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preliminary communication

Production of Citric Acid from a New Substrate, Undersized Semolina, by *Aspergillus niger*

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Summary

The production of citric acid from fermentation medium (mass per volume ratio 0.01 % of undersized semolina) by *Aspergillus niger* was studied by shake culture method. The effects of initial pH (4.5, 5.5 and 6.5), methanol (volume fraction 1.0, 2.0 and 3.0 %) and ammonium nitrate (mass per volume ratio 0.01 %) on the production of citric acid were investigated. Citric acid concentration, biomass concentration and the amount of total carbohydrates (as glucose) were determined during fermentation period. About 0.04, 0.0448 and 0.0506 g/L of citric acid was produced in fermentation medium with initial pH=4.5, 5.5 and 6.5, respectively. Citric acid and biomass concentrations were increased with the addition of methanol and ammonium nitrate. The yield of citric acid based on consumed sugar was about 21.9 % in the presence of 3.0 % methanol and 0.01 % ammonium nitrate in fermentation medium with initial pH=6.5.

Key words: citric acid, undersized semolina, *Aspergillus niger,* fermentation

Introduction

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is produced by fermentation (*1–5*). The filamentous fungus *Aspergillus niger* is the most commonly used microorganism for citric acid production (*5–7*). Citric acid is used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, antioxidation, flavour enhancement, preservation, plasticizer and as a synergistic agent (*8–10*).

Citric acid is produced commercially either by submerged fermentation or surface fermentation employing various carbohydrate sources (*4,11*). Molasses, carob pod extract, rape seed oil, corncobs, apple and grape pomace, kiwifruit peel, mandarin orange and brewery wastes have been used as substrates in citric acid production (*1–6,10–13*). It is necessary to use inexpensive and readily available raw materials in industrial production processes (*8*). From this point of view, large volumes of starchy materials are suitable substrates for the production of citric acid since they are cheap and renewable (*10*). There is little information on the utilization of wheat wastes rich with starch such as undersized semolina for citric acid production. Approximate composition of undersized semolina was 13–15 % moisture, 63–64 % starch, 11.9–13.2 % protein, 0.15–0.33 % cellulose, 1.7–2.5 % fat, 1.5–2.0 % sugar and 0.67–0.95 % minerals (*14*). Undersized semolina is generally produced during the production of macaroni from durum wheat (*Triticum durum*) (*15*). About 56 000 tonnes per year of undersized semolina is produced as a waste product in Turkey (*7*), which is a cheap industrial waste of macaroni factories. Therefore, the use of undersized semolina (as fermentation medium) was taken into consideration as an alternative raw material for the produc-

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tion of citric acid. The effects of methanol and ammonium nitrate on citric acid production rate were investigated in this study.

Materials and Methods

Microorganism and inoculum preparation

Aspergillus niger ATCC 9142 (American Type Culture Collection, Rockville, MA, USA) was used throughout this study. It was maintained on potato dextrose agar (PDA, Difco, Detroit, USA) slants at 4 °C and subcultured monthly. Undersized semolina was supplied from a regional macaroni factory (Beslen Makarna, Gaziantep, Turkey).

Spore suspensions for inoculations were obtained from potato dextrose agar (PDA) slant culture incubated at 30 °C for 5 days. The sporulated culture on PDA slant was scraped off and mixed with 5.0 mL of sterile potato dextrose broth (PDB, Difco, Detroit, USA).

Fermentation media

Experiments were performed in 250-mL Erlenmeyer flasks containing 150 mL of fermentation medium. Fermentation media were prepared at pH=4.5, 5.5, and 6.5 in the presence of 0.01 % of undersized semolina (USS), 1.0, 2.0 and 3.0 % volume fraction of methanol and with and without ammonium nitrate (0.01 %). The media were sterilized at 121 °C for 15 min. Each flask was inoculated with 0.1 % of spore suspension. Inoculated flasks were placed into a bench type water bath shaker (ST-402 NÜVE, Sanayi Malzemeleri Imalat ve Ticaret A. Ş., Istanbul, Turkey) and wrist shaken at 30 °C for 14 days. At every two-day interval, the contents of the fermentation flasks were analyzed for citric acid, sugar and biomass content.

Analytical methods

The sample was filtered through Whatman No. 41 filter paper to remove mycelium. The filter cake on paper was washed three times with distilled water, dried at 105 °C to constant mass and weighed as the biomass (*1,16*). The amount of citric acid in the filtrate was measured by titration with 0.1 M NaOH against phenolphthalein, as an indicator (*10,17*). The concentration of citric acid was calculated from the total acidity minus that of the blank, assuming the acid was only composed of citric acid. The amount of citric acid was also determined by HPLC (LC-10AD VP SHIMATZU, Kyoto, Japan model) method (*18*). Total carbohydrate in the fermentation filtrate at various times was determined as glucose by anthrone-sulphuric acid method (*19*).

Results

Sugar content of the medium was reduced by *A. niger* during fermentation and the amount of citric acid production increased in proportion with the sugar utilization. In citric acid production rate, two phases were observed. In the first phase, citric acid production rate was higher (up to 4 days of fermentation). This phase can be considered as rapid production phase. In the second phase (up to 14 days of fermentation), citric acid concentration increased slightly and became constant. This phase can be considered as slow production phase. About 97.2 % of citric acid was produced, 60.0 % of sugar was consumed in the first phase in USS medium without the additive and with initial pH=6.5 (Tables 1 and 3). The amount of citric acid production and sugar consumption increased with an increase in methanol concentration. The highest amount of citric acid was produced in the presence of 3.0 % of methanol in the fermentation medium. As initial pH increased, citric acid concentration increased.

Two phases were also observed in biomass formation. In the first phase (up to 4 days) a rapid increase in biomass production rate was observed. About 77.0, 82.2 and 85.0 % of biomass concentration were produced in the first phase in the presence of 1.0, 2.0 and 3.0 % of methanol, respectively, in fermentation media with an initial pH=6.5. After rapid production phase, a slight increase was observed in biomass production until the end of fermentation period (14 days).

Table 1. Citric acid (CA) fraction (%) and yield (%) ($Y_{P/S}$) in undersized semolina only (USS), and in USS with methanol (Met.) and ammonium nitrate (0.01 %) during fermentation period

Initial pH	Only USS		USS+1.0 % Met.		USS+2.0 % Met.		USS+3.0 % Met.	
	w(CA)/%	Yield/%	*w*(CA)/%	Yield/%	*w*(CA)/%	Yield/%	*w*(CA)/%	Yield/%
4.5	95.0[a] (5.0)[b]	15.1	85.7[a] (14.3)[b]	16.4	98.6[a] (1.4)[b]	17.7	89.4[a] (10.6)[b]	21.3
5.5	96.4[a] (3.6)[b]	15.2	75.0[a] (25.0)[b]	16.8	90.0[a] (10.0)[b]	17.8	81.0[a] (19.0)[b]	21.7
6.5	97.2[a] (2.8)[b]	16.2	83.8[a] (16.2)[b]	16.9	81.4[a] (18.6)[b]	17.9	79.4[a] (20.6)[b]	21.9

Yield (%) based on sugar consumed, [a]citric acid fraction in the first phase, [b]citric acid fraction in the second phase

Table 2. Biomass fraction (%) and biomass yield (%) ($Y_{X/S}$) in undersized semolina only (USS), and in USS with methanol (Met.) and ammonium nitrate (0.01 %) during fermentation period

Initial pH	Only USS		USS+1.0 % Met.		USS+2.0 % Met.		USS+3.0 % Met.	
	w(biomass)/%	Yield/%	*w*(biomass)/%	Yield/%	*w*(biomass)/%	Yield/%	*w*(biomass)/%	Yield/%
4.5	63.6[a] (36.4)[b]	80.0	69.5[a] (30.5)[b]	81.0	83.2[a] (16.8)[b]	91.0	88.4[a] (11.6)[b]	91.5
5.5	73.4[a] (26.6)[b]	76.0	55.3[a] (44.7)[b]	83.0	67.4[a] (32.6)[b]	94.0	82.2[a] (17.8)[b]	94.5
6.5	73.0[a] (27.0)[b]	81.0	58.4[a] (41.6)[b]	84.0	84.0[a] (16.0)[b]	95.2	87.0[a] (13.0)[b]	98.5

Yield (%) based on sugar consumed, [a]biomass fraction in the first phase, [b]biomass fraction in the second phase

Table 3. Sugar consumption (%) in undersized semolina only (USS), and in USS with methanol (Met.) and ammonium nitrate (0.01 %) during fermentation period

Initial pH	Only USS	USS+1.0 % Met.	USS+2.0 % Met.	USS+3.0 % Met.
	sugar consumption/%			
4.5	69.9[a] (30.1)[b]	59.5[a] (40.5)[b]	70.1[a] (29.9)[b]	72.4[a] (27.6)[b]
5.5	68.3[a] (31.7)[b]	86.7[a] (13.3)[b]	87.5[a] (12.5)[b]	88.3[a] (11.7)[b]
6.5	60.0[a] (40.0)[b]	73.4[a] (26.6)[b]	88.6[a] (11.4)[b]	90.0[a] (10.0)[b]

[a]sugar consumption (%) in the first phase, [b]sugar consumption (%) in the second phase

The influence of ammonium nitrate on citric acid production rate was also investigated in this study. After the addition of ammonium nitrate, a rapid increase in citric acid production was observed up to the 4th day of fermentation. After this period, it increased slightly. The production of citric acid was about 0.0506 g/L in USS medium (initial pH=6.5) without additives, while it was 0.068 g/L in USS medium with ammonium nitrate and 3.0 % methanol. *A. niger* produced 0.051, 0.052 and 0.054 g/L of citric acid at the end of 14 days of fermentation with 1.0, 2.0 and 3.0 % of methanol, respectively, in fermentation media with initial pH=6.5. After the addition of ammonium nitrate, about 0.052, 0.055 and 0.068 g/L of citric acid was produced in the presence of 1.0, 2.0 and 3.0 % of methanol, respectively. The highest amount of citric acid was produced in media with an initial pH=6.5, 3.0 % methanol and ammonium nitrate. As reported by other researchers, the addition of methanol with ammonium nitrate increased the production of citric acid (*20*). During fermentation period sugar content of the medium decreased with the increase of the citric acid production. Biomass concentration increased rapidly in the first phase (4th day) and then a slight increase was observed. This was due to the exhaustion of nitrogen supply (*21*). Since the dry weight continued to increase, it is clear that nitrogen exhaustion did not stop the cells from growing. The supply of nitrogen in the medium was exhausted early and the subsequent increase in dry weight was due to an accumulation of carbon by the cells (*22*).

Product yield

Citric acid ($Y_{P/S}$) and biomass yield ($Y_{X/S}$) based on the sugar consumed at the end of 14 days of fermentation were calculated (Tables 1 and 2). Citric acid and biomass yield were defined by the amount of citric acid produced divided by the amount of sugar consumed, and the amount of biomass divided by the amount of sugar consumed, respectively (*17,23*). A citric acid yield of 16.2 % was observed in USS medium with initial pH=6.5 in the absence of methanol and ammonium nitrate after 14 days. Maximum yields of 16.9, 17.9 and 21.9 % citric acid were obtained in USS medium containing 1.0, 2.0 and 3.0 % of methanol with ammonium nitrate, respectively, with initial pH=6.5. Citric acid yield increased with an increase in methanol concentration.

Biomass yield was about 81 % in USS medium with the initial pH=6.5. Maximum biomass yield (98.5 %) was observed in USS medium with ammonium nitrate and 3.0 % of methanol with the initial pH=6.5. Biomass yield increased with an increase of the initial pH of USS medium and of methanol concentration.

Discussion

In this study, considerable amounts of citric acid were produced from undersized semolina using *Aspergillus niger* by submerged fermentation. Citric acid concentration, sugar consumption, biomass concentration and citric acid yield increased in the presence of methanol. Methanol has an enhancing effect on the fungal production of citric acid (*6,13,24*). Roukas and Kotzekidou (*20*) reported that the addition of methanol at a concentration of 1.0 to 4.0 % resulted in a marked increase in the amount of citric acid production by *A. niger* in spent grain liquor and brewery wastes. In other research, it was also reported that the addition of 3.0 to 4.0 % of methanol concentration retarded growth, delayed sporulation and increased citric acid yields (*1*).

Nitrogen is a limiting factor in the citric acid production. Nitrogen is usually supplied in the form of ammonium nitrate, which was completely metabolized during fermentation periods. Citric acid started to appear when the nitrogen concentration fell below a low limiting value. It appears that the citric acid was produced by carbon-storing cells. Low dry weight might have been caused by the drastic reduction and denaturation of some enzymes active in the accumulation of carbon in the used pH range. The sugar concentration decreased throughout the fermentation period. This indicated that the cells were still viable. Therefore, there seems to be a link between the storage of carbon and the production of citric acid (*18,19*).

Conclusion

The amount of the citric acid produced, sugar consumption and biomass concentration increased with an increase in methanol concentration and addition of ammonium nitrate. In fermentation medium with the initial pH=6.5, with and without methanol and ammonium nitrate 21.9 and 16.2 % of citric acid yield was obtained, respectively. In citric acid production rate, two phases were observed and citric acid fermentation was found as Type II fermentation.

For the application of this study in industry, further studies have been planned based on the use of different undersized semolina concentrations and *A. niger* strains at different fermentation conditions to increase citric acid yield.

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Proizvodnja limunske kiseline s pomoću *Aspergillus niger* na podlozi s usitnjenim grizom

Sažetak

Ispitivana je proizvodnja limunske kiseline tijekom fermentacije na tresilici u podlozi koja je sadržavala 0,01 % griza usitnjenog ispod određene veličine s pomoću *A. niger*. Praćen je utjecaj početnog pH (4,5, 5,5 i 6,5), metanola (1,0, 2,0 i 3,0 %) i amonijeva nitrata (0,01 %) na proizvodnju limunske kiseline. Tijekom vrenja određivana je koncentracija limunske kiseline, biomase i količine ukupnih ugljikohidrata (računato kao glukoza). U podlozi s početnim pH-vrijednostima od 4,5, 5,5 i 6,5 dobiveno je oko 0,04, 0,0448 i 0,0506 g/L limunske kiseline. Dodatkom metanola i amonijeva nitrata povećava se koncentracija limunske kiseline i biomase. Iskorištenje limunske kiseline, s obzirom na utrošeni šećer, iznosilo je oko 21,9 % u prisutnosti 3,0 % metanola i 0,01 % amonijeva nitrata u fermentacijskoj podlozi s početnom pH-vrijednosti 6,5.