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Fed-batch Production of Gluconic Acid by Terpene-treated *Aspergillus niger* Spores

Sumitra Ramachandran · Pierre Fontanille · Ashok Pandey · Christian Larroche

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Abstract *Aspergillus niger* spores were used as catalyst in the bioconversion of glucose to gluconic acid. Spores produced by solid-state fermentation were treated with 15 different terpenes including monoterpenes and monoterpenoids to permeabilize and inhibit spore germination. It was found that spore membrane permeability is significantly increased by treatment with terpenoids when compared to monoterpenes. Best results were obtained with citral and isonovalal. Studies were carried out to optimize spores concentration (10^7–10^{10} spores/mL), terpene concentrations in the bioconversion medium and time of exposure (1–18 h) needed for permeabilization of spores. Fed-batch production of gluconate was done in a bioreactor with the best conditions [10^9 spores/mL of freeze-thawed spores treated with citral (3% *v*/*v*) for 5 h] followed by sequential additions of glucose powder and pH-regulated with a solution containing 2 mol/L of either NaOH or KOH. Bioconversion performance of the spore enzyme was compared with the commercial glucose oxidase at 50, 60, and 70 °C. Results showed that the spore enzyme was comparatively stable at 60 °C. It was also found that the spores could be reutilized for more than 14 cycles with almost similar reaction rate. Similar biocatalytic activity was rendered by spores even after its storage of 1 year at −20 °C. This study provided an experimental evidence of the significant catalytic role played by *A. niger* spore in bioconversion of glucose to gluconic acid with high yield and stability, giving protection to glucose oxidase.

Keywords *Aspergillus niger* · Fungal spore · Gluconic acid · Glucose oxidase · Citral · Monoterpenes

S. Ramachandran · P. Fontanille · C. Larroche (✉)
Laboratoire de Génie Chimique et Biochimique (LGCB), Polytech'Clermont
Ferrand–Université Blaise Pascal, 24, avenue des Landais, B.P. 206, Aubière Cedex 63174, France
e-mail: christian.larroche@univ-bpclermont.fr

A. Pandey
Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR,
Trivandrum 695 019, India

Introduction

Spores play an important role in the life cycle of fungi as an agent for reproduction. They are known to be highly resistant to extreme environmental conditions (high temperature, high pressure, toxic chemicals, and mechanical shocks) due to their exceedingly rigid structure [1]. Properties, advantages, and various applications of spores are summarized in the review by Ramachandran et al 2007 [2]. Although spores were considered as dormant and metabolically inert, several studies suggested that the spores contained enzymes as in the vegetative cells at a comparable level of activity [3]. Thus, the interest of exploiting spores as enzyme bags and promising biocatalysts developed. Replacement of vegetative cells by spores certainly possesses several advantages. In fermentation or biotransformation processes, use of spore instead of mycelium results in less viscous medium, further leading to easier aeration and product recovery. Moreover, spores are generally easier to store, and the lack of pellet formation results in greater homogeneity of the biocatalyst [4]. In some cases, biotransformation reactions are carried out exclusively by spores; in others, vegetative cells perform the biotransformation less efficiently and for a considerably shorter period of time. Other significant advantages include easier recovery of the products, elimination of the lag period, reuse of spores as catalyst for many cycles, and elimination of the production of other undesired products.

Gluconic acid is produced from glucose through a simple oxidation reaction catalysed by glucose oxidase. It is a multifunctional organic acid applied in food, detergent, pharmaceutical, and construction industries. Worldwide consumption of gluconic acid was about 87,000 tonnes in 2004 with the total market value of about US$333 million (Business communication Co., 2004). Properties, production techniques, and applications are summarized in the review by Ramachandran et al 2006 [5].

Our initial study demonstrated that *Aspergillus niger* spores acted as a reservoir of enzyme glucose oxidase [6]. With our previous investigations [7–9] as background, we further studied on the improvement of the process to utilize *A. niger* spores as catalyst in the bioconversion of glucose to gluconic acid. Bioconversion could not be carried out by fresh unpermeabilized spores and gluconic acid produced by them was close to zero. Hence spores should be permeabilized to enhance the transfer rate to act as catalyst in the bioconversion of glucose to gluconic acid [7]. Moreover, spore germination should be completely inhibited during the reaction to attain a high yield. So as to maintain these two conditions, sodium azide, an inhibitor of respiratory chain was used which prevented spore germination, and freezing was used to permeabilize spores. These treatments resulted in higher molar yields and with reaction rate of 1.5 g/L h [7]. Glucose oxidase activity of spore was found to be stable and active for a long time (3 months) without loss of the enzyme activity adding specific advantage to the bioconversion process [8] however, later studies showed that sodium azide was as an inhibitor to glucose oxidase [9]. Thus it was replaced by citral, a monoterpenoid, which could successfully inhibit spore germination and permeabilize spores. Here, in this study, we tried using several monoterpenes and terpenoids to treat spores before bioconversion. Further, fed-batch production of gluconic acid was carried out.

Solid state fermentation (SSF) was performed for the production of spores. SSF is a well-suited fermentation technique for spore production, as it often gives rise to better yields of homogenous and high volume of spores [10]. SSF has been termed as more efficient with particulate substrates. Buckwheat seeds were used as substrate for the study due to its particulate nature. It is a starchy substrate, with excellent mechanical properties (retention of structure, lack of agglomeration). Apart from its starchy nature, it also has high

protein content than rice, wheat, millet, maize, and sorghum. Amino acid in buckwheat protein is well-balanced and is rich in lysine and arginine [11].

Materials and Methods

Microorganism

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

Solid Substrate Preparation

Buckwheat seed, obtained from Fertinature Semences, Montluçon (France), was used as substrate. Buckwheat seeds (300 g) were 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 tissues. After this, water was drained off and the seeds were autoclaved (121 °C at 15 psi for 15 min). This gave a material containing initial moisture of 50 g water/100 g substrate.

Spores were dislodged from the 7-days-old PDA Petri dish using distilled water containing 0.1% (*v*/*v*) Tween-80 under aseptic conditions. A suspension containing 10^8 spores/mL was prepared and used as inoculum (10^8 spores/100 g of buckwheat seeds). It was aseptically transferred into the SSF medium.

Fermentor Design

Thermostated fixed bed glass column fermentor (inner diameter, 5 cm; height, 21 cm) 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^{-1} (free of CO_2, by passing it through a KOH solution column). Cultivation was carried out at 30 °C for 200 h.

Recovery of Spores for Bioconversion

Spores were harvested at 200 h of SSF and a spore suspension was obtained by adding 0.1% (*v*/*v*) Tween 80 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 spores collected were dispensed in distilled water.

Gluconic Acid Production

Bioconversion was carried out in 250 mL Erlenmeyer flask with 25 or 50 mL medium containing glucose (50 g/L). Calcium carbonate slurry (50 g/L) was used as neutralizing agent and was separately added to the medium after sterilization. The flasks were incubated at 30 °C on a rotary shaker at 200 rpm. The pH of the media was 6.5. Fed-batch production of gluconic acid was carried out in 2-L fermenter (Biostat Braun, Germany). Medium contained only glucose with an initial concentration of 100 g/L and periodically glucose

was added in powder form. pH was continuously adjusted to 5.8 with a 2 M sodium hydroxide solution or potassium hydroxide. The reactor was fed with air free of CO_2 by passing it through a KOH pellets column. The aeration rate was 0.15 slpm and stirring speed was 600 rpm. Unless and otherwise mentioned spore concentration in the media was 1×10^9 spores/mL.

Use of Different Terpenes as Permeabilizing and Spore-Inhibiting Agent

Fresh spores (10^{10} spores/mL) after the recovery from SSF medium were treated with terpenes such as alpha pinene, alpha pinene oxide, beta pinene, beta pinene oxide, carvone, carveol, citronellal, limonene, limonene oxide, linalool, myrcene, myrtenol, isonovalal, orcinol, and terpineol. Spores were treated with 10% of these chemicals for 18 h at 30 °C in a rotary shaker at 200 rpm unless and otherwise mentioned. Bioconversion was carried out with 25 mL of medium by adding the substrate and neutralizing agent as mentioned above.

Fresh and frozen spores (10^9 spores/mL) were treated with different concentrations of citral (2%–5%) for different time period (2–6 h) for optimizing bioconversion conditions. Further bioconversion was carried out with various concentrations of spores (10^7–10^{10} spores/mL) using the optimized concentration of citral.

Effect of Terpenes on the Glucose Oxidase Activity at Different Temperatures

Citral, limonene, and isonovalal at the concentration of 1% and 10% were added to the solution containing commercial glucose oxidase (1 g/L). A control experiment was carried out with commercial glucose oxidase prepared in distilled water without addition of terpenes. Bioconversion was started by the addition of substrate and the neutralizing agent as mentioned above. Also, bioconversion was carried out with citral-treated spore and commercial glucose oxidase (control) at 50, 60, and 70 °C.

Analysis

Glucose was estimated by dinitrosalicylic acid method [12]. Gluconic acid produced was measured by the method of Moellering and Bergmeyer [13] using the Boehringer assay kit (R-Biopharm, France). Spore count was performed using hematocyte counter (Malassez cell). Measurement of O_2 consumption and CO_2 production rates were done by a gas analyser Servomex Xentra 4100 (Servomex Company Inc, Norwood, MA, USA). Scanning electron microscopy (SEM) tasks were prepared by utilizing JEOL JSM-820 (Tokyo, Japan) equipped with secondary electron detector.

Results and Discussion

Use of Different Terpenes as Permeabilizing and Spore-Inhibiting Agent

Monoterpenes such as myrcene, limonene, α-pinene, and β-pinene and terpenoids such as linalool, orcinol, myrtenol, terpineol, carveol, carvone, isonovalal, citronellal, α-pinene oxide, β-pinene oxide, and limonene oxide were tested for their ability to permeabilize and inhibit spores for the use as catalyst in the bioconversion of glucose to gluconic acid (Figs. 1 and 2). Out of all the terpenes tested isonovalal and terpineol permeabilized the spores better and resulted in higher bioconversion rate. However no terpene could

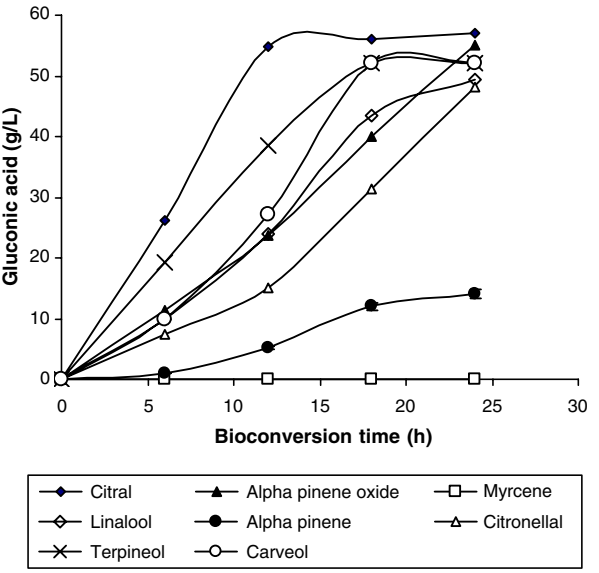

Fig. 1 Bioconversion of glucose to gluconic acid by spores permeabilized by some monoterpenes and monoterpenoids

permeabilize spore as that of citral. Cytoarchitectural difference was visualized in the images captured by SEM of the spores treated by citral and isonovalal (data not shown). It was interesting to note that monoterpenoids (α-pinene oxide, β-pinene oxide, limonene oxide) permeabilized spores better than monoterpenes (α-pinene, β-pinene, limonene). It was seen that terpenoids could basically permeabilize and inhibit the germination of spores irrespective of its different functional group such as alcohol (terpineol), aldehyde (citral, isonovalal), etc. Basically, monoterpenes are hydrocarbons consisting of multiple

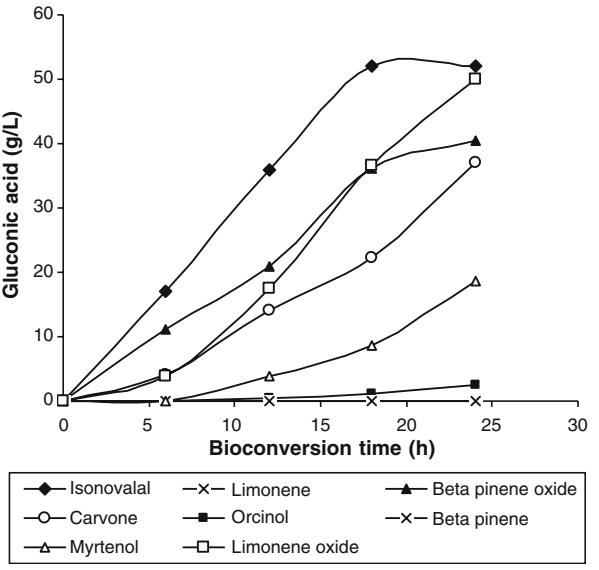

Fig. 2 Bioconversion of glucose to gluconic acid by spores permeabilized by monoterpenes and monoterpenoids

isoprenoids. They are of hydrophobic nature and studies on their antimicrobial action generally focus on cell membrane as primary target [14]. Owing to their lipophilic nature, they appear to accumulate in the microbial cell membrane (hydrophobic part) and play an important role in the mechanism of the toxic action [15] and also increase their permeability, resulting in leakage of enzymes and metabolites. However, in this case glucose oxidase was not excreted out from the spores. Terpenes acted on the spore membrane and increased the membrane fluidity, further helping in the transfer of the substrate and product in and out of the spore. Penetration of apolar compounds across the membrane is a simple diffusion process. Accumulation of such lipophilic compounds enhances their availability to the cell causing their toxicity. Even when citral was removed from the spores and resuspended in fresh glucose solution, spores were unable to germinate. This showed that citral was adhered to the hydrophobic spore and was able to stop the growth. However fungistatic effect was reversible when citral-treated spores were plated onto PDA. Thus, citral was selected to treat spores before bioconversion for further studies. Citral is a mixture of two geometric isomers, geranial (*trans* confirmation, approx. 55%–70%) and neral (*cis* confirmation, 35%–45%). There are reports where citral was tested as antifungal and antibacterial agent [16, 17].

The mechanism of action of terpenes cited in the literature comments that terpenes affect the proteins and specific enzymes. But in this case, glucose oxidase activity of the spore was not affected. Terpenes had an effect only on the viablility of *A. niger* spores and not on the biocatalytic activity. This was contrary to the result of Wolken et al 2002 [18], where terpenes were found to have effect on both the viability and the enzyme activity.

Further studies were carried out to optimize its concentration and time of exposure to inhibit germination and permeabilization. Both spores initially permeabilized by freezing thawing process and unpermeabilized fresh spores were taken for study. Higher concentration of citral and longer time of exposure were required to permeabilize fresh spores (Fig. 3). On the contrary frozen-thawed spores required lesser concentration of citral and shorter duration to get permeabilized, yet resulted in comparatively better biocatalytic activity (Fig. 4). Treatment of freeze-thawed spores with 5% citral and 3% citral resulted in better bioconversion, however treatment of spores with later concentration for 5 h was adopted since the concentration was affordable and the exposure time was practical. Different types (frozen and unfrozen), concentrations of spores (10^7–10^{10} spores/mL) were tested using this condition (3% citral for 5 h). Too low numbers of spores (10^7 and 10^8 spores/mL) were not sufficient for the bioconversion. Bioconversion increased with increase of spore concentration. Increase in the number of spores ensured better bioconversion with high reaction rate. However, bioconversion was comparatively better with 10^9 spores/mL than 10^{10} spores/mL. With very high concentration (10^{10} spores/mL) of spores, treatment of citral resulted in paste-like spore suspension, which probably leads to transfer limitation. Thus it was noted that 10^9 spores/mL treated with 3% citral for 5 h was the optimal spore concentration for the bioconversion reaction (Fig. 5). The extent of the inhibitory effect and permeabilization is depended strongly on the ratio between the terpene concentration and nature of the biomass (spores). Spores cultured by SSF are hydrophobic and are highly durable [19]. Spores used in this study was found to be resistant to organic solvents, detergents, mechanical cell permeabilization methods such as use of sonication, and high pressure. They were unable to permeabilize spores of *A. niger* [7].

Commercial glucose oxidase treated with citral, limonene and isonovalal was unaffected by the action of terpenes. The activity was similar to that of control (without addition of terpene in the medium). This proved that terpenes did not have any effect on the enzyme activity of glucose oxidase (data not shown).

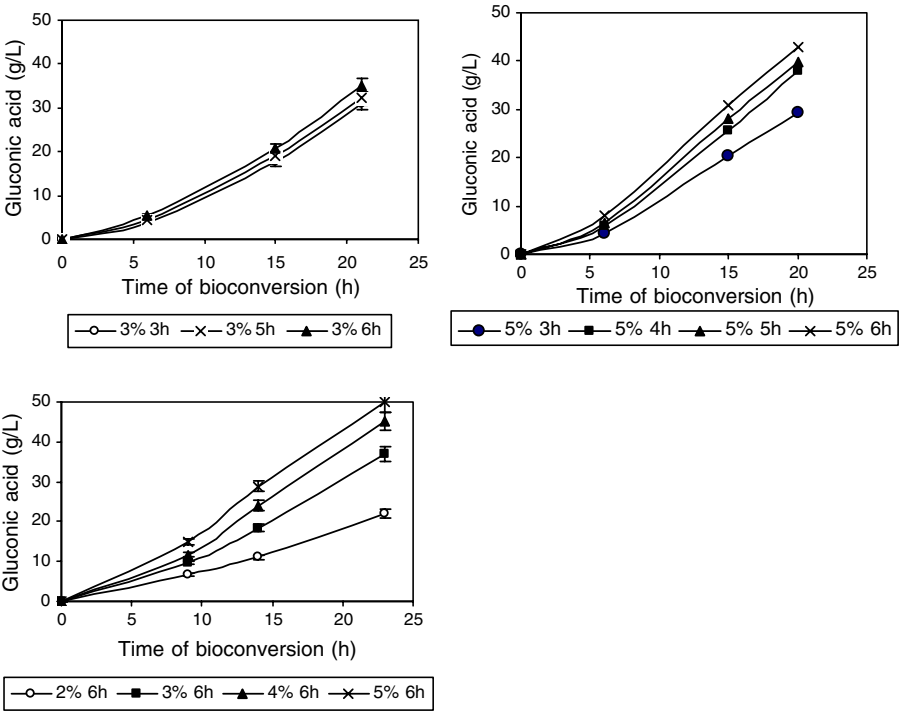

Fig. 3 Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (fresh, unpermeabilized). Spore concentration, 10^9 spores/mL; initial glucose concentration, 50 g/L; temperature, 30 °C; volume, 25 mL

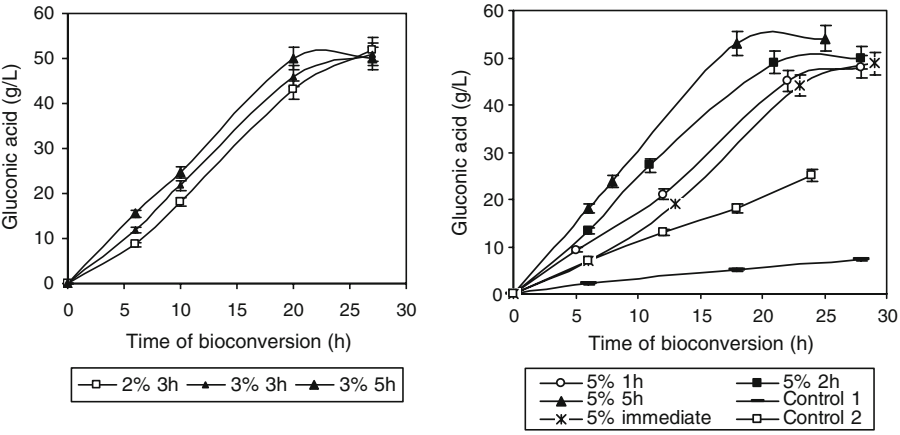

Fig. 4 Glucose oxidation catalysed by spores treated with different concentration of citral for permeabilization of spores (frozen, permeabilized). Control 1, frozen spores without citral treatment and no addition of sodium azide for inhibition of germination. Control 2, Frozen spores without citral treatment and inhibition of germination done by addition of 0.01 g/L of sodium azide. Spore concentration, 10^9 spores/mL; initial glucose concentration, 50 g/L; temperature, 30 °C; volume, 25 mL

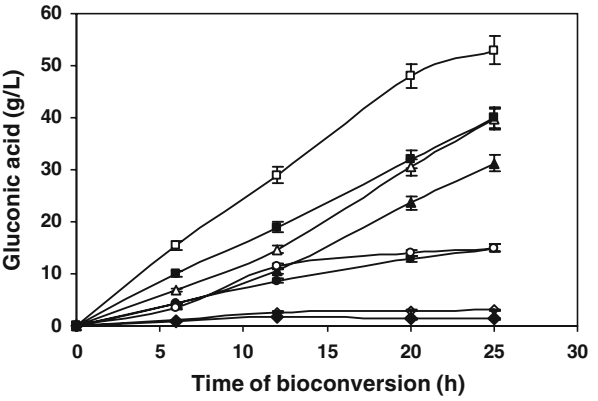

Fig. 5 Bioconversion of glucose to gluconic acid with frozen and unfrozen spore at different spore concentration [10^{10} frozen (*open triangle*), 10^{10} unfrozen (*filled triangle*); 10^{9} frozen (*open square*), 10^{9} unfrozen (*filled square*); 10^{8} frozen (*open circle*), 10^{8} unfrozen (*filled circle*); 10^{7} unfrozen (*open diamond*), 10^{7} frozen (*filled diamond*)].Spore concentration, 10^{9} spores/mL; initial glucose concentration, 50 g/L; temperature, 30 °C; volume, 25 mL

Fed-batch Production

Attempts were carried out to scale-up the process using the optimized conditions as above and feed the substrate once it gets exhausted. Before bioconversion, treatment of the spores with citral was carried out in the bioreactor. Bioconversion was commenced by the addition of glucose supplied in powder form and pH-regulated by sodium hydroxide. Bioconversion started immediately with a very high reaction rate of 5.26 g/L h (Fig. 6). This was comparatively higher with that of the reaction rate registered when bioconversion was carried out in Erlenmeyer flask. This could be explained because of the regulation of other parameters such as oxygen supply, high stirring speed etc. Reaction rate was slightly reduced when glucose was about to exhaust, and was restored once it was refed. At 18 h of reaction, 86 g/L of gluconic acid was produced with 14.4 g/L of residual glucose. There was neither protein synthesis nor carbon dioxide evolution noted. There was no glucose oxidase excreted out from the spore. When glucose was supplemented at the concentration of 100 g/L for the second time, reaction rate again increased as before. However at the end of the reaction, spore concentration was found to decrease due to the addition of sodium

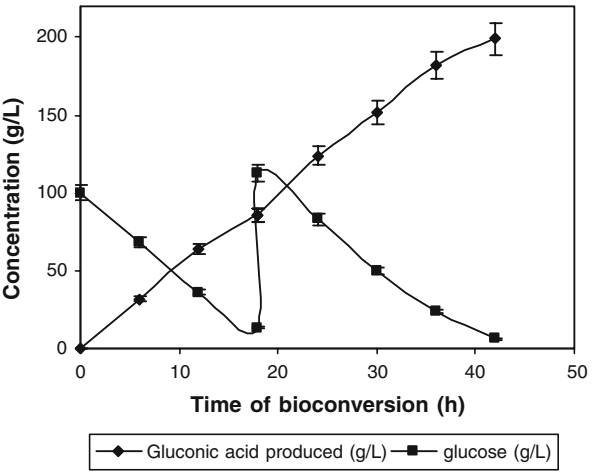

Fig. 6 Fed-batch production of gluconic acid with permeabilized spores of *A. niger*. Spore concentration, 10^{9} spores/mL; initial glucose concentration, 100 g/L; temperature, 30 °C; aeration, 0.15 slpm; stirring, 600 rpm; volume, 500 mL

hydroxide for neutralization. This was gradually reflected in the reaction rate at the end of the reaction. Thus the difference in the reaction rate noticed was due to the exhaustion of glucose and the decrease in the spore concentration. Similar results were found when potassium hydroxide was used as neutralizing agent (data not shown). It was noted that rate of reaction was increased with increase in initial glucose concentration of glucose however the yield always remained the same (100%).

Effect of Terpenes on the Glucose Oxidase Activity at Different Temperatures

Spores could carry out bioconversion reaction at temperatures 50 and 60 °C; however, rate of reaction at these temperatures were not similar to that of 30 °C (Fig. 7). On the other hand, commercial glucose oxidase activity was found to decrease with increase in temperature (50, 60 °C). Half-life of commercial (Sigma) glucose oxidase from *A. niger* exposed to 67 °C is 4.5 min [20]. Optimum temperature of *A. niger* glucose oxidase is 40 °C [21] and the denaturation point of periodate oxidized enzyme of *A. niger* is 72.8 °C [22]. Heat resistance of enzymes is markedly enhanced when they are present in spores rather in vegetative cells [1] as the spore structure is extremely rigid. Spore walls are resistant to extreme conditions and it is interesting to note that the stability of spore is transferred to its products. Glucose oxidase inside spores is comparatively thermostable when compared to the mycelial enzyme as it is well-protected by the rigid spore structure. Thus, enzyme inside the spores resembles a naturally immobilized enzyme protected from adverse environments, even at temperatures of up to 60 °C. It is also necessary to remark that this protective effect of the spore structure is suppressed when the temperature becomes higher than 60 °C (data not shown), which is probably the result of ultrastructural changes.

Spores were utilized repeatedly as biocatalyst for over 14 cycles of bioconversion. They were harvested after each cycle of bioconversion and the next cycle was started by transferring the washed spores into fresh glucose medium (data not shown). Gluconic acid production pattern was similar for each cycle without any appreciable loss of activity. This could be achieved as there is no enzyme leakage and enzyme in the spore closely resembled immobilized enzyme. Thus the biocatalyst could be used with considerable operational stability even after many cycles of bioconversion.

Biocatalytic activity of *A. niger* spores stored at −20 °C for one year followed the same pattern and comparable with that of spores stored at the same temperature for 1 day (data

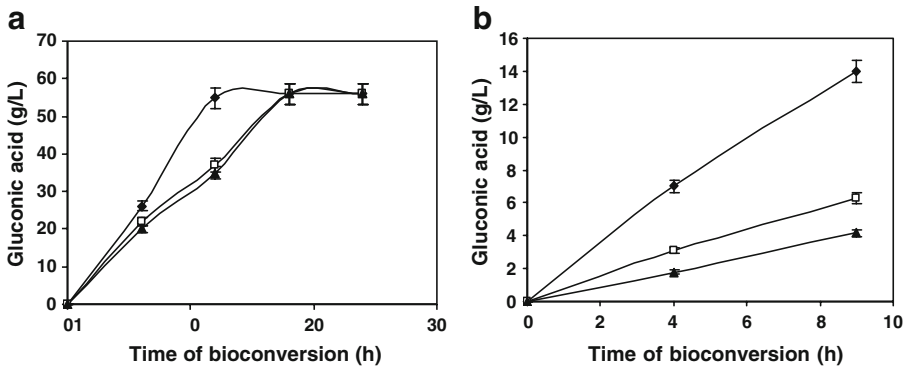

Fig. 7 Effect of different temperatures (*open diamond*, 60 °C; *open square*, 50 °C; *open triangle*, 30 °C) on biocatalytic activity of spores (**a**) and commercial glucose oxidase (**b**)

not shown). This indicated that there was no loss of enzyme activity during long storage time. Spores of *Aspergillus ochraceus* were stable for 1 year at −20 °C or 3 months at 4 °C without any detectable loss of hydroxylating activity. Commercial biocontrol agent 'green muscle' (spores) lasts for 3 years at 4 °C. The longevity of spore products has a direct consequence of their high resistance towards external factors [23].

Conclusions

The interesting fact about this system is its high reaction rate and its productivity. Spore stability, the protection rendered by spore to glucose oxidase even at high temperature (60 °C), reusability, its storage and the simplicity of the process without much regulation of other parameters adds advantages to the process.

The process could be made economical by using agro-industrial residues as a substrate for the spore production. Thus it could also be an environmentally friendly process by rendering value-addition to the agricultural wastes.

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