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Immobilization of *Aspergillus niger* in Polyurethane Foam for Citric Acid Production from Carob Pod Extract

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Abstract: *Aspergillus niger* strains MTCC 281 and KLP20 immobilized on polyurethane foam were utilized for the batch fermentation of citric acid from carob pod extract. Carob pods are the fruits of carob tree (*Ceratonia siliqua*). Carob pod extract with 40-50% sugars were inoculated with immobilized polyurethane foam and incubated at 30°C. Maximum citric acid yields of 23 and 33 g L^{-1} with free and immobilized cells in case of *A. niger* MTCC 281 and 27 and 38 g L^{-1} in case of *A. niger* KLP20 were obtained after 72 h of fermentation. From the studies *A. niger* KLP20 seems to be a potential strain among the two strains for the maximum citric acid production.

Key words: *Aspergillus niger*, citric acid, carob pod extract, immobilization, polyurethane foam

INTRODUCTION

Polyurethanes are one of the most versatile materials in the world today. They are known for being a perfect material for footwear, machinery industry, coatings and paints, rigid insulations, elastic fiber, soft flexible foam, medical devises. The unique properties of PU deserve popularity as PU offers elasticity of rubber, resistance to oils, solvents and fats. Polyurethane was found to be applicable in the biochemical and biotechnological fields as a perfect support for enzyme immobilization (www.polyurethane.org).

In the past few years, significant studies have been carried out on citric acid production and efforts to achieve higher volumetric productivity under conditions of submerged and surface modes of operation (Sankpal *et al*., 2001). Fungal morphology changes the physical problems of broth which causes numerous problems in industrial/fermenters with respect to gas dispersion (Sankpal *et al*., 2001) mass and heat transfer and homogenization. Cells have been commonly entrapped in a gel matrix through which substrates and products diffuse easily. Agar, agarose, kappa-carrageenan, collagen, alginate, chitosan or cellulose could be used as gel matrix (Park and Chang, 2000). Immobilized cells have been used for the production of organic acids, amino acids, antibiotics, enzymes, alcohol and other compound. Compared to free cell systems immobilized cells techniques have several advantages such a higher production rate and easier product separation (Jianlong, 2000).

In submerged cultures of filamentous fungi such as Aspergillus or Penicillum, cells form interlocking hyphae resulting in a very viscous solution as the cell mass increases. Agitation and aeration become difficult, which lead to poor oxygen mass transfer. Keeping filamentous fungi in pellet form or immobilizing them in solid supports reduces viscosity of the broth substantially, facilitating oxygen transfer with less agitation (Lee *et al*., 1989). The most widely used immobilization method is entrapment, but the adsorption method has two advantages namely its simplicity and better physiological conditions. However the selection of appropriate support material is important. To our

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knowledge, until now the reports on the immobilization of *A. niger* cells in polyurethane foam for the production of citric acid in carob pod extract is very scanty. The immobilization of *A. niger* cells in polyurethane foam for the production of citric acid has led to the development of a new process with the utilization of polyurethane foam as a immobilization matrix.

Citric acid was first isolated from lemon juice and crystallized as calcium citrate by Scheele in 1784. This acid is widespread in citrus fruits, pineapple, pears, figs and other fruits and tissues. Because of its high solubility, palatability and low toxicity citric acid is one of the most commonly used acids in the food and pharmaceutical industries greater stress has been placed on the increased citric acid production (Haq *et al*., 2002). Although citric acid can be obtained by chemical synthesis, the cost is much higher than fermentation. The worldwide demand for citric acid is about 1000,000 tons/annum (Soccol *et al*., 2003). Citric acid is produced by fermentation by many microorganisms, which include fungi, yeasts and bacteria. Among them *Aspergillus niger* is one of the most well known citric acid producers.

Carob pods are the fruits of the Carob tree (*Ceratonia siliqua*), which is mainly cultivated in the Mediterranean countries and in areas of North-America. The annual production is about 340,000-400,000 metric tons. The carob pod (kibble) contains the following expressed as g (100 g^{-1}) kibble: moisture 10-15, total sugars (glucose, fructose, sucrose and maltose) 40-50, protein 3-4, pectin 1-2, cellulose 7, hemicellulose 5; phenolic compounds 20, fat 0.5-1.0 and ash 2-3 (Roukas, 1999). Because of the high concentration of sugars in the carob kibble (pods) it is important to develop new uses for these sugars. Hence the present investigation was aimed to immobilize *A. niger* in polyurethane foam for the production of citric acid in batch fermentation.

MATERIALS AND METHODS

Chemicals

Pyridine, acetic anhydride and other chemicals required were procured from Qualigens Fine Chemicals, India. All the chemicals used were of analytical grade. Polyurethane foam was obtained from the local market. Carob pods were collected from trees in and around Gulbarga district.

Microorganisms

Aspergillus niger MTCC 281 was procured from IMTECH, Chandigarh was used as a standard strain throughout the investigation and KLP20 was isolated in our laboratory. The two strains were maintained on PDA (potato dextrose agar) slants at 4°C and sub-cultured every month.

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 0.01% Tween 80 to prepare the inoculum for further immobilization studies.

Fermentation Medium

Carob pods were collected locally in the month of March to April. These were deseeded and the kibbles were chopped into small pieces. Twenty five gram of the deseeded carob pods was taken in 100 mL distilled water for the preparation of extract. The extract was autoclaved at 121°C under 15 lb pressure for 15 min, and allowed to cool at room temperature. This extract had a total sugar concentration of 16° brix (initial sugars 50 g L^{-1}) and the pH of the extract was adjusted to 5.5 using 0.1 N (NaOH/HCl).

Immobilization and Fermentation Conditions

The polyurethane foam material used for immobilization of *A. niger* was obtained from the local market were cut into 2×2 mm sizes and washed with distilled water and sterilized. They were then

submerged in a flask containing fermentation medium and inoculated with *A. niger* spore suspension for immobilization and then allowed for fermentation at 30°C as described earlier (Lee *et al*., 1989).

Assay of Citric Acid

Samples were drawn aseptically at 24 h intervals and the citric acid was estimated spectrophotometrically by the method of Marier and Boulet (1958), which involved the color development with pyridine and acetic anhydride. The residual sugars were estimated by phenol sulphuric acid method of Dubois *et al*. (1956).

RESULTS AND DISCUSSION

Carob pod extract has been shown to be a potentially nutritive medium with sucrose in major proportion (Roukas, 1999), which aids in the citric acid production. Sucrose has been shown to be the best carbon source for citric acid production resulting in maximal yield of citric acid (Xu *et al*., 1989). Sterilized carob pod extract was inoculated with free and immobilized cells. Figure 1, 2 show the citric acid production pattern with concurrent decrease in the sugars. The analysis of the citric acid

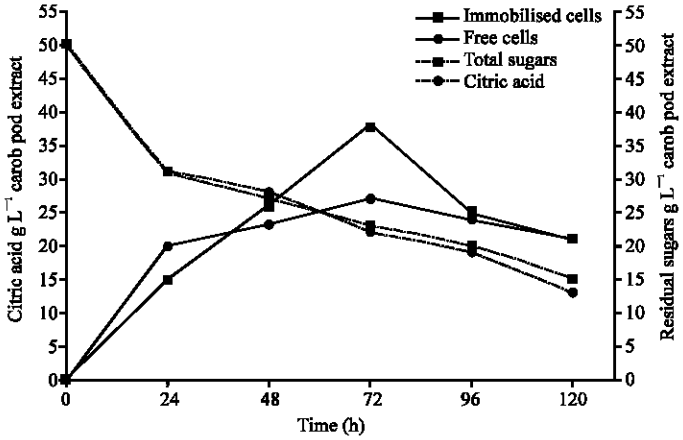

Fig. 1: Citric acid production by free and immobilized cells of *A. niger* KLP20

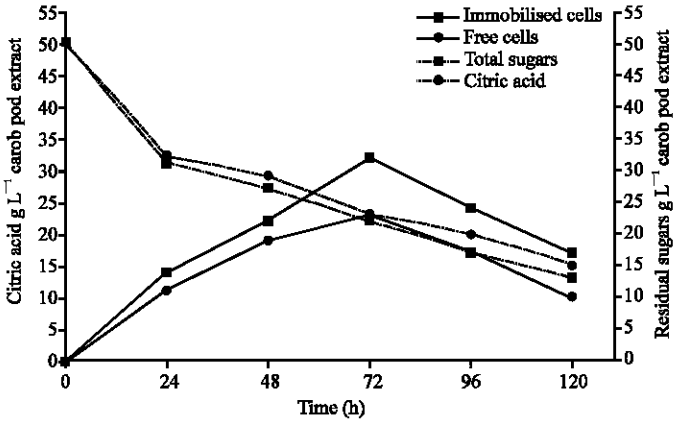

Fig. 2: Citric acid production by free and immobilized cells of *A. niger* MTCC 281

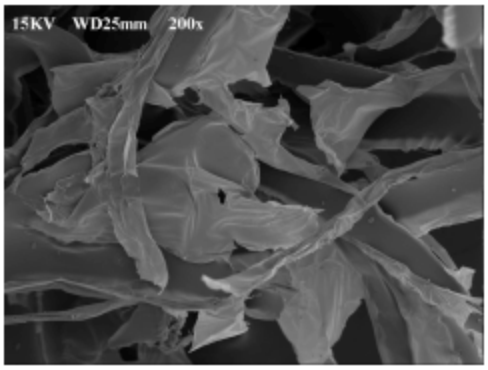

Fig. 3: Scanning electron micrographs of Polyurethane foam before immobilization

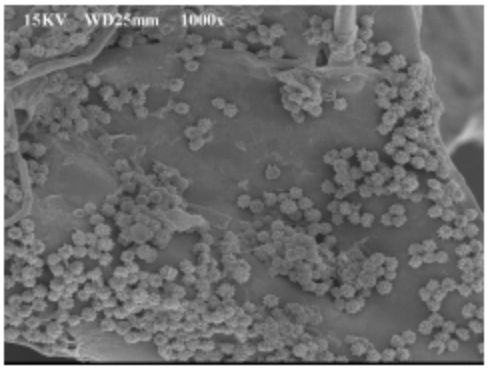

Fig. 4: Scanning electron micrographs of *A. niger* spores immobilized in Polyurethane foam

production was carried out at regular 24 h intervals. Maximal citric acid production was recorded at 72 h with citric acid levels of 23 and 33 g L^{-1} from free and immobilized cells of *A. niger* MTCC 281 and 27 and 38 g L^{-1} in case of *A. niger* KLP20, respectively. Polyurethane foam shown in Fig. 3 before immobilization and Fig. 4 shows the immobilization of polyurethane foam with *A. niger* spores. The readily available sugars were found to be utilized by both free and immobilized cells, thereby suggesting a decrease in the residual sugars was observed. The immobilized cells were able to produce approximately the same levels of citric acid consecutively upto 3 cycles tested and the immobilized cells were reusable for many more cycles without any loss of activity.

The above data indicate that maximum citric acid yield was observed at 72 h and decreased later. This may be due to the depletion of the essential nutrients required for the growth of the organism. Immobilized cells yielded 33 g L^{-1} citric acid as compared to the free cells yielding 23 g L^{-1} citric acid in case of *A. niger* MTCC 281 and 38 g L^{-1} citric acid when compared to free cells yielding 27 g L^{-1} in case of *A. niger* KLP20. The higher yields in case of immobilized cells could be due to the minimal growth of cells in the gel matrix and also due to the enzyme activities being retained at higher levels than that of the free cells. The interlocking hyphae of the filamentous fungi increase cell mass, which poses problems for oxygen transfer. Lee *et al*. (1989) reported a citric acid yield of 30 g L^{-1} in immobilized polyurethane foam by *A. niger* in sucrose medium. Honecker *et al*. (1989) reported 53 g L^{-1} by *A. niger* immobilized in alginate using 16% sucrose. Of the various industrial products utilized for citric acid production sugarcane bagasse seems to the most successful substrate for citric

acid production (Panda *et al*., 1984; El-Abyad *et al*., 1992; Haq *et al*., 2001). Polyurethane foam allows the immobilization of a large number of cells is the shortest time and highest bioactivity, this carrier was the most successful in comparison to the other matrices (data not shown) Thus we can justify from our studies, that citric acid production from the immobilized cells of *A. niger* in polyurethane foam practically seems to be a promising carrier/matrix for citric acid production with carob pod extract as a substrate.

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