Citric acid production on three cellulosic supports in solid state fermentation

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SUMMARY

Six strains of *Aspergillus niger* were screened in liquid medium and strain LPB 21 was selected for further studies on three different agro-industrial wastes/residues for production of citric acid by solid state fermentation. Cassava bagasse was found to be a better substrate than vegetable sponge and sugar cane bagasse. Citric acid production and yields were 13.64 g/100 g dried substrate and 41.78 %, respectively with the use of cassava bagasse. Studies on optimization of parameters for production of citric acid by *A. niger* LPB 21 in cassava bagasse medium indicated that 50% of initial moisture content, initial pH of 2.0, aeration rate of 60 ml/min/column and 26°C fermentation temperature are the optimum values. Under these optimized conditions, the production of citric acid was 280 g/kg dry substrate at 120 hours, which corresponds to the yield of 70% based on sugars consumed. Data on kinetics of pH changes, moisture level, loss of weight of the substrate, starch utilization and alpha-amylase production give insights in the process.

Keywords : citric acid, solid state fermentation, *Aspergillus niger,* cassava, bagasse, vegetable sponge, moisture, aeration, temperature.

RESUME

Production d'acide citrique par fermentation en milieu solide sur trois supports cellulosiques.

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Parmi les six souches *d'Aspergillus niger* qui ont été criblées en milieu liquide, l'une d'entre elles, la souche LPB 21 a été retenue pour les études de valorisation de trois sous-produits agro-industriels en vue de produire de l'acide citrique par fermentation en milieu solide. La bagasse de manioc s'avère le meilleur substrat par rapport à la bagasse de canne à sucre et à l'éponge végétale. La production d'acide citrique a été de 13,64 g / 100 g de substrat sec. Le rendement s'élève à 41,78%. Les différents facteurs intervenant dans cette production ont fait l'objet d'une étude d'optimisation. Les meilleures conditions retenues ont été les suivantes : une humidité initiale de 50%, un pH initial de 2.0, une vitesse d'aération de 60 ml/min/colonne et une température d'incubation de 26°C. Dans ces conditions, la production d'acide citrique a été de 280 g/kg de substrat sec après 120 h de fermentation, ce qui correspond à un rendement de 70% (sur la base des sucres consommés). Les résultats rapportés dans cette communication précisent l'évolution du pH; de l'humidité, de la perte de poids en substrat, de l'utilisation de l'amidon et de la production de l'alpha-amylase au cours de la fermentation en milieu solide.

Mots clés : Acide citrique, fermentation en milieu solide, *Aspergillus niger*, bagasse de manioc, éponge végétale, humidité, pH initial, aération, température de la fermentation, cinétique fermentaire.

INTRODUCTION

Citric acid was first isolated from lemon juice and crystallized as calcium citrate as early as in 1784 (Milson and Meers, 1985). Wehmer (1893) was the first to observe the presence of citric acid as a product of *Penicillium glaucum* growing on sugar. A great number of problems had to be overcome, before an effective fermentation process could be used commercialy (Lockwood and Schweiger, 1967).

Citric acid is of industrial interest, mainly used in the food industry (Rohr *et al*, 1983). Another important area of application is the pharmaceutical and the cosmetics industries. Increasing amounts of citric acid are being used in industry, e. g., as raw material for the manufacture of derivatives in the plastics industry. Actually, more than 300,000 tons of citric acid were produced per year, with a growing up demand of about 20 % per year. Most of the world's supply of citric acid is produced by carbohydrate fermentation with selected strains of *Aspergillus niger*. Citric acid is produced either by submerged or liquid surface fermentation processes, employing sugar beet molasses as substrate (Milson and Meers, 1985). About 80 - 85 % of the weight of the sugar used in the medium can be recovered as citric acid, the fermentation being usually conducted over a period of 5- to 14-day at 25 to 33°C (Peller and Perlman, 1979). However, solid state fermentation constitutes an other alternative with good yield, with the use of specific strains and the nature of the substrates employed (Flores, 1994; Shankaranand and Lonsane, 1993, 1994; Hang *et al.*, 1987; Hang and Woodames, 1984, 1986).

The objectives of the present work were to characterize the conditions of citric acid production by *Aspergillus niger* in solid state fermentation on different supports, such as vegetable sponge (Luffa cylindrica Roem), sugar cane and cassava (*Manihot esculenta* Crantz) processed residue. Therefore, several high citric acid producing strains of *Aspergillus niger* were studied in SSF under different parameters.

MATERIAL AND METHODS

MICROORGANISMS

Six citric acid producing strains of *Aspergillus niger* (LPB 21, LPB 28, LPB 32, LPB 34, LPB 70 and LPB 99) were used. All fungal strains were cultivated on agar plates of Czapek agar (50.5 g / L) and malt extract (40.0 g / L). After growth at 28°C for 7 days, the spores were harvested with platine loop under aseptic conditions and transferred into 20 mL test tubes, containing 10 mL of sterile 0.1% Tween 80. The spore suspension was mixed thoroughly and the number of spores per mL was determined using a hemacytometer. The spore suspension was stored at 4°C.

STRAINS SELECTION

Strain selection was carried out as described by Prescott and Dunn (1959). Inoculum was prepared in a 500 mL Erlenmeyer shake flask containing 150 mL medium, containing (g/L) sucrose, 140.0 ; NH_4NO_3, 2.231 ; KH_2PO_4, 1.0 ; $MgSO_4.7H_2O$, 0.23. The pH of the medium was adjusted to 1.6 - 2.2 with 1 N HCl solution. After sterilization at 121°C for 15 min and cooling to about 30°C, it was inoculated to contain 10^7 spores / mL. The flasks were shaken reciprocally at 100 rpm at 28°C for 10 days.

PREPARATION OF SUPPORTS

Solid materials were washed, dried in an oven at 60°C and sieved to obtain 8 - 20 mm fraction. Unless otherwise specified, they are moistened to 70% with a mineral solution containing (g / L) NH_4NO_3 , 4.46 ; KH_2PO_4 , 0.20 ; Mg $SO_4.7H_2O$, 0.46 ; $ZnSO_4.7H_2O$, 0.375 ; $CuSO_4.7H_2O$, 0.002. The pH of the medium was adjusted to 3.0 with 1.0 N HCl. When vegetable sponge and sugar cane bagasse were used, the medium was supplemented with sucrose (70 g/L). For cassava bagasse, residual solids from starch extraction process were used as carbon source.

SOLID-STATE FERMENTATION

Solid state fermentation was carried out in column type bioreactors (Raimbault and Alazard, 1980). The humidified substrate was inoculated with a suspension of 10^7 spores / mL placed in columns of 0.60 g/mL capacity and compacted (Soccol, 1994). The columns were placed in a temperature regulated water bath at 22°C and aerated at a rate of 100 mL/min. Studies were conducted using the same methodology as described above on the effect of initial moisture content, initial pH of the medium, aeration rate, temperature of fermentation and kinetics of citric acid production.

ANALYSES

Citric acid in the extract was determined by the colorimetric method of Marier and Boulet (1958) as modified by Hartford (1962). The analysis for starch was done by iodine-iodure method (Thivend *et al*, 1965) while the reducing sugar formed were estimated by the method of Bernfeld (1955). The total sugars were estimated by the method of Dubois *et al* (1956) after hydrolysis with HCl. Residual sugars were estimated by DNS method (Miller, 1959). Water content was measured, as described elsewhere (Soccol, 1992).

RESULTS AND DISCUSSION

SELECTION OF *ASPERGILLUS NIGER* STRAINS

After 10 days of submerged fermentation, all six strains of *Aspergillus niger* showed the ability for citric acid production. Strain LPB 21 produced the highest quantity of citric acid (Fig. 1) and hence was selected for futher studies.

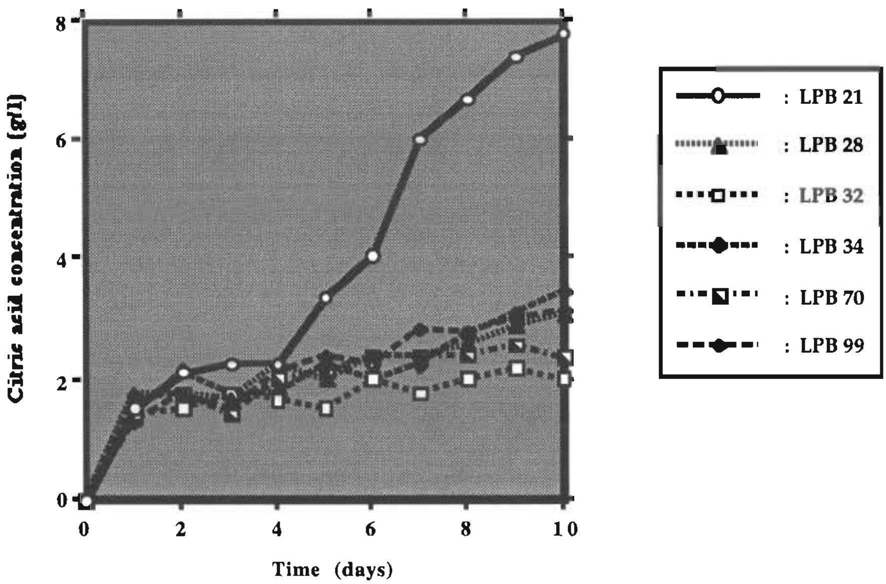


Fig. 1 - Comparison of citric acid production by several strains of *Aspergillus niger* in submerged fermentation.

SUPPORT CHARACTERISTICS

The three cellulosic materials were found to have good characteristics for use as support in solid state fermentation (Table 1). Cassava bagasse was characterized by a high content of total sugars. Consequently, when used, this support was not supplemented in sucrose.

Table 1. Physical and chemical characteristics of cellulosic materials used as support during solid state fermentation.

Support	Moisture (%)	Ashes (%)	Water absorption ($g\ H_2O$ /g support)	Total sugars (g/100 g sup.)	Starch (g/100 g sup.)
Vegetable sponge	11.54	0.33	3.25	0.75	-
Sugarcane bagasse	7.72	1.24	5.75	0.25	-
Cassava bagasse	14.04	1.72	4.07	46.50	41.55

SOLID-STATE FERMENTATION IN DIFFERENT CELLULOSIC SUPPORTS

Table 2 shows that cassava bagasse, in comparison to sugar cane bagasse and vegetable sponge, is the best support to produce citric acid by solid state fermentation. The citric acid production and yield were 13.64 g/100 g dry cassava bagasse and 41.78 %, respectively.

Table 2 - Solid-state fermentation in different cellulosic support.

Support	Produced citric acid (g / 100 g dried support)	Consumed sugar (g / 100 g dried support)	Yield (%)
Vegetal sponge	4.85	59.09	8.21
Sugar cane bagasse	5.64	60.16	9.34
Cassava bagasse	13.64	32.66	41.78

Initial Moisture : 70 %, Yield : Citric acid produced / Sugar consumed ; Initial sugar : cassava bagasse, 46,5 g / 100 g dried support ; sugarcane bagasse, 70.25 g / 100 g dried support ; vegetal sponge, 70.75 g / 100 g dried support.

EFFECT OF INITIAL MOISTURE CONTENT OF CASSAVA BAGASSE

The water content of solid support has been reported as an important limiting factor for solid state fermentation (Lonsane *et al*, 1992 ; Pandey, 1992). Consequently, the effect of moisture present in cassava bagasse was found to be in the range of 45-65 % initial moisture. Data showed that the production of citric acid at 8 days increased with an increase in initial moisture content of the medium up to 50% (Table 3).

Table 3. Effect of initial moisture content of cassava bagasse on citric acid production by *Aspergillus niger* LPB 21.

Moisture (%)	Citric acid produced (g / 100 g dried support)	Sugar consumed (g / 100 g dried support)	Yield (%)
45	16.35	36.92	44.28
50	27.25	40.10	67.96
55	25.32	36.83	68.75
60	22.15	39.63	55.89
65	19.39	37.19	52.14

Initial sugar : 46.5 g / 100 g dried support, Yield : g citric acid produced / g sugar consumed.

A further increase in the initial moisture content of cassava bagasse by 10% had an adverse effect on the synthesis of citric acid. Thus, the initial moisture content of 50% could be considered as ideal to produce a high quantity of citric acid (272 g/kg cassava bagasse). In this case, fermentation yield was approximately 70%.

EFFECT OF INITIAL PH

The medium pH for production of citric acid must be between 1.6 to 3.5 (Kolicheski, 1995). Table 4 shows the results for the production of citric acid by SSF using different pH values. Very low values of pH (1.0) reduces the production of citric acid. Higher values favour the production of citric acid and the maximum production was at pH 2.0, where the concentration of citric acid was 246 g/kg dried cassava bagasse with a yield of 71.45 %. These results confirm those obtained by different authors (Meers *et al*, 1991; Muller, 1981).

Table 4. Effect of initial pH of the medium on citric acid production by *A. niger* LPB 21 on cassava bagasse.

pH	Citric acid production (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
1	18.12	28.32	63.98
2	24.64	34.54	71.45
3	21.93	34.19	64.14

Initial sugar : 46.5 g/100 g dried support, Yield : Citric acid produced / sugar consumed

AERATION EFFECT

Aeration is an important factor in the production of citric acid in column type bioreactors because the air helps to dissipate metabolic heat and also provide the essential oxygen for the growth of the microorganism. The results of the aeration effect are shown in Table 5. The optimal aeration flow is between 50 and 60 ml/min and the aeration rate above or below these values leads to a fall in citric acid production.

Table 5. Effect of aeration rate on citric acid production by *A. niger* LPB 21 in cassava bagasse.

(ml/min/column)	Citric acid produced (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
40	13.60	38.36	35.45
50	25.42	36.13	70.36
60	27.12	37.54	72.24
70	19.45	37.51	51.85
80	17.32	32.61	53.11

Initial sugar : 46.5 g/100 g dried support, Yield : Citric acid produced/Sugar consumed

TEMPERATURE EFFECT

Optimum temperature for fermentation varies according the microorganism used, and is usually between 25 and 35°C (Prescott and Dunn, 1959). Highest production of citric acid occurred at 26°C (Table 6).

Table 6. Effect of temperature on citric acid production by *A. niger* LPB 21 in cassava bagasse.

Temperatures	Citric acid production (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
24	15.64	37.81	41.36
26	28.45	37.66	75.54
28	23.91	36.88	64.83
30	20.80	35.97	57.83
32	19.73	37.67	51.96

Initial sugar : 46.5 g/100 g dried support, Yield : Citric acid produced/Consumed sugar

KINETICS OF CITRIC ACID PRODUCTION

Fig. 2A shows the evolution of consumption of starch and citric acid production during fermentation. The production of citric acid starts in the initial period of fermentation although its concentration reaches 74 g/kg dried cassava bagasse at 48 h. Between 48 and 72 h fermentation, citric acid productivity reaches its highest point (201.34 g/kg dried cassava bagasse). Between 72 and 120 h, the final concentration in citric acid reaches a value of 280 g/kg dried cassava bagasse, which correponds to a yield of 70 % in relation with the starch consumed. These results are higher as compared to those of other authors working with different substrates (Hang *et al*, 1987; Omar *et al*, 1992; Shakaranand and Lonsane, 1993).

Alpha-amylase production is higher during the first 72 h fermentation (192 IU/g dried cassava bagasse) (Fig. 2B). Significant alpha-amylase production in the first 72 h is associated with higher consumption of starch in the same period (Fig. 2A).

pH is gradually reduced as citric acid is accumulated in the medium and reaches its lowest value (pH 0.5) after 96 h fermentation (Fig. 2B).

Fig. 2C shows the increase in moisture content of the medium and loss of weight of support during fermentation. An increase of moisture from 60.32 to 64.18% is due to fungal metabolism (Soccol, 1992 and 1994). The loss of weight is higher in the first 24 hours and it reaches about 7.93 %. After 120 hours of fermentation, the total loss of weight is 10.57%. This loss of weight from the support is due to starch tilization.

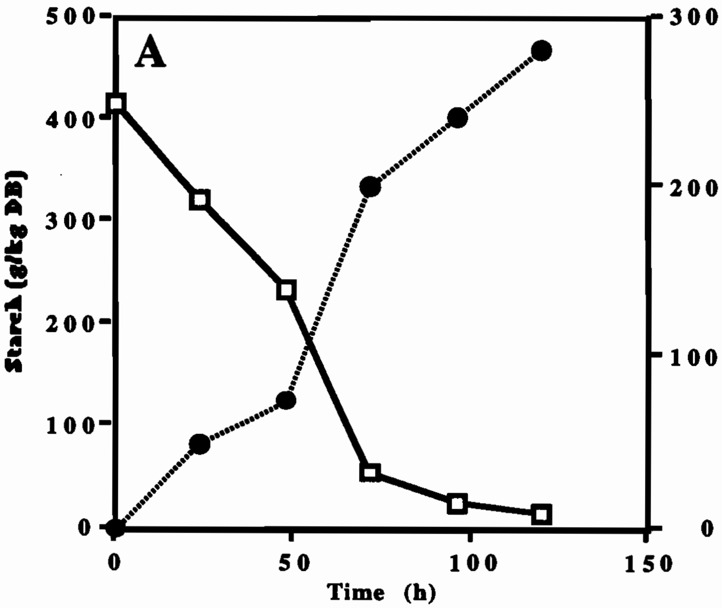
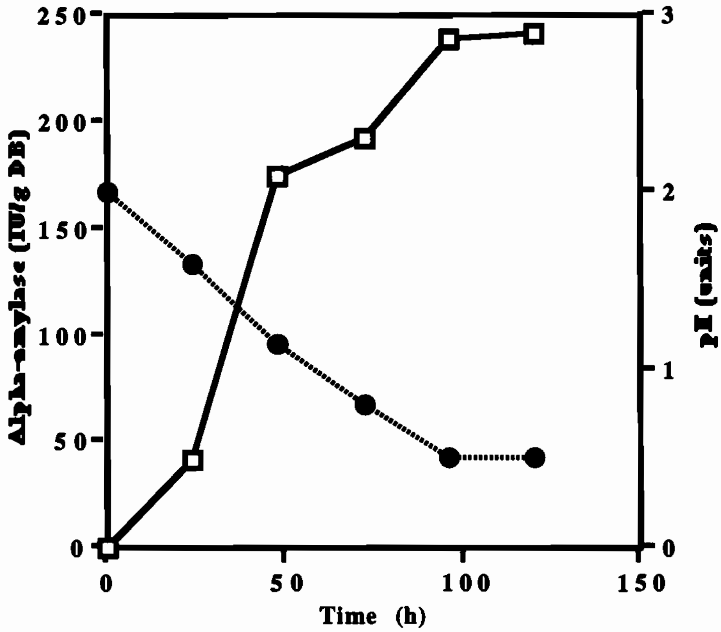
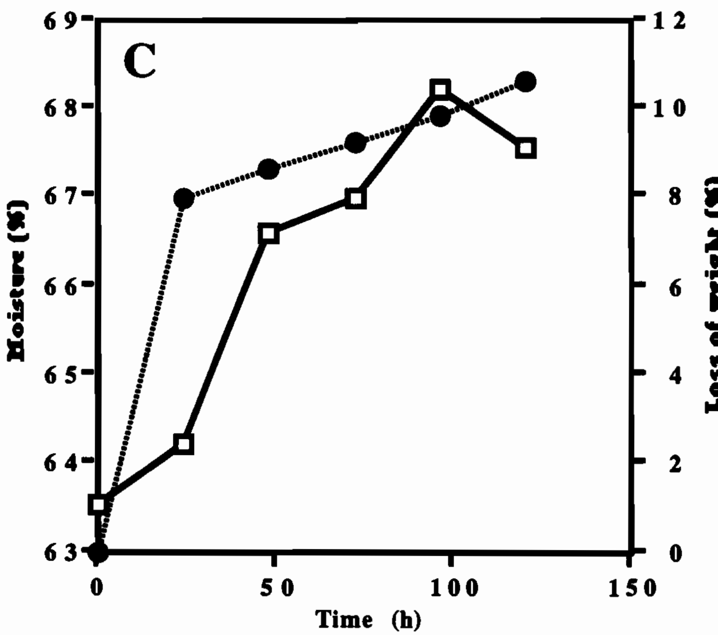


Fig. 2A. Kinetics of citric acid production by *A. niger* LPB 21 in SSF.





Figs. 2B-C. Kinetics of citric acid production by *A. niger* LPB 21 in SSF.

A : Starch utilization. B : Changes in pH and alpha-amylase levels. C : Changes in moisture and loss of weight during fermentation.

CONCLUSIONS

Aspergillus niger LPB 21 showed good potential for citric acid production in solid state fermentation based on the use of cassava bagasse as a substrate. The optimization allowed to improve the production and also the yield based on sugars consumed.

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