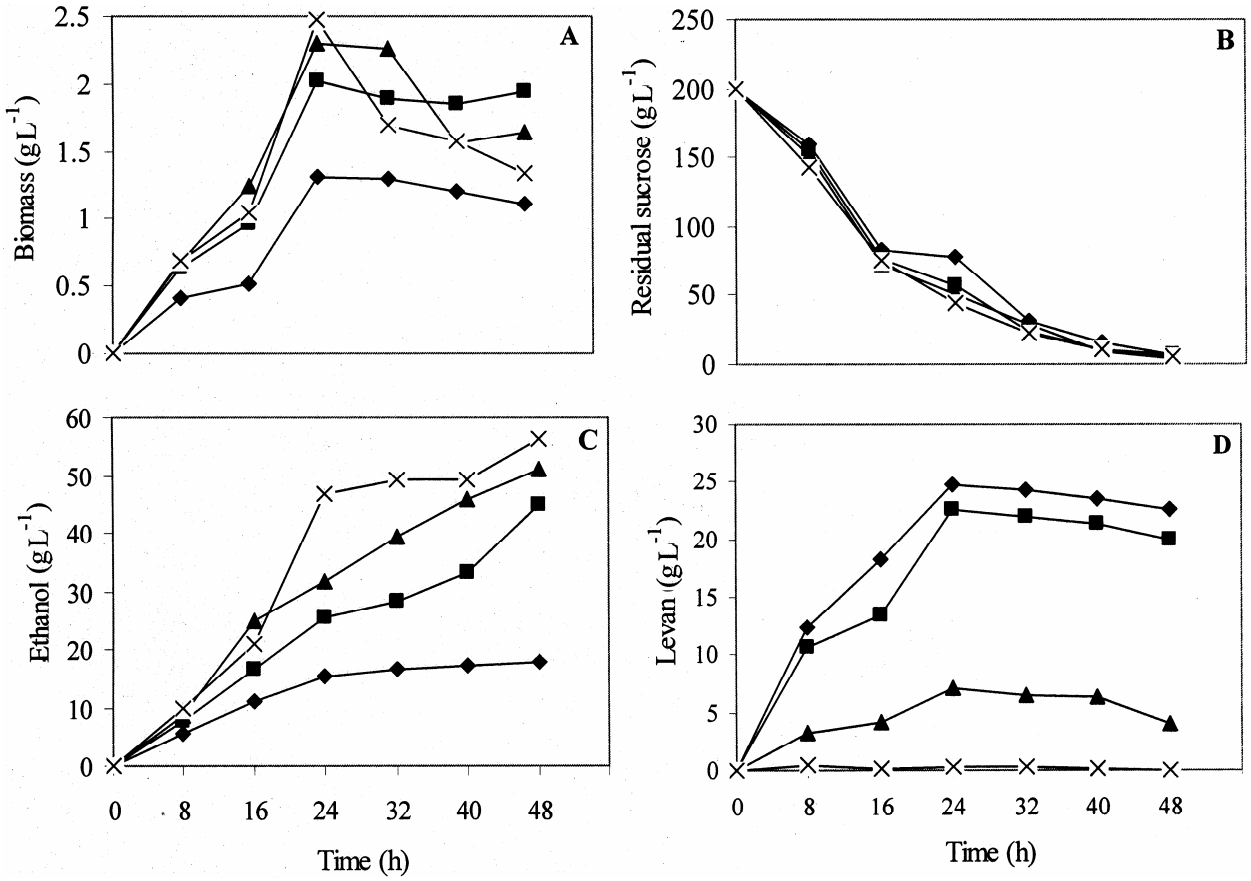

Fig. 3—Effect of fermentation temperature on levan production by *Z. mobilis* CT2 [(♦) 25°C (■) 30°C (▲) 35°C (×) 40°C]

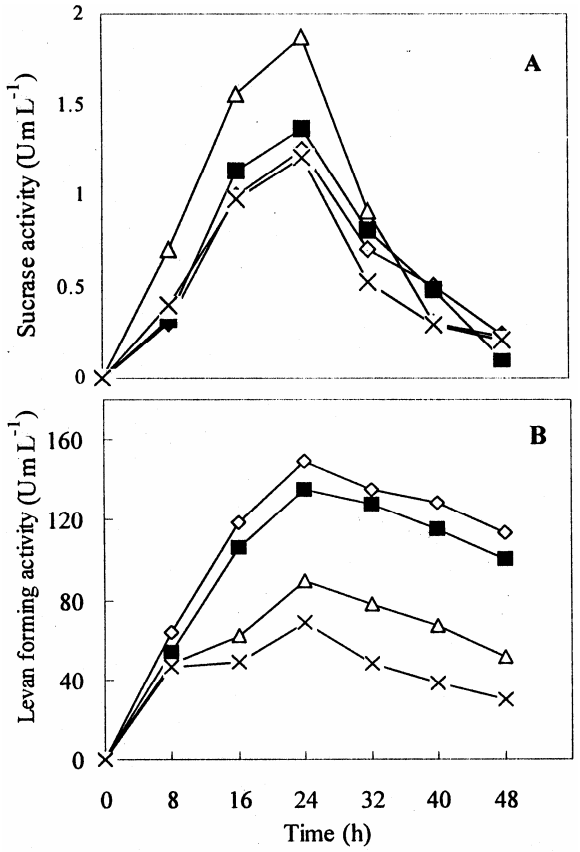

Fig. 4—Effect of temperature on synthesis of levansucrase by *Z. mobilis* CT2 (A)-Sucrose hydrolyzing activity (B)-Levan-forming activity [(◊) 25°C (■) 30°C (Δ) 35°C (×) 40°C]

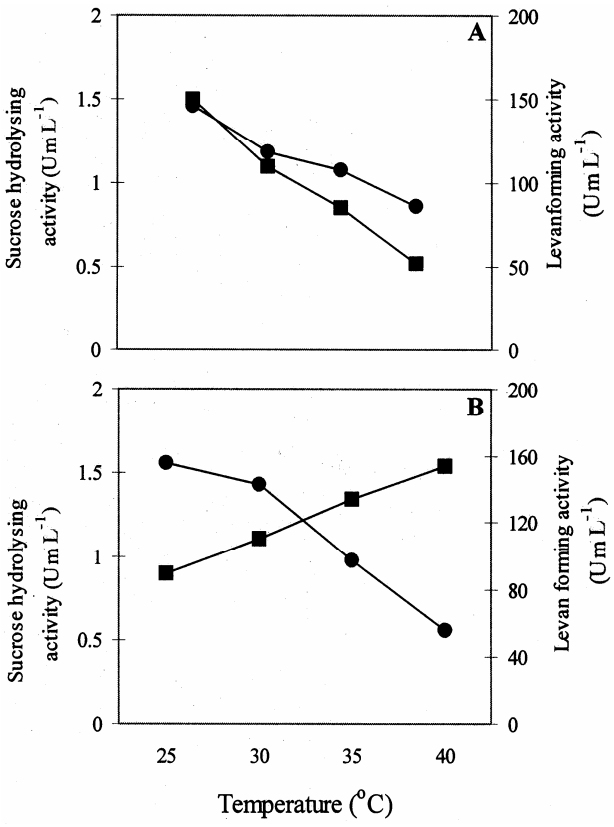

Fig. 5—Effect of temperature on levansucrase (A) Effect of temperature on levansucrase (B) Stability of the enzyme at different temperatures [(●) Levan forming activity (■) Sucrose hydrolyzing activity]

The results confirmed that levan forming activity is favoured at low temperature while hydrolysis is favoured at high temperature.

Effect of Fructose and Glucose on Levan Production

Levan production by *Z. mobilis* CT2 in fermentation medium supplemented with glucose or fructose was examined. Supplementation of either glucose or fructose to the fermentation medium decreased the levan production from 27.1 to 12.4 gL^{-1} (glucose) and 13.1 gL^{-1} (fructose) in 24 h (Table 2). However, ethanol concentration increased from 28.4 to 53.3 gL^{-1} (glucose) and 56.7 gL^{-1} (fructose) addition. Recently, it was shown that accumulation of ethanol decreases the levan production in the fermentation medium[4]. The levansucrase catalyze hydrolysis of sucrose and simultaneous transfructosylation reaction for the formation of levan. Therefore, addition of free fructose or glucose in the fermentation medium will not improve levan formation.

Table 2—Effect of addition of glucose or fructose on levan and ethanol production in fermentation medium (200 gL^{-1}) at 30°C after 24 h

Added substrate (gL^{-1})	Levan production (gL^{-1})	Ethanol production (gL^{-1})
Glucose		
50	16.3	51.1
100	12.4	53.3
Fructose		
50	17.3	53.6
100	13.1	56.7

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