INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2006/08–4–463–467
http://www. fspublishers.org

# Submerged Fermentation of Cheese Whey and Molasses for Citric acid Production by *Aspergillus niger*

S.A. EL-AASAR
*Botany Department, Faculty of Science, Zagazig University, Egypt*
E-mail: salahaasar@yahoo.com

## ABSTRACT

An isolate of *Aspergillus niger* was evaluated for citric acid production and enriched protein mycelium using whey and molasses for the fermentation medium. The results indicated that acid pretreated cane and beet molasses yielded 74.32 and 75.14% citric acid and 32.99 and 34.17% proteins. Ammonium nitrate (0.3, w/v) was the best additional nitrogen sources for protein and citric acid production in presence of whey and (15%; v/v) acid pretreated beet molasses. The pH 5.5 was optimum for dry cell mass (15.64 g $L^{-1}$) and protein content (45.8%) while; pH 6 was the optimum for citric acid production (47.63 g $L^{-1}$). Time course study during citric acid fermentation by *A. niger* showed that 4 days was optimum for dry cell mass, economic coefficient (EC) and protein conversion coefficient (PCC), while 6 for maximum protein and citric acid production. The chemical composition of *A. niger* grown under optimal culture conditions showed that the mycelium was rich in protein (29.04%), carbohydrates (soluble 10.32% & non-soluble 34.86%), crude lipids (6.37%), nucleic acids (RNA/DNA; 4.46/0.83%) and uric acid (0.43 mg $g^{-1}$). Toxigenic activity of *A. niger* showed no toxin production of B1, B2, G1 and G2. Profile of *A. niger* protein showed amino acids in sufficient amounts. Therefore, whey and beet molasses were optimized as the basal fermentation medium for maximal citric acid production as well as nutritional up-grading of these wastes for single cell protein (SCP) production by *A. niger* for animal feed.

**Key Words:** Citric acid; Fermentation; *A. niger*; Aflatoxins; Amino acids

## INTRODUCTION

Citric acid is colorless, odorless and easily soluble in water and alcohol with a pleasant taste, solid at room temperature and melts at 153ºC. It exists as an intermediate in the Krebs cycle when carbohydrates are oxidized to carbon dioxide (Haq *et al*., 2002). It is readily eliminated from the human body, as it easily metabolized and is therefore harmless (Reed, 1982). Citric acid is one of the most versatile industrial organic acids that are used in food preparations, cosmetics and pharmaceuticals. About 70% of citric acid is utilized in food industry, confectionary and beverages as an acidulant, flavor enhancer, preservative, chelator, buffer, emulsifier, stabilizer and antioxidant. About 10% is used in cosmetics and pharmaceuticals (Kubicek & Rohr, 1986; Lodhi *et al*., 2001).

Most of the commercially produced citric acid comes from *Aspergillus niger*. Various carbohydrate materials such as cane or beet molasses and crude unfiltered starch hydrolysate may be used in citric acid production by *A. niger* in submerged fermentation (Lodhi *et al*., 2001; Ali *et al*., 2002; Ishaq *et al*., 2002; Rehman *et al*., 2002, 2003; El-Holi & Al-delaimy, 2003; Meleigy & Matter, 2004). Moreover, large amounts of cheese whey milk permeate are produced as a by-product from dairy industry and generally considered as a waste and disposed off. The cheese whey has been used extensively in fermentation media by *A. niger* for citric acid production (Hossain *et al*., 1984; Banerjee *et al*., 1996; El- Samragy, 1996; Paul *et al*., 1996; El-Holi & Al- Delaimy, 2003; Meleigy & Matter, 2004).

The processing costs can be reduced by less expensive raw materials such as cheese whey and molasses for production of citric acid as well as nutritional up-grading of these wastes for single cell protein (SCP) production by *A. niger* for animal feed. To my knowledge from an intensive literature survey, lower organisms have the advantages over higher for protein production because of protein content of 40 - 80%, well–balanced amino acid composition, protein digestibility of up to 80% or more, rapid growth (it reproduce themselves from 1000 to 5000 times faster than plants & animals), easy to breed and to engineer genetically. When the microorganisms die, they disintegrate in the animal's stomach and used as a protein source. Concerning fungal mycelia, filamentous structure allows the product to be easily added to different types of foodstuffs. Fungal mycelia are less likely to cause adverse reactions on man, such as allergenicity or toxicity, than other single cell protein sources.

Industrial wastes like cheese whey, sugar cane and beet molasses, starchy green mills, agricultural residues and agro-industrial wastes, for economic reasons, are strongly involved in SCP and organic acids by many of fungal spp. (Gazaerly, 1983; El-Aasar, 1985; Helal, 1986; Paul *et al*., 1996; Arzumanov *et al*., 2000; Lodhi *et al*., 2001; Ishaq *et al*., 2002; Rehman *et al*., 2002; Ali *et al*., 2002; El-Holi & Al-Delaimy, 2003). Main objectives of the current study

were the utilization of cheese whey and cane molasses as the basic constituents in the fermentation media for economical large–scale citric acid production by protein–enriched *A. niger* for animal feeding.

## MATERIALS AND METHODS

**Fermentation organism.** *Aspergillus niger* isolated from sugar cane and beet bagasse by serial dilution method (Clark *et al.*, 1958) was evaluated for citric acid production. Best isolate was selected for further studies and raised on potato starch agar slants sporulation medium (Irshad, 1999) for the preparation of inoculums. The spores were transferred into 250 mL conical flasks containing 50 mL waste medium; cheese whey and cane or beet molasses only and adjusted to pH 6.0. The flasks were incubated at 30ºC with continuous shaking (200 rpm) for 6 days.

**Fermentation conditions.** Sugar cane molasses was obtained from EL- Howamdia Company, Egypt; beet molasses from EL-Dakahlia sugar factory, Belkas, Egypt. Cheese whey was kindly supplied by Faculty of Agriculture, Zagazig University. Pretreatment of molasses was carried out by three methods; centrifugation, sulfuric acid and calcium phosphate treatment according to the method adopted by Gazaerly (1983). Sterilized silicone oil (10%) was used to control foaming during fermentation. The study implicated the optimization of culture conditions using different levels of cane molasses (3, 5, 7 & 10%) and effect of varying concentration of nitrogen sources (0.2, 0.3, 0.4 & 0.5%) like $NH_4NO_3$, $(NH_4)_2SO_4$ and urea. Moreover, fermentation period and initial pH of fermentation media were optimized in independent experiments. All experiments were performed in triplicate.

**Analytical methods.** Biomass dry weight was determined using the method reported by Haq & Daud (1995). Sugars were estimated colorimetrically by Duboise method (1956). Citric acid was estimated using pyridine-acetic anhydride method as reported by Marrier & Boulet (1958), crude protein (Lowry *et al*., 1951), crude total lipids (Folch *et al*., 1957), moisture (Johnson & Ulrich, 1960) and ash (Brown & Zerban, 1948), while uric acid was extracted and determined according to Tinsley and Nowakoski (1957). Nucleic acids were extracted as described by Shibko *et al*. (1967) and the methods of Ashwell (1957) and Burton (1968) were applied for RNA and DNA determination, respectively. Mycotoxins were extracted and purified according to Parrish *et al*. (1965) and Eppley (1968). Qualitative and quantitative analysis were carried out using the method of Gimeno (1979).

## RESULTS AND DISCUSSION

Cheese whey and cane or/beet molasses were used as the basic fermentation media in this study. The proximate composition of cheese whey was found to be 4.6% lactose, 1.2% crude protein, 0.6% ash, 0.3% fat, 5.8% total soluble solid and 92.7% water, while cane molasses and beet molasses were; 16.9 and 18.8% water content; 46.7 and 49.6% total sugars; 1.87 and 2.33% crude protein; 1.1 and 0.9% total lipids; 10.7 and 8.9% ash content. Table I shows that the tested organism can produce satisfactory amounts of biomasses with satisfactory protein contents and citric acid yield by growing *A. niger* on economical media such as whey and molasses (crude or treated). It was observed that growth medium of whey and acid pretreated cane or/ beet molasses gave the greatest dry cell mass (11.76 & 12.26 g $L^{-1}$) in comparison with crude, centrifuged and calcium phosphate pretreated molasses. Moreover, the highest fungal protein content (388 & 419 mg $g^{-1}$) and citric acid production (32.82 & 35.23 g $L^{-1}$) were obtained using acid pretreated cane or beet molasses rather than the other used pretreatments. Gazaerly (1983) concluded that the removal of the mud from beet molasses reduced the concentration of both Zn and Cu elements by about 25%. Helal (1986) found that when beet molasses was clarified by $H_2SO_4$ and added into the fermentation medium as a sole carbon source, increased the dry weight yield with a little decrease in the nitrogen contents of *A. flavus* and *A. terreus.* Ali *et al.* (2002) selected *A. niger* GCBT7 as the best mould to support maximum production of citric acid using cane molasses without supplements. Also El-Holi & Al-Delaimy (2003) obtained low amount of citric acid (2.43 g $L^{-1}$) from whey alone. Addition of different sugars to whey enhanced citric acid production with a peak value of 106.5 g $L^{-1}$ with 15% sucrose and biomass yield 37.3 g $L^{-1}$ after 16 days. Rehman *et al.* (2002) studied *A. niger* ANABT using molasses medium containing sugar (150 g $L^{-1}$). Maximum amount of anhydrous citric acid obtained was 65.96 g $L^{-1}$ at 30ºC; the sugar consumption was 98 g $L^{-1}$ while mycelial dry weight was 14.55 g $L^{-1}$.

Growth media of waste cheese whey containing varying concentrations of acid pretreated beet molasses (10.0, 12.5, 15.0 & 17.5%) at initial pH 6.0 were fermented for 6 days at 30ºC under rotary shaking condition (200 rpm). *A. niger* showed maximum dry cell mass (14.28 g $L^{-1}$) and protein content (440 mg $g^{-1}$) on fermentation medium supplemented with 10.0% beet molasses while maximum citric acid production (42.17 g $L^{-1}$) was obtained with 15% beet molasses (38.52 g $L^{-1}$) at 17.5% (Table II). Ali *et al.* (2002), Ishaq *et al.* (2002), Rehman *et al.* (2002 & 2003), El-Holi & Al-Delaimy (2003), Demirel *et al.* (2005) and Kursat *et al.* (2005) reported that beet molasses were the best carbon source for *A. niger* mycelial growth and citric acid production.

Nitrogen sources had a profound effect on citric acid production. Growth media of waste whey and beet molasses containing varying concentration of ammonium sulphate, ammonium nitrate and urea at pH 6 were fermented for six days at 30ºC showed that *A. niger* preferred 0.3% $NH_4NO_3$ as a nitrogen source in the optimum growth medium, produced more protein and citric acid than other sources. Cheese whey amended with 15.0% beet molasses and

**Table I. Effect of whey and molasses on sugar consumption, dry cell mass, protein content and citric acid of *Aspergillus niger***

| | Dry cell mass g $L^{-1}$ | Sugar Consum–ption g $L^{-1}$ | Protein mg $g^{-1}$ | Protein % | E C | P C C | Citric acid g $L^{-1}$ | Citric acid % yield |
|---|---|---|---|---|---|---|---|---|
| **Whey + cane molasses** | | | | | | | | |
| Crude | 10.63 | 48.70 | 272 | 25.59 | 21.82 | 5.94 | 24.68 | 50.68 |
| Centrifuged | 10.87 | 46.82 | 313 | 28.79 | 23.22 | 7.27 | 27.75 | 59.27 |
| $H_2SO_4$-treated | 11.76 | 44.16 | 388 | 32.99 | 26.63 | 10.33 | 32.82 | 74.32 |
| Calcium phosphate treated | 8.93 | 40.18 | 165 | 18.47 | 22.22 | 3.67 | 24.61 | 61.25 |
| **Whey+ beet molasses** | | | | | | | | |
| Crude | 11.64 | 51.51 | 329 | 28.26 | 22.59 | 7.43 | 27.14 | 52.69 |
| Centrifuged | 11.36 | 49.76 | 377 | 29.66 | 22.83 | 8.60 | 33.61 | 67.54 |
| $H_2SO_4$-treated | 12.26 | 46.88 | 419 | 34.17 | 26.15 | 10.96 | 35.23 | 75.14 |
| Calcium phosphate treated | 9.48 | 41.64 | 286 | 30.17 | 22.76 | 6.51 | 22.81 | 54.78 |
| **Whey** | 2.66 | | 116 | | | | 4.63 | |
| Mean±SE | 10.87 | 46.21 | 318.63 | 28.51 | 23.53 | 7.59 | 28.58 | 61.96 |
| | ±0.41 | ±1.39 | ±28.44 | ±1.72 | ±0.64 | ±0.84 | ±1.66 | ±3.35 |

*Values are the average of triplicate samples; *12.5 %(v/v) molasses; initial pH 6.0; incubated at 30ºC in a rotary incubator shaker at 200 rpm for 6.0 days; Protein %= Fungal protein / dry cell mass; Economic coefficient (EC) = Fungal cell mass / sugar consumed X100; Protein conversion coefficient (PCC) = Fungal protein / sugar consumed X100; Citric acid % yield= Citric acid (g $L^{-1}$)/ sugar consumed (g $L^{-1}$) X100

**Table II. Comparison of growth yield, protein content and citric acid production of *A. niger* using whey amended with different levels of acid pretreated beet molasses**

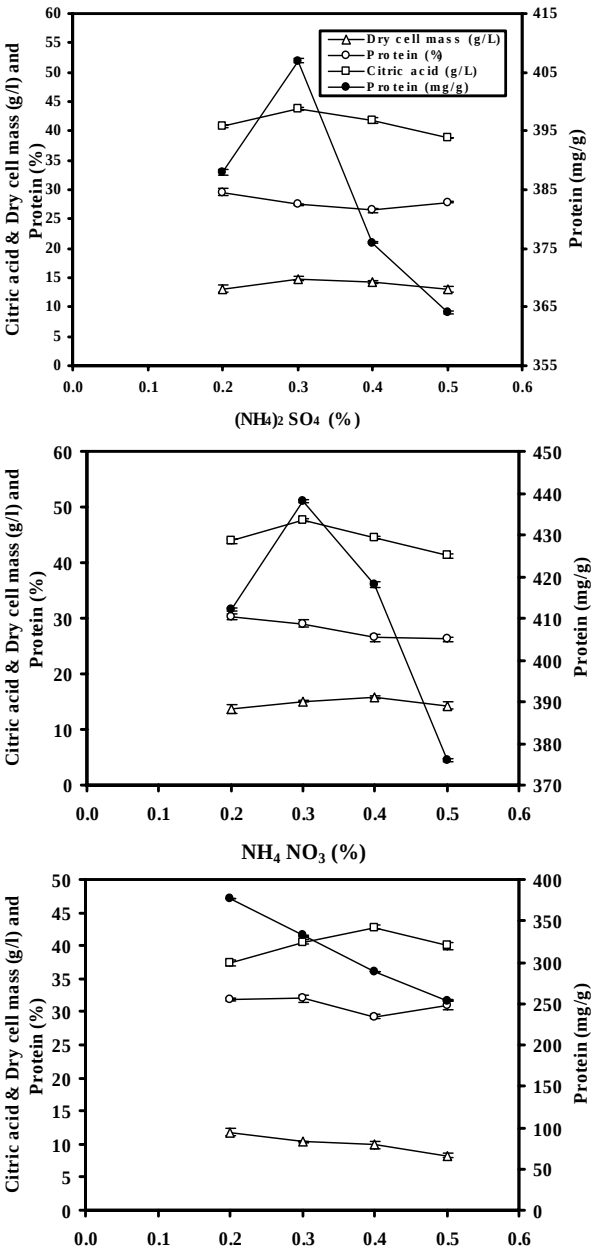
| Beet molasses levels | Dry cell mass g $L^{-1}$ | Sugar Consum–ption G $L^{-1}$ | Protein mg $g^{-1}$ | Protein % | E C | P C C | Citric acid g $L^{-1}$ | Citric acid %yield |
|---|---|---|---|---|---|---|---|---|
| 10.0 % | 14.28 | 37.18 | 440 | 30.81 | 38.41 | 16.89 | 26.64 | 71.65 |
| 12.5 % | 12.26 | 46.88 | 419 | 34.17 | 26.15 | 10.96 | 35.23 | 75.15 |
| 15.0 % | 11.32 | 54.73 | 374 | 33.04 | 20.68 | 7.73 | 42.17 | 77.05 |
| 17.5 % | 9.58 | 58.48 | 283 | 29.54 | 16.96 | 4.80 | 38.52 | 65.87 |
| Mean±SE | 11.86 | 49.32 | 379.00 | 31.89 | 25.55 | 10.09 | 35.64 | 72.43 |
| | ±0.98 | ±4.71 | ±34.84 | ±1.05 | ±4.68 | ±2.59 | ±3.32 | ±2.46 |

*Values are the average of triplicate samples.

fortified with 0.4 $NH_4NO_3$ was the best medium for mycelia dry weight production (Fig. 1). Maximum citric acid (47.63 g $L^{-1}$) was produced in the medium containing 0.3% ammonium nitrate followed by 0.3% ammonium sulphate and 0.4% urea. The difference of substrates used, organism and optimum conditions might be the source of variation. Abou-zeid & Muhammad (1984) observed that urea and ammonium salts could be used as source of nitrogen for citric acid production. Better nitrogen source among others for citric acid production are $NH_4NO_3$ (Garg *et al*., 1991) and urea (Lodhi *et al*., 2001).

The maintenance of a favorable pH is very essential for the greater mycelial production, protein content and citric acid by fungus. Effect of different initial pH (4.5 - 7.0) of the fermentation media was studied (Fig. 2) and maximum dry weight (15.64 g $L^{-1}$), protein content (458 mg $g^{-1}$) and citric acid (47.63 g $L^{-1}$) were obtained when the initial pH of the fermentation medium was adjusted to 5.5. Decrease in pH caused reduction in both protein and citric acid yield. It is likely that at low pH, the ferrocyanide ions were more toxic for the growth of mycelium and consequentially citric acid production. Ali *et al.* (2002) reported that a low pH in cane molasses medium is

**Fig. 1. Comparison of growth yield, protein content and citric acid production of *A. niger* using whey and beet molasses (15.0%, v/v) amended with different levels of different nitrogen sources**




inhibitory for the growth and citric acid productivity of *A. niger* GCBT7 and GCBT2, while higher initial pH leads to the accumulation of oxalic acid (Pessoa *et al*., 1982; Lodhi *et al*., 2001; Ali *et al.*, 2002; El-Holi & Al-Delaimy, 2003).

The incubation time requirement for maximal protein and citric acid production depends on the organism and fermentation conditions. Dry mycelial weight, protein content and citric acid biosynthesis were studied (Table III), where mycelial dry weight, EC and PCC were achieved (17.56, 36.72 & 14.85 g $L^{-1}$, respectively) after 4 days of

**Fig. 2. Relative tolerance of initial pH of the fermentation medium for citric acid production by A. niger enriched protein**

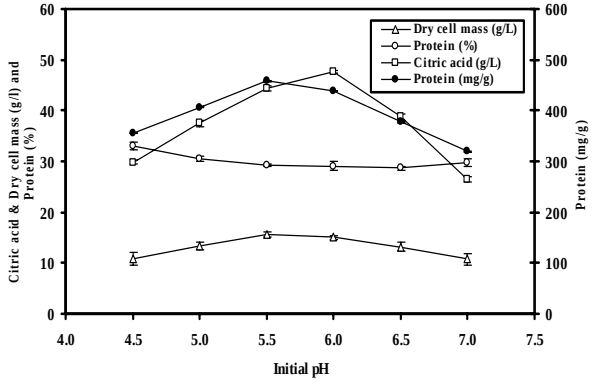



**Table III. Time course study during citric acid fermentation by *A. niger* enriched protein**

| Fermentation period(days) | Dry cell mass g $L^{-1}$ | Sugar consum–ption g $L^{-1}$ | Protein mg $g^{-1}$ | % | E C | PCC | Citric acid g $L^{-1}$ | %yield |
|---|---|---|---|---|---|---|---|---|
| 3 | 14.73 | 42.67 | 284 | 19.28 | 34.52 | 9.80 | 17.86 | 41.85 |
| 4 | 17.56 | 47.82 | 397 | 22.61 | 36.72 | 14.58 | 28.73 | 60.08 |
| 5 | 16.38 | 55.74 | 473 | 28.87 | 29.38 | 13.90 | 40.52 | 71.41 |
| 6 | 15.08 | 61.43 | 438 | 29.04 | 25.37 | 10.75 | 47.63 | 77.53 |
| 7 | 13.62 | 65.07 | 366 | 26.87 | 21.59 | 7.66 | 41.43 | 63.67 |
| Mean±SE | 15.47 | 54.55 | 391.60 | 25.33 | 29.52 | 11.34 | 35.23 | 62.91 |
| | ±0.68 | ±4.46 | ±32.44 | ±1.91 | ±2.80 | ±1.29 | ±5.31 | ±9.07 |

* Values are the average of triplicate samples.

fermentation incubation, while maximum production of citric acid (47.63 g $L^{-1}$) with % yield (77.53%) achieved after 6 days of incubation. Protein contents (47.63 g $L^{-1}$ & 77.53%) were also achieved after 6 days of incubation. Further increase in incubation period did not enhance protein and citric acid production. It might be due to decrease available nitrogen in fermentation medium, the age of fungus and depletion of sugar content (Wieczorek & Brauer, 1998; Lodhi *et al.*, 2001; Ali *et al*., 2002; El-Holi & Al–Delaimy, 2003; Demirel *et al*., 2005; Kursat *et al*., 2005). Ishaq *et al*. (2002) reported that the dry weight of mutant strain of *A. niger* GCB- 47, using molasses based medium was 17.39 g $L^{-1}$ and maximum production of citric acid 70.60 g $L^{-1}$ was obtained after 144 h incubation. Further increase in the incubation period resulted in the decreased production of citric acid. Thus it was concluded that 6 day incubation period, 30ºC incubation temperature and pH 6 as the initial pH of fermentation media are the most suitable conditions for fungal productivity and subsequently citric acid production with satisfactory amounts of fungal biomasses and protein contents.

Results of the previous experiments paved the way to determine the chemical constitution of *A. niger* produced after cultivation under the optimum conditions previously elucidated. The data revealed that dry *A. niger* cells contain about 16.11% water content (Table IV). The chemical analysis showed that the percentage of protein, total carbohydrates, crude lipids, nucleic acids, uric acid and ash contributed; 29.04, 45.18, 6.37, 4.46/0.83, 0.43 and 14.06%, respectively. Also, toxigenic activity of *A. niger* using TLC for qualitative analysis of aflatoxins B1, B2, G1 and G2 were carried out, which produced no mycotoxin under the condition used in this study. Interestingly, easier toxicity tests with *A. niger* strains used for food and feed have been extensively carried out and were found safe for this purpose (El-Aasar, 1985; Helal, 1986; Abd-Alla *et al*., 1997). Murad (1994) found that biomass yield was 11.0 g $L^{-1}$; 3.34% nucleic acid and 40.36% crude protein with 16 amino acids characterized by high levels of tyrosine and threonine in *Kluyveromyces lactis*, which were comparable with or higher than FAO reference protein.

Amino acid profile of the fungal protein of *A. niger* was determined, which was comparable with the FAO reference. Amino acids profile of *A. niger* protein revealed the presence of 17 amino acids; 9 of which are essential and the most abundant of these was aspartic acid (15.91 g 100 g $^{-1}$ protein) followed by iso-leucine, cysteine, proline, arginine, cystine, glycine, valine, threonine, methionine, tyrosine, lysine, leucine, tryptophane, phenylalanine, glutamic and alanine (Table V). It is worth to mention that *A. niger* protein contains most essential amino acids in sufficient amounts. Moreover, sulphur containing amino

**Table IV. Chemical composition of *A. niger* grown under optimal culture conditions (on dry weight bases)**

| Chemical Constituents | |
|---|---|
| Water content | 16.11% |
| Ash content | 14.06 |
| Crude protein | 29.04 |
| Carbohydrates | 45.18 |
| Soluble | 10.32 |
| Insoluble | 34.86 |
| Crude lipids | 6.37 |
| Nucleic acids (RNA/DNA) | 4.46/0.83 |
| Uric acid | 0.43 |
| Mycotoxins | |
| B1 | -ve |
| B2 | -ve |
| G1 | -ve |
| G2 | -ve |

* Values are the average of duplicate samples.

**Table V. Amino acids in the fungal proteins of *A. niger* compared to the FAO guide line values**

| Amino acids | g. amino acids / 100g protein | |
|---|---|---|
| | FAO guide line values | Aspergillus niger |
| DL-Alanine | - | 1.06 |
| L-Arginine monohydrochloride | - | 6.83 |
| DL-Aspartic | - | 15.91 |
| L-Cysteine hydrochloride | - | 8.36 |
| L-Cystine | 2.0 | 2.47 |
| L-Glutamic | - | 2.12 |
| GLycine | - | 6.36 |
| L-Leucine | 4.8 | 4.84 |
| DL-Iso-Leucine | 4.2 | 8.75 |
| L-Lysine monohydrochloride | 4.2 | 5.39 |
| DL-Methionine | 2.2 | 5.88 |
| DL-B-Phenylalanine | 2.8 | 2.27 |
| L-Proline | - | 7.08 |
| DL-Threonine | 2.8 | 5.93 |
| DL-Tryptophan | 1.4 | 2.72 |
| L-Tyrosine | 2.8 | 5.63 |
| Valine | 4.2 | 6.24 |

acids cysteine and methionine (5.36 - 5.88 g 100 g $L^{-1}$protein) were present in sufficient amounts. These results are in line with results obtained by Dokhan *et al.* (2005), who found that amino acid profile of *Rhizopus oryzae* NRRL 395 protein revealed presence of 17 amino acids, 8 of them were essential, with relative deficiency of sulphur amino acids. Moreover, Asgher *et al*. (1998) deduced that amino acid profile of *Arachniotus* sp contained reasonable amounts of essential amino acids and it was limiting only in lysine and methionine. Also, Banerjee *et al.* (1996) reported that amino acids content in mycelial biomass of *Tricholoma gigantium* produced in cheese whey medium was higher than that produced in optimal synthetic medium. The sulfur amino acids were not present in appreciable amounts. Paul *et al.* (1996) concluded that low temperature drying minimizes the loss of nutritionally available lysine of *Kluyveromyces fragilis* yeast grown in whey. In conclusion, using industrial wastes of cheese whey fortified with beet molasses enhanced and consistent economical large–scale yield of citric acid by protein-enriched *A. niger.* Chemical constitution of mycelial biomass proved to be protein-enriched and safe as SCP for animal feeding.

## REFERENCES


Abd-Alla, E.A., A.F. Shabab and S.E. Ali, 1997. Formation of mycotoxins by toxigenic fungi isolated from green coffee bean in Egypt. *Egypt J. Microbiol*., 32: 481–91

Abou-Zeid, A.Z. and A.A. Muhammad, 1984. Production of citric acid from yeast. *Agric. Waste*, 9: 51–7

Ali, S., I. Haq, M. Qadeer and J. Iqbal, 2002. Production of citric acid by *Aspergillus niger* using cane molasses in a stirred fermentor. *Electron. J. Biotechnol*., 3: 258–71

Arzumanov, T.E., N.V. Sishanova and T.V. Finogenora, 2000. Biosynthesis of citric aid by *Yarrowia lipolytica* repeat-batch culture on ethanol. *Appl. Microbiol. Biotechnol*., 53: 525–9

Asgher, M., M. Yaqub and A. Barque, 1998. Single cell protein production from delignified corn stover and its quality evaluation. *Pakistan J. Agric. Sci.*, 35: 7–10

Ashwell, G., 1957. *Methods in Enzymology III*. Interscince Publishers, Inc. NY

Banerjee, M., N. Samajpati, M. Banerjee and S. Maitra, 1996. Protein and amino acids contents in mycelial biomass of *Tricholoma lobayense*, *T. gigantium* and *T. crassum. J. Mycopathological Res*., 34: 173–7

Brown, C.A. and F.W. Zebran, 1948. *Physical and Chemical Methods of Sugar Analysis*, 3rd ed printing, P: 1020

Burton, K., 1968. *Methods in Enzymology*, 12B, Interscience Publishers, Inc. NY

Clark, H.E., G.E. Bordner, P.W. Kabler and C.B. Huff, 1958. *Applied Microbiology*, Pp: 27–53. International Book Company, New York, USA

Demirel, G., K.O. Yaykasli and A. Yasar, 2005. Production of citric acid by using immobilized *Aspergillus niger* A-9 and investigation of its various effects. *Food Chem.*, 89: 393–6

Dokhan, A.M., S.A. EL-Sayed and G.A. Amin, 2005. Direct fermentation of some starchy grain mill wastes to L (+) lactic acid and protein enriched fermented animal feed by *Rhizopus oryzae* NRRL 395. *N. Egypt J. Microbiol*., 10: 255–65

Duboise, K., 1956. Sugar determination by phenol sulphuric acid method. *Biotecnology and Bioengineering*, 10: 721–4

EL-Aasar, A.A., 1985. Single-cell protein from fungi. *M.Sc Thesis*, Botany Department, Faculty of Science, Zagazig University, Egypt

EL-Holi, M.A. and K.S. AL-Delaimy, 2003. Citric acid production from whey with sugars and additives by *Aspergillus niger. African J. Biotechnol.*, 2: 356–9

Eppley, R.M., 1968. Screening method for zearalenone, aflatoxin and ochratoxin A. *J. AOAC*, 51: 74–8

EL-Samragy, Y.A., M.A. Khorshid, M.I. Foda and A.E. Shihata, 1996. Effect of fermentation conditions on the production of citric acid from cheese whey by *Aspergillus niger. Int. J. Food Microbiol*., 29: 411–6

Folch, J., M. Less and G. Stanley, 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*., 222: 497

Garg, k., C. Sharam and G. kush, 1991. Repeated batch production of citric acid from sugar cane molasses using recycled solid state surface culture of *Aspergillus niger*. *Biotech. Lett.*, 13: 913–6

Gazaerly, M.A., 1983. The utilization of beet molasses for single cell protein production. *M.Sc Thesis*, Botany Department, Faculty of Science, Alexandria University, Egypt

Gimeno, A., 1979. Thin layer chromatographic determination of aflatoxins, sterigmatocystin, zearalenone, citrinin, T.2 toxin, penicillic acid, patulin and penitrem. *A. J. AOAC*, 62: 579–85

Haq, P.B. and D.A. Daud, 1995. Process of mycelial dry weight calculation for citric acid. *J. Biotechnol*., 9: 31–5

Haq, I., S. Ali, M. Qadeer and J. Iqbal, 2002. Citric acid fermentation by mutant strain of *Aspergillus niger* using molasses based medium. *J. Biotechnol.*, 5: 125-32

Helal, G.A., 1986. Protein and fats from halophilic fungi. *Ph. D. Thesis*. Department of Botany, Faculty of Science, Zagazig University, Egypt

Hossain, M., J. Brooks and I. Moddax, 1984. The effect of the sugar source on citric acid production by *Aspergillus niger. Appl. Microbiol, Biotechnol*., 19: 393–7

Irshad, M., 1999. Production of crude amylase from waste bread by *Aspergillus niger*. *M.Sc. Thesis* Department of Chemistry (Biochemistry). University of Agriculture Faisalabad, Pakistan

Ishaq, A., A. Sikander, I. Haq and M. Qadeer, 2002. Time course profile of citric acid fermentation by *Aspergillus niger* and its kinetic relations. *Online J. Biological Sci.*, 2: 760–76

Johnson, C.M. and A. Ulrich, 1960. California Agriculture Exp. Sta., Bull. 766. In: Wilde, S.A., G.K. Voigt and G.F. Iyer, (eds.) *C.F. Soil and plant Analysis for Tree Culture*, Pp: 112-3. Edited by Chesters, G.; Applied Science Publishers LTD. England

Kubicek, C.P. and M. Rohr, 1986. Citric acid fermentation, CRC Crit. *Rev. Biotech*., 3: 331–73

Kursat, O.Y., D. Gokhan and Y. Ahmet, 2005. Influence of alcohols on citric acid production by *Aspergillus niger*A-9 entrapped in polyacrylamide gels. *J. Food Engineering*, 70: 518–22

Lodhi, A.k., M. Asghar, M. Anjum, S. Ambreen and M. Asad, 2001. Production of citric acid from waste bread by *Aspergillus niger*. *Online J. Biological Sci.*, 1: 182–3

Lowry, O.H., N.J. Resebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem*., 193: 265–83

Murad, A., S. Abd-El-Ghani and K. El-Shenawy, 1994. Bioconversions of whey permeate into *Kluyveromyces lactis* biomass. *Egyptian J. Dairy Sci.*, 20: 261–71

Marrier, J.R. and M. Boulet, 1958. Direct determination of citric acid in milk with an improved pyridine, acetic anhydride method. *J. Dairy Sci*., 41: 1683

Meleigy, S.M. and Z.A. Matter, 2004. Citric acid productions from milk permeate supplemented with molasses and some mineral ions by *Yarrowia lipolytica* NRRL Y- 1095. *N. Egypt J. Microbiol*., 9: 77–93

Parrish, F.W., B.J. Wiley, E.G. Simmonas and L. Long, 1965. *A Survey of Some Species of Aspergillus and Penicillium for Production of Aflatoxins and Kojic Acid*. U.S. Army material commond, U.S. Army Natick Laboratories Natick. Massachusetts 01762

Paul, D., B. Chatterjee and A. Guha, 1996. Available lysine in protein in *kluyveromyces fragilis* yeast grown in whey. *Indian J. Dairy Sci.*, 49: 78–80

Pessoa, D.F., C. Diasde and C. Angela, 1982. Production of citric acid by *Aspergillus niger*. *Rev. Microbiol.*, 13: 225–9

Reed, G., 1982. *Prescott and Dunn`s Industrial Microbiology*, 4th ed. Pp: 709–39. AVI Publishers Co. USA

Rehman, A., S. Ali and I. Haq, 2002. Temperature optima for citric acid accumulation by *Aspergillus niger*. *Biotechnology*, 1: 108–10

Rehman, A., S. Ali and I. Haq, 2003. Selection of fermentation for citric acid in bioreactor. *Biotechnology*, 2: 178–84

Shibko, S., P. Koivistoven, C.A. Tratnyek, A.R. Newhall and L. Friedman, 1967. A method for sequential quantitative separation and determination of liver homogenate or from subcellular fraction. *Anal. Biochem*., 19: 514–28

Tinsley, J. and T.Z. Nowakoski, 1957. The determination of uric acid, particularly in Avian Excreta. *Analyst* (London), 82: 110–6

Wieczorek, S. and H. Brauer, 1998. Continuous production of citric acid with recirculation of the fermentation broth after product recovery. *Bioprocess Engineering*, 18: 1–6


**(Received 18 February 2006; Accepted 15 May 2006)**