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Supercritical CO_2 extraction of an immunosuppressant produced by solid-state fermentation

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ABSTRACT

Mycophenolic acid (MPA) is a secondary metabolite of *Penicillium* species with diverse biological properties and has clinical applications mainly as an immunosuppressive agent. Production of MPA was carried out using *Penicillium brevicompactum* (DSM 2215) by solid-state fermentation (SSF). Six different carbon sources (wheat bran, rice husk, rice bran, potato peel and rice husk - rice bran, rice bran-potato peel mixture) were examined by SSF in order to obtain the highest yield of mycophenolic acid. Rice bran-potato peel mixture was determined as the best solid substrate and used in scale up studies which was applied in the tray bioreactor. Supercritical CO_2 and soxhlet extractions were analyzed for the recovery of MPA from the fermentation broth. The highest recovery (0.47 g MPA/100 g substrate) was obtained by supercritical CO_2 at 30 MPa, 50 °C at a CO_2 flow rate of 15 g/min with 10% ethanol as co-solvent for 3 h. The results obtained by the scale-up in bioreactor and optimization of supercritical CO_2 entrained with ethanol can provide basis for the production of the immunosuppresant compound on industrial scale.

1. Introduction

Organ transplantation is preferably usedin many cases of treatment for chronic failure of major organs. In 1901, Karl Landsteiner discovered the existence of human AB0 blood groups so that the solid organ transplantation is not a phenomenon anymore [1,2]. After this development, the scientists focused on the reasons of the graft rejection as the major obstacle to successful transplantation, which is immune-responded rejection. Therefore, the studies were concentrated on effective immunosuppression. In the late 50′s, the total body irradiation was used for repressing the recipient immune system response and rejection but the consequences were constantly bad [3]. Following this, corticosteroids were used as for immunosuppression, but no effective results were obtained. Then cytotoxic agents were introduced acting as modified corticosteroids to block the immune system response after organ transplantation in the early 1960s [4,5]. The main classes of immunosuppressive drugs used in clinics to avoid tissue rejection include calcineurin inhibitors, target of rapamycin inhibitors, sphingosine-1-phosphate receptor modulators, cytotoxic agents, glucocorticoids and monoclonal antibodies [6,7].

In the development of early medicine, fungal metabolites have played an important role in drug discovery. The fungal compounds of antibiotics, antifungals, immunosuppressants and cholesterol-lowering agents have been used in the medical applications since 1950′s [8]. After cyclosporine was approved by FDA in 1983, the first fungal metabolite as clinically used immunosuppressant was available on the market. The main fungal metabolites for blocking the immune response were cyclosporine, mycophenolic acid (MPA) and mizorbine [5]. MPA is a fungal secondary metabolite and was initially isolated as a weak acid with antifungal activity from *Penicillium glaucum* and *Penicillium stoloniferum* culture filtrates (Alsberg ve Black; 1913). Gozio who discovered MPA, 6-(4-hydroxy-6- methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid from fermentation broth of *P. glaucum* in 1896, was not aware of its immunosuppressive property [9]. After that, a compound named as MPA was isolated from *P. stoloniferum* culture filtrate [10,11]. When the first animal studies were conducted, the compound was found to exhibit antiviral, antifungal and antitumor activities [12–14]. MPA is a non-competitive, reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) which is an enzyme catalyzing the oxidation of inosine monophosphate to the intermediate xanthine monophosphate [15]. In the *de novo* synthesis of these nucleotides, IMPDH acts as a key enzyme inducing the rate-limiting step in synthesis [16,17] and MPA depends on this pathway for the proliferation of lymphocytes [18]. MPA is produced by *Penicillium* species such as *P. brevicompactum*, *P. stoloniferum* and *P. roqueforti* and by some other microorganisms such as *Byssochlamys nivea* [19–24]. The production

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process depends on either submerged cultures with batch and continuous operating modes or solid-state fermentation [25–27]. When comparing different MPA productions, it was found that *Penicillium brevicompactum* is one of the best mycophenolic acid producing strain [28–30]. Therefore selected for the production of mycophenolic acid by solid-state fermentation (SSF).

Recently, utilization of various organic wastes in bioprocesses has become imperative due to the scarcity of natural resources. Several processes have been operated to utilize these organic wastes as raw materials for the production of value-added bioactive compounds such as antibiotics [31]. Among these, SSF has shown much promise in the development of several bioprocesses and products which involves microorganisms grown on solid materials without the presence of free liquid, thus creating a medium close to the natural environment to which microorganisms can easily adapt [32,33]. Therefore, the aim of SSF is to bring the cultivated fungi or bacteria into tight contact with the insoluble substrate to achieve the highest utilization of substrate for the production of target compounds [34]. This technique is more useful than submerged fermentation due to high concentration of the product, utilization of solid substrate, low demand for water, therefore less wastewater. Subsequent to SSF, fermentation products require downstream processes such as fractionation and extraction [35].

Lately, the development of new extraction methods has been accelerated while trying to minimize environmental loads [36]. Supercritical fluid extraction can be accounted as a promising new technology to recover valuable components from biological materials [37,38]. SFE aims to extract target analytes from solid and semi-solid matrices by adjusting temperature and pressure which leads the fluid to change its phase from liquid to supercritical [39]. One of the main reasons for implementation of this technology are the disadvantages of traditional extraction methods such as high costs originating from products and by products of organic solvents and environmental pollution. On the other hand, supercritical fluid extraction is environmentally friendly, residue free, selective, tunable and does not cause contamination in post-processes [40–44]. The diffusivity power of supercritical fluids is close to gases and at least two orders of magnitude higher than liquids [45] making them superior for extraction of non-polar compounds and separation of substances that are insoluble in supercritical medium [46,47]. Polarity of supercritical fluids can be modified using entrainers depending on the polarity of the compound to be extracted. Carbon dioxide (CO_2) is the most commonly used fluid. The solvating power can be easily adjusted with small changes in pressure and without phase transition and can be easily separated from the extract by reduction of pressure. In addition, the lower supercritical temperature of CO_2 is suitable for the extraction of temperature-sensitive compounds. Although CO_2 is non-polar, addition of polar organic solvents can enhance the extraction efficiency [48–50]. Therefore, supercritical carbon dioxide ($SC-CO_2$) extraction is an outstanding green technology with significant advantages over conventional counterparts [30,51]. Apart from extraction of biomolecules, the field is expanding to various life science applications leading to utilization of CO_2 for value-added products [52,53].

The aim of this study was to produce MPA by SSF utilizing industrial wastes such as wheat bran, rice husk, rice bran, potato peel and their various combinations in order to maximize bioconversion of these agro-industrial residues to MPA and scale-up the best combination to a bioreactor. $SC-CO_2$ extraction entrained with ethanol was optimized for the recovery of mycophenolic acid from the fermentation broth and compared with that of soxhlet extraction.

2. Materials and methods

2.1. Materials

A culture of *Penicillium brevicompactum* (DSM 2215) was used in this research. The active culture of microorganism was provided from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) culture collection. Fungal culture was stored at + 4 °C on slanted agar containing Potato Dextrose Agar (PDA) (Merck Ltd., Germany). In the production of mycophenolic acid by solid culture fermentation, wheat bran (Bioprocess Laboratory of Ege University, Faculty of Engineering, Department of Bioengineering), rice husk, rice bran (Ozserhat Gida San. Ltd. Sti. Ipsala, Edirne-Turkey) and potato peel (Ozgorkey Gida Urunleri San. Tic. A.S., Torbali, Izmir-Turkey) were used as substrates. The chemicals, Tween 80, ethanol 98%, NaOH, HCL and PDA (Potato Dextrose Agar) were purchased from (Merck Chemicals, Istanbul, Turkey). The Tween 80 solution was used at 0.1% (v/v) concentration to obtain spore suspension from *Penicillium brevicompactum* stock culture. The solution was sterilized in autoclave for 15 min at 121 °C before inoculation.

2.2. Methods

2.2.1. Pre-treatments applied to substrates

The potato peels were dried and then powdered using a grinder. In addition, the humidity contents of wheat bran, rice husk, rice bran and potato peel were determined by the moisture determination device (Mettler Toledo, Istanbul, Turkey).

2.2.2. Preparation of production medium

2.2.2.1. Preparation of stock culture medium. PDA (Potato Dehydrogenase) medium was used for stock culture of *Penicillium brevicompactum*. PDA content was indicated as potato infusion 4.0 g/L, D (+) glucose 20.0 g/L, and agar-agar 15.0 g/L. The PDA medium was prepared to be 1.95 g PDA in 50 ml distilled H_2O, sterilized at 121 °C, 1 atm pressure for 15 min and cooled at room temperature and made ready for inoculation.

2.2.2.2. Preparation of solid culture medium

2.2.2.2.1. Preparation of production medium in Erlenmeyer flasks. Four different solid substrates (wheat bran, rice husk, rice bran and potato peel) and mixtures of these substrates were used as solid culture media which were weighed to 20 g and placed in 500 ml Erlenmeyer with the aid of a spatula after moisturizing. Humidification of the solid culture media were adjusted with distilled water and solute to solvent ratios were adjusted according to material properties. Erlenmeyers were closed with rubber stoppers, then wrapped with aluminum foil and sterilized at 121 °C for 15 min. After sterilization, the samples were kept at room temperature and prepared for inoculation.

2.2.2.2.2. Preparation of bioreactor production medium. As a solid culture medium, a mixture of rice bran and potato peel substrate was used at a ratio of 1:1 (weight/weight). After weighing the final substrate to be 300 g, necessary amount of water was added to adjust 65% moisture. The ready to use substrate mixture was placed in the bioreactor trays, which were specially designed for solid culture. Then, the bioreactor was sterilized by steam sterilization at 121 °C for 15 min.

2.2.3. Scaling-up to bioreactor

Fermentation was scaled-up to a tray bioreactor (Designed at the Department of Bioengineering, Ege University, Izmir, Turkey and built by Bakon Machinery and Engineering, Izmir, Turkey) which was comprised of 5 perforated trays, 24.6 cm in diameter and 5 cm in thickness. A combination of potato peel and rice bran moisturized by distilled water was used as solid substrate for MPA production. In order to increase air diffusion in the solid culture medium, teflon cords were added into the substrate mixture. About 300 g of potato peel and rice bran combination was first autoclaved for 15 min at 120 °C with steam sterilization reaching a moisture content of 70% and inoculated with the liquid inoculum aseptically. After sterilization and cooling to room temperature, moisturized air was fed into the reactor from the bottom at an identified rate. The fermentation process was conducted at 28 °C for 9 days.