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Gelatin Net Hydrolyzate: A Novel Enhancer for Citric Acid Production by *Aspergillus niger*

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Abstract: This study aimed at enhancing citric acid production on pilot scale fermentors of 3600 l capacity with 2500 l working volume by *Aspergillus niger* UMIP 2563.04 using Gelatin Net Hydrolyzate (GNH), a by-product of soft gelatin capsules manufacturing. The results show that the addition of GNH to the fermentation medium has shortened the fermentation period from 212 to 152 h. The *A. niger* strain produced 175 ± 1 g L⁻¹ citric acid when grown on a medium containing beet molasses (200 g L⁻¹ total sugar) and GNH while it produced 70.65 ± 1 g L⁻¹ citric acid when grown on the same medium deprived from gelatin net hydrolyzate. On the basis of kinetic parameters, the efficiency of substrate uptake rate with respect to Q_s and q_s revealed that the strain grew significantly ($p < 0.05$) faster in the presence of GNH in the fermentation medium. Subsequent to the addition of GNH, the strain was significantly ($p < 0.05$) improved for the values of product yield coefficients ($Y_{p/s}$), ($Y_{p/x}$), citric acid productivity (Q_p) and citric acid specific productivity (q_p).

Key words: *Aspergillus niger*, pilot, fermentation, beet molasses, gelatin net hydrolyzate

Introduction

Citric acid is one of the few bulk chemicals produced by fermentation and is the most exploited biochemical product. Citric acid has a broad use in the household, in the preparation of numerous industrial products and in many industrial areas such as food, pharmaceutical and chemical industries and as a cleaning agent. The supply of natural citric acid is limited and the demand can only be satisfied by biotechnological fermentation processes. Citric acid has been produced using various fungi since 1917 and by yeasts since 1960s. Nowadays, *A. niger* is almost exclusively used for industrial scale production of citric acid. More than 600,000 metric tons are produced annually worldwide (Anastassiadis *et al.*, 2002).

Fermentation economics are driven by the profitability of a marketed product. A key component of this value is based on manufacturing cost per unit of product and the ability of a manufacturing process to meet additional demands without adding more production-scale fermentors (Parekh, 1999).

Soft gelatin capsules have been available since the nineteenth century since then they have been used to dispense a variety of liquids and solids with multitude of advantages over the conventional dosage forms (Stanley, 1986; Shah *et al.*, 1992). Gelatin net is an inexpensive and available by-product of pharmaceutical industries resulted from the production of soft gelatin capsules. Gelatin is a high molecular weight polypeptide derived from collagen. The most important sources of collagen for gelatin production are cattle horn, bovine hide, bones and pig skin (Nemati *et al.*, 2004).

The gelatin net has not been used so far for any purpose other than a cattle feed. Preliminary analysis of the gelatin net showed that it contains approximately 86% protein, 4% mineral salts and 10% water content. The amino acids profile of gelatin net comprises Alanine, Arginine, Aspartic Acid, Glutamic Acid, Glycine, Histidine, Proline, Hydroxyproline, Hydroxylysine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Serine, Threonine, Tryptophan, Tyrosine, Valine and minerals may stimulate citric acid yield. Hence the gelatin net was explored for its improvement of citric acid production by *A. niger* UMIP 2563.04.

In the present investigation, we identify growth and citric acid production kinetic parameters in beet molasses medium in the presence and absence of gelatin net hydrolyzate. In addition, we report for the first time the utilization of gelatin net hydrolyzate as an enhancer for citric acid production on pilot scale fermentation processes.

Materials and Methods

Microorganism

The organism used throughout this study is a citric acid producer *Aspergillus niger* strain. The strain was isolated from Egyptian soil and deposited in Pasteur Institute (Fungi Culture Collection) Paris, France under the number UMIP 2563.04. The strain showed a yield of citric acid of approximately 80±2 and 35±4% when grown on sucrose and beet molasses containing medium, respectively. The strain was selected due to its partial inability to utilize molasses as a sole carbon source in an attempt to raise its potent to produce citric acid through GNH enhancement.

Media

All media were sterilized at 121 °C for 15 min and the pH was adjusted as required. The following culture media (g L⁻¹) were employed throughout the work:

Sabouraud Dextrose Agar (SDA) for Cultivation (pH 5.6±0.2)

Mycological peptone, 10.0; glucose, 40.0; agar, 15.0.

Fermentation Medium for Citric Acid Production (pH 4±0.2)

Twenty five gram per liter calcium phosphate-pretreated sugar beet molasses, 350.0 and liquid ammonia (NH₄OH) was added during the fermentation run to keep its concentration not less than 250 mg L⁻¹.

Batch Culture Fermentor Studies

Inoculum level of 0.001% (W V⁻¹) of the *A. niger* spores were transferred to double jacket air lift pilot fermentors (Belach Bioteknik) of 3600 l capacity with 2500 l working volume. The fermentors are made entirely of stainless steel AISI 316 provided with pH and DO₂ probes to monitor both pH and dissolved oxygen during fermentation. The incubation temperature and aeration were carried out at 30 °C and 15500 mL min⁻¹, respectively. Kontramine® was automatically used to control foaming during the fermentation.

Determination of Citric Acid and Sugar Concentrations by HPLC

Citric acid, glucose and fructose were determined by Gilson HPLC instrument equipped with a Refractive Index Detector (RID), Aminex HPX-87H column (7.8×300 mm, Bio-Rad, USA) for citric acid analysis and Aminex HPX-87P column (7.8×300 mm, Bio-Rad, USA) for glucose and fructose analysis. The eluent used for analysis was 0.01N sulfuric acid solution. HPLC analyses were carried out under the following operation conditions: Pump flow, 0.6 mL min⁻¹; column temperature, 40 °C; sample amount, 20 µL and integration method, peak area. Concentrations were automatically calculated by Gilson Unipoint software. Sugar concentration was calculated as the sum of glucose and fructose concentrations.

Biomass Determination

Biomass concentration was determined according to Kirimura *et al.* (1988). Ten milliliter of the fermentation mash was filtered through a pre-dried (at 105 °C) and pre-weighed (A) filter paper. The filter cake was washed with 20 mL distilled water, put in a Petri-dish and dried for 48 h at 105 °C. The filter cake was allowed to cool in a desiccator and weighed (B).

$$\text{Mycelial dry weight (biomass) in g L}^{-1} = (B - A) \times 100$$

Estimation of Proteins

The amino acids present in the proteins develop a blue color with Folin phenol reagent and alkaline cupric tartarate, which is measured at 620 nm to estimate intracellular and extracellular proteins (Lowry *et al.*, 1951). To estimate intracellular proteins an aliquot of the filtrate (production medium) is used and for extracellular proteins microbial tissue extract (obtained by using a mortar followed by extraction with ice cold water) is used.

Estimation of Lipids

The residue obtained from the biomass on extraction with ice cold $\text{CHCl}_3/\text{CH}_3\text{OH}$ mixture is dissolved in H_2SO_4 and treated with vanillin reagent (0.2 g in 100 mL H_3PO_4) to get a red color which is measured at 510 nm (Lees and Sloane-Stanley, 1957).

Hydrolysis of Gelatin Net

Gelatin net was obtained from Amriya Pharmaceutical Industries Company, Alexandria, Egypt, chopped into small particles and milled in a cutter mill for 30 sec and sieved to a particle size less than 0.1 mm. Physically pretreated gelatin net was further treated with HCl (35% W V⁻¹) or H_2SO_4 (35% W V⁻¹) or NaOH (35% W V⁻¹) in a ratio 10:1 for 1 h at 120°C, adjust pH until neutrality.

Results and Discussion

The Effect of Different Gelatin Net Treatment on Citric Acid Production by *A. niger*

The gelatin net was treated physically and chemically as mentioned in the materials and methods section. The strain was allowed to grow in pilot fermentors, each containing 2500 l of fermentation medium and 10% (W V⁻¹) GNH previously treated as mentioned before. The fermentation cycles were performed at pH 4, 30°C and aeration rate of 15500 mL min⁻¹. The samples were harvested at various times and analyzed for total sugar consumption (sum of glucose and fructose concentrations), fungal biomass and citric acid formation. All cultures were continued until complete sugar consumption. The citric acid and biomass profiles using *A. niger* with physically pretreated gelatin net (PP), chemically hydrolyzed gelatin net by HCl (CPH), H_2SO_4 (CPS) and NaOH (CPSO) are shown in Fig. 1. The *A. niger* grown on HCl pretreated gelatin net (CPH) gave the maximum citric acid yield, whereas the higher biomass production was obtained in the culture deprived from GNH.

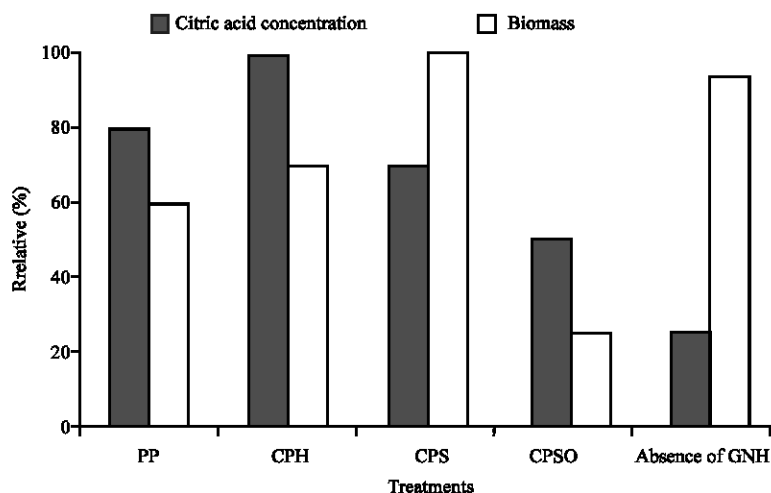


Fig. 1: The effect of different treatments of GNH on citric acid production by *A. niger*

Table 1: Results of lipid, protein and carbohydrate metabolism in *A. niger* UMIP 2563.04 strain with beet molasses as substrate in presence (P) and absence (A) of GNH

Incubation period (days)	Citric acid (g L ⁻¹)		Lipids (g 100 g ⁻¹)		Proteins (g 100 g ⁻¹)		Residual sugar (g L ⁻¹)		Biomass (g L ⁻¹)	
	P	A	P	A	P	A	P	A	P	A
7	175.00	49.30	15.10	14.60	12.10	9.90	0.00	31.60	24.00	28.90
6	155.00	34.50	27.10	13.00	15.30	8.10	25.50	59.40	23.00	21.70
5	125.00	26.90	16.90	11.20	14.20	6.80	60.00	84.50	19.60	18.80
4	98.70	18.50	12.40	7.80	11.70	4.20	88.00	108.00	15.30	15.70
3	67.50	10.30	10.10	5.30	8.90	4.00	125.00	147.00	8.20	11.90

The results of the lipid, protein and carbohydrate metabolism are given in Table 1. It was noticed that the metabolism was totally based on the sugar concentration of the substrate. The microbial tissues of the *A. niger* strain grown on beet molasses dispossess GNH and beet molasses containing GNH pretreated with hydrochloric acid produced 14.6 and 27.1 g 100 g⁻¹ of lipids and 9.9 and 15.3 g 100 g⁻¹ of proteins, respectively. It is obvious that the amount of lipids and proteins produced by the tissue in presence of GNH is higher than that produced in its absence. The fortification of fermentation media with GNH transform lipases and proteases present in *A. niger* to be active that their participation in the metabolism is more than the other culture under privileged of GNH. Consequently, the amount of acetyl coenzyme A (the precursor of the tricarboxylic acid cycle, TCA) supplied for the TCA cycle is also more. Accordingly, the amount of citric acid produced in presence of GNH was found to be more.

Time Course Study of Citric Acid Fermentation by A. niger UMIP 2563.04 Grown on Beet Molasses Fortified with and Deprived From GNH

The strain was allowed to grow in pilot fermentors of 3600 l capacity with 2500 l working volume. The fermentation medium was supplemented with 10% (W V⁻¹) GNH treated with 35% (W V⁻¹) hydrochloric acid (culture A). The basal fermentation medium containing beet molasses was examined as a control (culture B). The fermentation conditions were adjusted as mentioned before. The samples were harvested at various times and analyzed for total sugar consumption, biomass and citric acid concentration. All cultures were continued until complete sugar consumption.

The germination of *A. niger* conidia grown on beet molasses medium containing GNH was observed 9 h after inoculation. Swelling of the young hyphal agglomerates could be detected microscopically during the first 32 h of cultivation. This was the period of initial mycelial growth. After 40 h of cultivation, the microorganism was present in the form of spherical mycelial pellets that had developed from the swollen hyphal agglomerates, characterized by short, forked, bulbous hyphae. During the subsequent development only the diameter of the pellets slightly increased. There was no significant difference between the shape and size of culture (A) and (B) pellets. As shown in Fig. 2, the strain started to produce citric acid within 32 h while it started 16 h later when deprived from GNH. At the end of culture (A) fermentation run (152 h), the citric acid concentration was 175±1 g L⁻¹ with 100% sugar consumption. This amount of acid represents approximately 2.5-fold increase when compared to the amount of citric acid produced by culture (B) dispossessed from GNH at the end of its fermentation run (212 h). During the first period of fermentation, 40 h after inoculation, the development from spores to hyphae was characterized by low biomass content up to 4.1 g L⁻¹ whereas culture (B) showed 7.0 g L⁻¹ within the same period of fermentation. During the second fermentation period, the change of *A. niger* morphology to spherical pellets and the increase in biomass to 24±0.2 and 32.2 ± 0.2 g L⁻¹ in case of culture (A) and (B), respectively. The end mycelial dry weight of culture (A) represented approximately 75% of the biomass harvested from

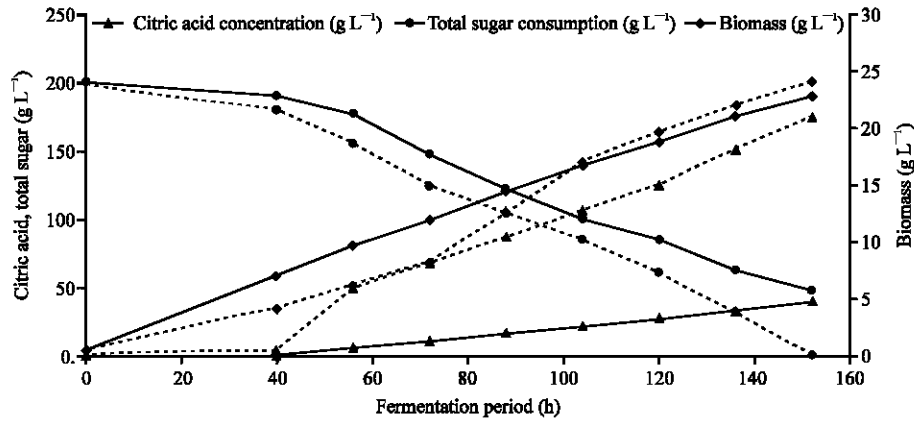


Fig. 2: Time course of citric acid production, sugar consumption and biomass formation by *A. niger* UMIP 2563.04 strain grown on beet molasses fortified with (..) and deprived from (___) GNH in pilot fermentors

culture (B). These results suggest that a high efficiency of substrate uptake by the strain when grown on GNH, which indicates a clear metabolic shift towards acid production. Cultures (A) and (B) consumed 200 g L^{-1} sugars and synthesized 175 ± 1.0 and $70.65 \pm 1.0 \text{ g L}^{-1}$ citric acid, respectively. These cultures also synthesized $24 \pm 0.2 \text{ g L}^{-1}$ and 32.2 ± 0.2 dry cell biomass.

Compared to these obtained results, maximum values for citric acid concentration (175 g L^{-1}) was several fold improved over those from other *A. niger* cultures or mutants used in researches or industrial fermentation processes (Snedecor and Cochran, 1980; Qazi *et al.*, 1990; Roukas and Alichanidis, 1991; Bennet and Klich, 1992; Kirimura *et al.*, 1992; Parvez *et al.*, 1998; Ikram-Ul *et al.*, 2003, 2004). Moreover, investigators normally use a sugar concentration range from 100 to 150 g L^{-1} in fermentation medium for citric acid production in order to avoid catabolic repression (Qazi *et al.*, 1990; Roukas and Alichanidis, 1991; Sakurai *et al.*, 1996; Parvez *et al.*, 1998; Ikram-Ul *et al.*, 2003, 2004). To the best of our knowledge no single report was obtained using a relatively high sugar concentration of 200 g L^{-1} in fermentation medium. Herein, *A. niger* UMIP 2563.04 was capable of tolerate relatively high concentration of sugar when the fermentation medium was supplemented with 10% GNH. The duration of fermentation period was also shorter than other *A. niger* strains used in citric acid production nevertheless their relatively lower initial sugar concentration in fermentation media (Snedecor and Cochran, 1980; Qazi *et al.*, 1990; Roukas and Alichanidis, 1991; Bennet and Klich, 1992; Kirimura *et al.*, 1992; Parvez *et al.*, 1998; Ikram-Ul *et al.*, 2003, 2004). Short fermentation runs (152 h) and withstanding of high sugar concentration (200 g L^{-1}) by *A. niger* UMIP 2563.04 in the presence of GNH provide a potent and economical process for citric acid production from beet molasses.

Kinetic Parameters for the Production of Citric Acid Following Growth of A. niger UMIP 2563.04 on Beet Molasses Containing 200 g L^{-1} Sugars in Presence and Absence of GNH

On the basis of kinetic parameters as shown in Table 2, the efficiency of substrate uptake rate with respect to Q_s and q_s revealed that culture (A) grew significantly ($p < 0.05$) faster than culture (B). Culture (A) was significantly ($p < 0.05$) improved for the values of product yield coefficients ($Y_{p/s}$) and ($Y_{p/x}$), citric acid productivity (Q_p) and specific productivity (q_p) over culture (B). The biomass formation rate (Q_x) of both cultures was only marginally different. Maximum growth in terms of specific growth rate of culture (A) was 0.33 h^{-1} greater than culture (B) during growth of both cultures. The *A. niger* strain when grown on beet molasses fortified with GNH exhibited enhanced production of citric acid over culture (B).

Table 2: Kinetic parameters for the production of citric acid following growth of *A. niger* UMP 2563.04 on beet molasses containing 200 g L⁻¹ sugars in presence and absence of GNH

Kinetic parameter	Symbol	Absence of GNH	Presence of GNH	Unit of parameter
Specific growth rate	μ	1.3±0.1	1.63±0.2	h ⁻¹
<i>Substrate consumption parameters</i>				
Substrate uptake rate	Q_s	0.94±0.07	1.32±0.09	g substrate L h ⁻¹
Specific Substrate uptake rate	q_s	6.2±0.3	8.3±0.3	g substrate g cells ⁻¹
Biomass formation rate	Q_x	0.15±0.03	0.16±0.03	g cells l h ⁻¹
<i>Citric acid formation parameters</i>				
Productivity	Q_p	0.33±0.05	1.15±0.05	g product l h ⁻¹
Specific productivity	q_p	0.01±0.004	0.05±0.002	g product g cells h ⁻¹
<i>Product and growth yield coefficient</i>				
Product yield coefficient	$Y_{p/s}$	0.35±0.07	0.88±0.06	g product g substrate ⁻¹
Product yield coefficient	$Y_{p/x}$	2.2±0.1	7.3±0.1	g product g cells ⁻¹
Growth yield coefficient	$Y_{x/s}$	0.16±0.04	0.12±0.03	g cells g substrate ⁻¹

± denotes standard deviation, among the replicates

Compared to our results, maximum values for Q_s , q_s , Q_p , $Y_{p/s}$ and $Y_{p/x}$ variables were several fold enhanced over those from other *A. niger* cultures or mutants used in citric acid production (Snedecor and Cochran, 1980; Qazi *et al.*, 1990; Roukas and Alichanidis, 1991; Bennet and Klich, 1992; Kirimura *et al.*, 1992; Parvez *et al.*, 1998; Ikram-Ul *et al.*, 2003, 2004). The work in this study has given a novel enhancer for production of citric acid by *A. niger* from beet molasses. More work is, however needed to investigate the reason for GNH induction of increased citric acid productivity on molecular genetic basis.

Conclusion

Citric acid is a metabolite of energy metabolism, the concentration of which will only rise to appreciable amounts under conditions of metabolic imbalances (Hutter, 1983). In our study, this objective was achieved by adding gelatin net hydrolyzate to the fermentation medium. Among the various pretreatment methods applied on GNH, hydrochloric acid treatment was found to give maximum citric acid yield. *A. niger* strain grown on beet molasses supplemented with GNH was significantly ($p \leq 0.05$) improved for the values of product yield coefficients ($Y_{p/s}$) and ($Y_{p/x}$), citric acid productivity (Q_p) and specific productivity (q_p) over the same strain when grown on beet molasses solely. Work in this study has given a novel enhancer for citric acid overproduction by *A. niger* grown on beet molasses in pilot scale submerged fermentation process. It is worthwhile to advise the citric acid industry sponsors to use such by-product to maintain high efficiency and profit bioprocesses.

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