Immobilization of *Aspergillus niger* in Hen Egg White for the production of Citric acid using carob pod extract

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__ABSTRACT__

Citric acid is produced by surface fermentation, submerged fermentation and solid state fermentation using different agro-waste substrates. Carob pod the fruits of the carob trees which are rich in fermentable sugars were used as substrate for citric acid production. Immobilization of cells and enzymes has been studied in different matrices. An immobilization method involving *Aspergillus niger* cells in hen egg white using glutaraldehyde as cross-linking agent was employed for the production of citric acid from carob pod extract. The carob pod extract were inoculated with 10g of immobilized cells and incubated at 30°C in (static position) batch fermentation. Maximum citric acid yields were 25g/l and 21g/l with free and immobilized cells in case of *A. niger* MTCC 281 and 28 g/l and 23 g/l in case of *A. niger* KLP20 respectively at 72 h of fermentation under batch fermentation.

__Key words: Aspergillus niger, carob pod extract, citric acid, hen egg white, immobilization.\_

__INTRODUCTION__

Citric acid (CA), an intermediate of the tricarboxylic acid cycle, is found in a variety of acidic fruit juices, particularly in the citric ones, although its extraction from natural sources, primarily lemon, was gradually replaced by biological procedures, mainly based on the use of microfungi, which are currently the most widely used. The production of CA was described in 1893 by Wehmer as a result of the metabolism of the fungus *Penicillium glaucum* [1]. Citric acid is one of the most commonly used organic acid in the food and pharmaceutical industry, which is produced mainly by submerged or surface fungal fermentation. *Aspergillus niger* fermentation is still the world’s leading source of commercial citric acid.

This process has thus been the subject of many studies [2-4]. The food industry consumes the largest amount of citric acid, as much as 70% of the total production, followed by approximately 12% for the pharmaceutical industry and 18% for other applications [5]. In most cases, the industrial production of CA by fermentation is done using *A. niger* strains, but also many other microorganisms are capable of accumulating CA, including other species belonging the same genus, *Penicillium janthinellum*, *Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis*, *Ustulina vulgaris* and various species of the genera *Botrytis*, *Ascochytta*, *Absidia*, *Talaromyces*, *Acremonium* and *Eupenicillium* [6].

Immobilization of microbial cells has received increasing attention in the past few years, and immobilized cells have been used for the production of organic acids, amino acids, antibiotics, enzymes, alcohol, and other compounds.
The production of citric acid has already been investigated on a laboratory scale with *Aspergillus niger* immobilized in a calcium alginate gel [10,11], polyacrylamide gel [12], and polyurethane foam [13].

Carob pod is the fruit of the carob tree (*Ceratonia Siliqua*) mainly cultivated in the Mediterranean countries and many areas of North America. The carob pod or kibble consists of 40-50 g/100g of sugars. These were till recently used exclusively animal fodder and human consumption. Because of its high concentration of sugars it is important for more attractive uses of the sugars [14].

Till date reports on the immobilization of *A. niger* cells in hen egg white for the production of citric acid is scanty. The immobilization of *A. niger* cells in hen egg white for the production of citric acid has led to the development of a new process. The present investigation was aimed to explore the practicability of citric acid production from carob pod extract by fermentation using immobilized *Aspergillus niger* cells.

**MATERIALS AND METHODS**

**Microorganisms:**
*Aspergillus niger* MTCC 281 (Procured from IMTECH Chandigarh, India) and *Aspergillus niger* KLP 20 locally isolated were used for the citric acid production and they were maintained at 4°C on potato dextrose agar slants.

**Inoculum:**
The cultures were incubated on potato dextrose agar slants at 30°C for 7 days. The spores obtained were suspended in 5 ml of sterile distilled water containing 0.1% Tween 80 for the preparation of inoculum.

**Immobilization method:**
The cells were immobilized in hen egg white following by the (modified) method of D'Souza et al., [15]. Leghorn variety of eggs was purchased from the local market for studies. 10 ml of hen egg white was mixed with 1 g of spores and injected with a syringe needle into a cold suspension (0-4°C) of 2% glutaraldehyde in toluene: chloroform (3:1) mixture, cross-linking was allowed to proceed for 2 h under stationary conditions. The beads were then removed and washed with water to remove traces of organic solvents and stored in buffer.

**Fermentation medium:**
Carob pods were collected locally in the month of March – April. These were desired and the kibbles were chopped into small pieces. 25 g of deseeded carob pods were taken in 100ml distilled water for the preparation of extract. The extract was autoclaved at 121°C for 15 min at 15 lb pressure and cooled to room temperature. The extract had a total sugar concentration of 16° brix (initial sugars 50 g/L) the pH of the substrate was adjusted to 5.5 using 0.1N NaOH and 0.1N NaCl. This extract was inoculated with 10% (w/v) immobilized cells and was placed for batch fermentation studies. For further repeated batch studies the extract was replaced at every 120 h intervals up to 3 cycles.

**Estimation of citric acid:**
Citric acid was estimated from the samples at every 24h interval following the pyridine acetic anhydride method [16]. The total sugars were estimated according to the method of [17].

**RESULTS AND DISCUSSION**

Carob pod extract has been shown to be a potential substrate for citric acid and also for the production of ethanol. The main component of the extract is sucrose [18]. Sucrose is known to be the best carbon source for citric acid production.

The carob pod extract inoculated with immobilized cells were analyzed periodically for both citric acid and total sugars at every 24 h intervals. Maximum citric acid in case of *Aspergillus niger* MTCC 281 was observed at 72 h (25 g/l and 21 g/l by free and hen egg white immobilized cells respectively) but with varying sugar conversion efficiency where free cells showed better sugar efficiency (65.71%) than immobilized cells (56.75%) and in case of *Aspergillus niger* KLP20 the citric acid production was 28 g/l and 23 g/l, with a variation in sugar conversion efficiency of (77.14%) and (63.88%) respectively. This was shown by the estimation of the total sugars and a
decrease in total sugars was observed. Similarly citric acid production decreased after 72 h due to depletion of sugars in the medium.

Fig 1: Citric acid production by free and immobilized cells of *A. niger* MTCC 281

Immobilization of cells for increased and rapid production of required product has been gaining importance in industrial processes. This can be performed or carried out by adopting various techniques, binding the cells to water soluble ion exchange materials, by cross linking from cell to cell carrier with a bi-functional reagent, or by trapping them in a polymer matrix where the cells are physically trapped. Because of the advantages of using immobilized cell systems, it has been used in the production of many different products. Bayraktar and Mehmetoglu [19]
reported the immobilization of conidia of *Aspergillus niger* in calcium alginate and reported 33% of citric acid production in synthetic medium. Kautola *et al.* [20] used cells of *Yarrowia lypolytica* A-101 cells in k-carrageenan and reported citric acid yield of 16.4 g/l. Ates *et al.* [21] reported the immobilization of *Aspergillus niger* NRRL-2270 in calcium alginate and used silicone oil as an oxygen vector and reported a citric acid yield of 13.1 g/l which were higher than the free cell systems. Demirel *et al.* [22] reported the immobilization of *A.niger* A-9 spores in calcium alginate and reported a citric acid yield of 2.03 g/l after 4 days of fermentation.

**CONCLUSION**

In the present study, free cells showed better citric acid production than the immobilized cells with both the *A. niger* strains MTCC281 and KLP 20. This may be due to the fact that the matrix may have resisted to some extent the entry of nutrients into and outside the matrix resulting in the decreased citric acid production. And the decrease in citric acid production may be due to the cross linking of the hen egg white matrix which may have created a stress wherein the oxygen uptake may have been reduced to the cells of both the strains in the study, thereby decreasing the citric acid production in immobilized cells than the free cells.

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