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A SEMI-PILOT-SCALE STUDY TO PRODUCE CITRIC ACID FROM HYDROL USING A MUTANT OF *ASPERGILLUS NIGER*

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Abstract

Citric acid is an edible acid with the current global demand of $1.7x10^6$ tons/annum. It is required to improve the industrial production of citric acid to satisfy the exponentially growing demands, using low-cost and renewable sources. Hydrol is a by-product of starch industry which contains approximately 70% fermentable sugars and can be exploited to produce citric acid. Here, the hydrol was subjected to fermentation in 23-L and 150-L fermenter to produce citric acid. Medium containing reducing sugar (S_0= 80-180 gL^{-1}) from hydrol was used as the basal fermentation medium using a derepressed mutant derivative of MUNAR 120 *Aspergillus niger*. Different process variables for the accumulation of citric acid were optimised. At optimum substrate concentration (150 gL^{-1}) citric acid titer of 130 gL^{-1}, the product yield of 0.86 gg^{-1}, the maximum volumetric productivity of 3.45 $gL^{-1}h^{-1}$, and specific productivity of 2.47 $gg^{-1}h^{-1}$ were achieved. In 23-L fermenter studies, 90-100% sugars were consumed at temperatures ranging from 22-38°C. Where, in a 150-L pilot-scale fermenter 80% of the theoretical yield was achieved. The experimental data and kinetic parameters have shown that the mutation has a dramatic impact on the metabolic activities. Enzymes inactivation phenomena implied a little increase in entropy during the activated state and suggested an improved protection conferred by mutant cell system. Overall metabolic activity of the mutant was significantly improved when compared to its wild-type parent.

Key words: Hydrol, *Aspergillus niger*, Mutant strain, Citric acid production, Waste utilization.

Introduction

Citric acid is an edible acid with the current global demand of 1.6 x 10^6 tons/annum which is still increasing exponentially (Dillon *et al.*, 2011). To fulfill its increasing demands, it is required to establish an alternative and cost-effective approaches to enhance the yield and productivities. A major part of its production is used in the food and beverages, while rest of it is used for other industrial purposes (Angumeenal & Venkappayya, 2013; Kwon *et al*., 2016). Due to its diverse applications, strains are being developed to minimize the cost of production and to enhance the production. The production process of citric acid is very complex and needs exploitation of several aspects simultaneously. Several enzymes of the metabolic pathway are required to be used harmoniously to regulate the flow of metabolites from cytosolic component to mitochondria for the enhanced formation of the product (Yang *et al*., 2017). To produce citric acid, both solid state and submerged fermentation techniques have been employed. The titre and nature of carbon source strongly effects the final yield of citric acid produced by *A. niger* (Show *et al*., 2015).Cane-molasses had been a feasible raw material to produce citric acid through fermentation. Molasses is a complex mixture of many components and attempts have been made to relate the presence of toxic components to fermentation performance (Sanjay & Sharma, 1994). Cost of molasses in the international market is increasing day by day. There is urgent need to exploit alternate feedstocks for its production. Hydrol, a high maltose corn syrup, is a by-product of starch industry and contains 70% fermentable sugars. This substrate can be exploited to produce citric acid.

Cloning of the target genes in homologous and heterologous hosts evolved recombinants but did not enhance product formation rate in the recombinants (Yang *et al*., 2015). Due to the negative feedback of catabolites in wild-type strains of *A. niger*, citric acid production is repressed (Lotfy *et al*., 2008), transport phenomena (Show *et al*., 2015) and enzymes of the metabolic pathway (Yin *et al*., 2017). For hyper-production, there is a need to overcome the barriers of catabolite repression and feedback inhibition by selecting 2-deoxyglucose-resistant (DG^r) and aspartate-requiring mutant strains of *A. niger* either through conventional mutation (as the first attempt) or through modern bioengineering design. The phenotype can be confirmed by serially transferring the putative variants on DG-sucrose-ox gall-selection medium and the selected variant can be characterized. These mutants should have increased productivity of citric acid from glucose, fructose, sucrose, and molasses as compared to their wild-type parent strains. Mutant strains capable of utilizing mixed sugars can be exploited for the enhanced citric acid production, but to install these robust abilities through bioengineering is not easy (Alvarez-Vesquez *et al.*, 2000). Resistance to DG was conferred by some alterations in the transport system for glucose (Papagianni *et al*., 1998), enhanced hexokinase activity, and glucose phosphate phosphatase activities (Ashok kumar *et al*., 2004; Sun *et al*., 2007).

A. niger was already mutatedfor higher production of citric acid using gamma ray-induced mutagenesis (Rajoka *et al.,* 1998) but the overall yield was low. In this study, auxotrophic strains of *A. niger* (dg^{-1} and asp^{-1}) were obtained after irradiation with gamma rays and strain was subjected to citric acid production using hydrol as the feedstock. The kinetic parameters including activation energy, midpoint temperature, enthalpy and entropy values of product formation process might elucidate the phenomena intricated in thermal inactivation of enzymes involved in the citric acid production. Numerical approaches suggested previously for their estimations (Aiba *et al*., 1973) were followed as applied to other fermentation systems (Converti & Dominguez, 2001) are also included. The optimization of process variables was performed to produce citric acid by this newly developed mutant strain of *A. niger* in the 23-L fermenter before up-scaling to the 150-L fermenter.

Materials and Methods

Fungal strain, growth media, and culturing conditions: In citric acid fermentation experiment, wild-type and mutant (*A. niger* dg^{-1} asp^{-1}) strains of *A. niger* were used. The autoclaved sterilized PDA (Potato Dextrose Agar medium; sliced potato 300 gL^{-1}, Dextrose 20 gL^{-1} and Agar 25 gL^{-1}, pH 5.5) was used to maintain the cultures. Hydrol was collected from Rafhan Maize Product Co. Faisalabad. Because of high glucose contents (70-75%), ash and protein content it is a good substrate for higher production of citric acid. Batch fermentations were performed in shake flasks and 23-L fermenter. For the production medium, pH was set to 6.5 and the optimum temperature was 30°C. To calculate all kinetic parameters, samples were collected at different time intervals including 0 h, 24 h, 48 h, 72 h, 96 h, and 120 h. Experiments were replicated three times to ensure the reproducibility. The 200 mL molasses medium (Sugar 1%, pH 6.0) was autoclaved in 1 L flask containing glass beads. Media were inoculated using a small number of spores from the slant and flasks were then incubated in shakers at 30°C, 200 rpm for 24 h.

Strain improvement: The cell suspension of *A. niger* was shifted from saline to sterile dioctylsulphosuccinate (Sigma) 0.005% solution. The colony forming units (c.f.u mL^{-1}) were maintained on oxgall-potato-dextrose-agar medium at 1.0 x 10^9 cells mL^{-1} for γ-irradiation and further studies. The cells were exposed to an exposure dose of irradiation ranging from 100 to 1200 G, then to select the radiation dose for mutation, survival curve was created. The mutant derivatives were enriched in medium containing 15 % carbohydrates in molasses + 1.5% DG, selected on molasses-based medium containing 1.5% DG, 0.01% Bromocresol green and 2.5% agar and characterized in the solid and liquid medium. Among several variants, the best mutant strain was picked and named as FAR 120, which was DG-resistant and aspartate- requiring variant from the mutated cells.

Fermenter studies: In fermentation experiments, the 23-L fermenter with a working volume of 15 L (B. Braun, Germany) was used, containing 80-180 gL^{-1}hydrol-sugars, 20 g corn steep liquor having pH 6.5. The 10% (v/v) inoculum was provided to the basal media based on the total working volume, and throughout the experiment (5 days). The temperature was adjusted to 30 ± 1°C, while a constant stirring and aeration were provided at 400 rpm and 0.5 vvm, respectively. Sterilized silicone oil was used as the anti-foaming agent. Similar conditions were used in the 150-L fermenter. Impact of different agitation (150-450 rpm) and aeration (0.25-1.0 LL^{-1} min) rates were studied on the overall production of citric acid by the mutant strain (*A. niger* $dg^{-1}asp^{-1}$).

Mycelial dry weight, sugars, and citric acid analyses: The dry mycelial weight was estimated by collecting the mycelia through filtration. The filtrate was subjected to further analyses, while sterilized distilled water was used to wash mycelia, then to get a constant weight, mycelia were oven dried at 80°C. Sugars were estimated calorimetrically by using DNS method (Miller, 1959) and taking glucose as a standard. Using glucose oxidase-peroxidase kit, glucose was measured. Sucrose was first converted into reducing sugars with 1 N HCl and reducing sugars were determined using DNS reagent. The values of sugars were rechecked on HPLC as described (Enquist-Newman *et al*., 2014).

Citric acid was precipitated as calcium citrate by adding hot (90°C) lime solution (pH 12.0) to the hot (90°C) fermented broth (1:1) and boiling for 10 min in a water bath and citric acid recovered with concentrated sulphuric acid (1 N) and decolorized by boiling with activated charcoal and was analyzed using HPLC as described (Rajoka *et al*., 1998). Impact of sugar concentrations (80-150 gL^{-1}), and impact of different nitrogen sources including ammonium sulfate, ammonium dihydrogen phosphate, ammonium nitrate, urea, fish meal and corn steep liquor used in equimolar concentrations was studied on citric acid production. All experiments were carried out in triplicates and the average of three independent experiments is presented in data.

Results and Discussion

To select high citric acid producing mutant, wild-type *A. niger* spore suspension was treated with γ-rays and expressed cells were spread on molasses-DG-oxgall-Asp (150 μg mL^{-1})-Bromocresol green-agar plates. Mutant strains were indicated by a larger yellow zone which appeared around eight colonies. Semi-quantitative plate studies revealed that three derivatives could produce the largest amount of acids which were further isolated and designated FAR 120, FAR 121, and FAR 122 and were subjected for citric acid production studies *In vitro*. Subsequent studies of above variants using shaken flasks revealed that variant FAR 120 was the best organism and was selected for fermenter studies.

Sugar concentration and citric acid production: The impact of sugar concentrations ranging from 80–160 gL^{-1} of hydrol on kinetic parameters of substrate consumption and product formation by *A. niger* FAR120 was studied in the 23-L fermenter (Table 1). The maximum volumetric productivity of citric acid (3.46 $gL^{-1}h^{-1}$) was achieved at 150 gL^{-1}. The overall sugar consumption, dry mycelial

weight, and the final pH of the fermentation medium were 150 gL^{-1}, 12.0 gL^{-1} and 3.5, respectively. A gradual decrease in citric acid production was observed in response to increasing or decreasing sugar concentration. The overgrowth of the mycelia in larger amount might be a possible reason for it, resulting into a more viscous media and mass transfer limitation. Max *et al*., (2010) reported that there was a reduction in the yield of citric acid with the increase of mycelia formation in the medium. A medium with higher sugar concentration (150-180 gL^{-1}) is needed for strains of *A. niger*.For citric acid production (in both shake-flask and fermenter studies), a 150 gL^{-1}hydrol-sugar medium was found to be optimum and was selected for further investigations.

Nitrogen source impact on citric acid production: Initial studies indicated that among various nitrogen sources, (ammonium sulphate, ammonium dihydrogen phosphate, ammonium nitrate, urea, fish meal, and corn steep liquor) the corn steep liquor was proved to be the best nitrogen source in shake flask studies. Its different concentrations (2%, 3%, 4%, 5%) were used with 15 % sugars of hydrol in fermenter studies. Corn steep liquor (2%, V/V) was ideal to provoke higher production of citric acid and was used in further studies. These studies indicated that sugars were completely consumed in 96 h of fermentation, though total substrate consumption could not be realized in shake flask studies (Table 2). Citric acid productivity was shown to be enhanced by two-fold in the fermenter studies when compared to the shake flask experiments (Table 2). Out of 150 g hydrol-sugars, only 112 g was consumed in citric acid synthesis in shake flask and the remaining was used in CO_2 evolution and was not fixed by pyruvate carboxylase. Several factors such as terminal pH, the age of fungi, reduced nitrogen in the fermentation medium, inhibitors production by fungi or feedback inhibition might have suppressed further synthesis of citrate in shake flask. In fermenter studies, 150 g sugars supported the synthesis of 125 g citric acid L^{-1} of fermentation medium. In comparison to the wild-type strain, all kinetic parameters were significantly improved.

Table 1. Impact of hydrol-sugar concentration on citric acid formation and substrate consumption in 23-L fermenter studies.

Parameters		Sugar concentration (w/v) in hydrol			
		8	12	15	18
Substrate Consumption	μ_m^* (h^{-1})	0.21 ± 0.02	0.23 ± 0.01	0.25 ± 0.02	0.18 ± 0.02
	Q_S^* (g $L^{-1}h^{-1}$)	3.50 ± 0.13	3.60 ± 0.11	3.90 ± 0.14	3.10 ± 0.11
	Q_X^* (g $L^{-1}h^{-1}$)	0.38 ± 0.01	0.37 ± 0.02	0.37 ± 0.01	0.36 ± 0.01
	q_S (g $g^{-1}h^{-1}$)	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03
	$Y_{X/S}$ (g g^{-1})	0.21 ± 0.03	0.24 ± 0.02	0.25 ± 0.03	0.18 ± 0.04
Product Formation	Q_{PCit}^* (g $L^{-1}h^{-1}$)	1.44 ± 0.10	1.86 ± 0.11	3.45 ± 0.09	2.16 ± 0.11
	q_{PCit}^* (g $g^{-1}h^{-1}$)	0.67 ± 0.04	0.90 ± 0.03	0.98 ± 0.02	0.48 ± 0.03
	$Y_{PCit/S}$ (g g^{-1})	1.00 ± 0.05	0.73 ± 0.06	0.91± 0.04	0.46 ± 0.05
	$Y_{PCit/X}$ (g g^{-1})	3.20 ± 0.12	5.00 ± 0.10	6.50 ± 0.11	3.80 ± 0.13

* These parameters were determined as described previously (Aiba *et al*., 1973)

Table 2. Kinetic parameters for substrate consumption and citric acid formation by *Aspergills niger* (Parent) and its mutant (Munar120) in fermenter on 15% sugars in hydrol.

Parameters		Parent	Munar120
Substrate Consumption	μ_m^* (h^{-1})	0.18 ± 0.01	0.22 ± 0.02
	K_S^* (%)	0.22 ± 0.02	0.18 ± 0.01
	m (g $g^{-1}h^{-1}$)	0.15 ± 0.01	0.11 ± 0.01
	Q_S^* (g $L^{-1}h^{-1}$)	1.50 ± 0.03	2.50 ± 0.05
	Q_X^* (g $L^{-1}h^{-1}$)	0.19 ± 0.02	0.37 ± 0.02
	q_S (g $g^{-1}h^{-1}$)	0.72 ± 0.03	1.00 ± 0.05
	$Y_{X/S}$ (g g^{-1})	0.11 ± 0.01	0.15 ± 0.02
	Y_{EG} (g g^{-1})	0.03 ± 0.01	0.04 ± 0.01
Product Formation	Q_{PCit}^* (g $L^{-1}h^{-1}$)	1.44 ± 0.01	3.45 ± 0.01
	q_{Pcit}^* (g $g^{-1}h^{-1}$)	1.11 ± 0.05	2.44 ± 0.07
	$Y_{Pcit/S}$ (g g^{-1})	0.60 ± 0.01	0.91 ± 0.03
	$Y_{PCit/X}$ (g g^{-1})	3.20 ± 0.11	6.50 ± 0.14
	α^* (g g^{-1} cells)	4.50 ± 0.05	5.50 ± 0.13
	ß (g $g^{-1}h^{-1}$)	1.25 ± 0.01	1.65 ± 0.12
	T_m	34.0 ± 1.31	36.0 ± 1.29

* All parameters were determined as described previously (Aiba *et al*., 1973)

Principally, citric acid synthesis is anaerobic fermentation and the micro-organism needs an abundant supply of oxygen beyond that required for growth. In both, growth-associated and non-growth associated modes, all culture media supported product formation and maximum production of citric acid in the mutant strain was substantially improved ($p \leq 0.05$) (Tables 1 and 2). As mentioned earlier, the substrate consumption parameters namely Q_s, q_s,$Y_{X/S}$, Q_X, and pH (Rajoka *et al*., 1998) were shown to regulate product formation. But this regulation occurred at the expense of product formation in these studies. Keeping in view the kinetic parameters for batch fermentation processes, the mutation resulted in doubling the specific and volumetric product formation rate. Fermenter based fermentation showed significant improvement in both volumetric and specific rates of substrate consumption and product formation.

Formation of citric acid was shown to be influenced by the growth-associated and non-growth associated factors (with lower values of α) and the mutant strain exhibited improved fermentation parameters over the wild-type strain. Out of total carbon consumed, 15 to 20 g were taken up for cell mass synthesis and maintenance functions (Table 2). Out of 1.8 g per g cells, 0.25 g per g cells was

taken up for maintenance functions. Similarly, out of 4.9 g citric acid per g cells, 1.72 g per g cells was growth-associated while 0.23 g per g cells per hour was non-growth-associated (β) in the case of the mutant organism. The parameters α or β or both expectedly have smaller values under genetic control, which do not support citric acid production; these parameters should have higher values when citric acid synthesis is derepressed. These values imply that greater the values of β, the greater is the non-growth associated citric acid production rate. Similarly, the value of α showed a similar trend for the growth associated citric acid production parameters.

Effect of agitation intensity and aeration rate: Two other parameters including agitation speed and aeration are very important to regulate product formation in the fermenter. Different factors including, the chemical composition of media, size of the fermenter and the organism under study determine the rate of agitation and aeration. Usually the higher oxygen demand of a culture becomes a limiting factor in the culture medium. Citric acid production of 87 gL^{-1} was attained after 72 h when the dissolved oxygen (DO) was kept above 20% saturation, however, the product formation was lowered when DO concentration was lower than 15%. Because, at lower DO, enzymes activities of the vital mitochondrial enzymes involved in glyoxylate cycle and citrate synthase would have been drastically lowered (Papagianni, 2007). Intercellular lipids profile is also altered which may result in loss of excretion of citric acid.

Both biomass and citric acid production were influenced by aeration and agitation rates. Citric acid production further increased to 109 gL^{-1} at an agitation speed of 400 rpm. The increase in acid production in response to increasing stirring speed would have been influenced by several factors including the improved rate of oxygen transfer, improved dispersion of the substrate and enhanced effective collisions. At stirring speeds higher than 400 rpm, both growth and citric acid production negatively affected, which might be because of shearing. Later, it was observed under a microscope (data not shown) that higher stirring speeds caused fragmentation of the mycelia.

Sugar consumption, mycelial dry weight, and pH of the medium were 130 gL^{-1}, 9.5 gL^{-1} and 2.1, respectively. The highest amount of citric acid produced based on the type of sugar used was 84 % and it was in accordance with the previous reports (Lotfy *et al*., 2008; Munshi *et al*., 2013). It is mostly believed that a fermenting culture demands higher oxygen leading towards the quick replenishment of the DO and hence requires efficient supply and mixing.

Citric acid production is anaerobic fermentation process. The need for high aeration rate has been recognized as a crucial factor influencing the citric acid accumulation using *A. niger* (Rodrigues *et al*., 2013). The citric acid production ranged from 28.83-50.20 gL^{-1} in response to aeration rates ranging from 0.25-1.5 vvm. The maximum titer (130 gL^{-1}) of citric acid was achieved at the constant aeration rate of 0.5 vvm at the stirring speed of 400rpm which might be due to the fact that aeration and mixing were efficient enough to meet the required DO level. The sugar was consumed at the rate of 150 gL^{-1}, the mass of dried mycelia was 9.5 gL^{-1}, and pH of the medium was 2.7. Hence, we have shown a 13.0% increment in citric acid when compared to previous reports.

Effect of pH and temperature on citric acid production: It was shown that a favorable initial pH should be maintained to get the improved citric acid production. Maximum productivity was achieved at an initial pH of 6.5 in both shake flask and fermenter (Tables 1 & 2). Thelower or higher pH values resulted in reduced citric acid production (Fig. 1) which might be due to the release/presence of some toxic ions. Therefore, pH 6.5 was selected for further studies.

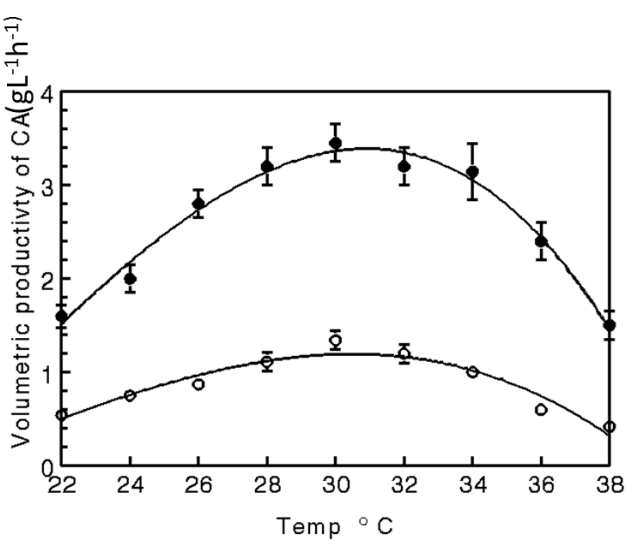

Fig. 1. The effect of pH of the medium on the volumetric rate of citric acid formation from the hydrol medium (15% carbohydrates concentration) in 23 L fermenter. The respective volumetric productivity values were plotted against pH to calculate optimum initial pH for citric acid production. Error bars show standard deviation among three replicates.

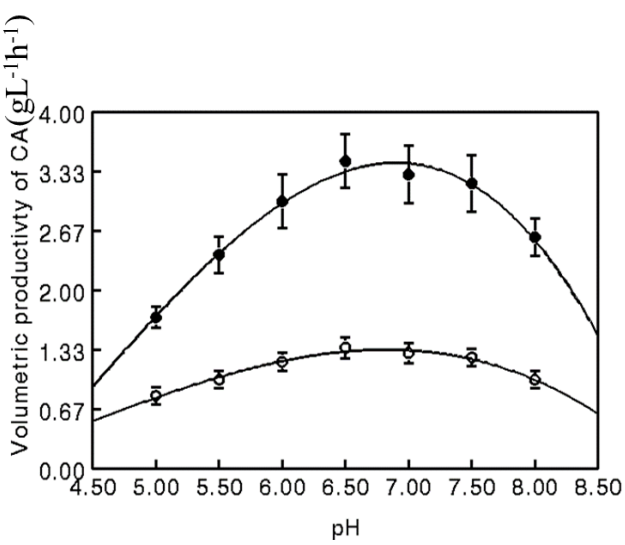

Fig. 2. The effect of fermentation temperature on the specific rate of citric acid formation from the hydrol medium (15% carbohydrates concentration) in 23 L fermenter. The respective specific productivity values were plotted against temperature to calculate the optimum initial temperature for citric acid production. Error bars show standard deviation among three replicates.

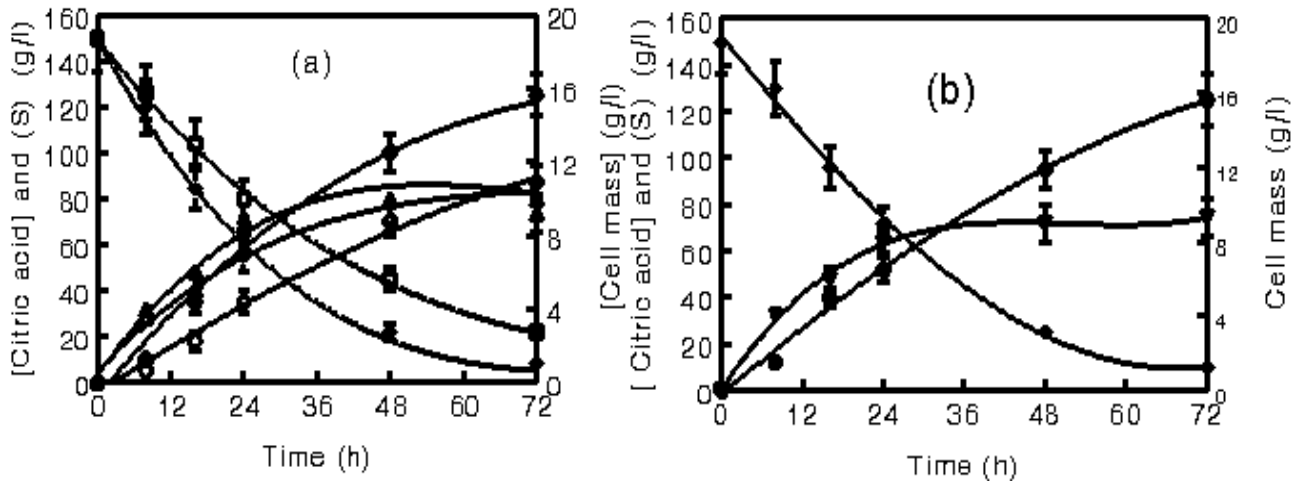

Fig. 3. Kinetics of production of citric acid (O), cell mass (Δ) and substrate (▯) in 23-l (a) and 150-l fermenter (b) during fermentation of hydrol medium by the parental organism (open symbols) and Mutant FAR120 (closed symbols) under optimum conditions of process variables. The initial pH of the medium was 6.5, inoculum size 10%, on glucose (1%), and temperature 30 °C. Corn steep liquor served as the nitrogen source. Error bars show standard deviation among three replicates.

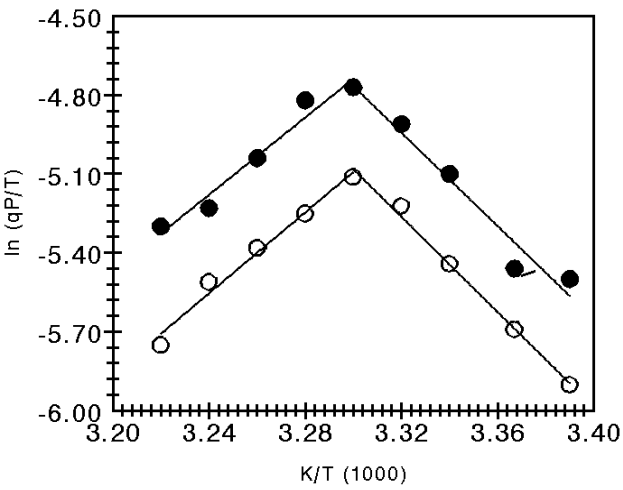

Fig. 4. Application of Arrhenius approach to calculate enthalpy and entropy for product formation and its inactivation following growth of parental (open circle) and recombinant (closed circle) organisms at different temperatures in 23 L fermenter under optimized fermentation conditions. Each point is a mean of three independent observations. Standard deviation among replicates was too small to be visible.

A temperature range of 20–38°C was used to see the impact of temperature on citric acid production, in both shake flasks and fermenter experiments (Fig. 2). At 25°C, production of citric acid was 49.0 gL^{-1} and 90 gL^{-1} in shake flask and fermenter, respectively. The highest rate of citric acid production was attained at 30°C which was 1.36 $gL^{-1}h^{1}$ and 3.45 $gL^{-1}h^{-1}$ shake flask and fermenter, respectively. The sugar consumption by the mutant was 100 gL^{-1} and 150 gL^{-1}, dry mycelial weight was 13.0 gL^{-1} and 8.5 gL^{-1}, in shake flask and fermenter respectively, while thefinal pH of the medium was maintained around 2.3. At lower temperatures, the enzyme activity was also low, giving no influence on the improvement of citric acid production. But at temperatures higher than 30°C citric acid synthesis was reduced which may be due to the general impact of enzyme denaturation. It has shown that 30°C is optimum for citric acid production and 40°C citric acid accumulation is completely blocked, and oxalic acid is accumulated instead (Amer *et al*., 1999). The cultures were re-grown under optimized fermentation conditions in time course studies to calculate all fermentation attributes in the fermenter (Fig. 3, Table 2) under optimized conditions respectively.

The mutation had a dramatic influence on the cell system as it lowered down the midpoint temperature, and activation energy values (Tables 1 & 2) and like thermo-stabilized enzymes (Vieille & Zeikus, 1996), the metabolic system of the mutant cells was thermo-stabilized by mutation. This was further confirmed by studying the thermal energy demand for product formation and its inactivation. To elucidate the relationship of temperature-dependent inactivation (reversible and irreversible) of citrate synthase and other associated enzymes, empirical approach of Arrhenius (Aiba *et al*., 1973) was employed. By using the extended application of Arrhenius approach to microbial phenomena, activation enthalpy of citric acid production was also calculated (Fig. 4):

$$q_{pCit} = T.\ k_B/h\ e^{\Delta S^*/R}\ e^{-\Delta H^*/R.T.} \quad \text{..... (1)}$$

$$\ln (q_{pCit}/T) = \ln(k_B/h) + \Delta S^*/R - \Delta H^*/R.1/T \quad \text{..... (2)}$$

where q_{pCit}, T, k_B, h, ΔS^* and ΔH^* are specific productivity, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation and gas constant, respectively. The thermodynamic parameters were calculated (Fig. 4) which are given in Table 3. The activation enthalpy of the citric acid formation (ΔH^* = 44 kJ mol^{-1}) was lower when compared to phytase production (70-80 kJ mol^{-1}) (Al-Asheh & Duvniak, 1994) but was favourable when compared to the ΔH^* of cell growth (74 kJ mol^{-1}) (Aiba *et al*., 1973; Converti & DelBorghi, 1997; Converti & Dominguez, 2001). Thermal inactivation of citric acid was caused due to inactivation phenomena which is indicated by an enthalpy (H_D^*) of 47 kJ mol^{-1} and is unusually lower when compared to citric acid production and lower when compared to previous reports i.e. 160-235 kJ mol^{-1} (Converti & Dominguez, 2001). These values

indicate that the decrease in productivity at high temperature might have happened due to the reversible denaturation of the key enzymes.

Table 3. Thermodynamic parameters estimated by Arrhenius approach for batch formation of citric acid from hydrol (15% carbohydrates) by *Aspergillusniger* and its mutant Munar120.

Parameter	Strain	Citrate formation	Thermal inactivation
Activation enthalpy (kJ mol^{-1})	Parental	75	44
	Mutant	64	47
Activation entropy (J mol^{-1} K^{-1})	Parental	8.8	-451
	Mutant	6.5	-461

The activation entropy of citric acid formation was 0.65 kJ mol^{-1} K^{-1} which was minimal and favorable when compared with the values from thermotolerant organisms and other fermentation processes by the thermotolerant organism (Aiba *et al*., 1973; Converti & Dominguez, 2001). While these values are consistent with the values for formation of a transition state with more rigid structure in respect of the reacting entities in the fermentation medium, they further suggest that the enzymatic reaction/s namely citrate synthase, pyruvate carboxylase, and phospho-fructo-kinase could have been rate-limiting factors in the citric acid synthesis (Converti & Dominguez, 2001).

The value of activation entropy of thermal inactivation was -0.461 kJ mol^{-1} K^{-1} which is low and reflected that the inactivation phenomenon inferred a slight impact on the activated state formation. Moreover, this value is lower when compared to some other enzymatic systems involved in product formation i.e. -0.248.40±16 and -297.2±12 kJ mol^{-1} K^{-1} (Ali & Haq, 2007) for mutant and the wild-type strain respectively , which suggested that mutated cell conferred some sort of protection against thermal inactivation. Finally, the optimized fermentation conditions were extended to a study conducted in the 150-L fermentor with a working volume of 100 L (Fig. 4). Maximum values of $Y_{PCit/S}$, $Y_{PCit/X}$, Q_{PCit} and q_{PCit} in fermenter remained almost unchanged than those obtained in the 23-L fermenter. These values are higher when compared to some other mutants of *A. niger* (Lotfy*et al*., 2008; Javeed *et al*., 2011; Umbreen *et al*., 2013) but the substrate consumption parameters are low.

Conclusion

A stable variant of *Aspergillus niger*, resistant to higher concentrations of de-oxy-D-glucose and aspartate was obtained. This mutant showed hyper-production of citric acid using hydrol medium. In the fermenter studies, 100% sugars were consumed, and mutant derivative supported 84% theoretical yield of the product. The citric acid productivity was considerably higher, and kinetic parameters indicated that enzyme system of the mutant strain (*A. niger* $dg^{-1}asp^{-1}$) was thermostable as compared to that of the wild-type strain. The selected organism supported higher production rate and resistance to feedback inhibition.

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