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COMPARATIVE STUDIES ON CITRIC ACID PRODUCTION FROM CHEESE WHY BY SUBMERGED AND IMMOBILIZED *ASPERGILLUS NIGER*

Sepideh A. Ghanbartabar, Ghasem D. Najafpour*, Maedeh Mohammadi

Biotechnology Research Lab., Faculty of Chemical Engineering, Noshirvani University of Technology, Babol, Iran. E-mail*: najafpour@nit.ac.ir

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ABSTRACT

Citric acid production from cheese whey using submerged culture and immobilized cell of *Aspergillus niger* was investigated. In this work, effect of substrate (lactose) and product (citric acid) concentrations, medium pH, sesame oil on production of citric acid was evaluated. In addition, the cell dry weight was measured during the fermentation process. For submerged culture of *A. niger*, maximum citric acid concentration of 4.45 g/l was obtained at pH value of 4, lactose concentration of 50 g/l, and 0.1% (v/v) sesame oil for incubation of 6 days. For incubation of 5 days, in immobilized cell operation at optimal pH value of 3 and lactose concentration of 70 g/l, maximum citric acid concentration of 8.4 g/l was achieved. Immobilized cells of *A. niger* had a significant impact on improvement of citric acid production and increased the yield up to 62.6%. The immobilized cells had about 3 folds increase in citric acid productivities in comparison with free cells of *A. niger.* The productivity of citric acid in immobilized cell system was 4 times greater than the free cells.

Keywords: *Aspergillus niger*; Citric acid; Immobilized cell; Submerged culture

INTRODUCTION

Citric acid (2-hydroxy 1,2,3 propane tricarboxylic acid) is an organic acid which naturally occurs in citrus fruits. Pure citric acid is colorless and readily soluble in water. In certain microorganisms under anaerobic condition, citric acid is produced as a by-product of the tricarboxylic acid cycle (TCA)[1]. More than a million tones of citric acid are manufactured every year due to its wide industrial applications; especially, about 72% of its usage is devoted to food industries. In food processing, citric acid is mostly used as an acidifier, a flavoring agent and chelating agent [2]. There is about 3.5-4% annual growth of its consumption rate, meanwhile the world wide demand for citric acid is increasing faster than its production, so implying the new production facilities and more efficient processes are required to produce more citric acid [3].

Selection of suitable and low cost substrate for production of citric acid is one of the most important parts. The desired approach in fermentation process for high yield of production is to utilize pure sugar; however, in commercial application it may not be economical because of costly substrate may even worth more than citric acid. Therefore, use of low cost and renewable carbon source has higher priority than synthetic substrate. Agricultural waste and industrial wastewater are considered as low cost and suitable type of carbon source. In fact, the substrate selection depends on its price, pollution, yield and rate of fermentation. In recent years using molasses are more common due to it availability and ease of use. It is considered as low cost raw material; it has 40-50% sugar content. However, nature of molasses consisted of invert and caramelized sugar, rich byproducts may offer challenges because of their more concentrated heavy metal content may not be desired for fermentative citric acid production [4, 5].

Cheese whey is abundant and also considered as low cost raw material; it is a desired carbon source that could be used for fermentation and citric acid production. Whey is a byproduct of the dairy industry representing 80 up to 90% of the total milk volume processed it is cheap and has about 55% of milk nutrient, which make it an interesting desired source of organic substrates because of its relatively high concentration of nutrients. Whey has a high biological (BOD) and chemical oxygen demand (COD), which exerts a significant oxygen demand on any wastewater treatment processes. It is a disposal challenge and the discharge of waste without treatment can cause serious disaster for environment. The bioconversion of whey to value-added products such as organic acids, ethanol, enzymes, PHB (poly-3-hydroxybutyrate), xanthan gum and biomass can reduced about 75% of its COD [6, 7].

Since 1970, enzyme immobilization was introduced and then expanded to cell immobile-zation. There are several techniques of cell immo-bilization; among them entrapment method is often applied in immobilized cell reactor [8]. Immobilizations are usually conducted with high molecular weight of hydrophobic polymeric gel such as alginate, chitosan, agar, poly acrylamide. In recent year, production of organic acid, amino acid, alcohol, and enzymes with immobilization technology are more favorable than conventional processes. High yield, stability, lower waste pro-duction, easier solid liquid separation, and high cell density without wash out even in high dilution rate are some of advantages of immobilized technique [9, 10].

The purpose of this paper is to investigate performance of immobilization of *A. niger* as an alternative strategy for the bioconversion of cheese whey to citric acid in compare to submerged fermentation. Both process parameters were opti-mazed and rate of production of citric acid as free and immobilized cells were compared.

MATERIALS AND METHODS

Inoculums preparation: *Aspergillus niger* PTCC 5010 was obtained from the Persian type culture collection (PTCC, Tehran, Iran) and was cultured in a flask with 100ml medium containing 1g glucose, 0.2g peptone, 0.5g yeast extract, 0.01g KH_2PO_4 and 0.01g $MgSO_4$. The flask was incubi-ted at 30°C and 130 rpm for 30 hours. The organism was cultivated on medium in plate agar and incubated at 30 °C for 72h, and then kept in 4°C as inoculum for citric acid production.

Medium preparation: The sweet cheese whey obtained from Kalleh Dairy Company (Amol, Iran). For removing proteins, the pH was adjusted to 4 using 1N HCl solution and then whey was boiled for 20 min. the pH was then adjusted to 7 using 1M NaOH solution. Proteins and other precipi-tated particles were removed by filtration [11]. The composition of pretreated cheese whey is summarized in Table 1.

Table 1. Composition of pretreated cheese whey

Fat	4 g/l
Lactose	50 g/l
Protein	369.62mg/l
Ash	0.6%
COD	52000 mg/l

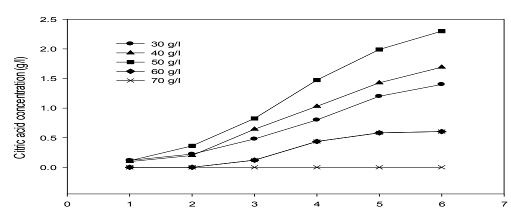
Cell immobilization: The cell immobilization was carried out by addition of 2% sterilized sodium alginate into culture of *A. niger* which was incu-bated in a 500ml flask at 30°C for 20h. Then, the mixture was extruded into 0.2 M $CaCl_2$ solution using a 1ml pipet to form droplet. Beads of calcium alginate with *A. niger* having particle dia-meter of 2.5-3.5mm were formed. The beads were washed for 3 times with distilled water to remove any excesses $CaCl_2$ salt solution. Then, beads were transferred to a sterilized sugar solution (3g/l lactose) and kept in refrigerator at 4°C for further use.

Fermentation parameters: The first step was determination of the best initial sugar concentra-tion, pH value and also investigate on growth stimulator (like sesame oil) on citric acid produc-tion using free *A. niger*. The optimization was carried out on basis of one factor at a time. The next step was evaluation of fermentation para-meters in immobilized cell. Finally, process yield and productivity of both techniques were compared. In all experimental runs, samples were aseptically removed in every 24 hours.

Analytical method: Concentration of produced citric acid was measured using the colorimetric method established by Marier and Boulet [12].The reducing sugars were determined by DNS method [13]. Cheese whey were characterized by quanti-fying the initial protein and fat concentration as well as COD content [14-16]. All analyses were performed in duplicates and average value was reported.

RESULTS AND DISCUSSION

Conventional citric acid production by free cells of *A. niger:* According to previous studies citrate productions could not occur at lactose concentration values below 25g/l [17]. Thus, in this study citric acid production was carried out on lactose concentration range of 30-70g/l. Figure 1(a) and (b) depict citric acid production and sugar utilization along with 6 days of incubation.



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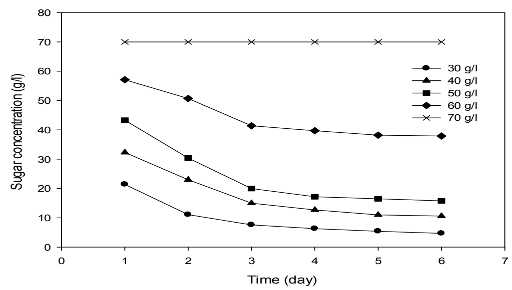

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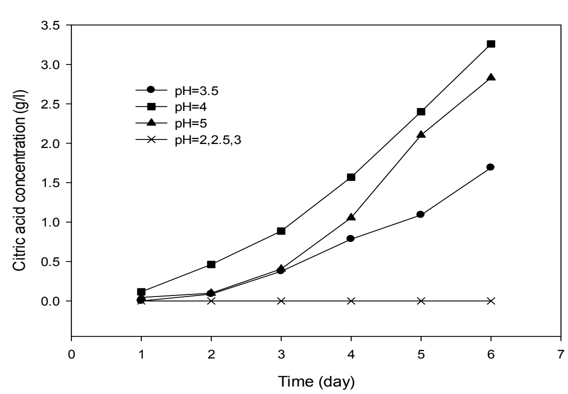
Fig. 1. Effect of initial concentration of lactose on (a) citric acid production (CAP) and (b) sugar utilization, along the process

In the first 3 days of incubation, growth rate were high (data are not shown), but citric production yield was relatively low. The citric acid production yield gradually increased until the nutrients in the medium were used up. This occurrence confirmed with the fact that citric acid is a secondary metabolite and should not be produced during the growth phase [18]. Maximum citric acid production yield was achieved in the medium which initially contains 50g/l lactose. *A. niger* growth was retarded in the medium with concentration more than 60g/l of lactose that was probably due to substrate inhibition.

Figure 2 represents the effect pH on the yield of citric acid production process. Previous studies indicated that in the pH range of 2≤ pH ≤3, the maximum values for citric acid production were achieved; because of the fact that the formation of oxalic acid as a byproduct is restricted in this pH range [19, 20]. However the strain of *A. niger* that be used in this study was unable to grow in the medium with pH ≤3. Therefore, citric acid production did not happen at all in pH less than 3.



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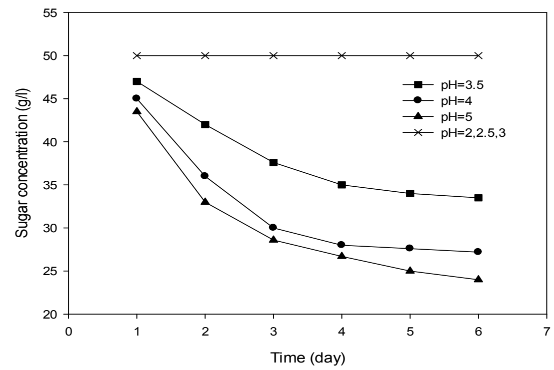

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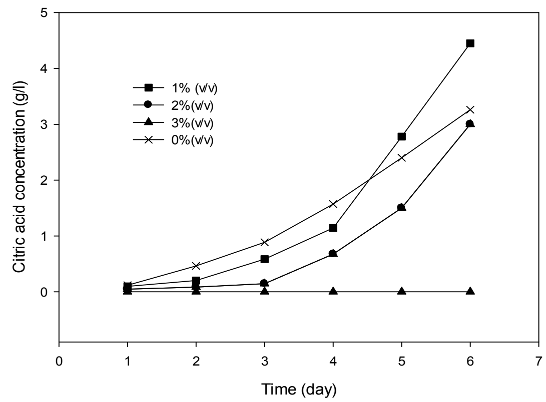
Fig. 2. Effect of pH on (a) citric acid production (CAP) and (b) sugar utilization along the process

Although at pH value of 5, the sugar consumption and growth rate was at its maximum point; but citric acid production was relatively low. That was the fact that most of the nutrients utilized and spent for cell growth. In batch culture, as it is illustrate at pH value of 4 maximum citric acid concentration of 3.26g/l was obtained. El-Aasar Sa used a complex media for the conversion of whey to citric acid; he has reported the optimum pH value was 5.5 [21]. Kim et al. [10] indicated that pH does not have direct influence on acid citric production mechanism; but, it can reflect on enzymes which respect to substrate utilization and possible product formation.

The effect of sesame oil on growth of free *A. niger* and citric acid production was also investigated. As a control experiment, fermentation for citric acid production without sesame oil was carried out at stage of optimization. Citric acid production and sugar consumption rate are shown in Figure 3. According to obtained experimental data, citric acid production was improved by adding 1 and 2% (v/v) sesame oil to the medium. Maximum citric acid production was achieved by addition of 1% (v/v) sesame oil concentration of with sugar conversion coefficient of 21.3% and final citric acid concentration of 4.45g/l. These results demonstrate that sesame oil had a profound effect on the metabolism of sugar by *A. niger*, but its effect is not exactly clear. It is reported in the literature that sesame oil stimulators the cells; as a result the permeability facilitate metabolites to be extracted [20, 22]. However its high concentration may cause growth inhibition.



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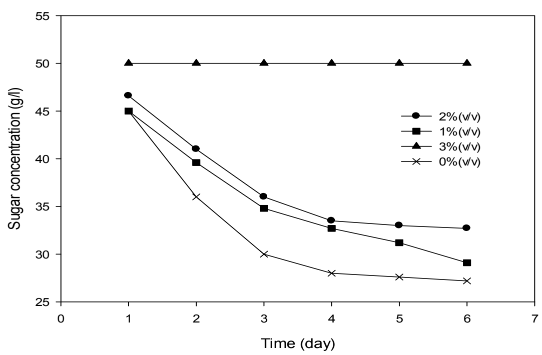

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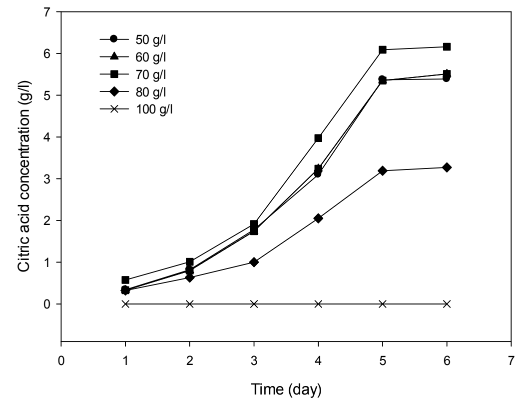
Fig. 3. Effect of sesame oil concentration on (a) citric acid production and (b) sugar consumption rate

Citric acid production by immobilized cells: Based on previous studies, immobilized *A. niger,* unlike free cells can grow in media with high lactose concentration even more than 60g/l [10]. Therefore, citric acid production using immobilized cells were carried out in sugar concentration in the range of 50-100g/l. Figure 4 illustrates the effect of different lactose initial concentration on citric acid production with immobilized *A. niger*. As it is shown, citric acid concentration was increased with increasing in lactose initial concentration up to 70g/l. However, the sugar concentration of more than 70g/l, the additional sugar concentration had negative impact on citric acid production that was probably due to substrate inhibition. In lactose concentration of 100g/l no citric acid production was observed.



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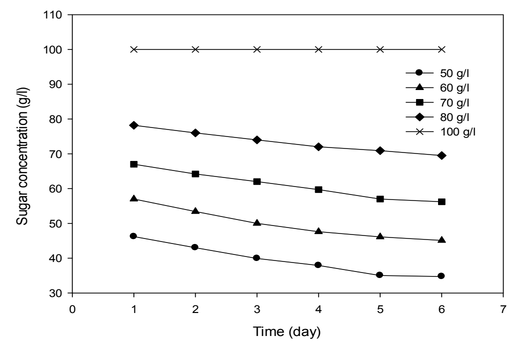

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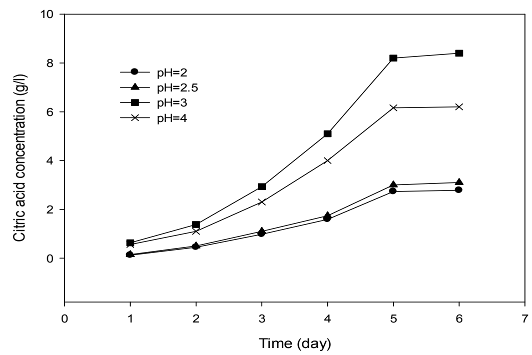
Fig. 4. Effect of initial lactose concentration on (a) citric acid production (b) sugar consumption, in immobilized cell

In initial stage, immobilized cell reactor was used; lactose initial concentration of 50g/l resulted in citric acid final concentration of 3.7g/l which is less than the value obtained with free cells in the same concentration of sugar. Such differences in citric acid production yield were most probably due to mass transfer limitation to immobilized cells of *A. niger* in compare to free cells of the organism.

In case of immobilized *A. niger*, maximum citric acid concentration of 6.1g/l and production yield of 46.8% was obtained in the medium with initial sugar concentration of 70g/l.

Furthermore, the effect of pH on citric acid production via immobilized cell was investigated. As it is shown in Figure 5(a) at pH value of 4, immobilized cells had higher citric acid production yield in compare to free cells. As it was expected, maximum citric acid production occurred at pH value of 3. The final citric acid concentration of 8.4 g/l and sugar conversion coefficient of 62.6% was achieved. But, in pH value below 3 immobilized cells were unable to utilize sugar in suitable manor, then, citric acid production was decreased.



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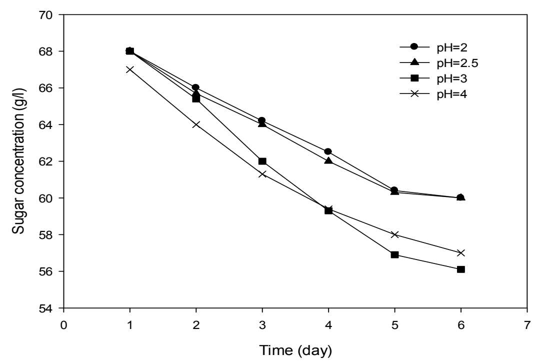

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Fig. 5. Effect of pH on citric acid production with immobilized cells on (a) acid citric production (b) sugar consumption rate

Adding 1 and 2% (v/v) sesame oil into medium has reduced the production of citric acid

(Figure 6). Since the positive effect of sesame oil on citric acid production was proved for free *A. niger*. The obtained results for immobilized *A. niger* demonstrated that sesame oil had devastating impact on calcium alginate gels permeability or it caused limitations in substrate transfusion. It is most probably true that the substrate was unable to penetrate into the beads; then the produced citric acid was much less than the expected amount.

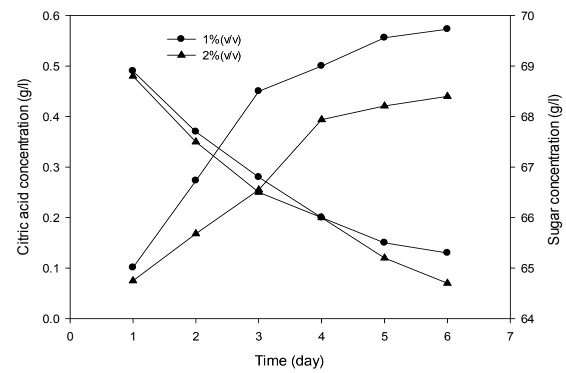


Fig. 6. Effect of additional sesame oil on citric acid production in immobilized cells

Table 2 summarizes the best medium composition and fermentation parameters for maximum acid citric production with free and immobilized *A. niger*.

Table 2: Best medium composition for citric acid production with free and immobilized *A. niger*

	pH	Lactose (g/l)	$(NH_4)_2Fe(SO_4)_2$ (g/l)	$MgSO_4$ (g/l)	Sesame oil % (v/v)
Free *A.niger*	4	50	0.5	0.1	1
Immobilized cell	3	70	0.5	0.1	0

Reuse of immobilized *A. niger* on citric acid production: One of the most noticeable benefits of immobilized cells is their potential for being reused for prolong duration. After 5 days of fermentation, immobilized cell was taken from the media and washed with sterile distilled water, and put into new sterile medium. This process was repeated for 7 times and the effect of each reuse on citric acid production was investigated. Experimental results are presented in Figure 7.

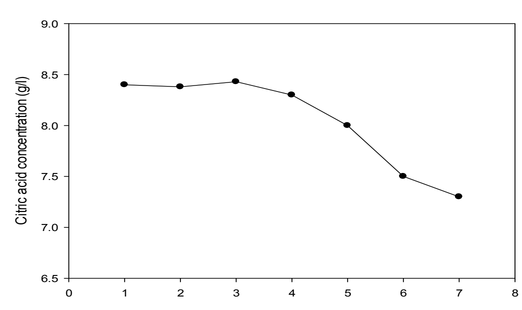
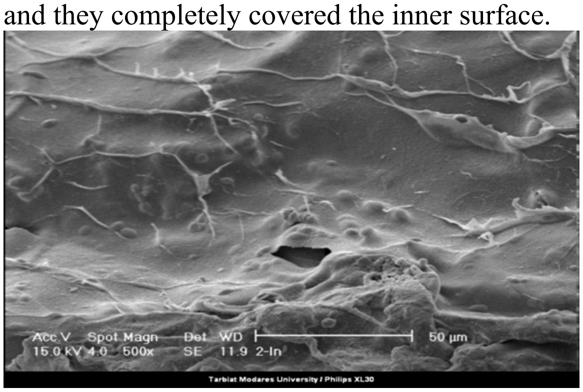


Fig. 7. Effect of number of reused immobilized cells on citric acid production

Immobilized cells have maintained its efficiency if it reused for 4 times. However if the number of reuses increase, citric acid production rate may decrease. Such reducing in citric acid production is because of the fact that population of *A. niger* inside the calcium alginate gel may increases or decease. Another, possibility might be due to clogging the porosity of the beads; therefore, the produced citric acid may not be able to diffuse outside the beads and acid accumulation inside the beads causes product inhibition[8].

The inner surface of the beads before and after use was examined under scanning electron microscope (SEM) (XL300, Philips, Netherlands) (see Figure 8). It is revealed that after 7 times reuse, cells apparently migrated from the inner side and new colonies have formed on the surface and they completely covered the inner surface.



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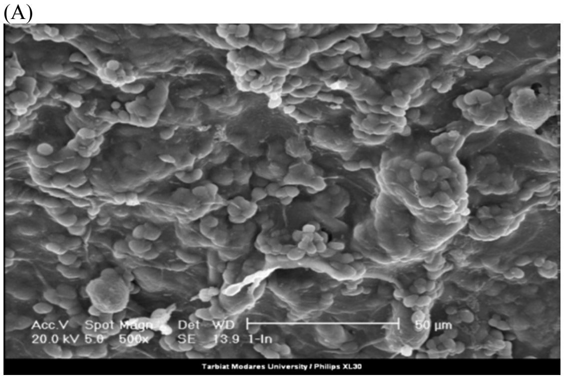

(B)

Fig. 8. SEM images of inner surface of calcium alginate gel (a) before being used (b) after 7 times reused

CONCLUSION

This study demonstrated that cheese whey has a great potential for citric acid production. Using *A. niger* immobilized cells had a significant impact on improvement of citric acid production and increased the yield up to 62.6%, that is about 3 folds in comparison with free cells of *A. niger*. Immobilized cells can be reused for 4 times without noticeable reduction in citric acid productivities. The best medium composition and fermentation parameter for maximum acid citric production within free and immobilized *A. niger* were defined.

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