View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Procedia Environmental Sciences 8 (2011) 396 – 402

Procedia Environmental Sciences

ICESB 2011: 25-26 November 2011, Maldives

# Development of Culture Inoculum for Scale-Up Production of Citric Acid from Oil Palm Empty Fruit Bunches by *Aspergillus niger*

Md. Zahangir Alam[aa], Md. Niamul Bari[a,b], Suleyman A. Muyibi[a], Parveen Jamal[a], Abdullah-Al-Mamun[a]

[a] *Bioenvironmental Engineering Research Unit (BERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM), Gombak, 50728 Kuala Lumpur, Malaysia*

[b]*Department of Civil Engineering, Rajshahi University of Engineering & Technology, Bangladesh*

**Abstract**

An extensive study on fungal inoculum was carried out to develop an effective culture (broth) inoculum for citric acid production from oil palm empty fruit bunches through solid state bioconversion. The effect of spores age, storage time of spore suspension inoculum, doses of spore suspension inoculum to prepare culture inoculum, ages of culture inoculum and doses of culture inoculum (concentration of biomass) on citric acid production were evaluated. It was found that the optimum age of spore was 3-4 days for the preparation of spore suspension inoculum and the maximum storage time of spore suspension inoculum was 2 days. Furthermore, the optimum dose of spore suspension inoculum was 20% (v/v) to prepare culture (broth) inoculum. The culture inoculum of 48 hrs age with biomass concentration of 0.82±0.3 g/L showed the highest production of citric acid compared to other percentage of spore suspension inoculum and culture age for all doses of culture inoculum. The highest production of citric acid of 367.4±2 g/kg-EFB was obtained with 25% (v/w) (0.21 g biomass/kg substrate) culture inoculum after 7 days of bioconversion.

© 2011 Published by Elsevier Ltd. Selection and/or peer-review under responsibility of the Asia-Pacific Chemical, Biological & Environmental Engineering Society (APCBEES) Open access under CC BY-NC-ND license.

*Keywords:* Culture inoculum, citric acid, solid state bioconversion

[a] Corresponding author. Tel.: +6 010 3649040; fax: +603 61964442.
*E-mail address*: zahangir@iium.edu.my.

1878-0296 © 2011 Published by Elsevier Ltd. Selection and/or peer-review under responsibility of the Asia-Pacific Chemical, Biological & Environmental Engineering Society (APCBEES) Open access under CC BY-NC-ND license.
doi:10.1016/j.proenv.2011.10.062

## 1. Introduction

Citric acid is commercially valuable fermentation product extensively used in different industrial purposes such as: acidulent, antimicrobial preservatives, antioxidant, prevent crystallization, anticoagulant, flavour stimulator, metal cleaner, etc. [1,2]. Large scale production is more challenging than laboratory scale production where inoculum is one of the major governing factors for any fermentation product. It is already established that citric acid production in laboratory scale is highly influenced by inoculum size (dose of spore) [3,4].

In laboratory scale production, spore suspension inoculum is usually used due to the requirement of less quantity and simplicity of its preparation [4]. Several researchers also carried out the experiments of pilot scale citric acid production by using spore suspension inoculum in packed bed, horizontal drum and tray type bioreactor [4,5] might be due to same reasons. However, large quantity of inoculum is required in case of large scale or commercial scale production. Furthermore, the preparation of large quantity of spore suspension inoculum is not easy as well as practicable. This situation leads to develop alternative inoculum without much hampering the product formation. Furthermore, product formation might vary with the variation of inoculum types, doses, age, and storage time as well as inoculum concentration.

The empty fruit bunches (EFB) is a new potential substrate for citric acid production which is abundant in Malaysia with the annual production of about 17 million tones [3,6]. The EFB, which is composed of more than 70-80% carbon source (cellulose and hemicellulose) is presently used for the plantation as mulch, composite boards, pulp, paper, cushions, landscaping and steam production by burning with little amount [7]. The rest of the biomass poses serious disposal problems in the environment that can be used as raw material for commercial production of citric acid.

So far our knowledge goes; this is the first time study in detail on the development of inoculum for the scale-up production of citric acid from solid substrate. Only a few reports are available on effect of spore age of inoculum on citric acid production which is also for spore suspension inoculum for laboratory scale experiment [8]. However, the use of culture (broth) inoculum, which could be produced in fermentation, might be the solution to fulfil the demand of large quantity of inoculum for large scale citric acid production especially through solid state bioconversion. Therefore, the aim of this study was to develop a suitable procedure for the preparation of effective culture inoculum for citric acid production from EFB through solid state bioconversion.

## 2. Materials and Methods

### *2.1. Major Substrate and Microorganisms*

The major substrate oil palm empty fruit bunches (EFB) was collected from Seri Ulu Langat Palm Oil Mill in Dengkil, Selangor, Malaysia and stored in a cold room at $4^0$C to avoid the unwanted bio-degradation by any microorganisms. The EFB samples were prepared by grinding to 0.5 mm particle size and below after washing vigorously with tap water and drying at $105^0$C for 24 hours. The ground EFB was dried at $60^0$C for 48 hours to get constant dry weight for the experimental study. A local isolate of *Aspergillus niger* IBO-103MNB (IMI396649) identified by the microbial identification organization, CABI Europ-UK, was selected for this study. This strain was obtained through series experiments of isolation, purification and screening against the citric acid production by using EFB as new substrate [6]. The fungal strain was maintained on 3.9% w/v of potato dextrose agar (PDA, Mark) slants, sub-cultured once in a month and stored at $4^0$C.

### 2.2. Preparation of Inoculum

The strain *A. niger* was grown on PDA medium in petri dishes at temperature of $32^0$C for 3 to 4 days. Spore suspension inoculum was prepared by washing the fungal culture grown on PDA plate according to procedure followed by Alam et al. [9]. About 8-10 ml of distilled water was poured each time onto the cultured agar plate and the spores were gently scrapped using sterile glass rod. Three plates of fungal culture were washed with 50 ml sterilized distilled water for maintaining the uniformity of spore concentration. The suspended fungal spores were then filtered using Whatman number 1 filter paper into an Erlenmeyer flask. The spore concentration (spore/ml) was determined by counting the numbers of spore with a haemocytometer to maintain its uniform strength. Spores concentration in the inoculum was maintained of $1\times10^8$ spores/ml. To prepare inoculum, all flasks, funnels, filter papers, distilled water and L-shaped glass rod were sterilized prior to use.

Culture inoculum (broth inoculum) was prepared by adding optimum dose of spore suspension in 1%(w/v) sugar solution, which was selected from the growth results of strain in different concentration (0-6% w/v) of sugar solution (data not shown). The inoculated solution was incubated at room temperature ($28\pm1^o$C) in rotary shaker at 150 rpm for their growth. The strength of culture inoculum was maintained by measuring the biomass concentration. The culture inoculum without biomass was obtained by filtering the culture inoculum with biomass using Whatman number 1 filter paper. Both culture inoculum with biomass and without biomass were tested to observed the effect of biomass on production of citric acid.

### 2.3. Experimental Procedure for Solid State Bioconversion

Bioconversion experiment for the evaluation of different inoculums was carried out in 250 ml Erlenmeyer flasks. Twenty grams of total substrate (wet basis) was prepared with major substrate – EFB (particle size ≤0.5 mm) and 6.4% (213.33 g/kg of EFB: w/w) sucrose (sucrose), 2% methanol (66.67 ml/kg of EFB: v/w) and 9% mineral solution containing 0.09 g/l $ZnSO_4.7H_2O$, 0.1 g/l $CuSO_4.5H_2O$, 0.4 g/l $MnSO_4$ and 5 g/l $MgSO_4.7H_2O$ [27 mg/kg of EFB $ZnSO_4.7H_2O$, 30 mg/kg of EFB $CuSO_4.5H_2O$, 120 mg/kg of EFB $MnSO_4$ and 1500 mg/kg of EFB $MgSO_4.7H_2O$], which was optimized in previous study (Bari et al., 2009) [6]. Moisture content was adjusted at 70% with mineral solution, inoculum, methanol and distilled water. Methanol and inoculum were added after sterilization of media by autoclaving at $121^0$C for 15 minutes. Initial pH was maintained at 6 and bioconversion experiment was conducted at incubation temperature of $32.5^o$C. All the experiments were performed in triplicate.

### 2.4. Harvesting and Extraction of Citric Acid

Harvesting and extraction of citric acid was carried out after 6 days of bioconversion (based on the previous studies) [3,6]. Fifty milliliter (50 ml) distilled water was added to the fermented substrate and mixed thoroughly to dilute properly. Diluted media was shaken for 1 hour at 150 rpm at room temperature ($28\pm1^0$C) in a rotary shaker [10]. The supernatant was collected by filtering with Whatman no. 1 filter paper and immediately analyzed to determine the content of citric acid.

### 2.5. Experimental Design for the Development of Culture Inoculum

The activity of inoculum might vary depending on the culture age (spore age) and inoculum storage time. Furthermore, the production of citric acid might vary on doses of suspension inoculum to prepare culture inoculum, age of culture inoculum and doses of culture inoculum for bioconversion. On the basis

of these assumptions, it is essential to determine the optimum conditions of culture inoculum for the production of citric acid. A series of sequential experiments were carried out to attain this target.

Spore suspension inoculum was prepared from 2, 3, 4, 5, 6 and 7 days old culture grown on PDA plate and tested against citric acid production. The most effective culture age was determined on the basis of comparison of yield. The prepared spore suspension inoculum (fresh) from best culture age was stored in chiller at $4^0$C for 30 days to observe the effect of inoculum storage time. The media was inoculated consecutively first four days (0, 1, 2 and 3 days) followed by every three days (6, 9, 12, 15, 18, 21, 24, 27 and 30 days) interval till 30 days. The maximum limit of inoculum storage time was determined on the basis of citric acid production.

The effect of age of culture inoculum was evaluated by varying from 1 to 5 days of culture age while suspension inoculum and culture inoculum dose were maintain at 5% (v/v) and 5% (v/w) respectively. The culture age was selected from this study to prepare inoculum for further study of suspension inoculum and culture inoculum dose. Suspension inoculum doses were varied as 5, 10, 15, 20 and 20% (v/v) to prepare five different culture inoculums. Each culture inoculum was examined against the production of citric acid production by varying the dose of 5, 15, 25 and 40% (v/w). Finally, effect of bioconversion time on citric acid production with culture inoculum was examined.

## 3. Results and Discussion

The requirement of inoculum for large-scale citric acid production through solid state bioconversion is huge compared to laboratory scale production in Erlenmeyer flask where spore suspension inoculum was used. However, the preparation of huge quantity of spore suspension inoculum is not practical and use of spore suspension inoculum is not usual practice. Furthermore, the characteristics of these two types of inoculum is different that might affect the production of citric acid. As a result a comprehensive study is essential for the development of culture inoculum to overcome or minimize the adverse effect on production. In this regards, sequential experiments were conducted to develop the culture inoculum concerning plate culture age (spores age), storage time of spore suspension inoculum, dose of spore suspension inoculum, culture inoculum incubation age and culture inoculum dose.

### *3.1. Effect of Age of Spores on citric acid production*

Fugal spore maturation and germination activity depend on the culture age of the fungi. Spore suspension inoculum was prepared from 2, 3, 4, 5, 6 and 7 days old culture grown on PDA plate and tested against citric acid production. The result shows that almost same production was obtained from 3 and 4 days old culture (spores) which showed the highest production (408.4-417.4 g/kg-EFB) compared to other lower and higher aged culture (Fig. 1). The lower production from the 2 days old culture might be due to immature spores. The highest production obtained from 3 and 4 days old spores might be due to young and enough maturity of spores which were sufficiently grown on media and produced citric acid. From 5 days to onward culture produced gradually lower citric acid due to their aging effect on germination and production. Similar study has been conducted by Khosravi-Darani and Zoghi [8] to observe the effect of age of spores on citric acid production from sugarcane bagasse and found that 6 days old spores of *Aspergillus niger* produced the highest citric acid.

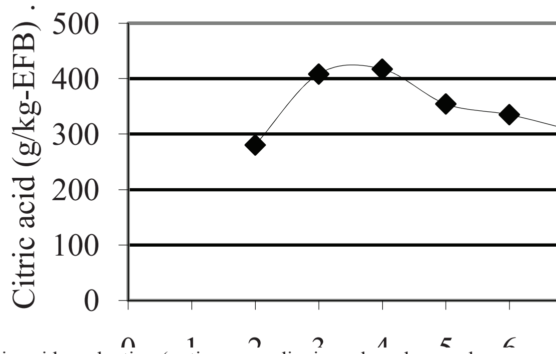


Fig. 1: Effect of culture age on citric acid production (optimum media, inoculum dose and process conditions were employed)

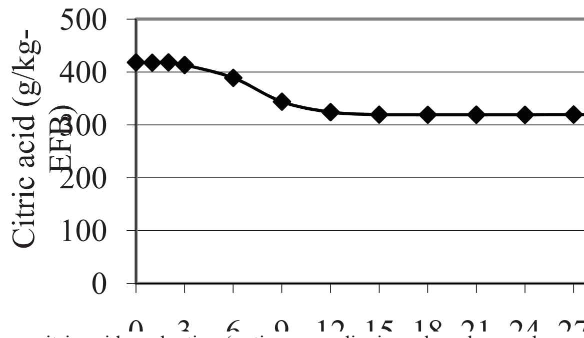


Fig. 2: Effect of inoculum storage time on citric acid production (optimum media, inoculum dose and process conditions were employed)

### *3.2. Effect of Storage Time of Inoculum on Citric Acid Production*

The prepared spore suspension inoculum was stored in chiller at $4^0$C for 30 days. The media was inoculated with spore suspension inoculum which was stored for 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days. It was observed that the production of citric acid was unchanged until first two days and decreased after that up to 15 days of storage time (Fig. 2). Within this time, production decreased about 24% of the highest production. The highest rate of production decreased from 6 to 9 days of storage time. The production did not decrease after 15 days of storage time. The most probable reason might be 15 days is the stability time of spore of that strain based on germination and production capability. It is therefore, the maximum storage time of inoculum is 2 days and it should be used within 2 days of preparation to obtain higher production of citric acid.

### *3.3. Effect of Doses of Suspension and Culture Inoculum on Citric Acid Production*

Different culture inoculums were prepared with 10, 15, 20, 25 and 30% spore suspension inoculum the doses. The substrate was inoculated with 10, 15, 20, 25 and 30% of each type of culture inoculum after incubation of 48 hrs. The results showed that the highest productions of citric acid were obtained for all doses of culture inoculum, which was prepared with 20% spore suspension inoculum (Fig. 3). The highest citric acid production of 367.88 g/kg-EFB was obtained with 25% culture inoculum among the all percentages of inoculum doses. The production of citric acid was almost same for 15 to 25% of culture

inoculum prepared with up to 15% of spore suspension inoculum dose. The lowest production was found for the culture inoculum prepared with 10% spore suspension inoculum.

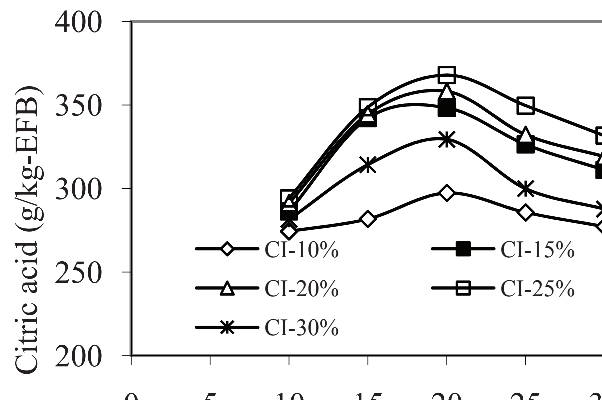


Fig. 3: Production of citric acid with the variation of suspension and culture inoculum doses

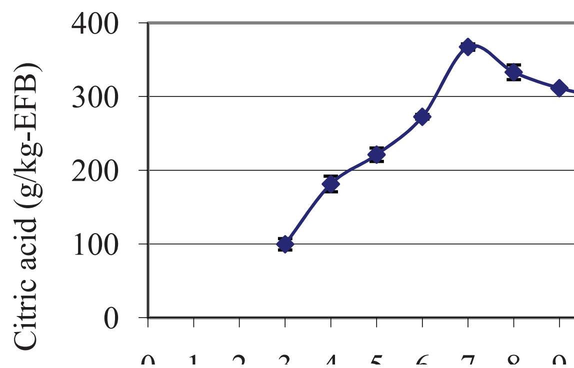


Fig. 4: Effect of bioconversion time on citric acid production with developed culture inoculum

### *3.4. Effect of Bioconversion Time on Citric Acid Production with Culture Inoculum*

Effect of bioconversion time on citric acid production with developed culture inoculum was examined to ascertain the bioconversion time for large scale production in bioreactor. The bioconversion experiment was carried out for 10 days and sample was harvested every day from 3 days of bioconversion. The variation of citric acid production with bioconversion time is presented in Fig. 4. The result showed that the highest production of citric acid of 367.4±4.3 g/kg-EFB was obtained after 7 days of bioconversion. The bioconversion time for highest production of citric acid with spore suspension inoculum was also same. The possible reason for same bioconversion time might be due to starting of new growth of fungi in each case after addition to the substrate.

It is found from literature that Kumar and Jain [5] carried out scale-up production of citric acid from treated sugarcane bagasse in packed bed bioreactor by using spore suspension inoculum ($2\times10^7$ spores/ml) and the highest production was found after 8 days of fermentation. Prado et al. [4] used spore suspension inoculum ($10^8$ spores/ml) of $10^7$ spores/g of dry substrate for citric acid production from cassava bagasse in tray type and horizontal drum bioreactor. The fermentation carried out for 5 and 6 days for tray and

horizontal drum bioreactor and production obtained of 263 and 269 g/kg dry cassava bagasse, respectively. Tran et al. [10] also used spore suspension inoculum ($1\times10^7$ spores/ml) for citric acid production from pineapple waste in rotary drum and tray type bioreactor

## 4. Conclusions

Culture inoculum was developed for scale-up production of citric acid in bioreactor from EFB through solid state bioconversion. The effect of spore age, spore suspension inoculum storage time, concentration of biomass and bioconversion time was evaluated. The results showed that spore suspension inoculum should be prepared from 3-4 days old plate culture. The prepared spore suspension inoculum should be used within 2-3 days to prepare the culture inoculum. The highest production of citric acid (367.4±2 g/kg-EFB) was obtained with 25% culture inoculum (48 hours old) with biomass concentration of 0.82±0.3 g/L, which was produced by 20% spore suspension ($1\times10^8$ spores/ml). The bioconversion time for highest production of citric acid was found to be 7 days.

## Acknowledgements

The authors are grateful to the Ministry of Science, Technology and Innovation (MOSTI), Malaysia, for financing the research project (No. 02-01-08-SF0050) under e-Science research grant and to the Department of Biotechnology Engineering, International Islamic University Malaysia for their support.

## References

[1] J-W. Kim, S. Barrington, J. Sheppard, B. Lee, Nutrient optimization for the production of citric acid by *Aspergillus niger* NRRL 567 grown on peat moss enriched with glucose. *Process Biochem* 2006, 41: 1253-1260.

[2] W. A. Lofty, K. M. Ghanem, E. R. El-Helow, Citric acid production by a novel *Aspergillus niger* isolate: I. Mutagenesis and cost reduction studies. *Bioresour Technol* 2007, 98: 3464-3469.

[3] M. N. Bari, M. Z. Alam, A. M. Suleyman, P. Jamal, A.A., Mamun, Improvement of production of citric acid from oil palm empty fruit bunches: Optimization of media by statistical experimental designs. *Bioresour. Technol*. 2009, 100: 3113-3120.

[4] F. C. Prado, L.P.S. Vandenberghe, A. L. Woiciechowski, J.A. Rodrigues-Leon, C.R. Soccol, Citric acid production by solid-state fermentation on a semi-pilot scale using different percentages of treated cassava bagasse. *Brizilian J. Chemical Eng.* 2005, 22 (4): 547-555.

[5] A. Kumar, V. K. Jain, Solid state fermentation studies of citric acid production. *African J. Biotechnol.* 2008, 7(5): 644-650.

[6] M. Z. Alam, M. N. Bari, A. M. Suleyman, P. Jamal, A.A., Mamun, Solid state bioconversion of oil palm empty fruit bunches for production of citric acid by wild *Aspergillus niger*. *Food Biotechnol*. 2009, 24(1): 19-36.

[7] A. B. Nasrin, A. N. Ma, Y. M. Choo, S. Mohamad, M. H. Rohaya, A. Azali, Z. Zainal, Oil palm biomass as potential substitution raw materials for commercial biomass briquettes production. *American J. Appl. Sci*. 2008, 5 (3): 179-183.

[8] K. Khosravi-Darani, A. Zoghi, Comparison of pretreatment strategies of sugarcane baggase: Experimenal design for citric acid production. *Bioresour. Technol.* 2008, 99(15): 6986-6993.

[9] M. Z. Alam, A. Fakhru'l-Razi, S.A. Aziz, A.H. Molla, Optimization of compatible mixed cultures for liquid state bioconversion of municipal wastewater sludge. *Water, Air, and Soil Pollutio*n 2003, 149: 113-126.

[10] C.T. Tran, L. I. Sly, D. A. Mitchell, Selection of a strain of *Aspergillus* for the production of citric acid from pineapple waste in solid-state fermentation. *World J. Microbiol. Biotechnol.* 1998, 14: 399-404.