Malaysian Journal of Microbiology, Vol. 9(2) 2013, pp. 161-165

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Production of citric acid by *Aspergillus niger* immobilized in *Detarium microcarpum* matrix

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Received 24 September 2012; Received in revised form 12 December 2012; Accepted 12 December 2012

ABSTRACT

Aims: This study investigated the potential use of *Detarium microcarpum* as a support matrix for the immobilization of fungal cell and its subsequent use in the production of citric acid.
Methodology and results: Conidia of *Aspergillus niger* were immobilized on *Detarium microcarpum* matrix crosslinked with glutaraldehyde (2.5% v/v). Effects of various immobilized conditions on citric acid yield were determined. Citric acid production was optimum at 9% gel concentration, 5.0 mm bead size and 400 mg spore load. Higher citric acid yield (82 g/L) was obtained by immobilized cells while free cells gave (61 g/L). Immobilized gels were stable for 15 days in the fermentation medium and retained about 75% of initial yield after five repeated cycles.
Conclusion, significance and impact of study: This study therefore shows that *D. microcarpum* is a novel matrix for cell immobilization and will enhance production of citric acid in batch system.

Key words: Citric acid; *Detarium microcarpum*; *Aspergillus niger*; cassava whey

INTRODUCTION

Citric acid is an important metabolite widely used in food industries. It is ubiquitous in nature and exists as an intermediate in the tricarboxylic acid cycle (Torres *et al*., 2002). Microbial production of citric acid had been achieved using free and immobilized fungal strains such as *Aspergillus niger* especially in submerged fermentation (Bayraktar and Mehmetoglu, 2000).

Immobilized microbial cells entrapped in polymeric matrices are currently receiving attention in the production of microbial metabolites due to fast and easy recovery of the cells and ability to reuse the cells. The technique also makes repeated batch and continuous process possible by allowing a consistent decrease in medium viscosity, enhanced nutrient and oxygen transfer (Jianlong, 2000; Osho *et al*.,2001).

Citric acid can be produced by cells entrapped in a gel matrix through which substrates and products diffuse in and out easily (Roukas, 2000). Among the matrices commonly used are calcium alginate (Bayraktar and Mehmetoglu, 2000), K-Carrageenan, Polyacrylamide gel (Horitsu *et al.,* 1985), polyurethane foam (Jianlong, 2000), cellulose microfibrils (Sankpal *et al.,* 2001). Some of these matrices are expensive and also have weak mechanical strength (Park and Chang, 2000). However, in the tropics, there are some natural polymers such as *Detarium microcarpum* with hydrocolloid properties comparable with conventional entrapment agents.

Detarium microcarpum is a member of the family leguminosae and the sub-family Caesalpiniacae of flowering plants (Enwere, 1998). Its seeds are used directly for soup thickening (Onweluzo, 1991). Although *D. microcarpum* has been reported as a good thickening agent or stabilizer, its industrial uses have not been well documented (Akpata and Miachi, 2001). Hence its potential use as immobilization matrix is being considered in this study.

MATERIALS AND METHODS

Microorganism

Strains of *A. niger* were screened quantitatively for the production of citric acid on Czapek-Dox agar medium at 30 °C for 96 h. The organism was maintained on Saboraud dextrose agar at 4 °C and sub-cultured bi-monthly.

Chemicals

Glutaraldehyde, formaldehyde and ethanol were from Sigma Ltd, UK. All chemicals were reagent grade.

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Substrates

Cassava whey was collected into sterile container from a cassava processing plant in Abeokuta, and stored at 4 °C until needed. *D. microcarpum* seeds were also obtained from a local market in Abeokuta, Nigeria.

Pretreatment of *D. microcarpum* seeds

D. microcarpum seeds were dehulled and grounded into powder form, the powder was defatted using the method of AOAC, 1995.

Immobilization of *A. niger*

Spores of *A. niger* were immobilized with *D. microcarpum* matrix using a modified method of Osho *et al.*, (2001). *D. microcarpum* powder (10 g) was crosslinked using glutaraldehyde (2.5%) v/v. Conidia of *A. niger* (250-500 mg) were mixed with 100 mL of crosslinked *D. microcarpum* slurry at 35 °C under vigorous stirring. The slurry was made into spherical beads (2.5 mm, 5.0 mm and 6.0 mm) by dropping through a syringe into ethanolic formaldehyde (40:60 v/v) for 24 h.

Production of citric acid production

Citric acid production by immobilized beads of *A. niger* was carried out in cassava whey medium (50 mL) containing glucose (10% w/v), ammonium phosphate (0.25% w/v) and methanol (1% v/v) at 30 °C for 24 h. Effects of different immobilization parameters (gel concentration, spore load and bead size) on citric acid production by *A. niger* were carried out in batch fermentations.

Assay method

Citric acid was determined titrimetrically (AOAC, 1995) by using 0.1 NaOH and phenolphthalein as indicator and calculated as g/L according to the formula.

$$\% \text{ citric acid} = \frac{\text{Normality x volume of 0.1 M NaOH x equivalent weight of citric acid x dilution factor}}{\text{Weight of sample (g) x 10}}$$

Reducing sugar content was determined using a refractometer and pH was also measured with a pH meter (Mettler-Toledo, Essex M3509 Type 340).

Statistical analysis

Data were means of triplicate determinations and analysed using SPSS 17.0.

RESULTS AND DISCUSSION

Immobilization of *A. niger* in *Detarium microcarpum* matrix

Studies on citric acid production by immobilized cells of *A. niger* on cassava whey medium was carried out. The effect of different gel concentration showed that citric acid yield was optimum at a gel concentration of 9% at 150 h of fermentation while a decrease in citric acid yield was noted in higher gel concentration (Figure 1) The low citric acid yield at higher gel concentration was due to diffusional limitations imposed by the solid nature of the hardened matrix (Osho *et al.*, 2001).

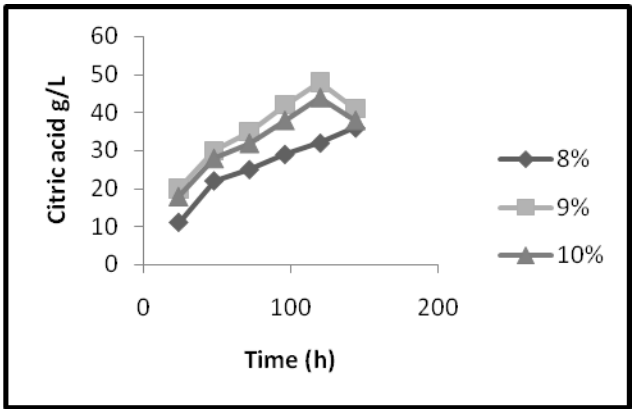


Figure 1: Effect of gel concentration on citric acid production by immobilized *A. niger* cells.

Bead size also has a significant influence on citric acid yield by immobilized *A. niger* cells (Figure 2). At a bead size of 5.0 mm, citric acid yield was maximum at 62 g/L while the largest bead size of 6.0 mm gave 49 g/L. At lower bead size, the gel matrix is thinner, which makes the fungal mycelia in the gel cavities more accessible to substrate than at higher bead size.

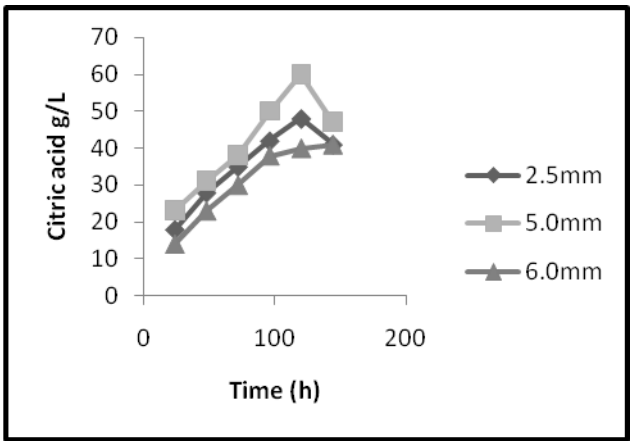


Figure 2: Effect of bead sizes on citric acid production by immobilized *A. niger* cells.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Effect of initial spore load showed that spore load had a very strong influence on citric acid production with maximum citric acid yield obtained at a spore of 400 mg (Figure 3).Spore load is known to play a vital role in the production of microbial metabolites, however higher concentration of spores did not lead to improved citric acid yield. This may be attributed to a decrease in mechanical strength of gel particles as cell loading increases (Dong *et al*., 2006).

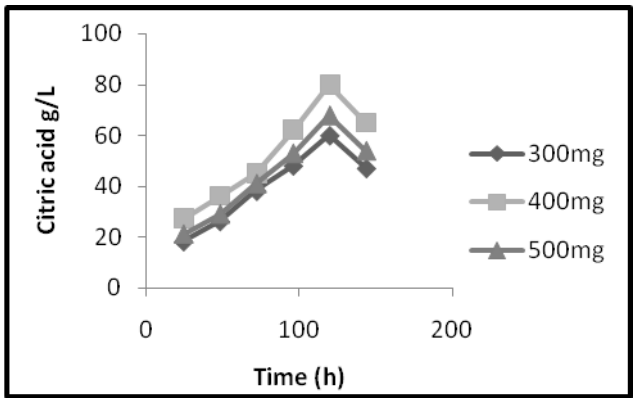
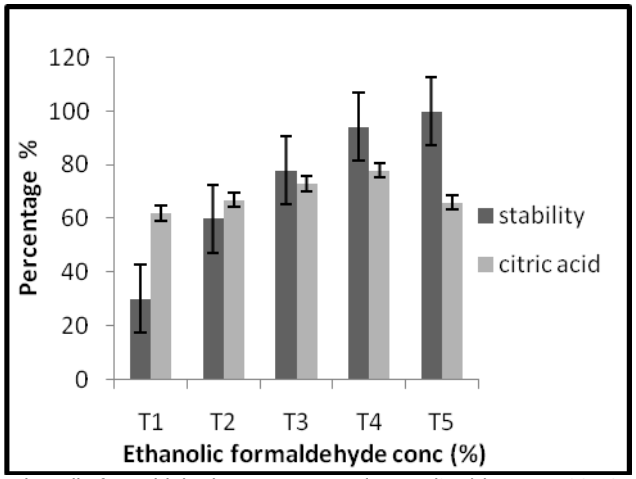


Figure 3: Effect of spore load on citric acid production by immobilized *A. niger* cells.

Stability of gel beads

Effect of various concentrations of ethanolic formaldehyde on gel stability was determined by monitoring percentage survival for 25 days. Results showed that hardening agents showed significant effects on both stability and citric acid synthesis ($p \leq 0.05$). Gels hardened with ethanolic formaldehyde (30:70% v/v) gave 100% stability up to the 15th day of fermentation (Figure 4).



Ethanolic-formaldehyde concentration (%v/v) T1=60:40; T2=50:50; T3=60:40; T4=30:70; T5=20:80

Figure 4: Effect of stabilizing agents on stability of immobilized beads of *A. niger* cells during citric acid production.

Glutaraldehyde provides a powerful covalent link between the microbial cell and its carrier matrix while solidification with formaldehyde enhances stability of the carbohydrate matrix and eventually overcomes the tendency to agglomerate or form a gel in aqueous solutions (de Alteris *et al*., 2001).

The repeated use of the immobilized *A. niger* cells showed that a decrease in citric acid production was observed as the re-use number increased (Table 1). This may be as a result of clogging of fungal spores immobilized within the matrix. Dong *et al*. (2006) reported that accumulation of the citric acid inside the cell inhibited activity of citrate synthase found in the citric acid cycle and thus led to decrease in citric acid production. However, immobilized *D. microcarpum* beads retained 75% of the initial citric acid synthesis after five repeated uses. One of the important characteristics of immobilized cells is their stability and reusability over an extended period of time (Shafei and Allam, 2010).

Table 1: Reuse of immobilized *A. niger* cells in five batch fermentations.

No of batch	Citric acid yield (%)
1	100.0
2	95.3
3	87.1
4	80.3
5	75.4

Data are means of triplicate, SD= ±0.36

Comparative citric acid yield by free and immobilized cells of *A. niger*

Comparative citric acid production by free and immobilized cells of *A. niger* was shown in Figure 5. Immobilized cells gave an improved citric acid yield (82 g/L) while free cells gave a lower yield of 61 g/L. The result agreed with Rymowicz *et al*. (1993) that the production of citric acid and fermentation efficiency were reportedly enhanced by hardening the alginate carrier beads with glutaraldehyde and by activation of the immobilized biocatalyst in a nutrient solution. Bayraktar and Mehmetoglu (2000) reported that immobilized cells offer several advantages over free cells such as decreased medium viscosity and enhanced oxygen and nutrient transfer, higher productivity, operational stability and decreased contamination of the product by free cells.

Residual sugar and pH profile during citric acid production

The residual sugar and pH profile during citric acid synthesis showed that at the end of the fermentation process, a significant reduction in residual sugar from 40 g/L to 10.2 g/L was obtained. The result agreed with Kareem *et al.* (2010) that there is a parallel relationship between citric acid production and the consumption of sugar. A decrease in the pH from 5.6 to 2.2 was also

ISSN (print): 1823-8262, ISSN (online): 2231-7538

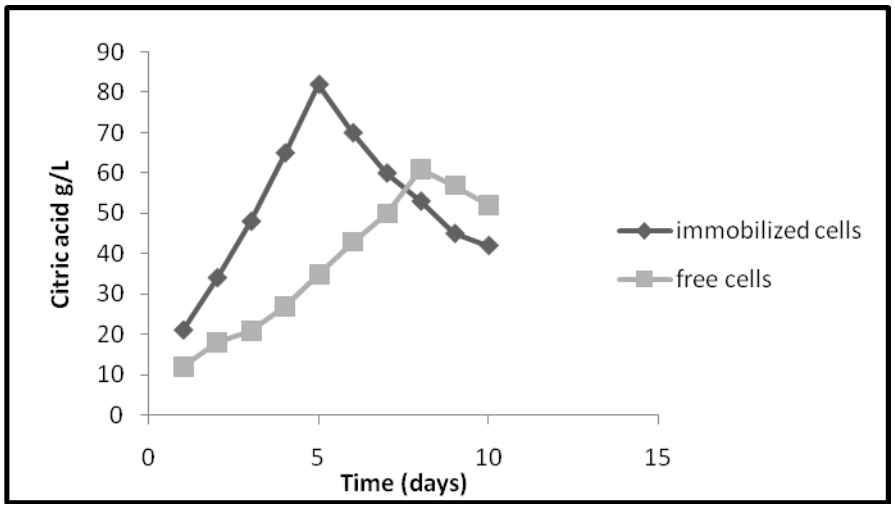


Figure 5: Comparative citric acid production by free and immobilized cells of *A. niger*.

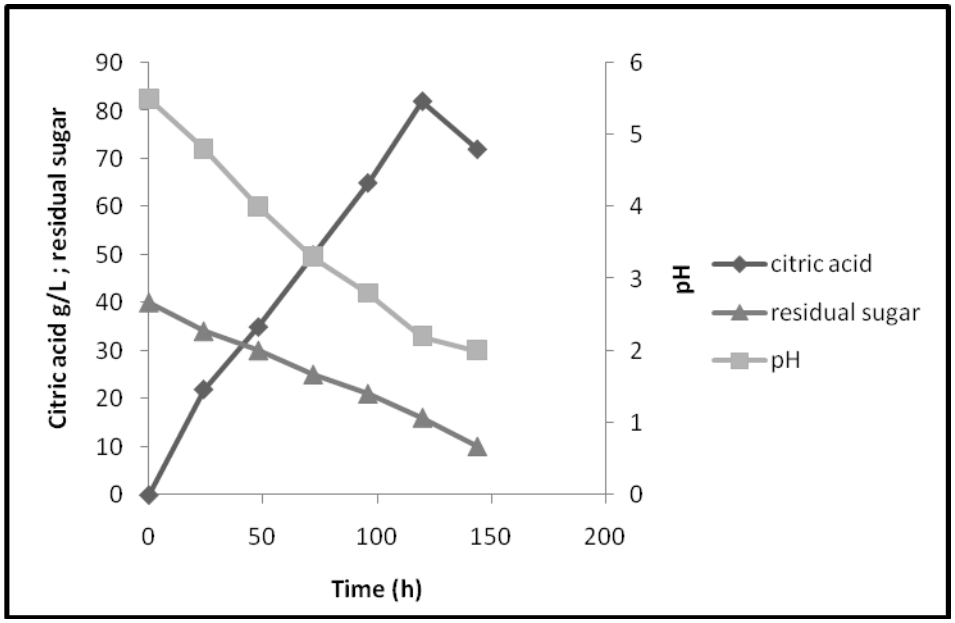


Figure 6: Residual sugar and pH profile during citric acid fermentation by immobilized *A. niger.*

observed as incubation period increased. Accumulation of citric acid during the fermentation process has been noted as a factor responsible for the drop in the pH of the fermented broth (Kareem *et al.*, 2010).

CONCLUSIONS

This study has presented the potential of *D. microcarpum* matrix as a suitable matrix for the immobilization of *A. niger* cells and its subsequent for citric acid production. In addition to higher citric acid yield, Immobilized cells were found stable and can retain about 75% of initial yield after five repeated uses. It is an abundantly available and environment friendly material which can promote large scale and economical production of commercially valuable organic acid.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538