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Research Paper

Citric acid production from brewers spent grain by *Aspergillus niger* and *Saccharomyces cerevisiae*

***Femi-Ola T. O. and Atere V. A.**
Department of Microbiology, Ekiti State University P. M. B. 5363, Ado-Ekiti, NIGERIA

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Abstract

Production of citric acid by submerged fermentation of brewers spent grain with *A. niger* and *S. cerevisiae* was investigated. The effect of the initial pH and presence of methanol on the rate of production was determined. The highest citric acid concentration was produced by *A. niger* at initial pH of 4.5 in the presence of methanol with a value of 0.512%, while fermentation with *S. cerevisiae* yielded a highest concentration of 0.312% citric acid. There were significant differences (P≤ 0.05) in the amount of citric acid produced and biomass generated during fermentation with and without methanol. However, there was no significant difference in the biomass production at different pH (P≥0.05).

Keywords Brewers spent grain, biomass, citric acid, fermentation, *Aspergillus niger, Saccharomyces cerevisiae.*

Introduction

Citric acid is an important multifunctional organic acid with a broad range of versatile uses in household and industrial applications. Citric acid (2- hydroxyl-1, 2, 3 propane tri carboxylic acid) exists in naturally in a variety of fruits and vegetable notably citrus fruits. It is most concentrated in lemons and limes, where it can comprise as much as 8% of the dry weight of the fruit [1, 2].

Today, essentially all of the commercial citric acid is produced by fermentation process employed are submerged fermentation by *Aspergillus niger* using a variety of substrates[3]. Substrates such as molasses, corn syrup, apple and grape pomace, pineapple waste, sugarcane juice have been used in the production of citric acid [3, 4, 5, 6, 7]. Citric acid is used in the food beverages, pharmaceutical, chemical cosmetic and other industries for applications such as acidulation, antioxidant, flavour, enhancement, preservation, plasticize and as a synergistic agent. Brewer's spent grain (BSG) is a by-product of beer brewing consisting of the residue of malt and grain which remains in the mash-kettle after the mashing and process. BSG consist primarily grain husks, pericarp, ferments of endosperm, starch, cellulose, glucans, and arabinoxylans [8]. Brewer spent grain has been utilized as animal feed, fertilizer and production of value added products such as organic acids microbial enzymes and in mushroom production. The use of this inexpensive substrate like BSG will reduce the cost of citric acid production.

This study evaluates the potential of brewers spent grain as substrate for citric acid production by *Aspergillus niger* and *Saccharomyces cerevisiae* and the effect of methanol on the fermentation process.

Materials and Methods

Microorganisms

Aspergillus niger and *Saccharomyces cerevisiae* were used in this study. These organisms are local isolates from spoiled orange and palm wine samples respectively. The organisms were cultured on Potato Dextrose agar (PDA) at 30^{o}C for five days. Pure cultures of both *A. niger* and *S. cerevisiae* on the PDA plate were scrapped off and mixed with 25 ml of sterile Potato Dextrose Broth. Approximately 2 ml of each of test organisms' suspensions containing 10^{6} spores/ ml were used as inocula.

Fermentation Media

The fermentation was performed in twelve 500 ml conical flask containing 200 ml of the fermentation medium containing (g/l) peptone, 2.0; yeast extracts, 1.5; KH_2PO_4, 2.0; $(NH_4)_2\ SO_4$, 2.0 and $MgSO_4.H_2O$, 2.0, brewers spent grain, 10.0. The fermentation media was adjusted to pH 4.5, 5.5 and 6.5 respectively. One percent volume fraction (0.2 ml) of methanol was added to half portion of the flasks containing the fermentation media. The pH of the fermentation media was adjusted using 0.2 M NaOH or 0.2 M H_2SO_4 and sterilized at 121^{o}C for 15 min. Each flask was inoculated with 0.1 % of spore suspension. Inoculated flasks were wrist shaken at 30^{o}C for 14 days. The content of the flasks were analyzed for citric acid, CO_2, microbial load and biomass content at 24 hours interval over a 14-day period.

Analytical methods

Proximate analysis

This was carried out on the brewer's spent grain sample for ash, moisture, crude fiber and fat using the method described by Association of Official Analytical Chemists [9]. Nitrogen was determined by the micro-Kjeldahl method reported by Pearson [10]. The percentage nitrogen was converted to crude protein by multiplying with 6.25.

Determination of Biomass Content

The suspension was filtered through Whatman No. 1 filter paper to remove mycelium. The filter cake paper was washed thrice with deionised water, dried at 105^{o} C to a constant mass in an oven (Gallenkamp), and weighed as the biomass.

Determination of citric acid content

Titrimetric method was employed for determining citric acid content [11]. The amount of citric acid in the filtrate was measured by titration with 0.1 M NaOH against phenolphthalein as an indicator. The concentration of citric acid was calculated from the total acidity minus that of the blank assuming that the acid was only composed of citric acid.

Determination of Carbon dioxide content

The CO_2 content was measured by the titrimetric method described by Caputi *et al*. [12]. Titration was carried out with 0.02M NaOH.

Determination of pH

This was determined using a digital pH and temperature meter (pH-25 Techmel & Techmel USA).

Statistical analysis

Analysis of variance and T-test for data was carried out using SPSS (Statistical Package for Social Science) version 11.0 software.

Results and Discussion

The chemical composition of the Brewers spent grain is shown in (Table 1). A gradual increase in biomass production was observed throughout the fermentation period. Fermentation flask containing 1 % methanol at initial pH of 4.5 produced the highest concentration of biomass (Table 2). The statistical analysis revealed that there was a significant difference in the biomass produced when 1% methanol was added (P≤0.05). However there was no significant difference in biomass produced at different pH values (P≥0.05). Maximum biomass concentration of 56% and 46% were observed for *Aspergillus niger* and *Saccharomyces cerevisiae* respectively. Citric acid production was maximum after the 96th hour of fermentation (0.512%/v) by *A. niger* (Table 3). Citric acid production in fermentation flask inoculated with *S. cerevisiae* increased at a steady state until a maximum production of 0.312% was reached at the end of the 7-day fermentation (Table 3). There was a significant difference (P≤ 0.05) when methanol was added. The rate of sugar consumption is shown in (Table 4). Samples inoculated with *A. niger* showed higher rate of sugar utilization even in the presence of methanol. The sugar content reduction correlates with the rate at which citric acid was produced.

Table 4 shows the rate at which CO_2 was produced during the fermentation process. A rapid increase was observed throughout the period of fermentation. The presence of methanol enhanced the production of citric acid, biomass and CO_2.

In this study, considerable amounts of citric acid were produced form Brewer's spent grain using *A. niger* and *S. cerevisiae* by submerged fermentation. Citric acid, biomass and CO_2 production increased generally in the presence of methanol (Table 3, 4 and 5). Methanol has an enhancing effect on the fungal production of citric acid [3]. Alben and Ezeronye [13] reported that the addition of methanol at a concentration of 1.0 to 4.0 percent resulted in a marked increase in the amount of citric acid produced by *A. niger* in spent grain liquor is brewery wastes. The reducing sugar reduces rapidly between the first and the fourth day. This was an indication that as the organisms are growing rapidly they make use of the available reducing sugar for the production of biomass [9]. *Aspergillus niger* produced a considerable amount of titratable citric acid. The production of citric acid increases rapidly from the first day to the fourth day of fermentation. After the fourth day, there was a sharp drop in citric acid and a surge increase in pH. Hang *et al*. [14] described this phenomenal by saying that *A. niger* oxidized the accumulated citric acid upon exhaustion of the fermentable sugar.

There was an increase in the percentage of biomass as the fermentation day progresses. The statistical analysis revealed that there was a significant difference in the biomass produced at (P≤0.05). However there was no significant difference in biomass produced at different pH used (P≥0.05). It was also observed that as the CO_2 production increased, the amount of citric acid produced also increased generally in all samples but at higher rates in samples inoculated with *A. niger*. The T –test carried out on the production of citric acid using *Aspergillus niger* and *Saccharomyces cerevisiae* reveals that there was a significant difference (P≤ 0.05) when methanol was added. Femi-Ola *et al*. [5] earlier reported that the addition of 3.0 to 4.0 percent of methanol does not only increase the citric acid production but also the pH, biomass, CO_2 production and sugar consumption. According to Alben and Erkmen [3] methanol may serve as additional carbon-source for the microbial cells.

It appears that the citric acid is produced by carbon-storing cells. The sugar concentration decreases throughout the fermentation period. This indicated that the cells were still viable. There seems to be a link between storage carbon and the production of citric acid [13]. The initial rapid increase in CO_2 production between the second and fourth day indicated that the cells were in exponential phase of growth. This is also evident in the initial rapid increase in mycelia mass. The sample inoculated with *A. niger*, in the presence of methanol and initial pH of 4.5 produced both the highest concentration of citric acid and CO_2. This can also be seen as evidence of a link between the citric acid production, the biomass and the CO_2 concentration. The acidity of the citric acid invariably caused decrease in the pH as citric acid fraction increases. This research has revealed a significant difference in the pattern of citric acid production in *A. niger* and *S. cerevisiae* (Table 3). In the case of *Aspergillus niger,* it utilizes the reducing sugar faster and reaches a peak and then start to oxidize the citric acid after the fourth day. However *S. cerevisiae* continued producing citric acid throughout the fermentation period, although the rate of production almost

remained constant after the fourth day. The observation may suggest that the pH at this point did not support citric acid excretion but resulted into the assimilation of residual citric acid by *A. niger*.

The amount of citric acid produced, sugar consumption, CO_2 production and biomass concentration increased with the addition of methanol. In the fermentation medium inoculated with *A. niger* with initial pH of 4.5 with and without methanol produces, 0.512% and 0.376% mass per volume of citric acid respectively. The sample inoculated with *Saccharomyces cerevisiae* at pH 4.5 with methanol also produced 0.312% mass per volume of citric acid. In the citric acid production rate, the *A. niger* has its peak on the fourth day and a further fermentation result in reduction in citric acid concentration. For citric acid production, preferably *A. niger* strains should be majorly used since it produces the highest citric acid concentration.

Table 1: Chemical composition of Brewer's spent Grain

Constituents	Percentage composition (%)
Ash	4.49
Moisture content	9.13
Crude protein	36.90
Fat	1.33
Fibre	11.08
Carbohydrate	37.09

Table 2: Changes in biomass production (%) throughout the fermentation

Samples	pH	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A. niger	4.5	8	20	28	34	34	38	46
Brewer's	5.5	8	12	24	26	28	24	32
spent grain	6.5	4	10	14	16.0	18	20	26
Brewers' Spent grain +	4.5	10	18	28	40	46	52	56
1% methanol	5.5	8	16	22	32	36	40	50
	6.5	8	14	20	30	30	34	36
S. cerevisiae	4.5	8	12	14	24	28	30	32
Brewers Spent	5.5	4	10	16	20	22	24	28
Grain	6.5	4	8	10	16	18	18	20
Brewer	4.5	6	22	26	30	36	40	46
Spent grain +	5.5	4	12	22	30	30	32	36
1% methanol	6.5	6	8	16	24	26	30	32

Table 3: Changes in the citric acid concentration (%/v) throughout the fermentation period

Samples	pH	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A. niger	4.5	0.088±0.001	0.212±0.002	0.260±0.001	0.376±0.001	0.288±0.001	0.248±0.002	0.216±0.002
Brewer's	5.5	0.160±0.020	0.204±0.001	0.224±0.002	0.306±0.002	0.264±0.002	0.200±0.002	0.188±0.001
spent grain	6.5	0.152±0.002	0.192±0.002	0.232±0.002	0.284±0.001	0.264±0.002	0.200±0.001	0.184±0.001
Brewers'	4.5	0.160±0.001	0.384±0.003	0.488±0.002	0.512±0.002	0.312±0.001	0.296±0.003	0.192±0.002
Spent grain	5.5	0.168±0.003	0.264±0.002	0.344±0.002	0.400±0.001	0.288±0.002	0.244±0.002	0.220±0.003
+	6.5	0.156±0.002	0.176±0.002	0.200±0.001	0.296±0.001	0.264±0.001	0.240±0.002	0.184±0.001
1% methanol								
S. cerevisiae	4.5	0.144±0.002	0.164±0.002	0.168±0.002	0.200±0.002	0.208±0.002	0.220±0.003	0.256±0.002
Brewers	5.5	0.152±0.002	0.180±0.001	0.185±0.002	0.204±0.001	0.216±0.001	0.228±0.002	0.240±0.003
Spent	6.5	0.148±0.001	0.160±0.002	0.168±0.001	0.184±0.002	0.200±0.002	0.208±0.001	0.224±0.002
Grain								
Brewer	4.5	0.136±0.003	0.180±0.003	0.224±0.001	0.256±0.001	0.268±0.001	0.216±0.002	0.312±0.002
Spent grain +	5.5	0.160±0.002	0.172±0.003	0.192±0.001	0.216±0.002	0.248±0.002	0.268±0.002	0.288±0.001
1% methanol	6.5	0.148±0.002	0.168±0.002	0.188±0.002	0.208±0.002	0.224±0.001	0.240±0.002	0.252±0.002

NB: Initial citric acid (%m/v) in fresh sample = 0.072%

Table 4: Changes in CO_2 production (mg/L) throughout the fermentation period

Samples	pH	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A. niger	4.5	140.25±0.03	167.75±0.02	228.25±0.02	266.75±0.01	297.00±0.02	324.50±0.02	360.25±0.02
Brewer's	5.5	101.75±0.03	143.00±0.03	192.50±0.02	255.75±0.02	283.25±0.02	302.50±0.03	316.25±0.03
spent grain	6.5	35.75±0.02	110.00±0.03	159.50±0.03	193.50±0.01	242.00±0.03	264.00±0.03	275.00±0.03
Brewers'	4.5	46.75±0.02	165.00±0.02	269.50±0.03	341.00±0.03	481.25±0.02	503.25±0.02	536.25±0.03
Spent grain	5.5	38.50±0.02	156.75±0.03	255.75±0.03	330.00±0.03	401.50±0.03	420.75±0.03	462.00±0.03
+	6.5	30.25±0.03	143.00±0.02	253.00±0.02	324.50±0.02	368.50±0.03	407.00±0.02	429.00±0.02
1% methanol								

S. cerevisiae	4.5	88.00±0.03	148.50±0.03	187.00±0.03	200.75±0.03	209.00±0.03	244.75±0.02	255.75±0.03
	5.5	66.00±0.02	167.75±0.02	203.50±0.02	264.00±0.02	294.25±0.03	313.50±0.01	330.00±0.03
Brewers Spent	6.5	44.00±0.02	173.25±0.02	247.50±0.03	269.50±0.02	305.50±0.03	327.25±0.02	343.75±0.02
Grain								
Brewer	4.5	44.00±0.02	198.00±0.03	244.75±0.02	308.00±0.03	330.00±0.03	352.00±0.03	376.75±0.02
Spent grain +	5.5	30.25±0.03	178.75±0.03	261.25±0.03	264.00±0.03	302.50±0.03	327.25±0.02	343.75±0.02
	6.5	30.25±0.02	189.75±0.02	277.75±0.02	291.50±0.03	313.50±0.03	357.50±0.03	379.50±0.02
1% methanol								

Table 5: Glucose consumption (%) during fermentation of Brewer spent grain with and without methanol

Samples	pH	Day 1	Days 2	Days 3	Days 4	Day 5	Day 6	Day 7
A. niger	4.5	8.22±0.01	6.60±0.01	4.74±0.01	2.58±0.01	1.86±0.02	1.08±0.01	0.96±0.01
Brewer's spent grain	5.5	8.34±0.02	6.90±0.02	5.04±0.02	2.64±0.02	2.28±0.02	1.25±0.01	1.08±0.03
	6.5	8.94±0.02	6.96±0.01	4.38±0.02	2.22±0.01	1.50±0.01	1.50±0.01	0.84±0.02
Brewers'	4.5	8.22±0.02	6.36±0.03	4.74±0.01	2.52±0.02	1.14±0.02	1.08±0.02	0.84±0.02
Spent grain	5.5	8.10±0.01	5.98±0.02	3.90±0.01	1.68±0.02	1.50±0.02	1.38±0.01	1.02±0.02
	6.5	8.04±0.03	6.96±0.02	5.04±0.02	3.42±0.01	1.86±0.01	1.74±0.03	1.14±0.01
+								
1% methanol								
S. cerevisiae	4.5	8.64±0.03	6.36±0.02	4.54±0.03	2.64±0.01	1.44±0.01	1.20±0.02	0.96±0.02
	5.5	8.28±0.01	6.60±0.02	3.82±0.02	2.46±0.02	1.98±0.02	1.56±0.01	1.36±0.01
Brewers Spent	6.5	8.82±0.02	6.90±0.01	4.54±0.01	2.34±0.02	1.86±0.02	1.74±0.02	1.26±0.02
Grain								
Brewer	4.5	8.40±0.01	6.24±0.02	4.48±0.02	2.58±0.03	1.68±0.02	1.44±0.01	1.44±0.02
Spent grain +	5.5	8.10±0.02	6.36±0.02	3.48±0.02	1.92±0.01	1.74±0.01	1.02±0.02	0.90±0.01
	6.5	8.64±0.02	7.02±0.02	4.38±0.02	2.34±0.01	2.28±0.01	2.16±0.03	1.92±0.02
1% methanol								

Initial percentage of reducing sugar = 9.84%

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