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# Bio-synthesis of citric acid from single and co-culture-based fermentation technology using agro-wastes

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ABSTRACT

Agro-based materials are primarily composed of complex polysaccharides that strengthen microbial growth for the production of industrially relevant value-added products. Therefore, in the present study, solid state fermentation (SSF) was carried out using agro-based waste materials (apple pomace, peanut shell and a mixture of both apple pomace and peanut shell with 50:50 ratio) as carriers/support for SSF to enhance citric acid production from single and co-culture consortia of *Aspergillus ornatus* and *Alternaria alternata*. During initial screening trial it was observed that growth media supplemented with apple pomace under SSF process of co-culture consortia presenting the preeminent 0.46 ± 0.42 mg/mL of citric acid. On partial optimization co-culture showed the maximum citric acid yield (2.644 ± 0.99 mg/mL) in the presence of arginine as a nutritional ingredient at 30 °C in an apple pomace based medium at 50% moisture content with pH of 5 and substrate concentration (25 g) after 48th of solid state fermentation. In conclusion, a suitable addition of fermentative substrate to the SSF medium increased fungal growth, sugar utilization and citric acid production when used in lower concentrations.



## 1. Introduction

Citric acid is one among the most versatile organic acid and broadly used for multipurpose in different industries including food, cosmetics, pharmacy, beverages and many others (Blair and Stall, 1993). Apart from its consumption as a food additive, citric acid is also considered as an indispensable component of various pharmaceuticals, synthetic detergents, cosmetics, and many other value-added products. Sales of citric acid worldwide are divided amongst the principle fields of use including food, confectionery & beverages (75%), pharmaceuticals (10%) and other industries (15%). The use of citric acid as a food acidulate depends in part on its strength as an acid. However, its pleasant taste and its property of enhancing existing flavors have ensured its dominant position in market (Kapoor, Choudhry, & Tauro, 1982). There is an immense need to investigate the factors responsible for low citric acid yields and to develop strategies to increase yield and reduce production cost (Milson & Meers, 1985).

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A range of various physicochemical and nutritional factors are considered very critical which can ultimately influence the growth of microbial strains and the end product. Moisture content, substrate particle sizes, nutrient sources, incubation temperature, pH and inoculum size are among major factors (Bari, Alam, Muyibi, & Jamal, 2009; Bari, Alam, Muyibi, Jamal, & Al-Mamun, 2010; Ellaiah, Srinivasulu, & Adinarayana, 2004; Lotfy, Ghanem, & El-Helow, 2007). So far, the influence of fermentation types including SSF, SmF and LSF and concentrations of fermentative substrates, sugars, metal ions, and aeration on citric acid production have been studied and reported elsewhere (Al-Mahin, Hasan, Khan, & Begum, 2008; Bayraktar & Mehmetoglu, 2000; Xu, Madrid, Röhr, & Kubicek, 1989).

Enormous quantities of agro-based industrial waste materials are generated throughout the world from the processing of raw agriculture materials (Anwar, Gulfraz, & Irshad, 2014; Ghaffar et al., 2014). Thus, agro-based industrial residues from the processing of sugarcane, orange, coffee, and rice present suitable feedstock for bioconversion into chemicals, including citric acid by fermentation process, thereby adding value to what normally constitutes wastes products (Giese, Dekker, & Barbosa, 2008; Iqbal, Kyazze, & Keshavarz, 2013). Microorganisms have long played a major role in the production/development of food industry (dairy, fish and meat products) and alcoholic beverages (Buyukkileci, Tari, & Fernandez-Lahore, 2011). There is great interest in the development and use of natural food and additives derived from microorganisms, since they are more desirable than the synthetic ones produced by chemical processes. For industrial use, citric acid can be produced from several agricultural based wastes such as apple pomace and others.

By keeping in mind the current scenario, industrially relevant demands and biotechnological valorization of various agro-based waste materials, this study was designed to obtain a hyper production of an industrially relevant citric acid. The main objective of this study was to use renewable and easily abundant agro-based materials under SSF for enriched citric acid production at optimal level.

## 2. Materials and methods

### 2.1. Chemicals and substrates

All chemicals were of analytical grade unless otherwise specified and used as-received. Various agro-based substrates such as apple pomace and peanut shells were obtained from local fruit market, Gujrat, Pakistan. The aforementioned substrates were first washed using hot water and then crushed into small pieces, dried at 60 °C, ground to 40 mm and finally stored in an air-tight moisture-free plastic jars for the use of the whole experiment.

### 2.2. Pre-treatment of substrates

Moisture-free powdered substrates (20 g), separately, were pre-treated with 1% HCl in an Erlenmeyer flask (500 mL) at room temperature (30 °C) for 1 h, afterwards, the substrate slurry was filtered through four layers of muslin cloth, and both filtrate and residue were retained. Residues were washed several times with distilled water to remove extra acidity prior to being used for protease production and further analysis.

### 2.3. Microbial cultures and inoculum development

In the present study, both of the microbial strains i.e., *Aspergillus ornatus* and *Alternaria alternata* were obtained from the Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan, grown on Vogel's agar slants at 35 °C for 3 days and stored at 4 °C for the whole experiment. To develop a homogeneous inoculum suspension, a pure colony of each strain was transferred into 100 mL of Vogel's liquid medium supplemented with trace elements after sterilizing the liquid medium at 103 kPa and 121 °C for 15 min. The g/L ingredients of the trace element solution were: $ZnSO_4.7H_2O$, 1.0; $CuSO_4.5H_2O$, 0.50; $MnSO_4.H_2O$, 0.50 and $Na_2MoO_4.2H_2O$, 0.50. The inoculated culture was then incubated in a temperature-controlled shaking incubator at 30 °C for 20 h at 140 rpm for the development of a homogenous inoculum.

### 2.4. Fermentation protocol

During the initial substrate screening trial, 20 g of each of the aforementioned substrates was taken in a 500 mL Erlenmeyer flask, moistened with Vogel's media, and inoculated with the freshly developed fungal spore suspension of *A. ornatus*, *A. alternata* and a co-culture consortia. All of the inoculated experimental flasks were incubated at 30 ± 1 °C for 5 days. After the stipulated fermentation time (24 h), 100 mL of distilled water was added to the fermented cultures and was then incubated in a temperature-controlled shaking incubator at 30 °C for half an hour at 180 rpm. The homogenized media were then centrifuged at 10,000× g for 10 min at 4 °C to get clear supernatant containing product solution; the resultant clear supernatant was used for analytical studies.

### 2.5. Determination of biomass

The pellets obtained during the extraction process were re-suspended in 50 mM phosphate buffer at pH 7.0 and re-centrifuged at 10,000 × g for 10 min in pre-weighed falcon tubes and dried at 80 °C until reaching constant weight and final biomass weights in grams were recorded.

### 2.6. Determination of citric acid

Citric acid was analyzed in a reaction mixture containing 1 mL of culture filtrate and 1.30 mL pyridine using as reported method of Marier & Boulet, 1958. Briefly, the mixture was added in the test tube and swirled briskly followed by 5.70 mL of acetic anhydride addition in each tube. Test tubes were placed in water bath at 32 ± 0.25 °C for 30 min. The optical density was measured (405 nm) on a spectrophotometer and the mg/mL concentration of citric acid was calculated using a standard curve assay.