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CITRIC ACID FERMENTATION OF HYDROLYSED SWEET POTATO STARCH BY *ASPERGILLUS NIGER*

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ABSTRACT

The present study deals with the screening of *Aspergillus niger* from soil for citric acid production by submerged fermentation. *A. niger* IIB-A6 produced 23.32±1.44 g/l citric acid. Sweet potato starch was found as a better substrate compared to maize starch. The kinetic parameters $Y_{p/x}$ (1.96 g/g cells), Q_p (0.13 g/l/h) and q_p (0.014 g/g/h) were 8-10 fold higher with sweet potato starch than the maize starch. Sugar conc. (150 g/l), incubation period (168 h) and initial pH (6.0) were optimized. The maximum citric acid production obtained during the course of study was 28.40±1.07 g/l. Sugar consumption and dry cell mass were 121.33±0.91 and 14.29±0.88 g/l, respectively. The mycelia were small pellets.

INTRODUCTION

Various fungi, which have been found to accumulate citric acid in their culture media, include strains of *Aspergillus niger*, *Aspergilus awamori*,*Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis* and *Yarrowia lipolytica* (Arzumanov *et al*., 2000). *A. niger* however, remained the organism of choice for industrial production of citric acid. Citric acid fermentation has three major categories viz. surface culture, submerged and solid-state (Kurbanoglu 2004). Although submerged citric acid fermentation is more energy consuming process, which gives higher production. The optimal incubation period for citric acid production varies both with the organism and fermentation conditions (Iyer *et al*., 1999). An appropriate initial pH is also critical for successful fermentation process and varies from strain to strain. Fermentation media for citric acid biosynthesis should consist of substrates necessary for the growth of microorganism, primarily the carbon, nitrogen and phosphorus sources. Carbon sources, such as glucose, sucrose, cane or beet molasses, starch from various sources, hydrocarbons, alcohols, fatty acids and natural oils have been evaluated for citric acid production (Ali and Haq, 2005). The sweet potato (*Ipomoea batatas* Lam.) generally contains more starch than the Irish potato and the starch has properties that are particularly useful in many food products and manufacturing processes. One major disadvantage in using molasses for citric acid production is its high ash content that contains some trace metals, which inhibits efficient production. The fermentation from the mash of dried sweet potatoes is however, relatively simple as it does not need any pre-treatment to be purged. The present study deals with the screening of *A. niger* strains isolated from soil for citric acid production. Hydrolyzed sweet potato starch was used as a basal fermentation media using submerged technique.

MATERIALS AND METHODS

Isolation of organism: Twelve strains of *A. niger* were isolated from different soil

samples of Lahore by serial dilution method (Clark *et al*., 1958). The cultures were maintained on sterilized potato dextrose agar, pH 4.5 and stored at 4ºC. These strains were identified according to Onion *et al.,* method (1986), showing following typical characteristics; hyphae were septate and hyaline. Condidiophore (300-400 µm) were long, smooth and colourless with globose, dark brown heads having an average diameter of 550 µm. Biseriate phialids having chains of conidia were born on the metullae, which were fairly uniform. The conidia were globose and have 2.5-10 µm diameter.

Inoculum preparation: The conidial suspension of *A. niger* was prepared by adding 10 ml of sterilized 0.05 % (w/v) diacetyl ester of sodium sulfo succinic acid (Monoxal O.T.) to a 3-5 day old slant culture having profuse conidial growth on its surface. An inoculum needle was used to prepare a homogenous suspension.

Starch extraction and preparation of hydrolysate from sweet potatoes: Sweet potatoes purchased from the local market were cut into small pieces after washing and peeling. The pieces were blended in small quantity of distilled water to form a homogenous mixture and placed at 4ºC for 24 h. The starch settled down was separated from liquid and oven dried at 60ºC, overnight. A starch solution of 250 g/l was prepared and autoclaved at 5.0 lbs/in^2 pressure (115°C) for 5 min. To liquefy starch, alpha amylase (2.0 U/ml) was added and heated at 95ºC in a water bath for 15 min. For saccharification, amylogluco-sidase (2.0 U/ml) was added and heated at 55ºC while constant stirring for about 4 h (Jamai *et al.,* 2006).

Preparation of maize starch hydrolysate: Maize starch was purchased from local market. Liquefaction and saccharification was done as above following the method of Jamai *et al.,* (2006).

Fermentation technique: Submerged fermentation technique was employed in the present study. Fifty millilitre medium containing (g/l): starch hydrolysate 150, NH_4NO_3 2.5, KH_2PO_4 1.0, $MgSO_4$. $7H_2O$ 0.25, $CaCl_2$ 0.5 was taken in 250 ml conical flasks and pH was maintained at 6.0. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min. The sterilized flasks were inoculated with 1.0ml of the inoculum under aseptic conditions. Sterilized ferrocyanide (200 ppm free ions conc) was added to each flask. The flasks were placed in a shaker incubated at 200 rpm for 24 to 240 hours at 30ºC. The methanol (1.0 ml of 3.0 %) was added after 24 h of the inoculation. All the experiments were run parallel in triplicates.

Assay methods: The mycelial morphology was determined on an aliquot extended on the petriplates and dry cell mass was determined according to Haq and Daud (1995). The sugar contents were estimated by Miller (1959) method. Citric acid was estimated following pyridine-acetic anhyd-ride method (Marrier and Boulet, 1958). Kinetics of research work was also studied (Pirt 1975). The statistical tests were based on Duncan,s multiple range (Snedecor and Cochran, 1980).

RESULTS

In the present study, 12 isolates of *A. niger* were screened for citric acid production using sweet potato and maize starch hydrolysates media by submerged fermentation (Table 1). The amount of citric acid produced ranged from 5.57-18.44 g/l for maize and 9.88-23.32 g/l for sweet potato starch. IIB-A6 produced better citric acid with sweet potato starch hydrolysate. Citric acid production increased with increase in sugar concentration in the fermentation medium

but up to 150 g/L (24.23±1.03 g/l) and then decreased (Table 2). Sugar consumption and dry cell mass were 108.48±1.24 and 10.36±0.92 g/L, respectively. Mycelial pellets were small. Citric acid production in the fermented broth increased gradually with the increase in incubation period with both hydrolysates (Fig 1). However, the maximum production (22.19±1.10 g/l with sweet potato) was achieved after 168h inoculation, which was 1.23 fold higher than maize starch. Sugar consumption and dry cell mass were 111.27±0.86 and 11.29±0.85 g/l, respectively.

The highest value for specific growth rate (μ = 0.31 h^{-1}) was noticed after 24h incubation with the sweet potato starch hydrolysate (Fig 2). But citric acid production with the sweet potato starch hydrolysate was the highest (22.19±1.10 g/l) after 168h incubation. Fig 3 shows a comparison of volumetric rates during citric acid fermentation by maize and sweet potato starch. After 168 h of fermentation, the value for Q_x with the sweet potato starch was noted 0.07 g cells/l/h. During the fermentation period, sweet potato showed higher values for volumetric rate of citric acid formation. The effect of different initial pH (4.0-7.0) on citric acid production by *A. niger* IIB-A6 was studied (Table 3). The maximum citric acid production (28.40±1.07 g/l) was obtained at an initial pH of 6.0. The sugar consumption and dry cell mass were 121.33±0.91 and 14.29±0.88 g/l, respectively. The mycelial morphology was in the form of small pellets. Any change in the pH other than 6.0, decreased citric acid production which become least at the neutral.

DISCUSSION

Sweet potato starch was found to be the best carbon source. It may be due to that the dry weight of sweet potato contained upto 80-90 % of carbohydrates. In the present finding, initial sugar concentration of 150 g/l gave maximum citric acid production. At this concentration, suitable amount of substrate was available to the organism till early stationary phase. Reduction in citric acid formation was observed when the sugar concentration was further increased. It might be due to overgrowth of the mycelium, which resulted in the increased viscosity of the medium (Matty and Allan, 1990). Pazouki *et al.,* (2000) pointed out that a sugar concentration higher than 160-180 g/l leads to a greater amount of residual sugars, making the process uneconomical, while a lower sugar concentration lead to a lower production of citric acid due to the accumulation of oxalic acid. In batch-wise fermentation of citric acid, the production starts after a lag phase of one day and reaches maximum at the onset of stationery phase (Vergano *et al*., 1996). The maximum production of citric acid was achieved, after 168h incubation. Further increase in incubation period resulted in the decreased citric acid production. It might be due to the decreased amount of nitrogen in the fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself and the depletion of sugar contents.

Maximum growth in terms of specific growth rate (μ in h^{-1}) was only marginally different during fermentation of sweet potato and maize starch hydrolysate on 150 g/l. However, when the culture was monitored for Q_s and q_s, there was a significant enhancement in these variables at optimal nutritional conditions. Similar kind of work has also been reported by Pirt (1975). Initial pH ranging from 4.0-5.5 citric acid production was not found encouraging. It might be due to that at a lower pH, the ferrocyanide ions were more

toxic for mycelial growth (Pessoa *et al.*, 1984). At an initial pH lower than 6.0, fermentation medium became acidic which was not favourable for fungal growth. The fermentation medium with an initial pH 6.0 resulted in the maximum citric acid production. A higher initial pH, however, might lead to the accumulation of oxalic acid (Shadafza *et al.*, 1976).

CONCLUSION

Sweet potato starch gave 1.2 fold higher citric acid production (22.19±1.10 g/l) than the maize starch after 168h conidial inoculation by *Aspergillus niger* IIB-A6. The cultural conditions such as sugar conc. (15 %, w/v), incubation period (168 h), and initial pH (6.0) were optimized. However, further studies to improve the culture by random mutation are pre requisite prior to scale up studies.

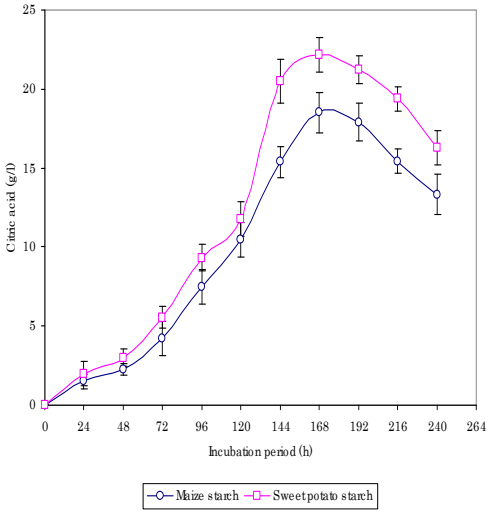

Fig-1: Comparison of citric acid production using maize and sweet potato starch hydrolysate*

*Sugar concentration 150 g/l, temperature 30ºC, initial pH 4.5. Y-error bars indicate standard deviation (±SD) among the three parallel replicates.

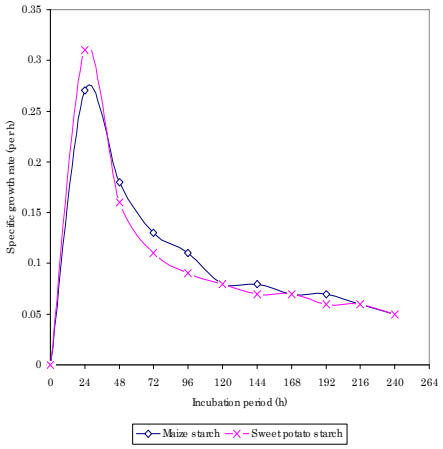

Fig-2: Comparison of specific growth rate for citric acid production using sweet potato and maize starch hydrolysate*

*μ (h^{-1}) = specific growth rate = g cells formed/h. Y-error bars indicate standard deviation (±SD) among the three parallel replicates

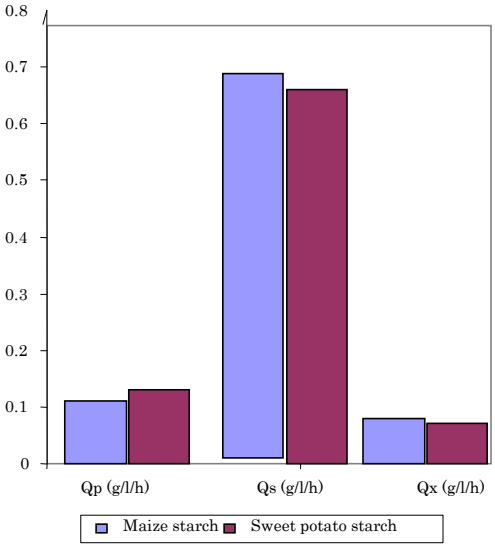

Fig-3: Comparison of volumetric rates* for cell mass formation by *A. niger* IIB-A6 with maize and sweet potato starch

*Volumetric rates, Q_x (g cells/l/h) = g cells formed/l/h, Q_p (g/l/h) = g citric acid produced/l/h, Q_s (g/l/h) = g substrate consumed/l/h

Table 1: Screening of *A. niger* isolates for citric acid production using sweet potato and maiz starch hydrolysates*

Isolates of *A.niger*	Sugar consumed (g/l)		Dry cell mass (g/l)		Citric acid (g/l)		Mycelial morphology	
	Maize	Sweet potato	Maize	Sweet potato	Maize	Sweet potato	Maize	Sweet potato
IIB-A1	110.17±0.76	92.63±1.16	11.86±1.34	8.45± 1.13	9.43± 1.03	16.03±1.31	Fine pellets	Small pellets
IIB-A2	112.52±0.99	113.46±1.11	9.60±0.88	11.67± 1.04	11.36±1.20	14.41±0.92	Large pellets	Intermediate pellets
IIB-A3	110.05±1.20	111.50±1.10	13.48±1.09	10.75± 1.33	9.36± 0.82	12.95±1.36	Fine pellets	Small pellets
IIB-A4	121.61±0.83	118.7±1.01	15.53± 0.83	11.79± 1.03	11.69± 0.67	9.88±0.99	Small pellets	Dumpy mass
IIB-A5	123.36±0.71	122.60±1.17	16.56± 0.97	15.71± 0.95	6.82±1.09	13.15±1.45	Viscous	Small pellets
IIB-A6	110.19±1.29	109.53±0.80	16.55±1.03	14.59±1.19	18.44± 0.90	23.32±1.44	Mixed pellets	Small pellets
IIB-A7	135.47±1.32	132.38±1.08	20.48±1.07	11.89±1.47	5.57± 0.99	10.49±0.93	Dumpy mass	Large pellets
IIB-A8	120.32±1.32	119.48±0.97	20.43±2.31	12.60± 0.86	10.57± 0.91	16.32±1.10	Large pellets	Mixed pellets
IIB-A9	117.36±1.29	113.31±1.19	18.76±1.08	12.14±1.56	12.59±1.19	14.02±1.27	Large pellets	Fine pellets
IIB-A10	141.67±1.66	133.53±1.04	19.69±1.14	11.64± 0.96	13.72± 0.86	17.44±1.23	Intermediate pellets	Small pellets
IIB-A11	131.17±1.04	130.56±1.20	21.49±1.34	19.47±1.17	12.34±1.01	14.54±1.12	Fine pellets	Mixed pellets
IIB-A12	116.35±0.97	114.16±1.12	14.33± 0.99	13.46± 0.99	14.39± 0.96	18.40± 0.69	Fine pellets	Small pellets

*Sugar concentration 150 g/l, fermentation period 168 h, temperature 30ºC, initial pH 4.5.
± Indicates standard deviation (±SD) among the three parallel replicates.

Table 2: Effect of initial sugar concentration on citric acid production by *A. niger* IIB-A6*

Sugar conc. (g/l)	Sugar consumed (g/l)		Dry cell mass (g/l)		Citric acid (g/l)		Mycelial morphology	
	Maize	Sweet potato	Maize	Sweet potato	Maize	Sweet potato	Maize	Sweet potato
75	71.42±1.03	70.37±0.73	9.28±1.12	8.42±0.88	11.31±1.11	14.92±1.16	Gelatinous	Viscous
100	94.46±1.13	92.47±1.33	10.37±1.06	9.36±1.05	14.58±1.34	21.31±1.03	Fine pellets	Small pellets
125	111.22±1.18	107.22±1.15	11.48±1.26	9.50±0.91	15.10±1.68	22.50±0.96	Mixed pellets	Small pellets
150	110.32±1.04	108.48±1.24	13.88±1.52	10.36±0.92	17.34±1.00	24.23±1.03	Large pellets	Small pellets
175	135.36±1.02	128.61±1.19	15.53±1.38	11.42±1.02	14.54±1.22	18.57±1.16	Large pellets	Mixed pellets
200	151.04±1.21	148.53±1.21	16.53±1.23	12.49±0.73	13.34±0.98	17.32±0.88	Large pellets	Mixed pellets

*Fermentation period 168 h, temperature 30ºC, initial pH 4.5.
± Indicates standard deviation (±SD) among the three parallel replicates

Table 3: Effect of initial pH on citric acid production by *A. niger* IIB-A6 using hydrolysed sweet potato starch medium*

Initial pH	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
4.0	88.33±0.84	9.96±1.19	11.43±0.67	Viscous
4.5	110.71±1.03	11.60±0.97	23.20±0.94	Fine pellets
5.0	115.39±1.30	12.36±0.68	25.57±0.74	Small pellets
5.5	119.70±1.09	13.51±0.88	26.27±1.16	Fine pellets
6.0	121.33±0.91	14.29±0.88	28.40±1.07	Small pellets
6.5	123.39±1.21	16.05±1.32	21.42±1.22	Mixed pellets
7.0	131.63±1.20	16.49±0.89	17.40±1.13	Large pellets

*Sugar concentration 150 g/l, fermentation period 168 h, temperature 30ºC. ± indicates standard deviation (±SD) among the three parallel replicates.

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