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Bioproduction and extraction optimization of citric acid from *Aspergillus niger* by rotating drum type solid-state bioreactor

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ABSTRACT

Solid-state citric acid fermentation was conducted in a 12-L rotating drum type bioreactor. The effect of inducers, ethanol and methanol were studied on citric acid bioproduction by *Aspergillus niger* NRRL 567 cultivated on apple pomace as a solid-substrate. Optimum conditions achieved for higher citric acid bioproduction (220.6 ± 13.9 g/kg dry solids, DS) were 3% (v/v) methanol, intermittent agitation of 1 h after every 12 h at 2 rpm and 1 vvm of aeration rate and 120 h incubation time. The response surface optimization proved effective for higher citric acid extraction from fermented solid-substrate. Higher citric acid extraction of 294.19 g/kg DS was achieved at optimum conditions: extraction time of 20 min, agitation rate of 200 rpm and extractant volume of 15 ml by response surface methodology.
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1. Introduction

Citric acid (CA) is the world's second largest fermentation product synthesized after industrial ethanol fermentation. CA dominates the organic acids category with an estimated bioproduction of more than 1.7 million tons per year and the volume of CA bioproduction is constantly rising at a high annual rate of 5% (Francielo et al., 2008) with future escalating trends. CA is an important multifunctional commodity chemical with a broad range of versatile applications, such as in: (1) biomedicine, e.g. synthesis of biopolymers for culturing a variety of human cell lines; (2) nanotechnology, such as drug delivery systems; and (3) agriculture, such as bioremediation of heavy metals due to its powerful sequestering action with various transitional metals (Dhillon et al., 2011a, 2011b). Considering high consumption rate and slight increase in price, the market value for this multifunctional acid was expected to exceed $2 billion in 2009 (Partos, 2005). Moreover, the demand of CA is increasing at faster pace due to various new applications coming to light which mandates the need for possible ways to increase its production. However, CA industry is currently facing challenges for economical and sustainable process development due to high substrate and energy costs.

The world's existing demand for CA is almost entirely (over 99%) met by fermentation processes, especially by *Aspergillus niger* submerged fermentation (~80%) (Francielo et al., 2008). However, in recent years, increasing interest has been shown in utilizing fruit pomace and other agro-industrial wastes by solid-state fermentation (SSF) for sustainable and economical CA process development (Karthikeyan and Sivakumar, 2010; Kuforji et al., 2010; Kumar et al., 2010; Dhillon et al., 2011c, 2011d). SSF is gaining worldwide attention for the production of CA due to the numerous advantages it offers, such as ability of filamentous fungus to grow efficiently and utilization of low cost agro-industrial solid waste as substrates.

In fact, there are two trends for the development of the feasible process for CA bioproduction: (1) use the abundant low cost substrates, such as agro-industrial wastes; and (2) efficient extraction of CA from the fermented solid biomass. As evident from the literature, various researchers have attempted to optimize the fermentation medium and the process parameters for decreasing the production cost of CA for commercializing the process to industrial scale (Shojaosadati and Babaeipour, 2002; Rivas et al., 2008; Karthikeyan and Sivakumar, 2010; Kuforji et al., 2010; Kumar et al., 2010; Dhillon et al., 2011c, 2011d, 2011e). Besides, extraction of CA from fermented solid biomass is also an important aspect of SSF. However, there is a dearth of information for CA extraction studies from fermented solid substrate.

The present study involves the effect of lower alcohols using apple pomace (AP) to cultivate *A. niger* NRRL 567 for solid-state CA fermentation in rotating drum type bioreactor. The study also aims at efficient extraction of CA from the fermented solid biomass. We have not come across any report or published literature where CA extraction has been optimized using statistical design to improve

Abbreviations: ANOVA, analysis of variance; AP, apple pomace; CA, citric acid; CFUs, colony forming units; DS, dry substrate; EtOH, ethanol; gds, gram dry substrate; MeOH, methanol; OD, optical density; PDA, potato dextrose agar; SSF, solid state fermentation.

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CA extraction from fermented solid biomass. The present study was therefore carried out to optimize different extraction parameters, such as extraction time (min), agitation (rpm) and extractant volume (distilled H_2O) (ml/g substrate) for higher extraction of CA by response surface methodology (RSM).

2. Materials and methods

2.1. Microorganisms and inoculum preparation

The microorganism used in this study was *A. niger* NRRL 567, procured from Agricultural Research Services (ARS) culture collection, IL, USA. This strain was maintained and periodically subcultured on potato-dextrose-agar (PDA) medium at 4 ± 1 °C. The culture of *A. niger* was grown on PDA Petri plates and incubated for 7–8 days at 30 ± 1 °C and 200 rpm. The spores were harvested from the sporulation medium plates by adding 10 ml 0.1% Tween-80 solution to each plate. To recover fungal spores devoid of mycelial contamination, filtration of harvested spores was carried out by using glass wool. Quantitative estimation of spores was performed microscopically using Nauber chamber and stored in test tubes at -20 ± 1 °C for maximum of 4 weeks. The spore suspension adjusted to 1×10^7 spores/ml count was used as an inoculum.

2.2. Substrate procurement and pretreatment

Fresh apple pomace (AP) was procured from Lassonde Inc., Rougemont, Montreal, Canada. AP was selected as fermentation substrate for its potential to be used for CA production based on our previous study using flasks and tray bioreactor (Dhillon et al., 2011d). AP used in this study was already supplemented with 1% (w/w) rice husk as a common industrial practice during the extraction of juice for a better hold on the apples during meshing and filtration. AP was completely dried at 50 ± 1 °C in a hot air dehydrator till constant weight, grounded and was passed through sieves to get the desired particle size of 1.7–2.0 mm which was used for this study.

2.3. Solid-state fermentation

The fermentation was carried out in a 12-L rotating drum type solid-state bioreactor, Terrafor (Infors HT, Switzerland). Approximately, 3 kg of rehydrated AP (moisture 75%, v/w) was sterilized in autoclave (121 ± 1 °C, 15 psi for 30 min) and was transferred into the sterilized bioreactor under aseptic condition. After transferring the substrate into the bioreactor the sterilization was done again to ensure complete decontamination and was supplemented with 3% (v/w) of inducers, ethanol and methanol, respectively. The initial pH (3.5 ± 0.1) was taken as such without any adjustment. The inoculation was done with the spore suspension having 1×10^7 spores/g substrate. For final moisture content of 75% (v/w), the volume of inducers and spore suspension was also taken into account. The fermentation was carried out in a controlled environment at 30 ± 1 °C, intermittent agitation rate of 2 rpm (for one hour after every 12 h) and aeration rate of 1 vvm. The fermentation was carried out for 12 days, and the sampling was done periodically after every 24 h. Fermented samples were extracted with distilled water and analyzed for CA and total spore count analysis.

2.4. Citric acid extraction

For CA estimation, the samples were harvested from each flask after every 24 h under aseptic conditions. CA was extracted from a solution prepared with 3 g of a fermented sample macerated with 15 ml of distilled water and shaken for 30 min in an incubator shaker at 200 rpm at 25 ± 1 °C. The supernatant was filtered through glass wool for removal of solid substrate and fungal mycelia. About 100 μl sample was taken in Eppendorf tubes for viability (total spore count) assay using haemocytometer and the remaining sample was centrifuged (Sorvall RC 5C plus by Equi-Lab Inc., Québec, Canada) at $9000 \times g$ for 20 min and the supernatant was analyzed for CA concentration.

2.5. Central composite design for CA extraction optimization

In separate experiments, optimization of CA extraction parameters from 120 h MeOH induced fermented AP was carried out using RSM. Application of RSM for the optimization of parameters having impact on CA extraction will help in achieving higher CA extraction from fermented solid substrates. RSM helps in overcoming the limitations of time consuming conventional optimization method of 'one factor-at-a-time' (at each stage, a single factor is changed while other factors remain constant). Moreover, the statistical optimization method can evaluate the effective factors and help in building models to study interaction and select optimum conditions of variables for a desirable response. In the response surface method, the factors, such as extraction time (min) (X_1), agitation (rpm) (X_2) and extractant volume (ml/g) (X_3), were considered as independent variables and CA extraction (g/kg DS) as dependent variable.

To begin with, the screening experiments were carried out to determine the direction of optimal domain of each process. Once the provisional optimal values were determined, a central composite design (CCD) was used to find the optimal conditions of these three factors (X_1, X_2 and X_3). CCD is a first-order (2N) design augmented by additional center and axial points to allow estimation of the tuning parameters of a second-order model. In order to identify the significant factors that affect the responses, an attempt was made to improve the extraction of CA from fermented solid substrate by comparing different levels of several factors that were found to have more influence on dependant variable, i.e. CA extraction. A study of determination of the provisional optimal values of these independent variables was carried out by using the steepest ascent method and was found to be significant. In this regard, a set of 17 experiments including, 3 center points (0, 0, 0) and six axial points ($\alpha = 1.68$) and 8 points corresponding to a matrix of 2^3 which incorporates 8 experiments including 3 variables (+1, −1, 0), were carried out. Each variable was studied at two different levels (1, +1) and center point (0) which is the midpoint of each factor range. The levels of each factor along with their codes and values of the experimental design and full factorial plan are listed in Table 1 and Table 2, respectively.

Table 1
Experimental range of the three independent variables studied using central composite design (CCD) in terms of actual and coded factors.

Variables	Symbol	Coded levels				
		−1.682	Low (−1)	Mid (0)	High (1)	+1.682
Extraction time (min)	X_1	11.10	15	20	25	28.94
Agitation rate (rpm)	X_2	110.56	150	200	250	289.44
Extractant volume (ml/g)	X_3	6.10	15	20	25	23.94

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