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CITRIC ACID PRODUCTION BY WILD AND UV – TREATED STRAINS OF *ASPERGILLUS NIGER* ON TWO DIFFERENT MINERAL SALT MEDIA

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Abstract

Microbial production of citric acid by a novel *Aspergillus niger* EE-12 and its UV – treated strain (UV-1) were carried out in shake flask cultures using mineral salt media containing sucrose or fructose as the carbon and energy sources. The highest citric acid concentration (36.1±0.1g/l) was obtained with the UV – treated strain UV-1 after 144 hours in medium containing sucrose and this was significantly higher ($p<0.05$) than the concentration produced by its parent strain EE-12. Citric acid production using medium containing sucrose (Sucrose salt medium) was significantly higher ($p<0.05$) than from medium containing fructose (Fructose salt medium) for both organisms. Product and growth yield coefficients ($Y_{CA/S}$, $Y_{CA/F}$, $Y_{CA/X}$, $Y_{X/S}$, $Y_{X/F}$) and volumetric rates (Q_F, Q_S, Q_{CA}, Q_X) were studied and compared for each of the test organisms based on the media (sucrose and fructose salt media) using students t-test analysis. This research indicated that the fungi strains are efficient for citric acid production using sucrose based medium.

Key words: Citric acid production, *Aspergillus niger*, sucrose, improved UV-1 strain, kinetic parameters.

Introduction

Fermentation processes play a major role in the production of most organic acids. Different microorganisms have been exploited to convert carbohydrates to high yields of organic acids. This property is demonstrated by various filamentous fungi, yeast and bacteria (Yalcin *et al.*, 2010). Fungal fermentation acids produced commercially, or at least studied extensively include citric, fumaric, gluconic, itaconic, kojic and gibberellic acids (Casida, 1968; Magnuson and Lasure, 2004). Except for the production of citric acid which is entirely by fungal fermentation, there is frequently great competition between microbiological and chemical processes for production of the various organic acids (Crueger and Crueger, 1984; Magnuson and Lasure, 2004).

Citric acid occurs naturally as a component of many fruits (Papagianni, 2007). Around 70% of the citric acid produced is used in the food and beverage industry, approximately 12% in pharmaceuticals and about 18% in other industrial utilizations (Kapoor *et al.*, 2004; Yigitoglu, 1992). It is widely used in the food industry as an acidulant and flavouring agent in beverages, confectionery and other foods, and in leavening systems for baked goods because of its high solubility, extremely low toxicity, and a pleasant sour taste (Kapoor *et al.*, 2004). As a food constituent, its use is unrestricted because it has generally regarded as safe (GRAS) status (Waites *et al.*, 2001; Jamal *et al.*, 2005). Citric acid is used in maintaining metals in solution for electroplating, as a cleaning and 'pickling' agent for metals. It is employed commercially as a chelating and sequestering agent and as plasticizers (Alben and Erkmen, 2004). Recently, because of its easy biodegradability, this organic acid has also found a ready acceptance in the detergent industry in place of polyphosphates and in the removal of sulphur in stack gases, a process applicable to power stations and other facilities where sulphur must be removed (Kapoor *et al.*, 2004).

Citric acid production rates and yields are highly dependent on the type of microorganism, the type of substrate and culture conditions (Yalcin *et al.*, 2009a; Haq *et al.*, 2001). This study, aimed at producing citric acid from carbohydrates using a novel *Aspergillus niger* and its optimized strain. Authors also compared the production efficiency and growth yield of parent and mutant strains.

Materials and Methods

Fungal strain and inoculum preparation

Aspergillus niger EE-12 and its mutant strain UV-1 were used for this study. The parent strain EE-12 was

isolated from waste water of crude oil Exploration Company located in Imo State, Nigeria. The selection of the strain EE-12 was performed with a preliminary study in which qualitative citric acid production abilities of 20 strains isolated from different sites were investigated using the dye method of Kareem *et al.* (2010). Of the tested strains, the highest citric acid production was obtained by *A. niger* EE-12, which was chosen for further studies. The mutant strain UV-1 was developed and screened by the treatment of the conidial suspension of the parent strain EE-12, with ultra-violet irradiation (253nm and $1.6x10^2$ $J/M^2/S$) at a distance of 8cm for 10minutes (Mazhar *et al.*, 2003; Rao *et al.*, 2006). The treated cells were kept in the dark for 2hours to avoid recovery by photo reactivation. The fungi strains were stocked on Potato Dextrose Agar (PDA) slant at 4^0C in the refrigerator. All the culture media were sterilized in the autoclave at 15psi, 121^0C for 15minutes except otherwise stated.

The spore suspension (inoculum) as described by Jamal *et al.* (2005) was employed with slight modification. Cultures of the fungi isolates grown on PDA medium in petri dishes at 32^0C for 5days were transferred into Erlenmeyer flask (250ml) containing 100ml of sterile distilled water. The flasks were incubated on a shaker incubator at 200rpm for 24 hours. Thereafter, the cultures were decanted and the supernatant was used as inoculum after measuring its strength ($1.3x10^6$spores/ml) using Haemocytometer.

Fermentation technique

The methodology and fermentation medium composition were drawn from Yigitoglu (1992), Haq *et al.* (2001) and Kamzolova *et al.* (2011). Two different fermentation media were used. The first contained (% w/v) Sucrose 15, KH_2PO_4 0.1, $MgSO_4.7H_2O$ 0.025, NH_4NO_3 3.25 and pH 3.5. In the second medium Sucrose was replaced with Fructose. Fermentation was carried out in 1000ml Erlenmeyer flask containing 150ml of fermentation medium inoculated with 2% (v/v) (3ml) spore suspension in sterile distilled water. Flasks were incubated in the rotary incubator shaker (200rpm) at room temperature (28 ± 2^0C) for 144 hours. All the experiments were run in triplicates. Sample aliquots were withdrawn at intervals of 12 hours and used for Citric acid production, sugar (Fructose or Sucrose) consumption and Biomass estimations.

Assay methods

Samples were taken from the fermentation medium and centrifuged at 5000rpm for 20 minutes at room temperature. The clear supernatant was used for estimation. The amount of citric acid in the sample was estimated spectrophotometrically, using Pyridine–Acetic anhydride method (Marrier and Boulet, 1958; Kishore *et al.*, 2008). Sucrose in the fermentation sample was estimated by Dinitrosalicylic acid (DNS) method described by Kishore *et al.* (2008). Fructose in the supernatant was measured by the adopted Seliwanoff's method described by Ferreira *et al.* (2010). For biomass estimation, the modified methods of Alben and Erkmen (2004) and Kishore *et al.* (2008) was used. The sample was filtered through a pre-weighted whatman filter paper. After filtration, it was dried in an oven at 105^0C to constant mass, cooled in a desiccator and thereafter the final weight was measured. The difference between initial and final weight was the biomass.

Kinetic analysis of cell growth and citric acid production

Kinetic parameters of batch fermentation process were determined, adopting the procedures reported by Mazhar *et al.* (2003) and Yalcin *et al.* (2009a&b). Product and growth yield coefficients ($Y_{CA/S}$, $Y_{CA/F}$, $Y_{CA/X}$, $Y_{X/S}$, $Y_{X/F}$) and volumetric rates (Q_F, Q_S, Q_{CA}, Q_X) were determined.

Parameters: $Y_{CA/S} = Y_{CA/F}$ = g citric acid produced / g substrate consumed, $Y_{CA/X}$ = g citric acid produced / g cell formed, $Y_{X/S} = Y_{X/F}$ = g cell formed / g substrate consumed, $Q_F = Q_S$ = g substrate consumed / L / h, Q_{CA} = g citric acid produced / L / h, Q_X = g cell formed / L / h.

Where: CA = Citric acid, X = Biomass, S = Sucrose (substrate), F = Fructose (substrate).

Statistical analysis

The results indicate the mean ± standard deviation of three separate trials where three independent cultures were used in the experimental design. The findings were statistically compared using the student's t-test (SPSS - 16.0 and Microsoft Excel 2007).

Result and Discussion

The ability of a novel *A. niger* EE-12 and its ultra violet radiation improved strain, *A. niger* UV-1 to produce citric acid from sugar based mineral salt media in shake flasks were studied. Citric acid production was carried out in a batch submerged fermentation at 30^oC and initial pH 3.5 for 144 hours with 15% initial sugar concentration in a 250ml shake flasks at 200rpm. Advantages of submerged technique include the possible use of wide range of substrates and better control of fermentation. In addition, it is less labour intensive, gives a higher production rate and uses less space (Pazouki *et al.*, 2000). Nevertheless, the optimum time of incubation for maximum citric acid production varies both with the organism and fermentation conditions (Kubicek, 1998 and Ali *et al.*, 2002). Mineral salt medium is a synthetic one that

normally contains a carbon source, a nitrogen source, phosphorus and magnesium source. The concentrations of all these elements have a profound effect on the yield of citric acid (Ali *et al*., 2001, Haq *et al*., 2001). Thus, the optimal citric acid production depends on the type of fermentation media used (Singh *et al*., 1998).

During the fermentation period, variations in citric acid concentration, sugar consumed (residual) and dry biomass of the strains were determined at specific time intervals for both of the substrates (Sucrose and fructose) as shown in figures 1 and 2. Overall, citric acid production started after a lag phase of about 12 – 24 hours and reached maximum within and at the onset of stationary phase. This is in perfect alliance with the report of Rajoka *et al*. (1998). An inverse relationship between citric acid production and the consumption of sugar was observed and this is in agreement with the report of Kareem *et al*. (2010). Also, the initial sugar concentration decreased throughout the fermentation period for both strain on sucrose and fructose salt media. Citric acid production was significantly different ($p < 0.05$) in fig. 1 after 144hours while it was not in fig. 2. Strain UV-1 had the highest sugar consumption of 134.2±0.2g/l and 94.0±0.8g/l on fructose and sucrose salt media respectively after 144 hours while the parent strain had 91.0±0.8g/l and 72.1±0.2g/l respectively. Though there was a steady increase in dry biomass throughout the fermentation period, strain UV-1 equally had the highest biomass formation of 15.0±0.1g/l and 12.±0.1g/l on sucrose and fructose salt media respectively. This indicated that the cells were still viable. Thus, it suggests a link between storage of carbon, biomass formation and production of citric acid (Alben and Erkem, 2004). Kishore *et al*. (2008) reported a similar result in their work. Comparatively, biomass formation by strain UV-1 and its parent EE-12 strain on sucrose and fructose salt media were significantly not different (Table 2).

Citric acid production by *A. niger* EE-12 (parent) and its improved UV-1 strain on sucrose salt medium were significantly different ($p < 0.05$) while they had no significant difference ($p > 0.05$) on fructose salt medium. The citric acid produced by strain UV-1 was significantly higher ($p < 0.05$) than the level produced by EE-12 after 144 hours of growth (Table 1) on sucrose salt medium. The improved strain produced 1.14~fold higher citric acid level than its parent strain after 120 and 144 hours of growth on sucrose salt medium, with the difference in citric acid production being statistically significant ($p < 0.05$). Furthermore, citric acid productions by both strains on sucrose salt medium were significantly higher ($p < 0.05$) than the concentration produced on fructose salt medium. The parent strain produced 1.6~fold higher citric acid on sucrose salt medium than on fructose salt media while strain UV-1 produced 1.9~fold higher citric acid on sucrose salt medium against fructose salt medium. Strain UV-1 gave 13% improvement for citric acid production on sucrose salt medium. Mazhar *et al.* (2003) carried out mutational studies using *A. niger* and reported 15 – 22% improvement in citric acid production. So, the results obtained with our UV irradiated fungi isolate gave a lower improvement. This could be attributed to the fact that Mazhar *et al.* (2003) reported their best results after a combination of strain and process optimization. However, UV-irradiated DNA is unstable and recovers due to photo reactivation (Haq *et al*., 2001).

The utilization of the substrate in cells concerns the consumption of substrate for growth, maintenance and product formation, thus its kinetics was considered. Different kinetic parameters such as product and growth yield coefficients ($Y_{CA/S}$, $Y_{CA/F}$, $Y_{CA/X}$, $Y_{X/S}$, $Y_{X/F}$) and volumetric rates (Q_F, Q_S, Q_{CA}, Q_X) were studied and compared for each of the test isolates based on the media used (figures 3 – 6). Comparatively, the values for product and growth yield coefficients of the parent strain EE-12 and its improved UV-1 strain on sucrose salt medium were significantly higher than on fructose salt medium ($p < 0.05$). The above result could be as a result of higher preference for glucose component of sucrose by *A. niger* (Waites *et al*., 2001). Also, volumetric rate of citric acid production on sucrose salt medium are significantly higher than on fructose salt medium ($p < 0.05$). In figure 3, the volumetric rate of substrate consumption (Q_F and Q_S) were not statistically different after 144hours but were significantly different ($p<0.05$) after 24hours. This is the early exponential phase thus there is a high need for carbon source thereby resulting to rapid breakdown of sucrose into readily utilizable glucose or fructose. Equally, at the prevailing pH of the fermentation medium acid hydrolysis of sucrose could have contributed to the result. The volumetric rate of citric acid production (Q_{CA}) and the mass yield (Y_{CA}) of *A. niger* UV-1 and its parent strain EE-12 are comparable to those reported in the literature for citrate producing strains (Rajoka *et al*., 1998 and Mazhar *et al*., 2003). Biomass yield coefficients ($Y_{X/S}$, $Y_{X/F}$) and product yield coefficients ($Y_{CA/S}$, $Y_{CA/F}$); an index of the efficiency of conversion of a substrate into biomass and product respectively, as shown in figures 5 & 6 are considerably moderate. However, organisms may exhibit different yield coefficients for the same substrate, due primarily to the pathway by which the compound is metabolized (Waites *et al*., 2001).

Conclusion

This work confirmed the growth and citric acid production characteristics of two *A. niger* strains (EE-12 and UV-1) using mineral salt media containing sucrose or fructose as its carbon source. This showed the difference in citric acid production capabilities of the strains in these media, revealing that sucrose is a better carbon source for citric acid production. Natural sources of this sugar are therefore promising substrates for citric acid production processes. Citric acid production of the improved UV-1 strain was higher than that of its parent EE-12 strain. The data for kinetic parameters give an idea about optimizing the culture conditions in a possible scale-up of citric acid production process. Undoubtedly, strain optimization and selection are important stages for citric acid production process; thus finding new efficient and improved strains for an industry is a veritable innovation. A domestic Nigerian strain with minimal optimization strategies was confirmed to be efficient for citric acid production. This can be geared towards local content development. The authors strongly suggest further optimization research on these fungi strains.

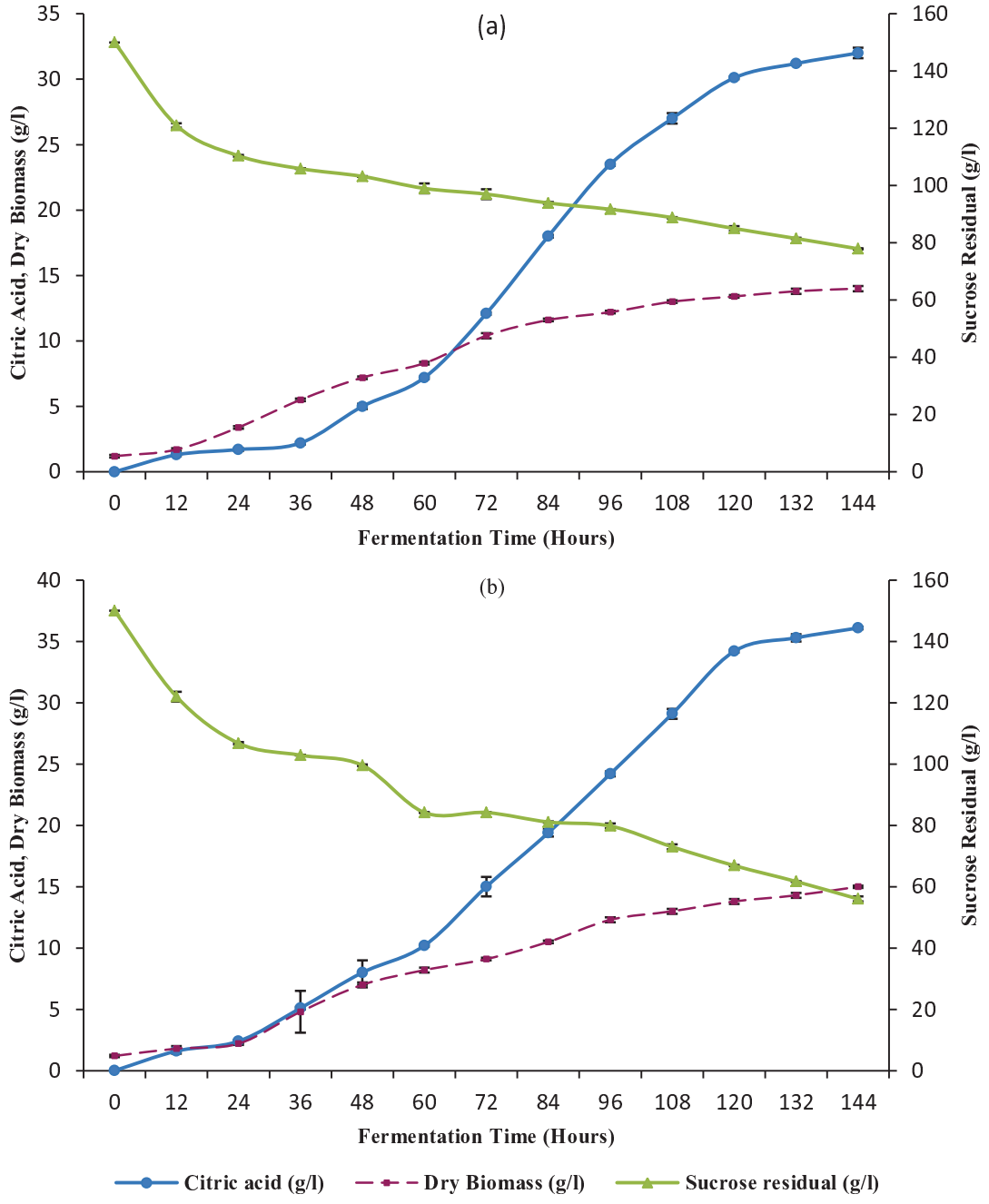

Fig. 1: Time course of Dry biomass, Sucrose consumption (Residual) and Citric acid production by *A. niger* EE-12 (a) and *A. niger* UV-1 (b) grown on Sucrose Salt medium. Y error bars indicate the standard deviation of the triplicates.

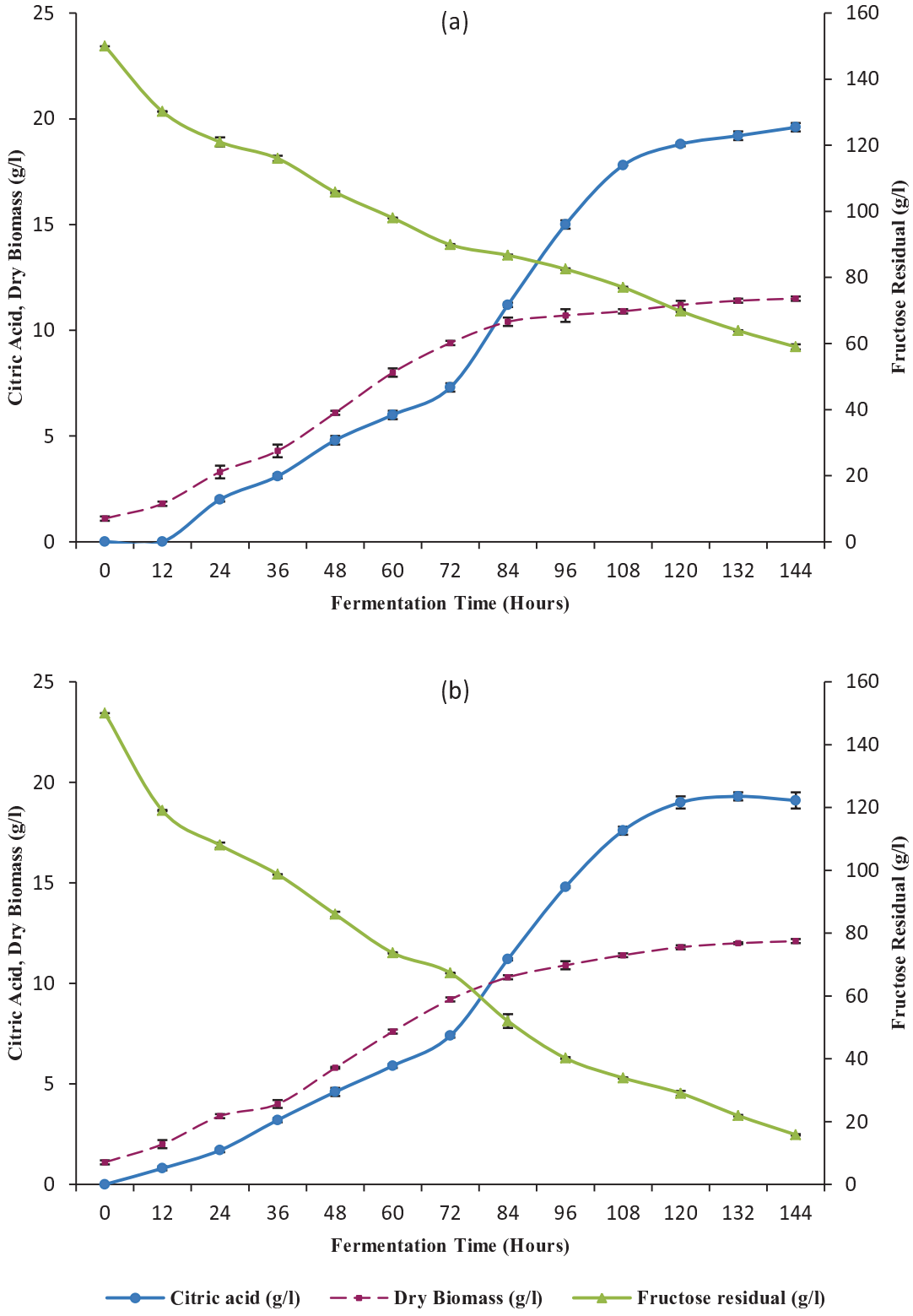

Fig. 2: Time course of Dry biomass, Fructose consumption (Residual) and Citric acid production by *A. niger* EE-12 (a) and *A. niger* UV-1 (b) grown on Fructose Salt medium. Y error bars indicate the standard deviation of the triplicates.

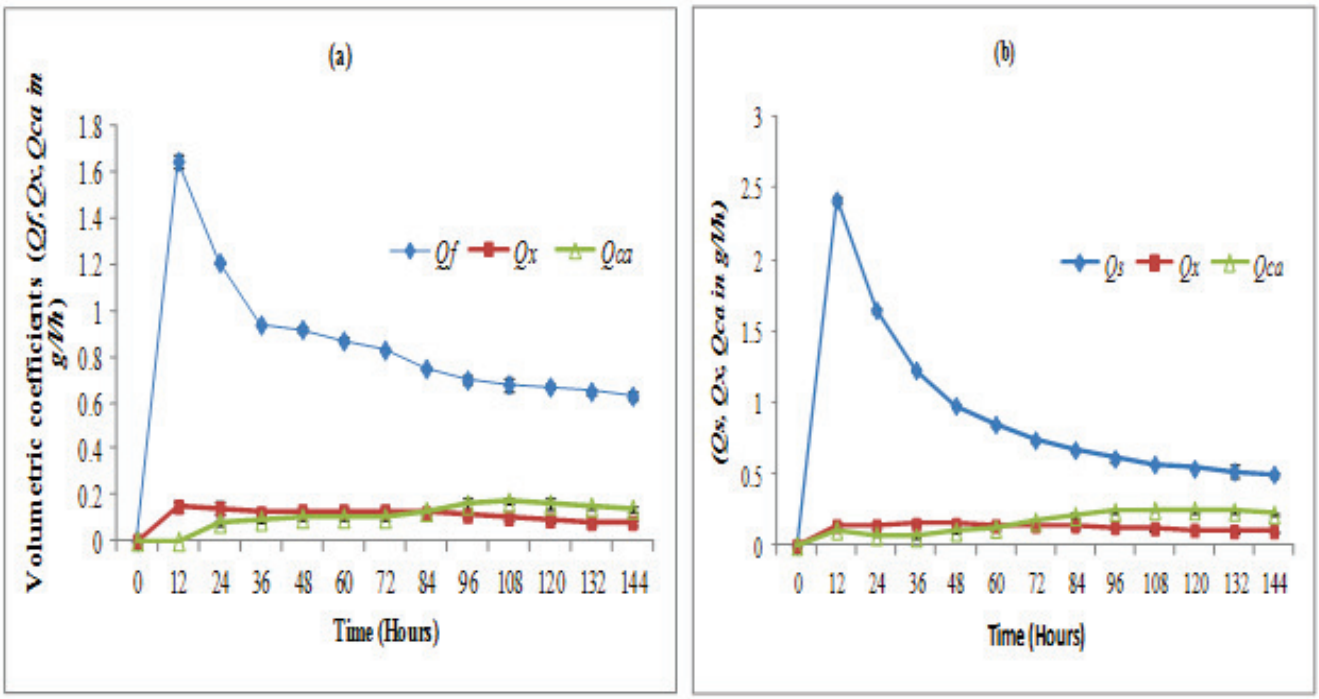

Fig. 3: Comparison of volumetric coefficients for citric acid production by *A. niger* EE-12 on Fructose salt medium (a) and Sucrose salt medium (b). Values for volumetric rate of substrate consumption (Q_F and Q_S) are not statistically different ($P > 0.05$) while values for volumetric rate of biomass formation and citric acid production (Q_X and Q_{CA}) are significantly different ($P > 0.05$) respectively.

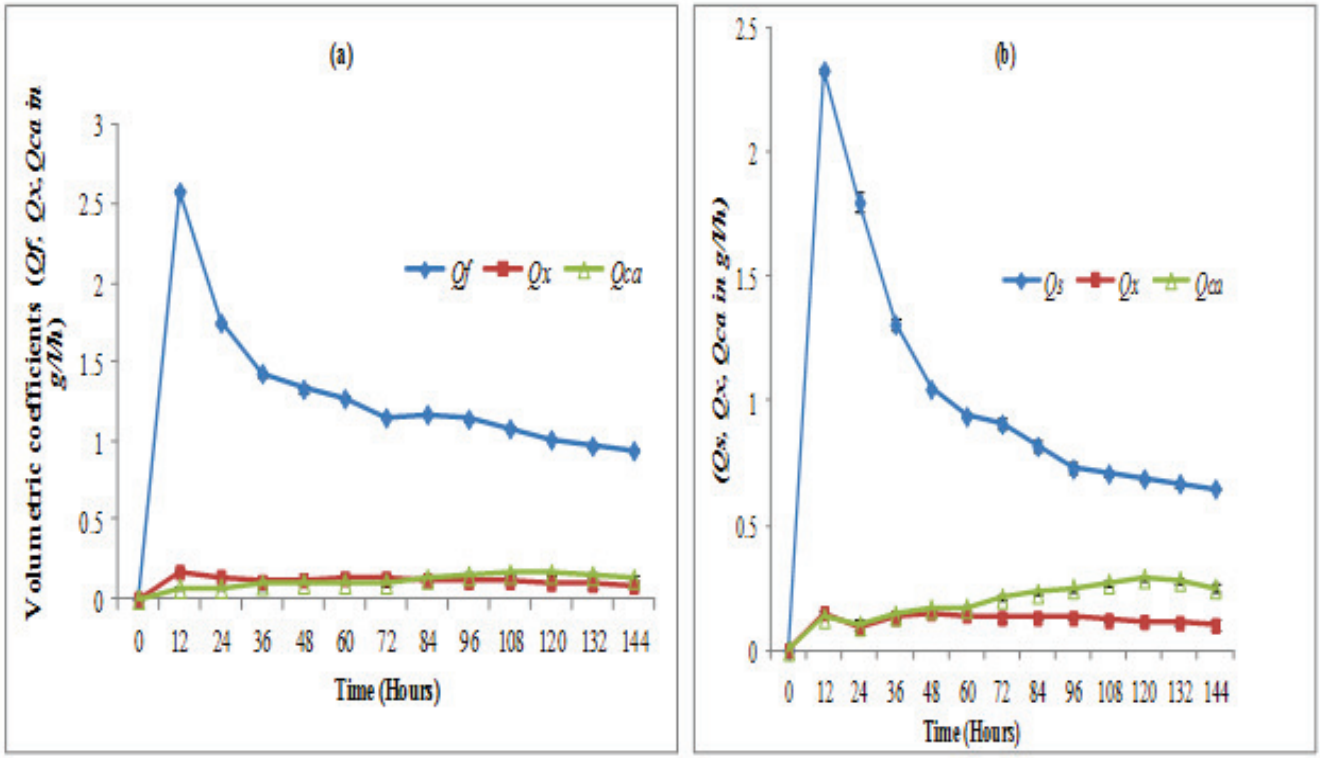

Fig. 4: Comparison of volumetric coefficients for citric acid production by *A. niger* UV-1 on Fructose salt media (a) and Sucrose salt media (b). Values for volumetric rate of substrate consumption (Q_F and Q_S) and citric acid production (Q_{CA}) are significantly different ($P < 0.05$) while values for volumetric rate of biomass formation (Q_X) are not statistically different ($P > 0.05$).

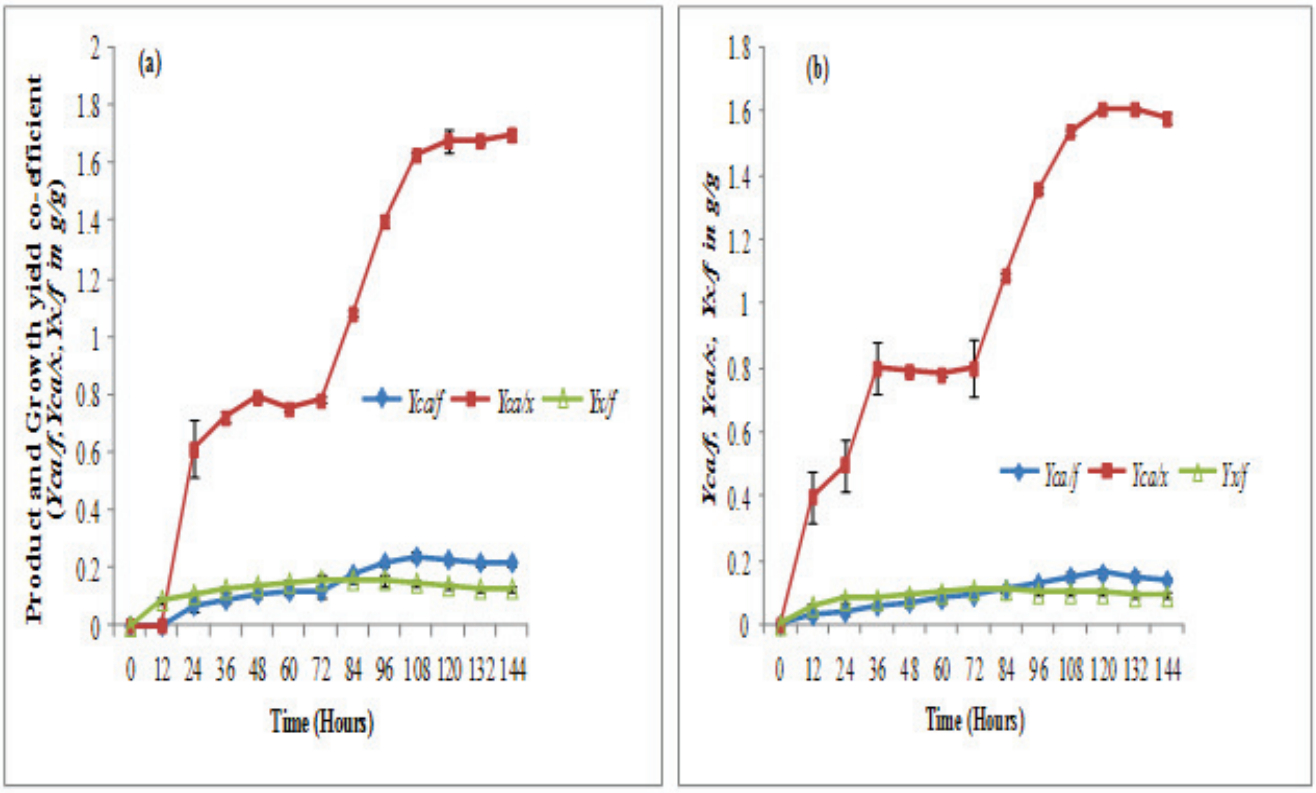

Fig. 5: Comparison of product and growth yield coefficients for citric acid production by *A. niger* EE-12 (a) and *A. niger* UV-1 (b) on Fructose salt medium. The values for *Yca/f* and *Yx/f* are significantly different ($p < 0.05$) respectively while values for *Yca/x* are not significant ($p > 0.05$).

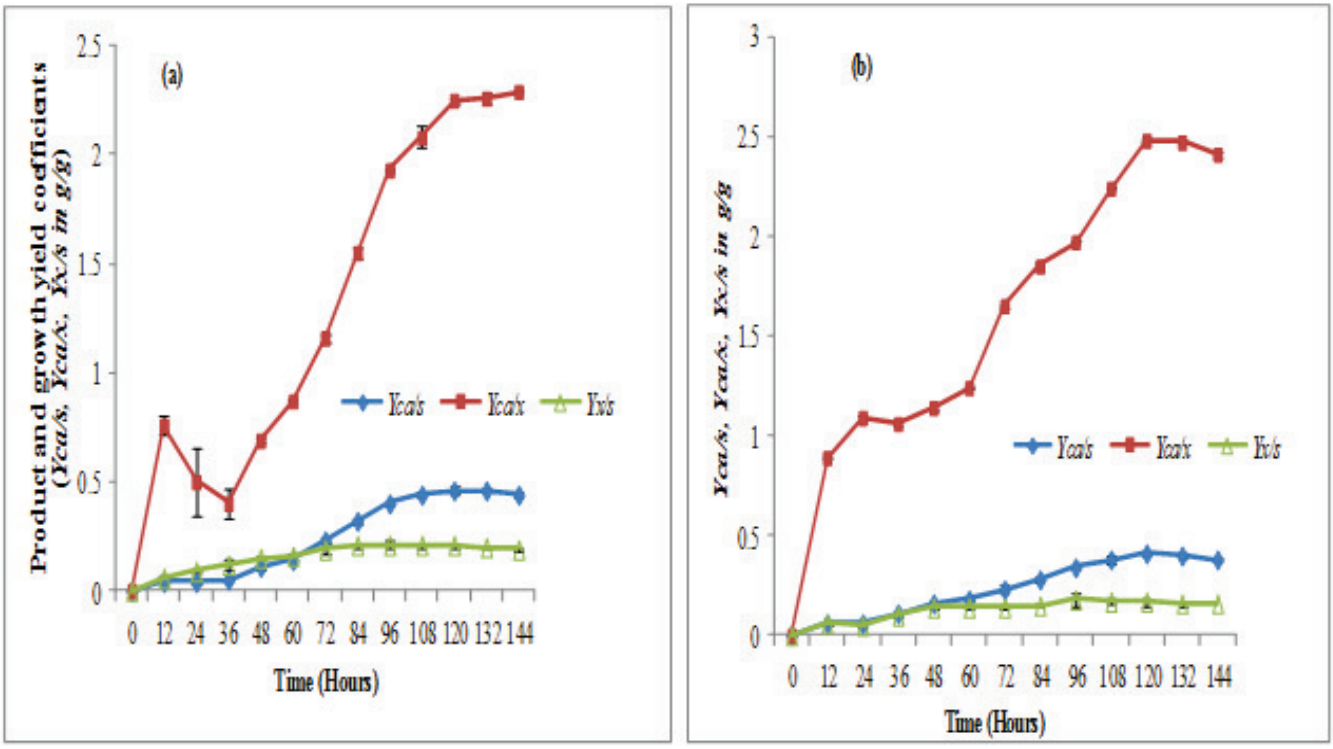

Fig. 6: Comparison of product and growth yield coefficients for citric acid production by *A. niger* EE-12 (a) and *A. niger* UV-1 (b) on Sucrose salt medium. The values for *Yca/x* and *Yx/s* are significant ($p < 0.05$) respectively while values for *Yca/s* are not significant ($p > 0.05$).

Table 1: Comparison of citric acid production between *A. niger* EE-12 and its improved (UV-1) strain after growth on two different fermentation media.

Fermentation time(h)	Citric acid concentration (gL^{-1})			
	Sucrose Salt Medium		Fructose salt medium	
	EE-12	UV-1	EE-12	UV-1
0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
24	1.7 ± 0.1^{a}	2.4 ± 0.1^{a}	2.0 ± 0.1^{a}	1.7 ± 0.1^{a}
48	5.0 ± 0.2^{a}	8.0 ± 1.0^{b}	4.8 ± 0.2^{a}	4.6 ± 0.2^{a}
72	12.1 ± 0.1^{a}	15.0 ± 0.8^{b}	7.3 ± 0.2^{a}	7.4 ± 0.1^{a}
96	23.5 ± 0.1^{a}	24.2 ± 0.2^{a}	15.0 ± 0.2^{a}	14.8 ± 0.05^{a}
120	30.1 ± 0.1^{a}	34.2 ± 0.1^{b}	18.8 ± 0.1^{a}	19.0 ± 0.3^{a}
144	32.0 ± 0.4^{a}	36.1 ± 0.1^{b}	19.6 ± 0.2^{a}	19.1 ± 0.4^{a}

Concentrations are mean of three independent set-up ± standard deviation, subscripts in the same row of data that have different letters are statistically different at $p< 0.05$ using student's t-test.

Table 2: Comparison of biomass formation by *A. niger* UV-1 (improved) and its parent (EE-12) strain grown on two different fermentation (sugar) media.

Fermentation time(h)	Dry biomass concentration (gL^{-1})			
	Sucrose Salt Medium		Fructose salt medium	
	EE-12	UV-1	EE-12	UV-1
0	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}
24	3.4 ± 0.1^{a}	2.2 ± 0.1^{a}	3.3 ± 0.3^{a}	3.4 ± 0.1^{a}
48	7.2 ± 0.1^{a}	7.0 ± 0.2^{a}	6.1 ± 0.1^{a}	5.8 ± 0.05^{a}
72	10.4 ± 0.2^{a}	9.1 ± 0.1^{a}	9.4 ± 0.1^{a}	9.2 ± 0.1^{a}
96	12.2 ± 0.1^{a}	12.3 ± 0.2^{a}	10.7 ± 0.3^{a}	10.9 ± 0.2^{a}
120	13.4 ± 0.1^{a}	13.8 ± 0.2^{a}	11.2 ± 0.2^{a}	11.8 ± 0.1^{a}
144	14.0 ± 0.2^{a}	15.0 ± 0.1^{a}	11.5 ± 0.1^{a}	12.1 ± 0.1^{a}

Concentrations are mean of three independent set-up ± standard deviation, subscripts in the same row of data that have a common letter are not statistically different at $p > 0.05$ using student's t-test.

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