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Levoglucosan kinase involved in citric acid fermentation by *Aspergillus niger* CBX-209 using levoglucosan as sole carbon and energy source

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Abstract

Conditions were optimized for the production of citric acid by a mutant *A. niger* CBX-209 using levoglucosan derived from pyrolysis of wastepaper as a sole carbon and energy source in a simple medium. The optimum concentration of levoglucosan and wheat bran in the medium was 8% and 3%, respectively, at an optimum initial pH between 5.5 and 6.0 at 35°C. During fermentation, direct formation of glucose 6-phosphate from levoglucosan in the presence of ATP and Mg²⁺ as observed by HPLC in the reaction with both cell extracts and partially purified enzyme, suggested that the enzyme acting on levoglucosan is a kinase. Time-course changes in the levels of this special levoglucosan kinase in *A. niger* CBX-209 grown on levoglucosan and glucose revealed that levoglucosan kinase was an inductive enzyme. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Levoglucosan (1,6-anhydro-β-D-glucopyranose) (LG), which is very scarce in nature, can be obtained by the destructive distillation of starch [1,2] and has been used for the synthesis of other carbohydrates including sugar derivatives, dextrin-like polymers, and as a research tool for determining the configuration of anomeric glucosides [3]. Levoglucosan is of special interest now, since it can be derived through pyrolysis

of cellulosic materials of very low cost [4–6]. Its production and potential use as a fermentative carbon and energy source in the fermentation industry could lead to the possible commercial utilization of large quantities of cellulosic materials such as wastepaper, waste wood products, straws, etc.

Although it has been recognized that cellulosic materials can be converted by pyrolysis into oils containing high concentrations of LG, the commercial application of this anhydrosugar has been hampered by the presence of substances [7] in the pyrolysis oil that are toxic to the growth or metabolism of microorganisms. Since levoglucosan can now be isolated in crystalline form from pyrolysis mixture [2,8,9], it may be used in fermentation industries.

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Studies have shown that many eucaryotic microorganisms are capable of utilizing LG to grow and even produce useful metabolites even though it is not a natural product in nature. Till now, most tested eucaryotic microorganisms, except two yeast species, can grow on LG media [7]. As to products from LG, *Aspergillus terreus* converts purified LG to itaconic acid with the same yield and rate as in the conversion from glucose [10]. Five yeast strains tested had the ability to ferment activated charcoal treated pyrolysis tar (containing high concentrations of LG) to ethanol, but their ethanol yields were considerably lower than on glucose [7]. With regard to the metabolism of LG, Kitamura et al. [11,12] found that all tested eucaryotic microorganisms capable of growing on LG directly converted it by phosphorylation into glucose 6-phosphate with a specific kinase. But their metabolites from LG were not reported. Very few procaryotic organisms were found to utilize LG. Yasui et al. [13] selected six colonies from soil that assimilated LG completely in the liquid culture and found that all of them seemed to be coryneform bacteria. Nakahara et al. [14] identified one of these colonies to be the genus *Arthrobacter* and discovered that a novel enzyme, LG dehydrogenase, catalyzed the dehydrogenation of LG to form 1,6-anhydro- β -D-ribohexopyranose-3-ulose, using NAD^+ as an electron acceptor. Glucose was then formed after the action of at least two other enzymes. However, no useful products have been reported by procaryotic organisms growing on LG.

According to the reports from Prosen et al. [7] and Nakagawa et al. [10], *Aspergillus niger* can grow well on purified or partially purified LG, but its products have not been investigated. This paper describes citric acid fermentation of LG, the verification of the direct formation of glucose 6-phosphate from this special intramolecular glucoside catalyzed by LG kinase, and the changes of activity in LG kinase during the fermentation.

2. Materials and methods

2.1. Microorganisms

The mutant, *Aspergillus niger* CBX-209, was derived by γ -ray irradiation of spores of parent strain

CBX-2, which has high citric acid productivity using starch as carbon and energy source at an industrial scale [15]. The strain was maintained on LG agar slant at 4°C and was subcultured every month. The medium composition for conidia preparation on the agar slants per liter was: LG, 20 g; peptone, 1 g; K_2HPO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; and agar, 20 g.

2.2. Fermentation

The fermentation media contained only LG and wheat bran with an optimum initial concentration of 8% and 3%, respectively. The media were dispensed in 50 ml aliquots in 250 ml Erlenmeyer flasks and autoclaved for 20 min at 121°C. Flasks were inoculated, each, with 1 ml suspension having about $(1-3) \times 10^5$ conidia, incubated at 35°C on a rotary shaker at 400 rpm. The initial pH of the medium was adjusted to 6.0.

2.3. Substrates and other reagents

LG, which was obtained through the pyrolysis of wastepaper and purified by the method described by Zhang et al. [8], was recrystallized to more than 98% purity. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose oxidase, peroxidase and glucose 6-phosphatase were purchased from Sigma Co. Ltd. (US). All other reagents were of analytical grade.

2.4. Assays

Glucose 6-phosphate and citric acid analysis was performed at 30°C on HPLC system (Gilson 712 system, Gilson Medical Electronics Inc., US) equipped with an Elite ODS-18C or a Biorad Aminex HPX-87H column, respectively, and both detected by a refractive index detector. The mobile phase was doubly distilled water or 8 mM H_2SO_4 at 1 ml/min, respectively. The titration method was also used for routine determination of citric acid. The microbial growth biomass was measured by weighing mycelia filtered, washed twice with distilled water and then dried at 100°C to constant weight.

LG-phosphorylating activity was assayed by measuring glucose 6-phosphate formation with the

glucose 6-phosphate dehydrogenase coupling system described by Maitra et al. [16] with some modifications, i.e. the reaction mixture consisted of 100 mM LG (or glucose), 2 mM ATP, 1 mM NADP⁺, 10 mM MgCl₂, 1 unit of glucose 6-phosphate dehydrogenase and triethanolamine buffer was replaced by a 50 mM CHES (2[N-cyclohexylamino]ethanesulfonic acid) buffer (pH 9.3). A non-rate-limiting amount of 6-phosphogluconate dehydrogenase was also added to this system when crude cell extracts were used as the enzyme preparation to avoid the excessive estimation of the activity, which was caused by the additional reduction of NADP with the action of the endogenous 6-phosphogluconate dehydrogenase. Then the value was divided by two. The increase in absorbance at 340 nm was continuously measured with a constant temperature cell compartment. One unit of LG-kinase activity was expressed as the amount of enzyme catalyzing the reduction of 1 μmol of NADP per min at 30°C at pH 9.3. Protein was measured by the Coomassie brilliant blue dye-binding method described by Bradford [17] with bovine serum albumin as the standard.

LG-hydrolyzing activity was assayed by measuring glucose formation with the glucose oxidase and peroxidase coupling system described by Papadopoulos et al. [18]. Crude cell extracts of 1 ml volume or culture supernatant were mixed with 100 mM LG and incubated in several buffer systems, e.g. 100 mM citrate buffer (pH 2.0 and 5.0), 50 mM Tris–HCl buffer (pH 7.1), 100 mM triethanolamine–HCl/NaOH buffer (pH 8.0), or 50 mM CHES buffer (pH 9.3) at 30°C. The mixture was then heated in a boiling water bath for 5 min to stop the reaction, and the glucose concentration was measured.

2.5. Detection of the changes of LG kinase and hexokinase during citric acid fermentation

Mycelia of *A. niger* CBX-209 were grown on glucose (or LG) medium for 24 h and were transferred to an LG (or glucose) medium and cultivated. Samples during fermentation were centrifuged and washed three times with 50 mM extraction buffer composed of 50 mM CHES (pH 9.3), 1 mM β-mercaptoethanol, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The cells were then ground in liquid nitrogen with a mortar and pestle. The cell powder was resuspended

by the addition of the same extraction buffer at the rate of 3 ml per gram of wet cells, briefly ground again and then centrifuged at 18,000 × g. The supernatant was assayed to determine LG-kinase activity.

3. Results and discussion

3.1. Citric acid fermentation on LG

3.1.1. Optimization of the carbon and nitrogen concentration for *A. niger* CBX-209

Previous studies with *A. niger* CBX-2 suggested that an initial corn starch concentration between 13.5% and 14.4%, equivalent to glucose concentration between 15% and 16%, was optimum for maximum citric acid production [15]. As further studies showed that an initial glucose and wheat bran concentration of 8% and 2%, respectively, was optimum for citric acid conversion, the LG concentration was first fixed at 8% to determine the optimum nitrogen (wheat bran) concentration for this mutant (Table 1). Citric acid yield increased (while residual LG decreased) with an increase in the initial wheat bran concentration over a range of 1–3%, and a conversion rate as high as 87% was obtained at a concentration of 3%, after which, although the amount of LG utilized during fermentation continued to increase, there was a decline in the citric acid yield.

Since results above showed that the optimum nitrogen concentration in the LG medium for this mutant was higher than that in glucose medium, further experiments were carried out to verify whether initial LG concentration of 8% was optimal for this mutant. By fixing the initial wheat bran concentration at 3%,

Table 1
Effect of wheat bran concentration on citric acid production by *A. niger* CBX-209

Initial wheat bran (%)	Residual LG (%)	Citric acid yield (%)
1.0	2.90	45.0
2.0	2.03	66.9
2.5	1.12	78.3
3.0	0.30	87.0
3.5	0.25	60.1
4.0	0.15	43.8

Table 2

Effect of LG concentration on citric acid production by *A.niger* CBX-209

Initial LG (%)	C/N ratio	Residual LG (%)	Citric acid yield (%)
4	6.7:1	0.43	68.8
6	10.0:1	0.33	82.5
8	13.3:1	0.28	87.5
10	16.7:1	3.40	55.0
12	20.0:1	8.90	12.1

Fig. 1. Effect of initial pH and incubation period on citric acid fermentation on LG by *A. niger* CBX-209. (■) pH, and (●) incubation periods.

the optimum initial LG concentration was estimated (Table 2). Table 2 showed that increasing the initial LG concentration from 4% to 8% resulted in an increased conversion of LG into citric acid, but beyond this point, the yields decreased sharply, as was the case with both CBX-209 and CBX-2 glucose (citric acid) fermentation (data not shown). Optimum initial sugar concentration for *A. niger* CBX-209 in the LG medium was just the same as that in glucose medium, but best conversion of LG into citric acid occurred at a C/N ratio of 13.3:1 compared with that of 20:1 in glucose medium.

3.1.2. Effect of initial pH, incubation temperature and time

The optimum pH for citric acid production by this mutant in LG medium was examined over a range of 3.0–7.0 (Fig. 1). This mutant had no growth at pH 3.0. Citric acid production occurred with increasing

Fig. 2. Detection of the formation of glucose from LG in cell extracts and culture fluids at 30°C and an initial LG concentration of 100 mM. (■) cell extracts (pH 7.1), (●) culture fluid (pH 7.1) and (▲) culture fluid (pH 2.0).

pH, with maximum citric acid production occurring between pH of 5.5 and 6.0.

Experiments carried out similarly (not shown) to test the effect of incubation temperature revealed that the optimum temperature was 35°C, which is nearly the same as that in the glucose media.

Fig. 1 also gave the time course of CBX-209 shake fermentation. Citric acid concentration reached the maximum value on the 5th day of incubation at 35°C, after which there was a slight decline.

3.2. Detection of LG-kinase in *A. niger* CBX-209

3.2.1. Absence of LG-hydrolyzing activity in *A. niger* CBX-209 during its citric acid fermentation

Since LG can be hydrolyzed to glucose at low pH, experiments were carried out to verify whether *A. niger* CBX-209 employed a special type of LG-hydrolyzing enzyme to utilize LG as carbon and energy source during its citric acid fermentation at a low pH of about 2 (Fig. 2). Both cell extracts and culture fluids were assayed. Fig. 2 shows that there was no formation of glucose when cell extracts or culture fluids were incubated with LG in pH 7.1 buffer systems. In other buffer systems at pH 5, 8 or 9.3, results were almost the same (data not shown). In culture fluids of pH 2 (Figs. 2 and 3), glucose concentration increased steadily with an increase in both time and temperature. However, the increase of glucose concentration was due to acid hydrolysis and was not caused by LG-hydrolyzing enzyme, because

Fig. 3. Detection of glucose from LG in culture fluids of pH 2 at an initial LG concentration of 100 mM.

Fig. 4. Detection of glucose 6-phosphate by HPLC equipped with an ODS-18C column.

there would have been a peak of enzyme activity with the increase of temperature if this special enzyme was present.

3.2.2. Verification of LG kinase

The reaction mixture composed of cell extracts and LG at pH 9.3, in which no formation of glucose was detected, was mixed with 2 mM ATP and 10 mM MgCl₂, and then was incubated for 30 min at 30°C. The product was analyzed by HPLC (Fig. 4). The first peak eluted had the same retention time as that of a standard glucose 6-phosphate. Using a glucose 6-phosphate dehydrogenase coupling system, formation of glucose 6-phosphate in the reaction

Fig. 5. Detection of glucose by HPLC (equipped with an ODS-18C column) formed by the addition of glucose 6-phosphatase to the reaction mixture composed of LG and partially purified LG phosphorylation enzyme.

mixture above containing ATP and Mg²⁺ could also be detected. Furthermore, after incubating for 2 h, no LG could be detected and the concentration of glucose 6-phosphate reached its maximum value.

Further experiments were carried out to characterize this LG phosphorylation enzyme and the product. In these experiments, LG-phosphorylating enzyme, partially purified by an ion-exchange chromatography, was used instead of the crude cell extracts in order to eliminate the effect of phosphoglucomutase and 6-phosphoglucuronate dehydrogenase on the LG-phosphorylation enzyme detection. After a complete reaction for 2 h, the reaction mixture was stopped by heating in a boiling water bath for 5 min, mixed with glucose 6-phosphatase, incubated for 30 min at 30°C, and the product was detected by HPLC (Fig. 5). The second peak eluted in Fig. 5 had the same retention time as that of a standard glucose. Since this product could also be detected by glucose oxidase and peroxidase coupling system (data not shown), it was considered to be glucose.

Results above showed that the activity of this enzyme acting on LG depended on the presence of Mg²⁺ and ATP, and that the product formed was glucose 6-phosphate. Therefore, *A. niger* CBX-209 should have one active protein phosphorylating LG, that can be considered to be an LG kinase, during its citric acid production.

Fig. 6. Time-course changes in LG kinase and hexokinase activities of *A. niger* CBX-209 during citric acid fermentation on LG. (■) Growth, (●) LG kinase activity and (▲) hexokinase activity.

Fig. 7. Time-course changes in LG kinase and hexokinase activities of *A. niger* CBX-209 during citric acid fermentation on glucose. (■) Growth, (●) LG kinase activity and (▲) hexokinase activity.

3.3. Regulatory properties of LG kinase and hexokinase in *A. niger* CBX-209

A. niger CBX-209 precultured on glucose for 24 h was transferred and cultivated on the LG medium with shaking at 35°C and LG kinase and hexokinase activities were calculated (Fig. 6). From this figure, it can be seen that LG kinase activity increased along with the cell growth, reached the maximum at the late exponential growth phase, and then decreased. Also, as shown in Fig. 6, the specific activity of LG phosphorylation exceeded that of glucose phosphorylation. These results indicated the inductive nature of LG kinase synthesis and the constitutive nature of hexokinase synthesis, respectively. Fig. 7 showed the time-course changes of LG kinase and hexokinase in *A. niger* CBX-209 growing on glucose. When the cells

grown on LG were used as the seed culture, LG kinase activity decreased rapidly until the middle exponential growth phase on glucose, and then maintained at a low level during the fermentation. It can also be seen from this figure that although both LG kinase and hexokinase activities decreased until the middle exponential growth phase, the rate of decrease in LG kinase was larger than that in hexokinase activity. Thereafter, hexokinase activity increased gradually.

4. Conclusion

The development of mutant CBX-209 for citric acid production has shown the possibility of evolving appropriate fungal mutants for citric acid production in the LG medium. For other fungi that could grow on LG, development of their mutants might also be necessary, because low or even no yields of their useful metabolites might be produced when using LG as carbon and energy source.

The mutant, *A. niger* CBX-209, needed a higher nitrogen concentration to achieve maximum citric acid yield in an LG medium than that on glucose medium. On the other hand, like glucose, high concentrations of LG > 8% were inhibitory to citric acid production. Further studies for application to fed-batch culture might be expected to improve the production efficiency of this mutant and to lower the cost.

As to the metabolism of LG during its citric acid fermentation, although LG could be easily hydrolyzed to glucose with acid, LG-hydrolyzing activity could not be detected, either in cells of this mutant or in its culture fluids. Otherwise, direct phosphorylation to glucose 6-phosphate from LG was detected by HPLC in the reaction with the cell extracts or partly purified enzyme. Since this enzyme also needed ATP and Mg^{2+} like hexokinase in this mutant, it was believed to be a special kind of kinase, i.e. LG kinase.

LG kinase and hexokinase in *A. niger* CBX-2 were regulated in different ways when it was grown on different carbon sources. The level of LG kinase activity changed depending upon the carbon sources used, showing an inductive nature of its synthesis by LG. Since LG kinase had quite a strict substrate specificity and phosphorylated LG exclusively, it could not contribute to citric acid production in the glucose medium. However, in an LG medium, although hexokinase had

no effect on LG, it might have a slight contribution to the overall citric acid conversion, because a little amount of LG was converted into glucose during fermentation, especially when pH of the culture was lowered to about 2. *A. niger CBX-2* cultivated in LG medium needed a longer fermentation time to consume the substrate, possibly owing to the slower rate for this mutant to take LG into the inner cells than that of glucose.

5. Patent

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For further reading

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