Pak. J. Bot., 46(5): 1899-1903, 2014.

PROCESS OPTIMIZATION OF CITRIC ACID PRODUCTION FROM *ASPERGILLUS NIGER* USING FUZZY LOGIC DESIGN

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Abstract

The inherent non-linearity of citric acid fermentation from *Aspergillus niger* renders its control difficult, so there is a need to fine-tune the bioreactor performance for maximum production of citric acid in batch culture. For this, fuzzy logic is becoming a popular tool to handle non-linearity of a batch process. The present manuscript deals with fuzzy logic control of citric acid accretion by *A. niger* in a stirred tank reactor using blackstrap sugarcane molasses as a basal fermentation medium. The customary batches were termed as 'control' while those under fuzzy logic were 'experimental'. The performance of fuzzy logic control of stirred tank reactor was found to be very encouraging for enhanced production of citric acid. The comparison of kinetic parameters showed improved citrate synthase ability of experimental culture ($Y_{p/x}$ = 7.042 g/g). When the culture grown on 150 g/l carbohydrates was monitored for Q_p, Q_s and $Y_{p/s}$, there was significant enhancement in these variables over the control. Specific productivity of culture (q_p = 0.070 g/g cells/h) was several fold increased. The enthalpy (ΔH_D = 70.5 kJ/mol) and entropy of activation (ΔS = -144 J/mol/K) of enzyme for citric acid biosynthesis, free energies for transition state formation and substrate binding for sucrose hydrolysis of experimental were substantially improved.

Key words: Citric acid, Microbial fermentation, *Aspergillus niger*, Fuzzy-logic, Kinetics.

Introduction

Citric acid fermentation by *Aspergillus niger* is a fairly mature technology, and it is unlikely to expect a radical breakthrough (Andersen *et al*., 2009). It is one of the rare examples in fermentation where academic discoveries have worked in tandem with industrial know-how, in spite of an apparent lack of collaborations to give rise to a very efficient process (Nandi *et al*., 2003, Ali & Haq, 2005). However, two areas where new technology might impact will be on composition of nutrient medium and the physiological conditions of fermentation design. The fermentation products including citric acid have usually been analyzed offline after completion of the batch or in some cases even during the progress of batch. The batch processes have been characterized by flexible, unsteady, limited duration operation and kinetic behaviour (Kasperski & Miskiewicsz, 2002; Hawranik & Sorensen, 2010). The process operating variables must not be disturbed to obtain consistent product quality. But the disturbances arise from deviation in the specified trajectories, variation in the impurities and errors in charging the bioreactor vessel, leading batch-to-batch variation, thus affecting the product quality. The inherent non-linearity of the citric acid accretion by *A. niger* renders its control difficult (Hensen & Seborg, 1992; Karaffa & Kubicek, 2003). So, there is always a need to fine-tune the bioreactor performance for maximum production of citric acid.

A novel approach to control the batch process has been evolved which is called 'Intelligent Control'. There are many methods, which fall under this category, but fuzzy logic has become a popular tool to handle non-linearity of a batch process (Dhillon *et al*., 2011). Therefore, work is needed to optimize the kinetic control of citric acid fermentation by *A. niger* in a stirred tank reactor using a fuzzy logic design.

Materials and Methods

Organism: The culture *A. niger* IIB-28 was obtained from *Microbe Culture Bank (MSB 2.8), Institute of Industrial Biotechnology, GC University Lahore, Pakistan* and maintained on potato dextrose agar medium, pH 4.8 (E-Merck, Germany). Sub culturing was carried out every 2 weeks and the slants were stored at 4°C in a lab-cool (MP-153, Sanyo, Tokyo-Japan).

Molasses pre-treatment: Blackstrap molasses obtained from Pattoke Sugar Mills, Pakistan was clarified according to the method of Panda *et al*., (1984). Sugar contents of molasses were about 45% (w/v). Thirty-five millilitre of 0.1 N H_2SO_4 was added to 1 L molasses medium and placed in water bath at 90°C for 1 h. After cooling to room temperature, the medium was neutralized with lime (CaO) and left overnight. The clear supernatant was diluted to 15% (w/v) sugar.

Inoculum preparation: A volume of 45 ml of Vogel's medium (containing 2% glucose, 0.5% trisodium citrate, 0.5% KH_2PO_4, 0.2% NH_4NO_3, 0.4% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.1% peptone, 0.2% yeast extract, pH 5.5) was dispensed in a 250 ml conical flask. Chromic acid washed marble chips (12-15 in number) were added to the flask (to break mycelial pellets) and sterilized. The flask was inoculated with 1.2×10^6 spores/ml of *A. niger* under aseptic conditions. Inoculum was allowed to grow at 30°C in a rotary shaking incubator (160 rpm).

Bioreactor operating conditions: The fermentation experiments were carried out in a laboratory scale 7.5-L batch stirred tank reactor (New Brunswick Scientific Inc., USA) with a working volume of 5-L. Fermentation medium consisting of pre-treated blackstrap molasses containing (%, w/v): sugar 15.0, NH_4NO_3 0.025, ash contents 0.45, trace metals like iron, zinc, aluminium 0.035 and $K_4Fe(CN)_6$ 200 ppm (initial pH 6) was sterilized at 15 lbs in^{-2} pressure (121ºC) for 20 min. The

reactor was equipped with monitors, which were used to measure and control foam, temperature, pH, stirring rate and dissolved oxygen. The vessel of reactor was equipped with a four-blade turbine with two extra rotating shafts. A peristaltic pump was used to control the foam by automatic addition of an antifoam silicone agent. The inoculum was used at a level of 4% (v/v). For tests with automatic pH control, a system operating with an ingold sterilizable electrode and automatic addition of 0.1 N HCl / KOH solutions through peristaltic pump was used. Controls were performed at different time intervals (12, 24, 48, 72, 96, 120, 144, 168, 192, 216 h).

Control of fuzzy logic design: The fuzzy logic control of stirred tank reactor was used to control the fermentation process (Fig. 1). The input variables of the fuzzy logic control were error and error rate in biosynthesis of citric acid (P). The universes of discourse of these variables were three fuzzy sets i.e.; negative (NE), positive (PO) and zero (ZE). The lower and higher values of operating parameters (Q) were selected as fuzzy output variables. The universes of discourse of these variables were divided into three fuzzy sets which were linguistically called as negative (NE), positive (PO) and zero (ZE). An initial rule base was made by stating a number of rules that were operating in the bioreactor. The rules were written down and investigated for their correction and a rule base was formed by an interactive process (Nandi *et al*., 2003). These rules were considered to generate the control action at lower and higher values of operating parameters (Q). Different methods have been used for defuzzification but in present studies centroid method was used to obtain the crisp value in the production rate. The fuzzy control provides two crisp values, one lower and other higher. One suitable value was selected for the implementation of the fuzzy controller.

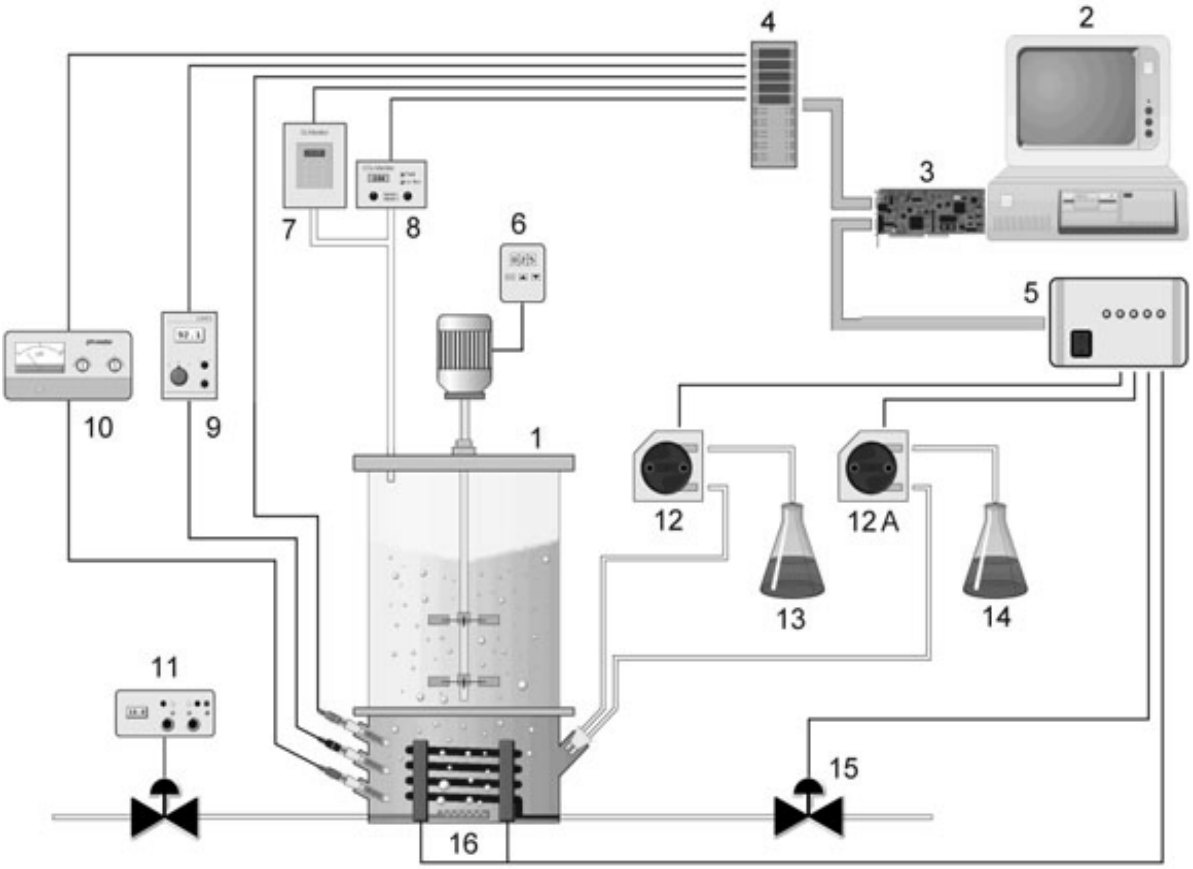

Fig. 1. Schematic diagram of the 7.5 L stirred bioreactor.
Process control through, 1) software, 2) acquisition board, 3) modules, 4) relays, 5) inverter, 6) oxygen probe, 7) CO_2 monitor, 8) oxygen sensor, 9) pH meter, 10) air flow controller, 11) peristaltic pumps, 12,12A) water reservoir, 13) sugar feed reservoir, 14) water valve, 15, 16) heaters.

Analytical techniques: Dry cell mass was determined gravimetrically using dry weight method following the method of Haq & Daud (1995). Citric acid, succinic acid, fumaric acid, malic acid and sugars in fermented broth were determined with isocratic high performance liquid chromatography (Perkins Elmer, USA) using U.V. detector for acids as described earlier and DNS method for sugars (Marrier & Boulet, 1958, Miller, 1959). Kinetic parameters for batch fermentation process were determined after Pirt (1975). The empirical approach of Arrhenius equations was used to describe the relationship of temperature-dependent irreversible inactivation of

product. The temperature ranged from 26-40°C. Specific rate of product formation (q_p, enzyme units/g cells/h) was used to calculate different variables using equation I and II. The plot of $\ln(q_p/T)$ vs $1/T$ gave a straight line whose slope was $\Delta H/R$ and intercept was $\Delta S/R + \ln(K_B/h)$, where h (Planck's constant) = 6.63×10^{-34} Js and K_B (Boltzman constant $[R/N]$=1.38×10^{-23} J/K where N (Avogadro's No.) = 6.022×10^{23} mol^{-1}. Activation enthalpy of ∝-amylase production was determined following equations I and II.

$$q_p = T \,.\, kB \,/\, he^{\Delta S\,/\,Re\text{-}\Delta H\,/\,RT} \quad \ldots\ldots\ldots \text{I}$$

$$\ln(q_p \,/\, T) = \ln\,(kB \,/\, h) + \Delta S \,/\, R - \Delta H \,/\, RT \quad \ldots\ldots\ldots \text{II}$$

Results and Discussion

In the fuzzy logic design, productivity (P) was taken as controlled variable, operating parameters (Q) as manipulated variables and initial substrate concentration as disturbable variable. The performance of the fuzzy logic control of the stirred tank reactor for citric acid production by *A. niger* IIB-28 with input multiplicities in the operating parameters was evaluated using the closed loop block diagram (Fig. 2). The block diagram was prepared by using Matlab and its related Simulink and Fuzzy Logic toolboxes. It was found that the performance of fuzzy logic control for a continuous batch bioreactor with input multiplicities was much better as compared to the uncontrolled conditions where the maximum production of citric acid was only 72.76 g/l. The work is substantiated with the findings of Nandi *et al*., (2003) and Kasperski & Miskiewicsz (2002), however our work regarding the fuzzy logic bioreactor design and citric acid productivity rate is approximately many fold improved compared to the previous investigations.

In batch-wise fermentation, citric acid biosynthesis by *A. niger* starts after a lag phase of about 12-24 h and reaches maximum at the onset of stationary phase. In the present study, citric acid biosynthesis was increased with the increase in fermentation period (12-216 h). A comparison of experimental with control batch cultures for dry cell mass, sugar consumption and citric acid biosynthesis is shown in Fig. 3abc. The customary batches were termed as 'control' while those under block fuzzy logic were 'experimental'. The maximum amount of citric acid (48.06 g/l) was obtained with the experimental 96 h after inoculation (168 h in case of control) which was 5.4 fold improvement. The enhancement in acid production was thus substantial. Dry cell mass and sugar consumption were 12.96 and 94.32 g/l, respectively. Further increase in incubation period (beyond 96 h) greatly reduced citric acid production, however, biomass and substrate consumption were almost constant up to 168-180 h regardless of incubation, followed by a steady decline afterwards. Similar kinds of findings have also been reported by Adachi *et al*., (2003) and Dhillon *et al*., (2011). Thus, citric acid biosynthesis, substrate consumption and time of incubation were altered with the new loop of fuzzy logic biocontrol. Succinic and fumaric acid were also produced but their highest productivities were 0.14 and 0.02 g/l only. Majolli & Aguirre (1999) reported maximum yield of citric acid (13.68%) using molasses based medium, 292 h after inoculation, which is commercially not feasible. It was considered in the present study that oxalic acid was produced as an undesirable by product and there is little net CO_2 formation during the production phase (Kasperski & Miskiewicsz, 2002).

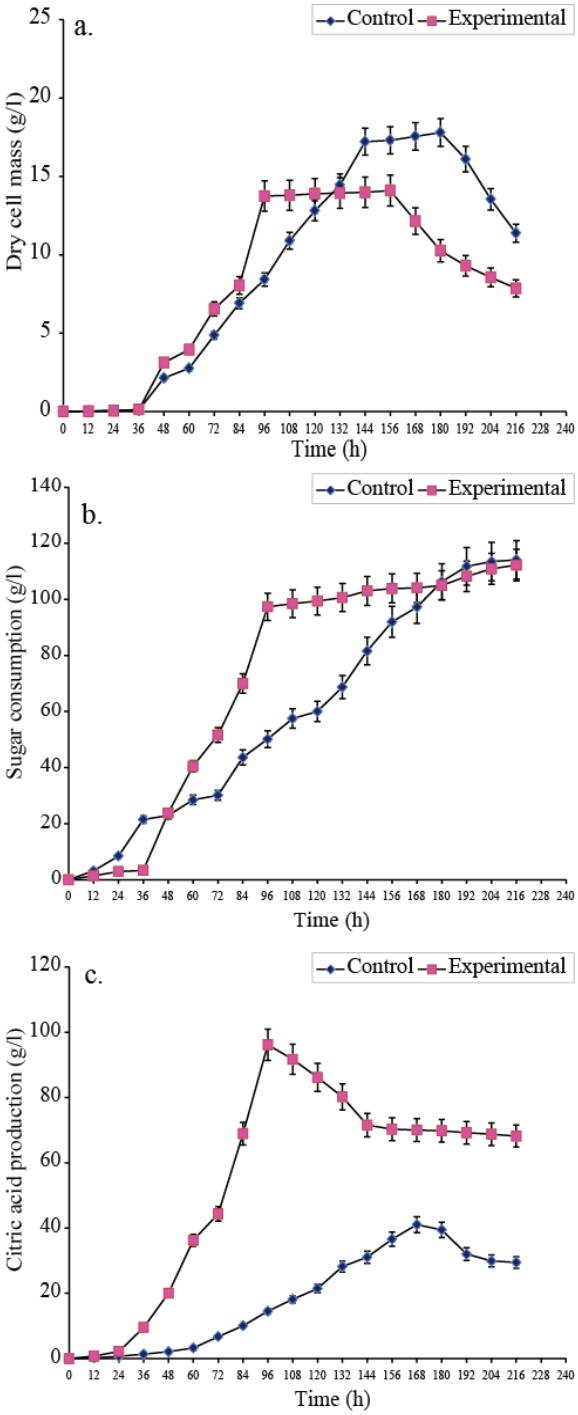

Fig. 3. Comparison of control and experimental for citric acid production by *A. niger* IIB-28 in stirred tank reactor.
Temperature 30°C, initial pH 6, 1 vvm air (0.6% DO level), sugar 150 g/l.

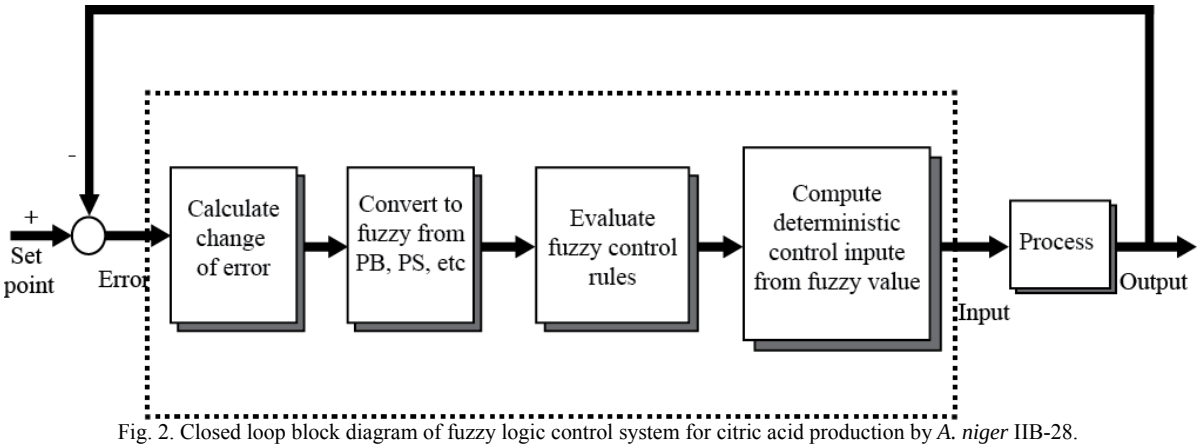

Fig. 2. Closed loop block diagram of fuzzy logic control system for citric acid production by *A. niger* IIB-28.

The comparison of kinetic parameters on citric acid biosynthesis by *A. niger* IIB-28 was also made (Table 1). All the values for the experimental were significantly improved for $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$ over to the control, 16, 24 or 32 h after the inoculation. Maximum growth in terms of specific growth rate (μ h^{-1}) was only marginally different during fermentation at 16 or 24 h. However, when the culture was monitored for Q_p and q_s, there was significant enhancement in these variables after 24 h over to 16 h or all other rates examined. The value for q_p (i.e., specific productivity) after 24 of incubation was highly significant (*HS*, LSD~0.562, 2.48 fold improved at 16-32 h of incubation). Pera & Callieri (1997) identified the relationship between growth kinetic parameters and citric acid production under low oxygen tension with supplementation of additional mineral nutrients. The rate coefficients were found proportional to biomass. However, in the present study the rate coefficient of citric acid production proportional to growth rate was almost constant. Product yield coefficients ($Y_{p/s}$ & $Y_{p/x}$) were several fold improved as compared with Pirt (1975) and Karklins *et al*., (1999).

Temperature affects the normal functioning of enzymes and many of these enzymes control the nutritional requirements of cell and consequently its composition (Ali & Haq, 2005). *A. niger* IIB-28 was cultivated at different temperatures (26-40°C) and based upon this thermal tolerance the enthalpy and entropy of activation was determined (Table 2). The activation entropy of enzyme citrate synthase by the culture was recorded to be (-)144 J/mol/K. In the present study, thermal inactivation of product (26-40°C) characterized by an activation enthalpy (ΔH_D) of 70.5 kJ/mol was remarkably higher than the control. The value of ΔH_D (42 kJ/mol) was significantly lower than the values of some other cultures (Hensen & Seborg, 1992; Nandi *et al*., 2003; Ali & Haq, 2005). The activation entropy of enzyme formation by the culture (-144 J/mol/K) was very low and comparable with that for gluconic acid production by thermotolerant bacteria, which reflects that this inactivation phenomenon implies a little disorderness during growth and subsequent enzyme formation. Practically this value is lower than other systems (Priede *et al*., 2002; Khosravi-Darani & Zoghi, 2008), which suggest more protection exerted by culture cell system against thermal inactivation. Thus *A. niger* IIB-28 has the capability to produce citric acid at higher temperature (even at 38-40°C).

Table 1. Comparison of kinetic parameters of citric acid fermentation by *A. niger* IIB-28 by control and experimental.

*Kinetic parameters	Control			Experimental		
	96 (h)	120 (h)	144 (h)	96 (h)	120 (h)	144 (h)
Product formation						
Q_p (g/l/h)	0.784	1.006	0.902	0.412	0.722	0.600
$Y_{p/s}$ (g/g)	0.802	0.965	0.941	0.423	0.603	0.512
$Y_{p/x}$ (g/g)	6.556	7.042	5.583	3.235	4.026	2.243
q_p (g/g cells/h)	0.062	0.070	0.054	0.037	0.058	0036
Substrate consumption						
μ (h^{-1})	0.132	0.143	0.161	0.093	0.108	0.111
$Y_{x/s}$ (g cells/g)	0.123	0.134	0.176	0.062	0.096	0.138
Q_s (g/l/h)	1.094	1.049	0.951	0.996	0.823	0.430
q_s (g/g cells/ h)	0.082	0.072	0.061	0.079	0.052	0.032
LSD	0.390	0.562	0.326	0.262	0.315	0.191
Significance level <p>	*S*	*HS*		-	*S*	

*Kinetic parameters: Q_p = gram citric acid produced per litre per hour, $Y_{p/s}$ = g citric acid produced per g ram substrate consumed, $Y_{p/x}$ = gram citric acid produced per gram cells formed, q_p = gram citric acid produced per gram cells per hour, μ (specific growth rate) = per hour, $Y_{x/s}$ = gram cells per gram substrate utilized, Q_s = gram substrate consumed per litre per hour, q_s = gram substrate consumed per gram cells per hour.

Table 2. Thermodynamic parameters determined by Arrhenius approach for formation and inactivation of enzyme.

Thermophilic culture (*A. niger* IIB-28)	Enzyme thermal inactivation		Protein content (mg/ml)
	Control	Experimental	
Activation enthalpy, ΔH_D (kJ/mol)	21.4	70.5	11.76
Activation entropy, ΔS (J/mol/K)	(-) 87	(-) 144	14.45

*Thermodynamic parameters were determined using the following equation, $Ln(q_p/T)=Ln(k_B/h)+\Delta S/R-\Delta H_D/R.1/T$, where q_p, T, k_B, h, ΔS, ΔH_D and R are specific activity, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation and gas constant, respectively. The values of k_B, h and R are 1.38×10^{-23} J/K, 6.63×10^{-34}Js and 8.314 J/K//mol, respectively. ΔH_D was calculated as slope and $Ln(k_B/h)+\Delta S/R$ as intercept.

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(Received for publication 23 April 2012)