

## Stability of Glucose Oxidase Activity of *Aspergillus niger* Spores Produced by Solid-State Fermentation and Their Role as Biocatalysts in Bioconversion Reaction

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Received: July 10, 2006

Revised version: January 16, 2007

Accepted: June 15, 2007

### Summary

The aim of this work is to demonstrate the role of conidial spores as a reservoir of glucose oxidase and their stability as a biocatalyst in the bioconversion reaction for the production of gluconic acid. Solid-state fermentation (SSF) was carried out in fixed-bed column bioreactor for the production of *Aspergillus niger* spores. Growth parameters, sporulation and kinetics of gluconic acid production were analysed at different time intervals during the course of SSF. Spores of different age (48–216 h) were used as biocatalysts in the bioconversion reaction. Spores harvested at a later period of SSF (196 h) produced high titres of gluconic acid (30 g/L) in the bioconversion medium when compared to the spores harvested at early (48 h) stages of SSF (2.2 g/L). Spores (harvested at 200 h and stored in freezer for 91 days) exhibited the same glucose oxidase activity and served as an active and stable catalyst when compared to the fresh spores, showing that aging (storage) did not affect enzymes present in the spores, which suggested that the spores acted as an efficient enzyme reservoir. Yields close to 93 % were obtained with 98 g/L of gluconic acid production, corresponding to an average productivity of 1.7 g/(L.h). The stability of the enzyme in the spores and the ability of conidia to be stored for a long time without the loss of activity add specific advantage to the bioconversion process.

*Key words:* *Aspergillus niger*, calcium gluconate, fungal spores, gluconic acid, glucose oxidase, sodium gluconate

### Introduction

Fungal spores are generally utilised for strain conservation and dissemination. In industrial large-scale fermentation, spores are used as inoculum, which after vegetative growth produces primary and secondary metabolites. However, spores could also accomplish a wide range of conversion reactions without being allowed to germinate. Methyl ketone production by *Penicillium ro-*

*quefortii* (1), the reduction of  $\beta$ -keto esters by *Mucor rouxii* and steroid production by *Aspergillus ochraceus* (2) are some of the reactions catalysed by the fungal spores. Our earlier studies (3) showed that the spores of *Aspergillus niger* can be utilised as a catalyst in the bioconversion of glucose to gluconic acid. Gluconic acid is a non-corrosive, non-volatile, non-toxic, mild organic acid, which is produced around 60 000 tonnes annually worldwide. It is widely used in pharmaceutical, cement, tex-

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tile and chemical industries. Oxidation of the aldehyde group on the C-1 of  $\beta$ -D-glucose to a carboxyl group results in the production of glucono- $\delta$ -lactone ( $C_6H_{10}O_6$ ) and hydrogen peroxide. Glucono- $\delta$ -lactone ( $C_6H_{10}O_6$ ) is further hydrolysed to gluconic acid spontaneously. Glucose oxidase ( $\beta$ -D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) catalyses this transformation reaction in fungi. *Aspergillus niger* NRRL 3 is a well known strain producing glucose oxidase and has been widely studied (4).

All known processes described in literature on gluconic acid production deal with the use of vegetative mycelial form of the fungi. The industrial process currently employed for the production of gluconic acid is also based on the use of mycelial inoculum of *A. niger* (5,6). However, Moksia *et al.* (3) showed that the process of gluconic acid production could be carried out by spores replacing the conventional conditions of using vegetative mycelial form. Gluconic acid production by spores could offer potential advantages compared to their corresponding vegetative cells. Fungal fermentation broths using mycelial vegetative forms become highly viscous, leading to difficulties in aeration and mixing of the fermentation medium. Since the reaction is an oxidation process, it would not be efficient if the aeration efficiency was not good. This problem could be overcome by the use of spores, which are relatively smaller in size, and are oval and round in shape, instead of long and fibrous vegetative forms (mycelia) of the same microorganism (7).

Fungal spores can be produced in solid-state fermentation (SSF), since it gives rise to better yields of homogeneous spores (8). SSF supports well the growth of fungi and their spore production due to the accurate values of water activity observed in this system (8,9). There are only a few reports on the production of gluconic acid using SSF. Roukas (10) reported the production of gluconic acid by SSF on figs and achieved a yield of 63 %. Singh *et al.* (11) performed SSF by using HCl-pretreated sugarcane bagasse and the highest level of gluconic acid (107 g/L) with 95 % yield was obtained. However, in this study, SSF was carried out just for the production of spores. Later, these spores were used in liquid medium for the bioconversion of glucose (3).

The aim of the present study is to investigate the kinetics of growth parameters and sporulation behaviour of *A. niger* in SSF in a fixed-bed column fermentor using buckwheat seeds as solid substrate. Also, the ability of *A. niger* spores in acting as a reservoir of the enzyme and their stability and longevity in carrying out the biotransformation was investigated.

## Materials and Methods

### Microorganism

A strain of *Aspergillus niger* NRRL 3 was used in this study. It was maintained on potato dextrose agar medium and subcultured fortnightly.

### Solid substrate preparation

Buckwheat seed, obtained from Fertinature Semences, Montluçon (France) was used as substrate. A mass

of 300 g of buckwheat seeds was washed with distilled water and allowed to cook with equal amount of distilled water in a boiling water bath (100 °C for 15 min) to soften the tissue. After this, the water was drained off; the heat resistant antibiotic chloramphenicol (1 mg/g of dry buckwheat seed) was added to it and the seeds were autoclaved (121 °C at 1.055 kg/cm<sup>2</sup> (15 psi) for 15 min). This gave a material containing initial moisture of 50 g water per 100 g of substrate.

### Inoculum preparation

The spores were dislodged from the 7-day-old PDA Petri dish using distilled water containing 0.1 % (by volume) of Tween 80 under aseptic conditions. A suspension containing 10<sup>8</sup> spores/mL was prepared and used as inoculum (10<sup>8</sup> spores per 100 g of buckwheat seeds). It was aseptically transferred into the SSF medium.

### Fermentor design

Thermostated fixed bed glass column (5 cm i.d., 21 cm height) fermentor with a jacket for circulation of water to control the temperature was used for the study. The column was packed with pre-inoculated substrate with a bed height of 21 cm. The fermentor was supplied with continuous aeration from the bottom of the bioreactor at 0.075 L/min (free of CO<sub>2</sub>, by passing it through a KOH solution column). Cultivation was carried out at 30 °C for 216 h. Sampling was performed at regular intervals of 24 h. Initial moisture of the substrate was 0.5 g H<sub>2</sub>O/g IDM (initial dry mass).

### Analysis

Samples collected from the column at intervals were appropriately diluted with 0.1 % Tween 80 and homogenised with an Ultra Turrax blender. This homogenised mixture was utilised for further analysis. For the determination of starch content, the homogenised mixture was treated at 100 °C for 15 min, followed by the hydrolysis at 55 °C for 20 min with 0.1 mL of 1 g/L of amyloglucosidase (Sigma-Aldrich, France). The supernatant obtained after centrifugation (10 000×g for 5 min) was assayed for reducing sugar using the dinitrosalicylic acid (DNS) method (12). Soluble protein content was measured by using the modified Lowry method (13). Spore count was performed using haematocyte counter (Malassez cell). Carbon dioxide evolution from the fermentor was measured by collecting the effluent gas in 0.8 M KOH, and periodic assay of this solution for its carbonate content using titrimetric method was done (14).

### Recovery of spores for bioconversion

SSF medium was harvested at different time periods and was stored at -20 °C for 48 h. An aliquot of the frozen mass was thawed at room temperature (25 °C). Spore suspension was obtained by adding 0.1 % (by volume) of Tween 80 solution to the thawed medium (concentration of 1 g/mL) and shaken at 180 rpm for 1 h. It was then filtered using gauze cloth to remove the buckwheat seeds. The filtrate was collected and centrifuged at 10 000×g for 10 min. The supernatant was discarded and the pellet was used as spore source.

### Gluconate production

Bioconversion was carried out in 500-mL Erlenmeyer flask with 50 mL of medium containing (in g/L): glucose 40 and sodium azide 0.01. Calcium carbonate slurry (40 g/L) was used as neutralising agent and was separately added to the medium after sterilization, which resulted in calcium gluconate production. The flasks were incubated at 30 °C on a rotary shaker at 200 rpm. The pH of the medium was maintained at 6.5. For sodium gluconate production, a bioreactor (Applikon, the Netherlands) of 1.5-litre working volume was used. The medium contained (in g/L): glucose 100 and sodium azide 0.01. pH was maintained at 6.5 throughout the process with 5 mol/L of sodium hydroxide solution. Bioconversion reaction was carried out at 600 rpm, with aeration rate of 0.075 L/min and temperature of 30 °C. In both cases, spore concentration in the medium was  $10^{10}$  spores/mL.

Glucose was estimated by DNS method (12). Gluconic acid produced was measured by the method of Møllering and Bergmeyer (15) using the Boehringer assay kit (R-Biopharm, France).

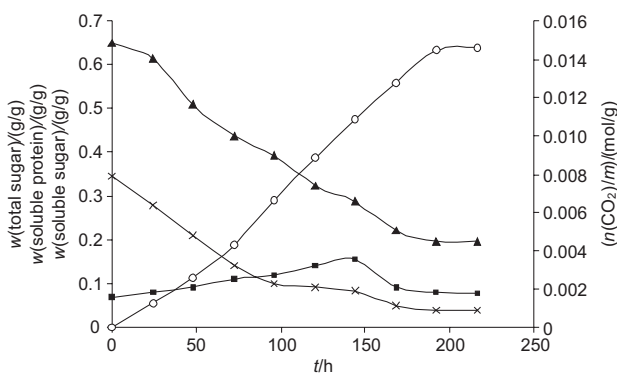
### Stability of spores as reservoir of glucose oxidase

Spores harvested at 200 h were stored in a freezer (−20 °C) for 91 days. Glucose oxidase activity of the spores was investigated by using these spores in the production of sodium gluconate.

## Results and Discussion

### Growth and sporulation behaviour of *A. niger* during solid-state fermentation

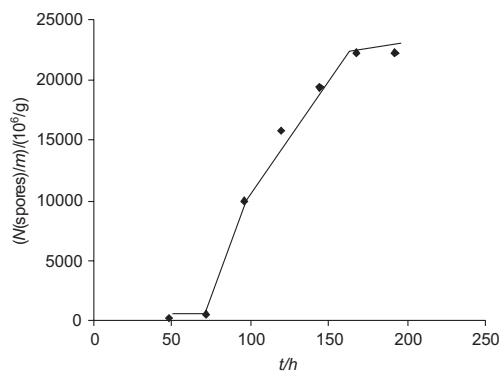
Buckwheat seed is a starchy substrate, with excellent mechanical properties (retention of structure, lack of agglomeration) and known to produce spores of *Penicillium roquefortii* (16). The time course of the consumption of total reducing sugar, free reducing sugar, protein content and carbon dioxide evolution rate are given in Fig. 1. Spore germination took place at the initial hours



**Fig. 1.** Time course of various growth parameters during solid-state fermentation. Total sugar (▲), soluble protein (■), soluble sugar (x) and carbon dioxide evolution (o). Experimental conditions: buckwheat seeds 300 g (wet mass), aeration 0.075 L/min, temperature 30 °C, initial moisture 0.5 g H<sub>2</sub>O/g IDM (initial dry mass)

of SSF, followed by the uniform colonization of the substrate with mycelium. There was active growth phase from 24 to 168 h, when utilization of starch took place. Accumulation of reducing sugars continuously decreased until 168 h, the time at which sugar consumption stopped. This behaviour could be attributed to the water limitation, just like in the case of *Penicillium roquefortii* grown on the same substrate (16). Sporulation started during the active growth phase (72 h), when maturation of the older mycelium took place. Growth and sporulation patterns were homogenous in different segments of the column and there were no differences, as reported by Pandey *et al.* (17). There was an initial lag phase of sporulation extending until 72 h, after which there was a rapid increase of spore production extending until 200 h of the cultivation. At the end of cultivation (196–216 h), spore production reached a stationary phase, along with the decrease of carbon dioxide evolution rate. Profile of the produced CO<sub>2</sub> revealed a strong decrease in the production rate after 196 h, corresponding to the beginning of the stationary phase. Carbon dioxide production could be associated with growth as its release corresponded to the fungal respiration. Its evolution rate continued after the aging of the mycelium too. This might be due to endogenous respiration and to spore formation since not only mycelium was formed during growth of fungi in SSF, but spores were also produced (18).

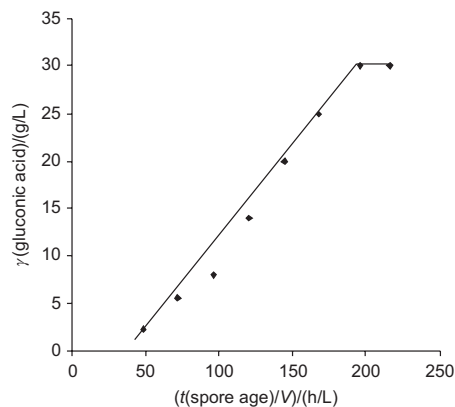
Time course of sporulation of *A. niger* on buckwheat seed is shown in Fig. 2. Sporulation behaviour of filamentous fungi generally depends on the nutrients and moisture content of the medium. It has been reported that nutrient limitation and increased moisture content aid in achieving good sporulation yield (16). However, a nutrient limitation, which is always possible even during an active growth phase, has not been clearly evidenced in this work.



**Fig. 2.** Sporulation of *A. niger* during solid-state fermentation on buckwheat seeds. Experimental conditions as given in the legend of Fig. 1

### Bioconversion carried out with spores recovered at different time periods of solid-state fermentation

Spores obtained at different time of SSF cultivation (48–216 h) were frozen for 48 h and studied for their ability to catalyse the bioconversion of glucose to gluconic acid. Results are shown in Fig. 3. It was interesting to note that the overall initial specific enzymatic activity



**Fig. 3.** Gluconic acid production after 24 h of reaction by the spores recovered at different time periods of SSF, referred to as spore age. It must be pointed out that figures on the y-axis can be related to an average specific activity of the biocatalyst; a concentration of 10 g/L corresponds to an activity of 0.42 mg/h·10<sup>10</sup> spores. Initial glucose concentration was 40 g/L and spore content was 10<sup>10</sup> spores/mL

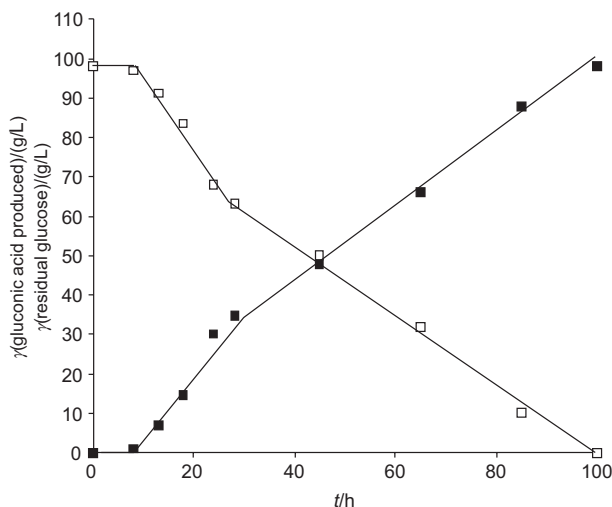
increased with the increase in the age of spores. This feature revealed a heterogeneity in the conidial material, which could result from the presence of at least two kinds of conidia bearing different enzymatic activity. A similar phenomenon has already been reported with *A. candidus* grown on buckwheat seeds, where spores were shown to have different protein contents (19). However, irrespective of the age of spores, product yield was nearly 100%. Spores of 196 h produced 30 g/L of calcium gluconate at 24 h of bioconversion; similar pattern was shown by spores harvested at 216 h. The enzyme produced by the mycelial stage of the fungus was transported to its spores without any loss of enzyme into the solid medium. The transport of the enzyme from the mycelia to the spores was fully accomplished with the aging of the spore. Thus, older spores could carry out better the bioconversion reaction when compared to the younger ones. During SSF, glucose oxidase was likely to be present in both fungal forms (mycelia or spores); however, gluconic acid was not produced in the medium at any time period, which indicated that no enzyme release in the medium took place during the cultivation.

From the study, it was demonstrated that spores of *A. niger* contained an active enzymatic system holding glucose oxidase and all the accessory enzymes such as peroxidase for the production of gluconic acid, similar to its corresponding mycelium. Once the spore germination process was effectively controlled, the spores served as enzyme reservoir and were efficiently used as biocatalyst in the bioconversion reaction directly. Thus, the role of spores as biocatalyst eliminated the extraction and purification process of the enzyme from the spores, making the process simple and economical.

#### Bioconversion using 91-day-old spores

Solid substrate medium after 200 h was kept in frozen dry state (−20 °C) for 91 days; it was thawed (25 °C) and the spore suspension was made to be used for the bioconversion (sodium gluconate), as mentioned before. It was seen that the spores were active, stable and resulted in obtaining a yield of 1.01 g of gluconic acid,

which is close to the stoichiometric value (1.09 g/g). The yield was very similar to that of the spores stored in a freezer for 48 h. Time course of the bioconversion reaction is shown in Fig 4. The rate of gluconic acid production varied with the period of the reaction. There was a negligible amount of gluconic acid conversion until the 8th hour of bioconversion. This lag phase could not be associated with preparative stage or growth, as there was no nitrogen source in the medium and sodium azide was incorporated in the medium as well. Thus, normal spore germination process could not take place and the glucose in the medium was not consumed. After 8 h, there was a constant rate of reaction (8–28 h), which had a value close to 1.7 g/(L·h). Another linear curve of gluconic acid production, with a productivity of 0.91 g/(L·h), was noticed from the 45th hour of reaction extending until the end. These two phases exhibited the same molar yield of 93%. During the course of reaction, no net protein synthesis took place due to the lack of nitrogen in the medium and the presence of sodium azide. During the bioconversion, some of the spores were found sticking onto the walls of the bioreactor. However, this did not affect the rate of conversion significantly, probably because they remained in contact with the reaction medium. The differences in the observed reaction rates could be due to the changes in the rate of transfer of substrate and product in and out of the spores.



**Fig. 4.** Bioconversion carried out with 91-day-old spores recovered at 200 h of solid-state fermentation. Residual glucose (□) and gluconic acid production (■) at 24 h of bioconversion. Spore content was 10<sup>10</sup> spores/mL

The longevity of spore products has a direct consequence of their high resistance towards external factors (20). Spores of *A. ochraceus* were stable for one year at −20 °C without any detectable loss of hydroxylating activity (21).

#### Conclusions

Conidia of *A. niger* acted as a reservoir of enzyme glucose oxidase. This study brings into light the possibility of exploitation of *A. niger* spores as a stable catalyst in the bioconversion of glucose to gluconic acid. The



test carried out to analyse the feasibility of the direct use of *A. niger* spores as biocatalyst after their storage for longer duration in the bioconversion reaction was successful. This process could minimise the steps such as enzyme extraction from the spores and its further purification. The stability of the enzyme, which can be attributed to the lack of active protease in the conidia, and the longevity of spore stability add advantage to the process. It is simple, versatile, easy to handle, reliable and economical. This could serve as an innovative process and could compete efficiently with the conventional use of mycelial form of the fungus in gluconate production.

### Acknowledgements

Sumitra Ramachandran acknowledges the Doctoral Fellowship awarded by French Embassy, India.

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