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Original Article

Citric Acid Production by *Aspergillus niger* through Solid-State Fermentation on Sugarcane Bagasse

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Four isolates of *Aspergillus niger* (*viz.*, CA16, 14/20, HB3 and 318) were used for citric acid production using sugarcane bagasse as a substrate and sucrose solution as a moistening agent. Using 3 g of sugarcane bagasse as substrate, the highest citric acid production was obtained when 10 ml of 14% sucrose solution was used as moistening agent. Maximum citric acid production was found after 11 days fermentation for all isolates of *A. niger*. Both sugar utilization as well as citric acid production was found highest in the presence of Prescott salt by *A. niger* 318 (196.21 µg/g), followed next by *A. niger* 14/20 (103.06 µg/g). However, in absence of Prescott salt both sugar utilization and citric acid production was highest (50.01 µg/g) by *A. niger* 14/20. In general, extension of the fermentation (up to 11 days) resulted in an increase in citric acid, total titratable acid and biomass, and decrease in both residual sugar concentrations.

Keywords: Citric acid, Solid-state fermentation, *Aspergillus niger*, Sugarcane bagasse

Introduction

Citric acid is one of the most commonly used organic acid in the food and pharmaceutical industry, which is produced mainly by submerged or surface fungal fermentation. *Aspergillus niger* fermentation is the world's leading source of commercial citric acid. This process has thus been the subject of many studies[1-3]. Recent work by Kumar *et al.*[4] and Anupama and Ravindra[5] showed successful citric acid production through solid-state fermentation. Solid-state fermentation is regarded as better option for citric acid production because of lower energy requirement, higher product yield with little risk of bacterial contamination, generation of less waste water and environmental concerns regarding the disposal of solid-waste[6]. Another benefit for solid-state fermentation is that, inhibition of citric acid production due to presence of metal ions are ineffective in solid-state fermentation[7-8].

Until about 1920, all commercial citric acid was produced from lemon and lime juices[9]. Röhr *et al.*[10] reported that citric acid can be produced by fermentation process using species of microorganisms namely *Aspergillus niger*, a fungus, which was used commercially for the first time in 1923. The factors that affect the production of citric acid by fermentation include the nutritional composition of the media, environmental conditions, deficiency of manganese and other metals, pH, and dissolved oxygen tension[10]. The influence of types and concentrations of sugars[11-12], chelating effect on metal ions[13], ammonium nitrate and aeration[14] on citric acid production by *A. niger* have also been studied. At present time citric acid is produced commercially using mutant strains of *A. niger*, and with a significant amount by *Saccharomycopsis lipolytica*[15], *Pencillium simplicissimum*[16] and *A. foeitidus*[17]. Other carbohydrates and wastes that have been considered, experimentally, to produce citric acid by *A. niger* includes inulin, date fruit syrup, sugar cane molasses, soya whey, kumara, carob pod and cheese whey[18].

Large amounts of sugarcane bagasse are produced world wide as a by-product of sugar industries. Sugarcane bagasse has been successfully utilized as a carrier for solid-state fermentation for citric acid production by several investigaors[4,19] In our country bagasse is mainly used as a fire fuel, so this agricultural waste can be easily used for citric acid production. The present work was undertaken to investigate the possibility of using sugarcane bagasse as a substrate fortified with different quantity of sucrose solution as moistening agent and additional carbon source for solid-state fermentation for citric acid production by four isolates of *A. niger*.

Materials and Methods

Microorganisms used

Four citric acid-producing isolates of *Aspergillus niger* were used in the present study. An isolate designated as CA16[20-21] used as the original parent organism, which was a natural isolate from local soil. Other three isolates of *A. niger* designated as 14/20, HB3 and 318[22-23] were the radiation mutant of *A. niger* CA16. The

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isolates were maintained on agar slant medium containing 1% malt extract, 1% yeast extract, 1.5% dextrose and 2% agar, stored at 4°C and sub-cultured fortnightly.

Citric acid production medium

Sugarcane bagasse collected from Rajshahi Sugar Mill was sun-dried; cut into small pieces, grounded and screened to collect a particle size of about 1.2-1.6 mm. Bagasse (3 g) was taken in 250-ml Erlenmeyer flasks and moistened with different volume of 14% sucrose to set the desired moisture level. Separate sets of media were enriched with Prescott salt (NH_4NO_3, 2.23 g/l; K_2HPO_4, 1.00 g/l and $MgSO_4.7H_2O$, 0.23 g/l). The pH of the medium was adjusted at 3.5 and autoclaved.

Inoculum

Spore suspension of the concentration 2 x 10^7 spores/ml prepared from 8 to 10-day-old cultures of *A. niger* was used as inoculum in each experiment.

Solid-state fermentation

Each flask containing citric acid production medium was inoculated with 1 ml of spore suspension followed by mixing and incubation at 30°C in the a humidity-controlled incubator for 11 days.

Analytical techniques

Initial and residual sugar concentrations, total titratable acidity (TTA) and citric acid produced by fermentation were estimated by the method of anthrone-sulphuric acid method[24] and Marrier and Boulet method[25] respectively from 5-11 days. In addition to these, dry weight of medium after fermentation was also carried out.

Results and Discussion

The present work was a feasibility study of citric acid production by solid-state fermentation using sugarcane bagasse. Potentiality of four isolates of *Aspergillus niger* for citric acid fermentation was also determined. An important parameter for solid-state fermentation is moisture content of the substrate. To standardize the optimum moisture content, sugarcane bagasse was moistened with different volume of 14% sucrose solution, which was the only moisture source of the medium as well as it also fortified the medium with readily available sugar. *A. niger* 318 was used to carry out this test and the results are shown in Table 1. After 11 days of fermentation, *A. niger* 318 produced 139.65, 140.03, 145.04, 187.75, 173.51, 197.75 and 65.79 µg/g of citric acid when sugarcane bagasse was moistened with 2, 3, 4, 5, 8, 10 and 12 ml of the sucrose solution respectively. Both the total titratable acidity value (TTA) and citric acid production was found highest with 10 ml sucrose solution.

Hossain *et al*.[26] explained that the nature of sugar source has a marked effect on citric acid production by *A. niger*. Sucrose is the traditional commercial substrate for citric acid production[18]. Glucose, fructose and maltose have also been used as substrates for citric acid production[12]. Sucrose is of relatively low molecular weight and readily transported into microbial cells for hydrolysis by intracellular enzymes[27]. Kumar *et al*.[4] showed that lower moisture reduces substrate availability to the fungus resulting lower citric acid production. However, although higher moisture increases substrate availability it reduces porosity of the medium and hence the heat and mass transfer resulting low citric acid production. In this experiment also the maximum citric acid was found at a moderately higher moisture (10 ml) level, which was optimal and selected for further experiment.

Table 1. *Effects of moisture content using 14% sucrose solution on citric acid production by Aspergillus niger 318*

Sucrose solution (ml)	Total titratable acidity (TTA) (µg/g)	Citric acid production (µg/g)
2	4.69	139.65
3	4.50	140.03
4	6.43	145.04
5	6.69	187.75
8	6.88	173.51
10	7.27	197.75
12	6.43	65.79

Initial dry weight of sugarcane bagasse used as the substrate of solid-state fermentation for citric acid production was 3.0 g. After enriched with 10 ml of 14% sucrose and incolulated with spore suspension of *A. niger* the biomass was increased with the increase of duration of fermentation (Table 2). The biomass increase was mainly due to production of mycelial body and their sporulation. Biomass production was not same for all the isolates. In the presence of Prescott salt, the biomass content was highest (4.00 g) in case of *A. niger* 318. In the absence of Prescott salt, the biomass yield was highest in case of *A. niger* 14/20. Increasing biomass content during citric acid production by solid-state fermentation was also reported by Anupama and Ravindra[5].

Table 2. *Dry weight of biomass produced by solid-state fermentation by Aspergillus niger at different days*

Aspergillus niger isolate	Mycelial dry weight (g) after fermentation				
	Day 0	Day 5	Day 7	Day 9	Day 11
With Prescott salt					
318	3.00	3.60	3.74	3.90	4.00
HB3	3.00	3.12	3.25	3.36	3.58
CA16	3.00	3.00	3.24	3.28	3.46
14/20	3.00	3.14	3.23	3.44	3.52
Without Prescott salt					
318	3.00	3.20	3.41	3.45	3.40
HB3	3.00	3.10	3.25	3.36	3.48
CA16	3.00	3.20	3.20	3.34	3.38
14/20	3.00	3.42	3.42	3.50	3.75

Sugar utilization rate was different for different *A. niger* isolates during citric acid production (Table 3). Prescott salt was also found to have effect on sugar utilization for citric acid production.

In presence of Prescott salt in the fermentation medium, the sugar utilization rate was higher as compared to that without Prescott salt during production of citric acid. Initial sugar concentration in the sugarcane bagasse enriched with 10 ml of 14% sucrose solution was 857.34 mg/g substrate. With the increase of fermentation period, sugar concentration in the medium was reduced and maximum reduction was found on day 11. In presence of Prescott salt, residual sugar found in the fermentation medium by *A. niger* CA16 on day 5, 7, 9 and 11 was 521.68, 477.50, 450.25 and 427.35 mg/g respectively. This residual sugar by *A. niger* HB3 on day 5, 7, 9 and 11 was 422.07, 399.70, 368.27 and 292.07 mg/g respectively. *A. niger* 14/20 did not utilize 420.75, 372.50, 290.78 and 312.48 mg/g sugar after 5, 7, 9 and 11 day of fermentation respectively. Highest amount of sugar was utilized by *A. niger* 318 in presence of Prescott salt. The residual sugar in the medium was 410.31, 312.07, 136.55 and 64.78 mg/g on the day 5, 7, 9 and 11 respectively.

Table 3. *Sugar utilization in solid-state fermentation by Aspergillus niger during citric acid production*

Aspergillus niger isolate	Estimated sugar concentration (mg/g) at different period of fermentation				
	Day 0	Day 5	Day 7	Day 9	Day 11
With Prescott salt					
318	857.34	410.31	312.07	136.55	64.78
HB3	857.34	422.07	399.70	368.27	292.07
CA16	857.34	512.68	477.50	450.81	427.76
14/20	857.34	420.45	372.50	312.48	290.78
Without Prescott salt					
318	857.34	526.82	441.47	388.71	325.86
HB3	857.34	575.70	474.06	435.26	387.93
CA16	857.34	672.68	627.68	593.54	574.14
14/20	857.34	491.90	412.76	326.64	279.78

On the other hand, in absence of Prescott salt, residual sugar found in the fermentation medium by *A. niger* CA16 on day 5, 7, 9 and 11 was 672.68, 627.68, 593.54 and 574.14 mg/g respectively. This residual sugar in case of *A. niger* HB3 on day 5, 7, 9 and 11 was 575.70, 474.06, 435.26 and 387.93 mg/g respectively. *A. niger* 14/20 did not utilize 491.90, 412.76, 326.64 and 279.31 mg/g sugar after 5, 7, 9 and 11 day of fermentation respectively. Highest amount of sugar was utilized by *A. niger* 14/20 in absence of Prescott salt. The residual sugar in the medium was 526.82, 441.47, 388.71 and 325.86 mg/g on the day 5, 7, 9 and 11 respectively in case of *A. niger* 318 without presence of Prescott salt. Earlier report of Islam *et al.*[23] showed higher utilization of sugar by *A. niger* 318 in both presence and absence of Prescott salt during citric acid production in liquid fermentation. However, here in solid-state fermentation in absence of Prescott salt *A. niger* 14/20 utilized more sugar than *A. niger* 318.

Total titratable acidity (TTA) value of citric acid against 0.1 *N* NaOH was determined at different fermentation period during citric acid production by four strains of *A. niger* (Table 4). The TTA value was found just proportional to the utilization of sugar in the fermentation medium. In presence of Prescott salt, the TTA value was found higher than in the absence of Prescott salt. In presence of Prescott salt, the highest TTA value was found in case of *A. niger* 318 throughout the fermentation period, whereas without Prescott salt, the highest TTA value was found by *A. niger* 14/20 throughout the fermentation period. In presence of Prescott salt, the TTA value for citric acid was found to be 3.72, 3.86, 4.11 and 4.24 µg/g by *A. niger* CA16; 5.53, 5.79, 5.92 and 7.07 µg/g by *A. niger* HB3; 5.86, 6.11, 7.20 and 7.45 µg/g by *A. niger* 14/20; 7.24, 7.27, 8.61 and 8.62 µg/g by *A. niger* 318 on day 5, 7, 9 and 11 respectively. Again, without Prescott salt, the TTA value for citric acid was found to be 2.51, 3.92, 4.24 and 4.50 µg/g by *A. niger* CA16; 4.24, 4.56, 5.14 and 5.40 µg/g by *A. niger* HB3; 5.53, 5.66, 5.92 and 6.17 µg/g by *A. niger* 14/20; 4.56, 5.02, 5.40 and 5.66 µg/g by *A. niger* 318 on day 5, 7, 9 and 11 respectively.

Table 4. *Total titratable acdity (TTA) in solid-state fermentation by Aspergillus niger during citric acid production*

Aspergillus niger isolate	TTA value at different period of fermentation (µg/g)				
	Day 0	Day 5	Day 7	Day 9	Day 11
With Prescott salt					
318	0.00	7.24	7.27	8.61	8.62
HB3	0.00	5.53	5.79	5.92	7.07
CA16	0.00	3.73	3.86	4.11	4.24
14/20	0.00	5.86	6.11	7.20	7.45
Without Prescott salt					
318	0.00	4.56	5.02	5.40	5.66
HB3	0.00	4.24	4.56	5.14	5.40
CA16	0.00	2.51	3.92	4.24	4.50
14/20	0.00	5.53	5.66	5.92	6.17

Citric acid production was also different with different *A. niger* isolates. Citric acid production was found to increase with the increase of fermentation period and maximum citric acid found on day 11 (Table 5). Citric acid production was found to be higher in Prescott salt than in the absence of Prescott salt. In presence of Prescott salt, the highest citric acid production was found in case of *A. niger* 318 throughout the fermentation period, whereas without Prescott salt, the highest citric acid production was found in case of *A. niger* 14/20 throughout the fermentation period. In presence of Prescott salt, citric acid titre was 35.68, 36.35, 58.67 and 62.51 µg/g sugarcane bagasse by *A. niger* CA16; 42.12, 72.71, 76.94 and 79.06 µg/g sugarcane bagasse by *A. niger* HB3; 65.59, 76.17, 92.24 and 103.78 µg/g sugarcane bagasse by *A. niger* 14/20; 94.06, 135.62, 149.47 and 196.21 µg/g sugarcane bagasse by *A. niger* 318 on day 5, 7, 9 and 11 respectively. On the other hand, without Prescott salt, estimated citric acid value was 22.69, 25.0, 26.73 and 34.62 µg/g sugarcane bagasse by *A. niger* CA16; 35.58, 38.85, 41.74 and 43.09 µg/g sugarcane bagasse *A. niger* HB3; 38.28, 41.93, 48.28 and 50.01 µg/g by *A. niger* 14/20; 37.51, 38.08, 42.89 and 44.24 µg/g sugarcane bagasse by *A. niger* 318 on day 5, 7, 9 and 11 respectively.

Table 5. *Citric acid titre in solid-state fermentation by Aspergillus niger during fermentation*

Aspergillus niger isolate	Citric acid production (µg/g) at different period of fermentation				
	Day 0	Day 5	Day 7	Day 9	Day 11
With Prescott salt					
318	0.00	94.06	135.62	149.47	196.21
HB3	0.00	42.12	72.71	76.99	79.00
CA16	0.00	35.68	36.35	58.67	62.51
14/20	0.00	65.59	76.17	92.24	103.06
Without Prescott salt					
318	0.00	37.51	38.08	42.89	44.24
HB3	0.00	35.58	38.85	41.74	43.09
CA16	0.00	22.69	25.00	26.73	34.62
14/20	0.00	38.28	41.93	48.28	50.01

In conclusion, we found in this study that using sugarcane bagasse as a natural fermentation medium fortified with 10 of 15% sucrose as additional carbon source and moistening agent for citric acid production was superior when supplemented with Prescott salt. Maximum citric acid was produced by *A. niger* 318 on sugarcane bagasse in solid-state fermentation.

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