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Cultivation of *Aspergillus phoenicis* with high superoxide dismutase-like activity

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ABSTRACT

Free radicals are known to be highly harmful to cellular components, particularly superoxide anion radical ($O_2^{\bullet -}$) which is a precursor of the more reactive oxygen species, contributing to various diseases. Although the amount of superoxide anion radicals can be regulated by superoxide dismutase (SOD), the scavenging function of SOD supplied from external sources is difficult to exploit because owing to the restraint of its molecular weight. An alternative approach adopts low-molecular-weight compounds with SOD-like activity. This study screens strains for obtaining molecules with high SOD-like activity, and optimizes the conditions for generating SOD-like metabolites. An *Aspergillus* phoenicis strain with highly potent of SOD-like antioxidant producing was obtained, and further applied in different lab-scale jars with batch and fed-batch fermentation. The medium composition and culture parameters for the strain with high SOD-like activity were also studied. The SOD-like activity of *A. phoenicis* was 10 U/mL when cultivated in PDB medium. For more medium investigation, 15% glucose and 2% beef extract were served as carbon and nitrogen sources, respectively, for their benefit of less cost and higher productivity. Response surface methodology (RSM) was then used to determine the optimal growth conditions for *A. phoenicis* to produce SOD-like activity. The optimal temperature and pH were found to be 30 °C and 5–6, respectively. The SOD-like activity of *A. phoenicis* in a batch fermentation using a 5 L fermentor was 420 U/mL with a biomass of 5 g/L dry cell weight. With fed-batch cultivation, although the SOD-like activity of *A. phoenicis* achieves a similar performance with 400 U/mL, the biomass could be observably improved to 21 g/L after fermentation for 96 h.

1. Introduction

A free radical is a molecule having an unpaired electron in its outer orbit. Free radicals are essential molecules in the role of life and biological evolution, including signal transduction, gene transcription and regulation of enzyme performance in cells (Fang *et al.*, 2002). They are also important mediators to the immune response. For example, O_2 is converted to $O_2^{\bullet -}$ through NADPH oxidase complex, if phagocytes are activated. However, excess free radicals cause nucleotide oxidation and dimerization, and ultimately lead to mutations during replication for their unstable and very reactive properties (Behl and Moosmann, 2002). These reactive species may also associate with the membrane lipids, especially the unsaturated carbohydrates side chains of phospholipids, and cause membrane dysfunction and cell lysis owing to lipid oxidation. The damaging consequences of free radicals are known to a variety of very different human diseases, such as ischemia and reperfusion, arthritis, chronic gut inflammation and immune complex-induced pulmonary injury (Conner and Grisham, 1996; Nordberg and Arner, 2001). Similar situations are also associated with biochemical and physiological response in plans. In report by Jaleel *et al.* (2007), the result indicates paclobutrazol make a influential contribution toward salinity stress. And the addition of triadimefon could enhance the antioxidative enzymes to overcome the damage of free radicals subjected to sodium chloride treatment (Jaleel *et al.*, 2008).

Different dietary antioxidants have been developed during the past decades, and have served as an effective nutrition support to lower the deleterious impact of free radicals through enzymatic and non-enzymatic protection (Chen *et al.*, 2005; Kim *et al.*, 1994). For example, coenzyme Q_{10} is widely used as an antioxidant preventing lipid peroxidation and can be produced by microbial conversion (Choi *et al.*, 2005). L-Carnitine helps cells generate energy, which is crucial for fat metabolism and also stimulated production remarkably by genetic engineering (Bernal *et al.*, 2007). Glutathione (GSH), also known as γ-glutamyl cysteineglycine, is a key antioxidant in human body. It shows an essential role in protecting cells from oxidative injury and regulating the immune

Abbreviations: g, gram; L, liter; mL, milliliter; nm, nanometer; mM, millimolar; min, minute; rpm, revolution per minute; d, day; h, hour; U, activity unit; μm, micrometer; kDa, kilodalton.

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system as well. For more and more claims made, the production of glutathione with baker's yeast draws much attention regarding the optimal method for raising the intracellular amount (Lai *et al.*, 2008). Another powerful free radical scavenger, resveratrol, is also an intensive research from microorganisms in response to oxidative stress (Beekwilder *et al.*, 2006).

Among those reactive species, superoxide anionic radical ($O_2^{\bullet -}$), which is a precursor of the more reactive oxygen species, is usually generated *in vivo* in the electron-rich aerobic environment, especially in epithelial cells, macrophages and neutrofils (Fang *et al.*, 2002). In living cells, $O_2^{\bullet -}$ can be transformed into hydrogen peroxide by superoxide dismutase (SOD), a defense enzyme protecting cells from cellular damage caused by reactive oxygen species (Conner and Grisham, 1996). SOD regulates the concentration of superoxide anionic radical, and receives much attention because of its protective effect against oxygen toxicity (Milesi, 2007). Hence, SOD has attracted extensive interest from the pharmaceutical and food industries (Barriere *et al.*, 2001; Meyer *et al.*, 2005; Pan and Ye, 1997).

However, SOD is inactivated by either digestive enzyme or gastric juice when it is orally administered, since its enzyme properties with molecular weight exceed 30 kDa, and it cannot be absorbed into the gastrointestinal tract (Zielinski *et al.*, 2006). An alternative means of achieving this goal is to find low-molecular-weight compounds that mimic SOD behavior and can act as healthier alternatives. SOD-like compounds, such as flavonoids, polyphenol, tannin and carotene, have natural radical-scavenging ability, and can be absorbed into the gastrointestinal tract (Fridovich and Darr, 1993; Hattori *et al.*, 1995; Matsuo, 1997). Therefore, such targets are useful candidates for designing functional foods. Various studies focus on the screening of potential sources for SOD-like activity from rice wine, fruits and vegetables, as well as microorganisms (Kim *et al.*, 2004; Miyake *et al.*, 2003), and hope to help human SOD activity richer. SOD-like compounds extracted from fermentation broths by microorganisms are generally more promising than plant or animal source for consideration of raw material provision (Doblado *et al.*, 2005; Osawa, 2003; Yen and Chang, 2003).

The fermentation conditions and metabolites of *Aspergillus phoenicis* have been widely examined, focusing on enzymes such as β-glucosidase, xylanase, β-xylosidase, and α-D-mannosidase (Athanasopoulos *et al.*, 2005; Rizzatti *et al.*, 2004). Gutierrez-Correa and Tengerdy (1997) concluded that *A. phoenicis* co-cultured with fungal cultures enhances combined biomass production, and along with increasing in cellulose, endoglucanase and β-glucosidase activities. All these enzymes have high potency in applications of food and feed industries, pulp/paper processes and enzymatic saccharification of cellulosic materials (Suwasono and Antranikian, 1997). For the flavour industry, β-glucosidase plays an important role in the release of aromatic compounds, since it hydrolyzes the monoterpenyl glycosides without altering the aromatic attributes (Jager *et al.*, 2001). Additionally, α-D-mannosidase from *A. phoenicis* can be used to synthesize several oligosaccharides for special purposes (Suwasono and Rastall, 1998).

Besides those enzymes, some *Aspergillus* strains have been adopted to exploit natural antioxidants. Antioxidants help prevent some diseases. Microbial sources have recently been found to be a plentiful source of natural antioxidants (Chen *et al.*, 2005; Kawasumi *et al.*, 1999; Yen and Chang, 2003). However, the potential of *A. phoenicis* to produce antioxidants, particularly SOD mimic materials, is still little known. This investigation measures the antioxidant ability, defined as ability to scavenge free radical, of broths achieved by submerged fermentation of *A. phoenicis*. *A. phoenicis* was screened from the Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute (FIRDI).

Experimental design can significantly improve process development (Nawani and Kapadnis, 2005). This work explores how medium composition and strain culture parameters could raise SOD-like activity by using response surface methodology (RSM). *A. phoenicis* cultured in a 5- and 20-L fermentor under optimal conditions was investigated.

2. Materials and methods

2.1. Microorganism

A. phoenicis was screened from among tens of candidates in the Bioresources Collection and Research Center (BCRC), which is part of the Food Industry Research and Development Institute.

2.2. Medium

The aqueous media in which the SOD-like compounds were produced were PDA medium, composed of 2% (w/w) potato, 0.2% dextrose and 0.6% agar; PDB medium, containing 2% potato and 0.2% dextrose, and PDB modified medium, with 5% glucose, 1% peptone, 0.5% yeast extract, 0.25% $MgSO_4{\cdot}7H_2O$ and 0.5% KH_2PO_4 under 1 L deionized water.

2.3. Experimental design

A partial factorial two-variable, three-level experimental design was used, with temperature for 20–30 °C. Along with three-variable, two-level experimental design, glucose, yeast extract as well as peptone were also examined tautologically to discover the optimum cultivation medium corresponding to concentration ranges of 5–10, 0.5–1 and 1–2%, respectively. Response surface methodology (RSM) was also introduced to the analysis of experimental data. Design Expert software (Version 6.0), a commercial statistical package, was employed to establish the tabulation format and three-dimensional plot.

2.4. SOD-like activity assay (Kim et al., 1995)

0.1 mL of pyrogallol solution (2 mM in 10 mM HCl solution) was added to 0.1 mL of distilled water and 0.8 mL of a 55.6 mM Tris–cacodylic acid buffer (TCB, pH 8.2) containing 1.1 mM diethylene triamine penta-acetic acid (DTPA), and the absorbance at 420 nm was monitored at 25 °C. The auto-oxidation rate of pyrogallol as control was determined by the slope of the absorbance curve for the first minute of the reaction, while absorbance change was controlled in the range 0.06–0.07 min^{-1}. To analyze the SOD-like activity, 0.1 mL of a sample was added in place of distilled water. The pyrogallol auto-oxidation inhibition rate (%) can be calculated as: $(A - B) \times 100/A$, where A and B denote the auto-oxidation rates of pyrogallol in the control and presence of a sample, respectively. A single unit of SOD-like activity was defined as a pyrogallol auto-oxidation inhibition rate of 50%.

2.5. Cultivation

Case for flask culture, stock culture was grown on potato dextrose agar (PDA) at 25 °C. Spore suspension of *A. phoenicis* was prepared with sterile water, and stored at −80 °C. The spore suspension (0.5 mL, 10^7 spores/mL) was then inoculated into a 500 mL flask containing 100 mL of medium. The flasks were cultivated at 30 °C by shaking at 150 rpm. For 5 L jar fermentation, the flasks were first inoculated at 30 °C by shaking at 150 rpm for 39 h on a rotary shaker. The inoculum (100 mL) was then transferred into a 5 L (BioFlo 3000, New Brunswich Scientific) fermentor containing 3 L of medium (∼3.3% inoculum). Fermenta-