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Production of L-Glutamate Oxidase and *In Situ* Monitoring of Oxygen Uptake in Solid State Fermentation of *Streptomyces* sp. N_1

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ABSTRACT

Effects of water content and carbon and nitrogen sources on the production of L-glutamate oxidase (GOD) by solid state fermentation (SSF) of *Streptomyces* sp. N_1 were investigated in a 250-mL shake flask. The results show that in the solid medium containing wheat bran 98% (w/w), KCl 0.2% (w/w), and $MgCl_2$ 0.2% (w/w), addition of 2.0-mL water per gram solid medium and 0.4% (w/w) $(NH_4)_2SO_4$ was the best for GOD production. In this work, we also developed a simple technique for *in situ* measuring oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) in SSF in a shake flask based on the principle of Warburg manometer. The method was successfully applied to determine OUR and CER values in SSF of *Streptomyces* sp. N_1. The results indicate that the largest OUR value was detected about one or two days ahead of the highest GOD activity reached depending on the fermentation conditions, and the OUR may be used as an *in situ* indicator of GOD production in the SSF process.

Index Entries: Carbon and nitrogen sources; L-glutamate oxidase production; *in situ* monitoring of oxygen uptake; solid state fermentation; *Streptomyces* sp. N_1; water content.

INTRODUCTION

After World War II, submerged fermentation (SmF) of microorganisms became very popular in the fermentation industry for the production of antibiotics, ethanol, organic acid, and other useful substances. In con-

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trast to the rapid progress in SmF technology, solid state fermentation (SSF), which had been used for thousands of years, has shown slow progress and very limited application. However, compared with SmF, SSF does have some advantages, such as simplicity of fermentation equipment and lower cost of operation. In addition, SSF has attractive applications in the fields of biomass energy conversion, solid waste treatment or composting, and in the food industry.

L-Glutamate oxidase (GOD) is a relatively newly discovered enzyme *(1–4)* which specifically catalyzes a reaction which forms one mole of hydrogen peroxide, one mole of α-ketoglutaric acid and one mole of ammonia from one mole of L-glutamic acid, one mole of oxygen, and one mole of water. It has been used to determine L-glutamate or to construct biosensor for L-glutamate determination *(5)*, which is important in L-glutamate fermentation industry, food and clinical chemistry for fermentation process measurement, evaluation of food quality and diagnosis of several kinds of diseases *(3)*.

Until now, there are only a few reports regarding the isolation, purification, and production of GOD from different sources, and the microbial fermentation studies for GOD production are scarcely reported *(1–4)*. In our laboratory, a strain of *Streptomyces* sp. N_1 was isolated from soil *(6)*, which can produce extracellular GOD in SSF. Although SSF with molds or yeasts has been widely studied and used for production of many important substances such as enzymes and ethanol *(7)*, reports on SSF with filamentous prokaryotes are very few. In this paper, effects of water content and carbon and nitrogen sources on the GOD production in SSF by *Streptomyces* sp. N_1 were investigated. In addition, we note that there is yet no research regarding the *in situ* monitoring of oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) of solid state fermentation in shake flasks. In this work, based on the principle of Warburg manometric method we developed a simple and convenient system for *in situ* monitoring of the parameters of OUR and CER of SSF in a shake flask, and successfully applied the system to the SSF process for L-glutamate oxidase production by *Streptomyces* sp. N_1.

MATERIALS AND METHODS

Microorganism

Streptomyces sp. N_1, which was isolated from soil for GOD production *(6)*, was used in this work. It was propagated on wheat bran agar slants for 7 d at 28°C. For short-term storage, the slants were maintained at 4°C.

Solid State Fermentation

Solid state medium components used (% w/w, dry matter) were: 98% wheat bran, 0.2% KCl and 0.2% $MgCl_2$. The solid medium of 4 g was mixed

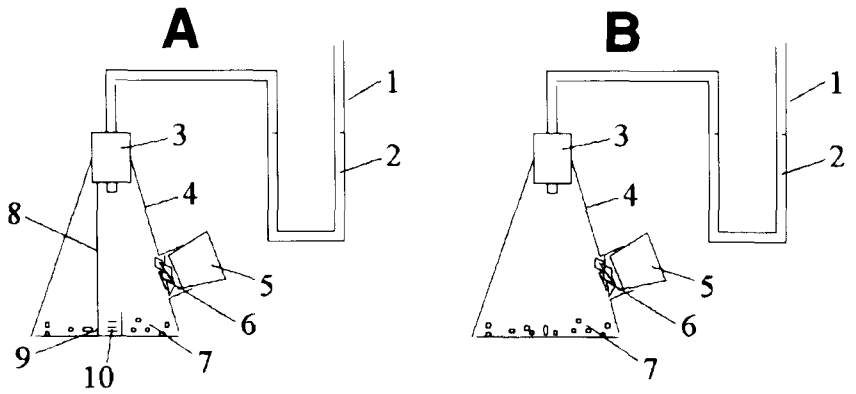

Fig. 1. Experimental system for *in situ* measuring oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) of solid-state fermentation (SSF) in a shake flask. A, system with a 20-mL alkaline solution-containing bottle; B, system without the bottle. 1, liquid column (glass tube); 2, water; 3, rubber bung; 4, 250-mL shake flask; 5, rubber bung; 6, cotton plug; 7, solid media; 8, stainless steel wire for securing of the bottle 9; 9, 20-mL bottle; 10, 10% KOH solution (ca. 3-mL).

with certain amount of water as required for investigation and then sterilized for 30 min at 121°C. The initial pH was adjusted to 7.0 with 1*M* NaOH solution. An inoculum of 10^8 spores in 1 mL water per 250-mL shake flask (containing 4 g dry matter) was used. Fermentation was carried out for 6–10 d at temperature of 28°C and relative air humidity of 96%.

Enzyme Assay

GOD activity was determined according to peroxidase catalyzed chromogenic reaction by modification of the method as described by Ishikawa et al. *(3)*. The reaction mixture was composed of: 1 mL 4-aminoantipyrine (2 m*M*), 2 mL phenol (3 m*M*), 0.1 mL horseradish peroxidase (60 U/mL) and 0.1 mL sample. The reaction was carried out at 37°C for 10 min, and the absorbance was measured at 505 nm. For enzyme activity, one unit was defined as the amount of enzyme which catalyzed the formation of 1 μmol hydrogen peroxide per min under the reaction conditions as described.

Development of a System for *In Situ* Measuring OUR and CER of SSF in a Flask

As shown in Fig. 1, an experimental system for *in situ* monitoring OUR and CER of SSF in a flask was developed based on the principle of Warburg manometer *(8)*. The whole system set in an incubation shaker is under temperature-controlled environment during fermentation, the microorganisms take up oxygen and give out carbon dioxide, that is rapidly absorbed by the alkaline solution in the little bottle in the flask (Fig. 1A). When OUR and CER are measured, the side arm of flask is closed with

rubber bung (i.e., "5" in Fig. 1). Thus, the OUR value can be obtained by monitoring the dynamic decrease of the pressure in the closed system, which is resulted from the consumption of oxygen inside the flask. The calculation formula are as follows:

$$\text{OUR} = K \cdot \mathrm{d}h / \mathrm{d}t \tag{1}$$

$$K = \rho \cdot g \cdot V / RT + S \cdot P / RT \tag{2}$$

where h is the height of liquid column in manometer; t is time; ρ is the density of the liquid in manometer; g is acceleration of gravity (a constant); V is the gas volume inside the whole system (determined as described below), R is gas constant, T is the temperature; S is the square of liquid column (at the right side) of the manometer; P is air pressure.

The value of V is determined by subtracting the volume of liquid in vessel(s) (i.e., 250-mL flask for Fig. 1B, and both 250-mL flask and 20-mL bottle for Fig. 1A) from the total volume within the system (Fig. 1). The total volume within the system is determined as follows: In a temperature-constant room (28°C), the clean dry whole system is weighed, and it is then filled with distilled water that has been boiled to remove dissolved gases and cooled to 28°C. Water is added until the level just reaches the reference point on closed arm of manometer, then the whole system is weighed. The water density at this temperature can be ascertained, thus the volume of the whole system can be calculated. To improve the accuracy of the method, in our case the V value is determined for different flasks, and the rubber bung used to seal the system is pushed in tightly to give the same internal volume for a specific flask.

For system B (Fig. 1B), the change of pressure inside the system is determined by the consumption of oxygen over the production of carbon dioxide. That is,

$$\text{If CER} > \text{OUR},\ K \cdot \mathrm{d}h/\mathrm{d}t = \text{CER} - \text{OUR} \tag{3}$$

$$\text{If OUR} > \text{CER},\ K \cdot \mathrm{d}h/\mathrm{d}t = \text{OUR} - \text{CER} \tag{4}$$

Because the OUR value is available from system A, the value of CER can be obtained from Eq. (3) or (4).

RESULTS AND DISCUSSION

Effect of Water Content on OUR and GOD Production

It is well known that the water content of substrate is an important factor affecting both cell growth and product synthesis in SSF *(7)*. Water causes swelling of the solid substrate and facilitate a good utilization of substrate by microorganisms. Figure 2 shows the dynamic changes of

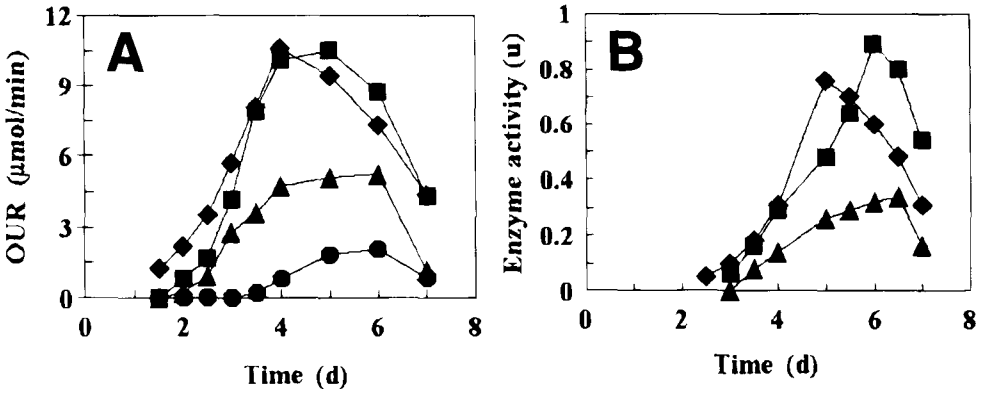

Fig. 2. Effect of water content in solid medium (4 g) on OUR and activity of L-glutamate oxidase (GOD) in SSF of *Streptomyces* sp. N_1 in a 250-mL flask. Symbols for water content (mL/g solid media): Circle, 0.75; triangle, 1.25; square, 2.0; rhombus, 3.5.

OUR value and GOD activity in the SSF process of *Streptomyces* sp. N_1 in media with different water contents. After 36-h fermentation, the OUR value determined in the medium with water content of 3.5 mL/g was 1.26 μmol/min but nearly zero in the other three media. The appearance of the maximum OUR value in the medium with water content of 3.5 mL/g (medium 1) was about one or two days ahead of that obtained from the media with water content of 2.0 mL/g (medium 2), 1.25 mL/g (medium 3) or 0.75 mL/g (medium 4). The OUR value obtained from the flask fermentation was increased with an increase in water content within 2.0 mL/g. Similar results have been reported in SSF of *Aspergillus niger* growing on cassava starch and several other filamentous fungi *(7,9)*. The value of CER is close to that of OUR, thus the value of respiratory quotient (RQ, i.e., CER/OUR), is near 1 (data not shown). GOD production may be related to the OUR value. GOD activity appeared at an OUR around 3.5 μmol/min and reached the maximum about one day later than the time when OUR reached the maximum. In addition, similar to OUR, the enzyme activity that appeared in medium 1 was nearly a half or one day ahead of that determined in the medium 2 or 3 (Fig. 2). The appearance of the maximum enzyme activity in medium 1 was also one or one and a half days ahead of that obtained from medium 2 or 3 (Fig. 2). No GOD activity was detected in medium 4 where the OUR value obtained was the lowest. The largest OUR value and the highest GOD production were both obtained in medium 2 among all the four kinds of media. Medium 2 was better than medium 1 for the microbial growth and enzyme production, although medium 1 possessed a higher water content. This may be caused by that more water limited oxygen transfer as demonstrated by Ramesh and Lonsane *(10)*. In their case, α-amylase production by SSF of *Bacillus licheniformis* M27 was also largely reduced by high water content.

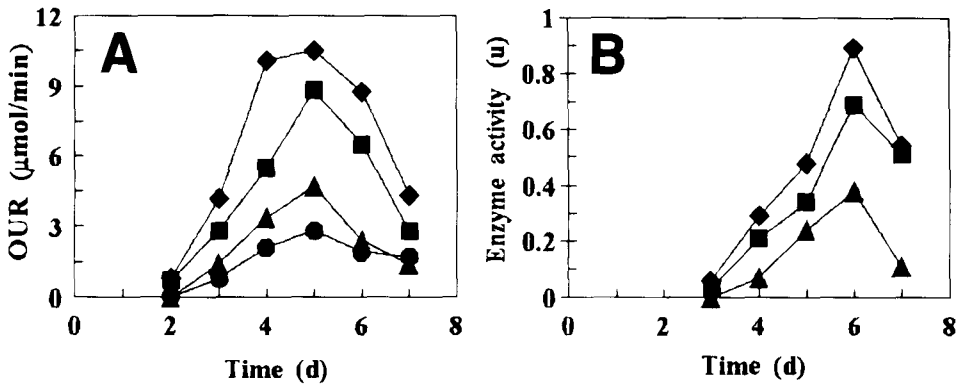

Fig. 3. Effect of glucose concentration in the solid medium on OUR and GOD activity in SSF of *Streptomyces* sp. N_1 in a flask. Symbols for glucose concentration (%, w/w): Circle, 10; triangle, 6; square, 3; rhombus, 0.

Effect of Carbon Source on OUR and GOD Production

The profiles of OUR with addition of various amount of glucose to the solid state medium containing the same water content (2.0 mL/g) are shown in Fig. 3. The OUR value obtained from the medium with a higher glucose addition was smaller than that obtained from the medium with lower or without glucose addition. GOD production also decreased with more addition of glucose, and no GOD activity was detected in the medium containing 10% (w/w) glucose. Several other kinds of carbon sources such as sucrose, glycerol, lactose, and soluble starch were also tested instead of glucose to observe their effects on both OUR value and GOD activity. The result indicates that compared with the control (without addition of carbon source), these carbon sources within 2% (w/w) did not have significant effect on OUR value and GOD production (data not shown).

Effect of Nitrogen Source on OUR and GOD Production

Several kinds of nitrogen sources such as $(NH_4)_2SO_4$, $NaNO_3$ and soybean powder were separately supplemented to the solid medium (containing wheat bran 98%, KCl 0.2% and $MgCl_2$ 0.2%, as a control) to test their effects on the microbial growth and GOD production. The results indicate that compared with the control, relatively larger OUR value and higher GOD production were obtained in the media with addition of 4% (w/w) soybean powder (data not shown) or 0.4% (w/w) $(NH_4)_2SO_4$ (Fig. 4). As shown in Fig. 4, with supplementation of 0.4% (w/w) $(NH_4)_2SO_4$ the maximum OUR and enzyme activity reached were 12 μmol/min after 4 d fermentation and 1.2 U after 6 d fermentation, respectively. However, a further increase of $(NH_4)_2SO_4$ addition (i.e., 0.8%) significantly reduced OUR value and GOD activity. In the SSF with addition of $NaNO_3$ (0.4% or 0.8%, w/w), the medium pH was increased to more than 8.0 after 4 d fer-

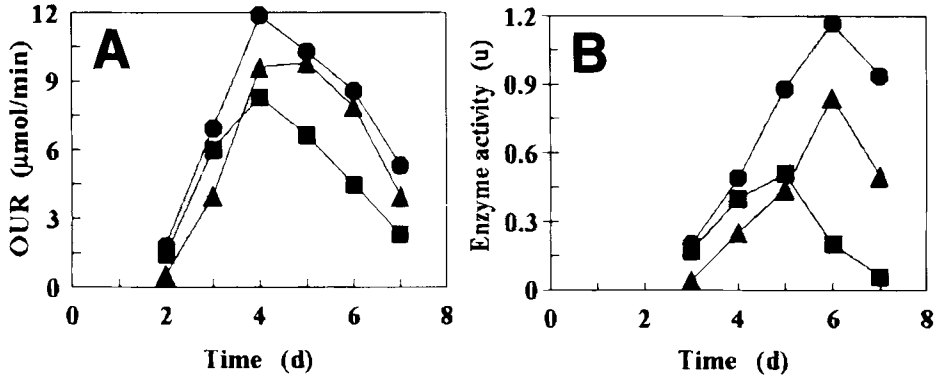

Fig. 4. Effect of $(NH_4)_2SO_4$ addition to the medium on OUR and GOD activity in SSF of *Streptomyces* sp. N_1 in a flask. Symbols for $(NH_4)_2SO_4$ concentration (%, w/w): Triangle, 0 (control); circle, 0.4; square, 0.8.

mentation, and both the enzyme production and OUR value were greatly decreased (data not shown).

CONCLUSIONS

In this work, the effects of water content, carbon and nitrogen sources on OUR and GOD activity were studied in SSF of *Streptomyces* sp. N_1, and the OUR values were shown to be related with the enzyme production. A relatively higher GOD production was accompanied with a larger OUR value, and the time of the maximum OUR value reached was about one or two days ahead of the appearance of the maximum GOD activity depending on the fermentation conditions. Thus, OUR value may be used as an *in situ* indicator of GOD production in the SSF process.

At present, one major problem in a SSF system is how to *in situ* or on-line monitor some important bioprocess parameters, especially microbial growth *(11)*. The technique for *in situ* measurements of OUR and CER as described here is simple and easy to operate. Since the biomass of many kinds of microorganisms can be estimated from the data of OUR or CER *(7,9,12)*, we consider that this OUR and CER measuring system is useful to *in situ* monitor microbial growth in a SSF process in a shake flask. Unfortunately, *in situ* monitoring of the microbial cell mass in our present system is impractical. This is because of the fact that it is difficult for us to separate the mycelia from solid-state medium and difficult to obtain the biomass value, thus it is impossible to establish a correlation between the biomass and OUR (or CER).

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