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Full Length Research Paper

Solid state fermentation studies of citric acid production

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Treated sugarcane bagasse supplemented with sucrose medium was found 1.7 fold (citric acid based on sugar consumption) better substrate than untreated bagasse carrier. The performance of packed bed reactor at aeration rate of 0.75 l/min and apparent packing density of 35.0 g/l was superior with citric acid yield of 55.90% (w/w), overall productivity of 0.087 g/100 g DS.h and specific growth rate of 0.055 h^{-1}. However, in flask fermentation citric acid yield of 41.56% (w/w) with overall productivity of 0.064 g/100 gDS.h and specific growth rate of 0.043 h^{-1} was observed. The system confirmed that citric acid production was Type-II fermentation. Citric acid recovery of 90.39% (w/w) was achieved from fermented broth.

Key words: *Aspergillus niger*, kinetics, packed bed reactor, sugarcane bagasse.

INTRODUCTION

Citric acid, a tricarboxylic acid is widely used as an acidifying agent and antioxidant in food, beverages and pharmaceutical industries (Kapoor et al., 1982). Conventionally, it is produced by submerged fermentation using molasses as a raw material. In recent years, considerable interest has been shown in solid state production of citric acid by *Aspergillus niger* using agroresidues like bagasse, corncob, carob pod (Hang and Woodams, 1998; Roukas, 1999; Vandenrberghe et al., 2000) and waste of food processing industries like apple and grape pomace and fruit peel (Hang and Woddams, 1986, 1987b, 1988; Hang et al., 1987a; Shojaosadati and Babaeipour, 2002) due to its several advantages like solid waste management, biomass energy conservation, production of high value products and little risk of bacterial contamination (Roukas, 1999; Tran and Mitchell, 1995). However, only a few approaches to the design, operation and scale up of bioreactors (Mitchell et al., 2000) and their development for different industrial applications have been reported (Durand and Chereau, 1988; Durand et al., 1996) because of heterogeneous nature of the substrate, particularly due to the separation of the biomass from the residual substrate. Citric acid production in different solid state bioreactors like flasks, tray and drum have also been compared (Vandenberghe et al., 2004). Relationship between citric acid production and rate of respiration in *A. niger* have been studied in solid state fermentation (Pintado et al., 1998; Prado et al., 2004).

The objective of present work is firstly, to observe the effect of bagasse pretreatment and further to compare the kinetic data of flask bioreactor and packed bed bioreactor after optimizing the aeration rate and bed packing density. Finally, the procedure to recover citric acid from fermented broth is discussed.

MATERIALS AND METHODS

Inoculum

Spore suspension of *A. niger* DS1, reported by Kumar et al. (2003) with concentration of 2 x 10^7 spores/ml was prepared by adding 25

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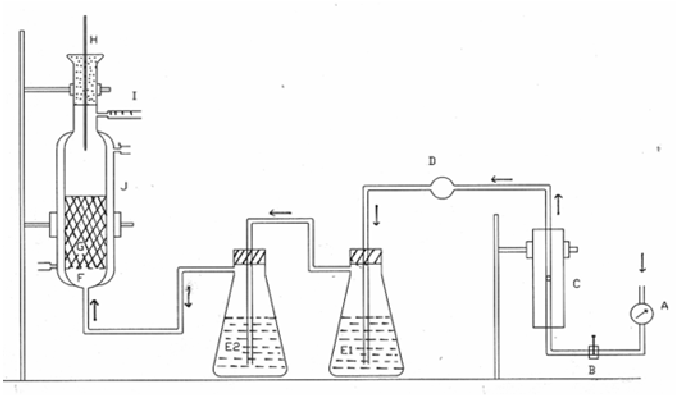

Figure 1. Schematic diagram showing packed bed bioreactor set up. A = Compressor, B = Gate Valve, C = Rotameter, D = PTEF Air Filter, E1 & E2 = Humidifying Bottles, F = Perforated Plate, G = Column Reactor, H = Beckman's Thermometer, I = Glass Wool Air Filter For Outlet of Exhaust Air, and J = Jacketed Wall of Reactor.

ml of sterile distilled water with Tween-80 (0.1%) on PDA slant and was shaken vigorously for a minute on vortex mixer. It was suitably diluted to obtain a spore concentration of 2 x 10^7 spores/ml.

Substrate

The carrier, sugarcane bagasse for solid state fermentation was procured from National Sugar Institute (NSI), Kanpur, India. It was dried in oven, ground and screened to collect the particle size of 1.2 - 1.6 mm. The carrier was treated overnight with 2 N HCl at room temperature and washed thoroughly with distilled water to get neutral washing, further dried and

Fermentation

Solid state fermentation in flasks was carried out using bagasse (treated/ untreated) with particle size of 1.2 - 1.6 mm, moistened with sucrose medium (g/l) (sucrose, 310; NH_4NO_3, 25; $MgSO_4.7H_2O$, 2.5; $CuSO_4$, 0.04 at pH 4.0), methanol 4% (v/w) and inoculum of concentration 2 x 10^7 spores/ml in a way so that the moisture level of 75% (v/w) could be sustained in the system. The flasks were incubated at 30°C maintaining humidity level inside the stationary incubator. One flask was harvested at alternate days for the estimation of residual sugar, citric acid and biomass concentration. Sampling was continued till almost all the sugar of the medium was consumed.

A jacketed glass bioreactor (4.5 cm diameter x 15 cm long) was fabricated with a provision to supply humidified air from the bottom. Treated bagasse supplemented with sucrose medium (TRBS) was prepared like above for using as substrate and inoculated with spore suspension sustaining 75% (v/w) moisture. It was transferred to the bioreactor with apparent packing density of 30, 35, 40 and 45 g/l. Aeration rate of 0.25, 0.75, 1.25 and 1.75 l/min was varied at different packing densities to optimize the PBR operating conditions. The incubation temperature of 30°C was maintained by circulation of water through the jacket of reactor and passing humidified air from the bottom at the desired aeration rate. The set up of packed bed reactor is shown in Figure 1. The column reactor was run in batch mode and was harvested at appropriate time intervals for further estimations.

Analytical techniques

The harvested fermented mass was dried at 90°C up to constant weight and designated as dry fermented mass (DFM). It was extracted three times with 100 ml of distilled water at 40°C and 300 rpm for 30 min so as to separate almost all the mycelia from the support. It was then filtered through muslin cloth, dried in oven up to constant weight and designated as dry solid residue (DSR). The filtrate (consisting of biomass, sugar and citric acid) was centrifuged at 6000 rpm for 15 min. The supernatant was used to estimate residual sugar using phenol-sulfuric acid method (Dubois et al., 1956) and the citric acid was analyzed by Reinart and Neisbitt Method (Reinart and Neisbitt, 1957). The weight of biomass was estimated by overall mass balance (Lu et al., 1995). The concentrations were reported on the basis of g/100gDS (dry substrate) and the yield of citric acid was reported in percentage (w/w) on the basis of sugar consumed.

Weight of biomass = weight of DFM – (DSR + Citric acid + Residual sugar)

The results reported are average values of the experiments conducted in triplicates with deviation of ± 1.0%.

Recovery of citric acid from fermented broth

Measured centrifuged supernatant volume of DFM having maximum citric acid yield was neutralized with NaOH and precipitated by dosing $CaCl_2$ at 90°C. The precipitated calcium citrate was filtered, dried and weighed. This filtrate was then dissolved in minimum distilled water and precipitated as $CaSO_4$ by adding concentrate H_2SO_4 of specific gravity 1.84. $CaSO_4$ was filtered and filtrate containing citric acid was further crystallized, re-crystallized, dried and weighed.

RESULTS AND DISCUSSION

Effect of pretreatment of bagasse

The comparative yields of citric acid production for bagasse and treated bagasse are shown in Table 1. The maximum citric acid production with untreated (UTRBS) and treated bagasse (TRBS) was 24.62% (w/w) and 41.56% (w/w) respectively on the basis of sugar consumed after 8 days of fermentation, which was 1.7-fold higher as compared to untreated carrier.

It appeared that high order of molecular packing of celluloses and hemicelluloses in crystalline regions of bagasse limited the growth of fungi and hydrolytic reactions only to the external surface of crystallites. Pretreatment increased susceptibility of cellulose by its structural modification; like disrupting lignin-carbohydrate complex and high order cellulose structures to increase the amenability of substrate to the microbial growth (Fan et al., 1982; Garg and Sharma, 1991). Based on the above results the treated bagasse supplemented with sucrose medium was selected for further studies.

Kinetic studies in flask using TRBS

Citric acid, residual sugar and biomass concentrations at

Table 1. Yield data showing citric acid production and microbial growth in flask experiments and packed bed reactor.

No. of days	Flask experiments				Packed bed reactor (treated bagasse as carrier)	
	Untreated bagasse as carrier		Treated bagasse as cCarrier			
	Yp/s	Yx/s	Yp/s	Yx/s	Yp/s	Yx/s
0	0	Negligible	0	Negligible	0	Negligible
2	4.14	2.76	4.70	2.40	6.75	3.08
4	7.89	6.01	18.54	6.97	23.51	11.56
6	20.77	4.58	35.87	6.47	45.52	9.66
8	24.62	4.24	41.56	6.02	55.90	8.77
10	24.39	4.79	40.98	6.12	55.06	8.97

Yp/s: citric acid g/100 g of sugar consumed, and Yx/s: biomass produced g/100 g of sugar consumed.

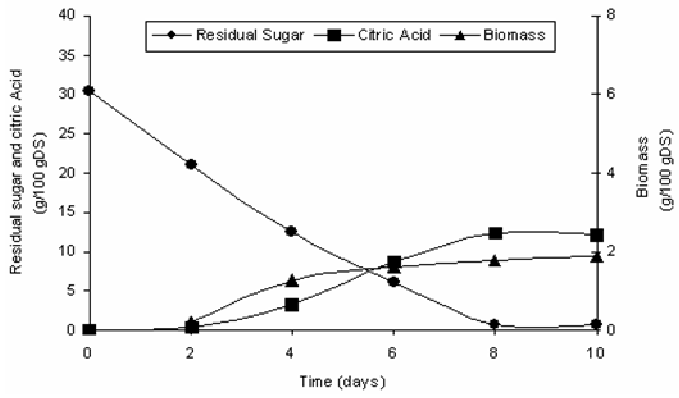

Figure 2. Microbial growth and citric acid production on treated bagasse supplemented by sucrose medium (TRBS) during solid state flask fermentation.

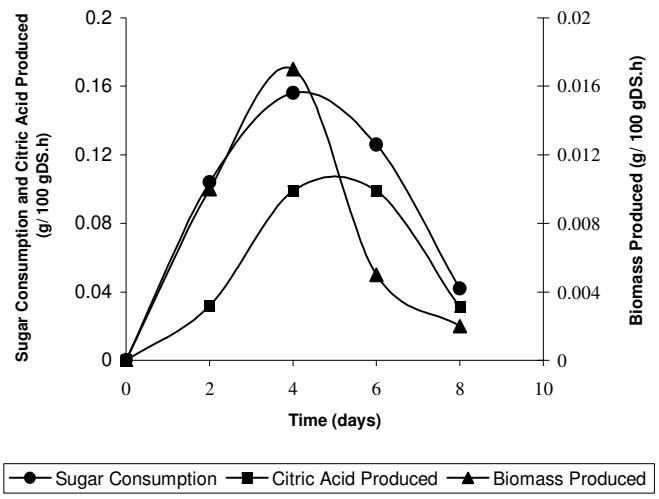

Figure 3. Kinetic rate data showing microbial growth, sugar consumption and citric acid production on TRBS in Solid State Flask Fermentation.

various time intervals during solid state fermentation of treated bagasse are shown in Figure 2. These data were used to calculate yield constants for citric acid and biomass production (Yp/s, Yx/s) as shown in Table 1. Figure 2 showed some lapse in microbial growth and product formation that indicated kinetically the citric acid fermentation came under Type-II fermentation according to the classification proposed by Gaden (Garg and Sharma, 1991; Aiba et al., 1973). In the 1st phase, growth was accompanied by minimal product formation while in 2nd phase, product formation reached at its maximum but growth rate was low. The maximum citric acid concentration of 12.35 g/100 gDS (DS: dry substrate) was obtained after 8 days of fermentation, while the biomass yield was maximum 6.97% (w/w) on 4th day of fermentation. The overall productivity of the process was 0.064 g/100 gDS.h. The sugar was almost consumed at the end of fermentation. Figure 3 showed the kinetics during the fermentation process expressed on the basis of original dry weight of treated bagasse supplemented with sucrose medium. The rate of citric acid and biomass production was found maximum on 4th day (0.099 and 0.017 g/100 gDS.h). However, the rate of biomass production decreases drastically on further fermentation, while the rate of citric acid production remains constant up to 6th day to obtain maximum yield at 8th day. This agrees with the flask experiments performed on kumara by Lu et al. (1995). Figure 4 showed the specific rate data expressed on the basis of dry weight of fungal biomass. The maximum observed specific rate data of citric acid production (q_p) was 139.13 mg/gDB.h (DB: dry biomass) with specific growth rate (μ) of 0.043 h^{-1} on 2nd day of fermentation, while the volumetric rate of citric acid production was maximum from 4th to 6th day of fermentation. This further confirms that the citric acid production falls under Type-II fermentation.

Optimization of aeration rate and packing density

Figure 5 showed that the maximum citric acid concentration of 16.76 g/100 gDS was obtained at aeration rate

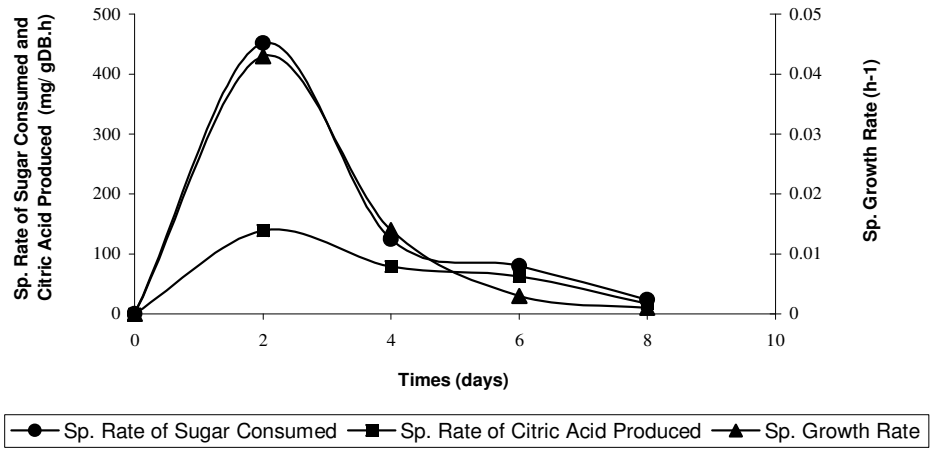

Figure 4. Specific rate data showing microbial growth, sugar consumption and citric acid production on TRBS in solid state flask fermentation.

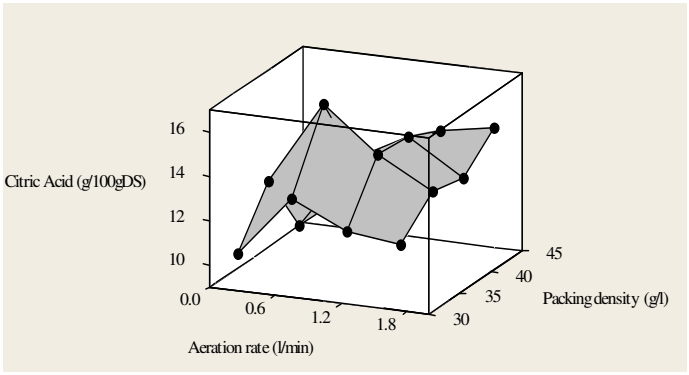

Figure 5. Surface plot of aeration and packing density on citric acid production in packed bed reactor.

of 0.75 l/min and packing density of 35 g/l. It was evident that these parameters influenced citric acid production due to proper heat and mass transfer. Although, aeration with low bed density enhanced the citric acid yield but higher aeration rate with excessive voidages inhibited its production due to shear stress in the packed bed. This had harmful effect on filamentous fungus morphology and channeling of packed bed. Similarly, high packing density restricted the airflow and inhibited the fungal growth. A minimum aeration was required so that the metabolic activity of the filamentous fungi was not affected. Comparable results were obtained for protein enrichment of lignocellulosic substrate and citric acid production from apple pomace in multi layer packed bed bioreactor (Shojaosadati, 1999; Shojaosadati and Babaeipour, 2002).

Kinetic analysis in packed bed reactor

The kinetic data of bioreactor using aeration rate of 0.75 l/min with apparent packing density of 35 g/l for various

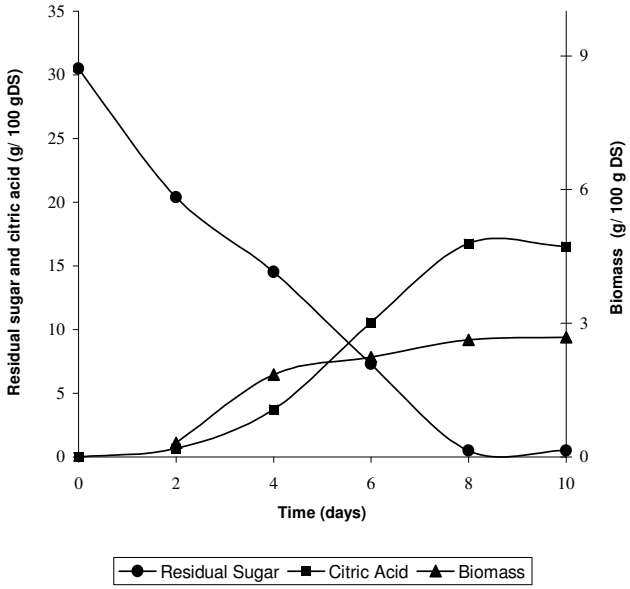

Figure 6. Microbial growth and citric acid production on TRBS in packed bed reactor.

time intervals are shown in Figure 6. The maximum citric acid concentration achieved was 16.76 g/100 gDS with a yield of 55.90% (w/w) after 8th day of fermentation. The remaining sugar concentration was almost negligible. The yield of biomass (Yx/s = 11.56% w/w) was maximum on 4th day of fermentation. The overall productivity of the system was found 0.087 g/100 gDS.h. Figure 7 showed the kinetic rate data during fermentation on the basis of original dry weight of treated bagasse supplemented with sucrose medium. The rate of biomass production (0.020 g/100 gDS.h) was highest on 4th day of fermentation and decreases drastically on further fermentation, while the rate of citric acid production (0.130 g/100 gDS.h) was

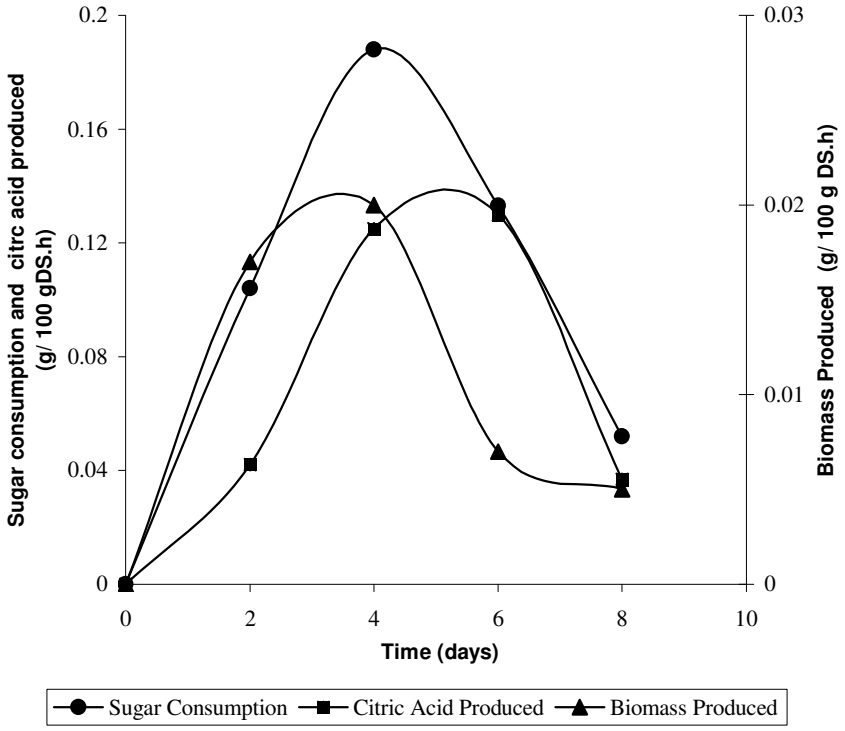

Figure 7. Kinetic rate data showing microbial growth, sugar consumption and citric acid production on TRBS in packed bed reactor.

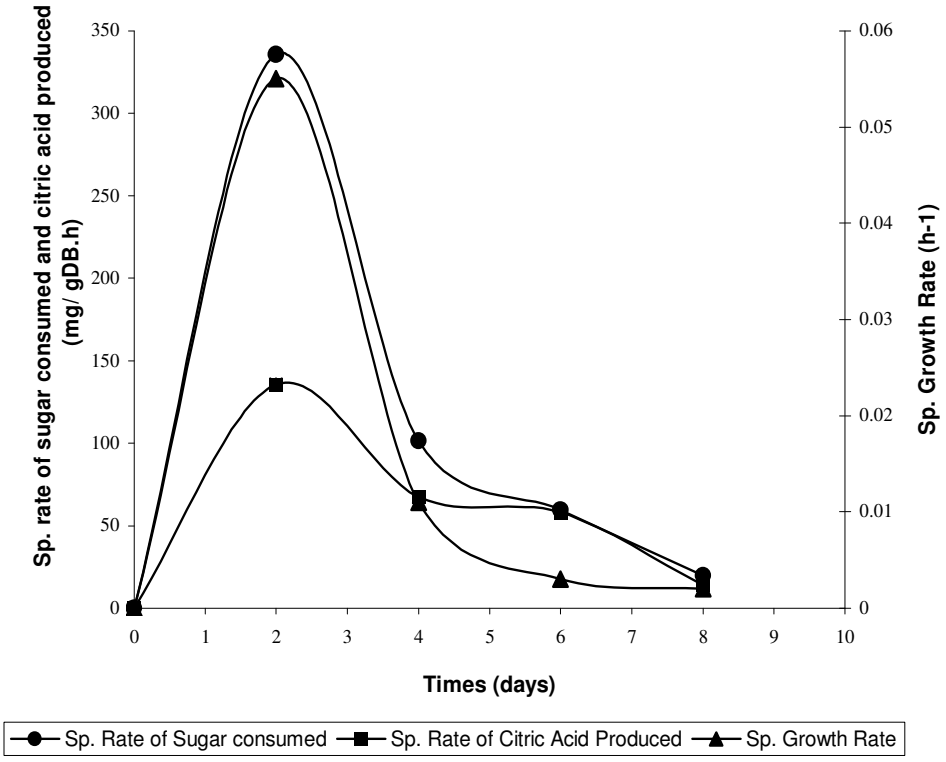

Figure 8. Specific rate data showing microbial growth, sugar consumption and citric acid production on TRBS in packed bed reactor.

Table 2. Comparison of results from the previous literature.

Substrate	Yield (g/100 gDS)	Overall Productivity (g/100gDS.h) or (g/100ml.h)	Maximum Overall Productivity (mg/gDB.h)	Sp. Growth Rate (h^{-1})	Reference
Kiwi fruit peel	>60	0.104	---	---	Hang and Woodams (1987)
Apple pomace (PBR)	---	0.108	---	---	Hang and Woodams (1988)
Kumara	58	0.048 *	122	0.042	Lu et al (1995)
Amberlite (PBR)	---	0.0135	---	---	Rojas et al (1995)
Cane bagasse (PBR)	14.6	---	---	---	Vandenberghe et al (2000)
Cassava bagasse (PBR)	20.6	---	---	---	ibid
Apple pomace (PBR)	80	---	u---	---	Shojaosadati and Babaeipour (2002)
Cane bagasse	41.56	0.064	139.13	0.043	Present work
Cane bagasse (PBR)	55.90	0.087	135.48	0.055	Present work

*Wet weight basis.

maximum after 4th day of fermentation (0.125 g/100 gDS.h). Figure 8 showed the specific rate expressed on the basis of dry weight of fungal biomass. It revealed that the maximum specific rate of citric acid production was 135.48 mg/gDB.h with specific growth rate of 0.055 h^{-1} on 2nd day of fermentation, while the volumetric rate of citric acid production was significantly higher on 4th and 6th day of fermentation. These results showed that the trend of kinetic growth was similar with flask fermentation with improved kinetic data and further confirmed that the product fermentation started after the sufficient growth had taken place (Garg and Sharma, 1991).

Recovery of citric acid from fermented broth

Measured centrifuged supernatant volume of DFM having maximum citric acid yield was used to recover citric acid.

Weight of DFM (PBR) = 18.02 g.
Weight of citric acid obtained after recovery = 2.73 g.
Corresponding weight of citric acid/100-g DS (from graph based on fermented solution) = 15.15 g/100 g DS.
Weight of citric acid calculated by the quantitative estimation = 16.76 g/ 100 g DS.
Recovery (%) of citric acid from the fermented broth= (15.15 x 100)/ 16.76 = 90.39% (w/w).

Conclusion

Packed bed reactor fermentation has more kinetic potential for citric acid production when compared to simple flask fermentation. Aeration and packing density of packed bed reactor not only helps in heat and mass transfer but also assists in removal of metabolic heat to provide proper environment for microbial growth, resulting in higher product yield. More than 90% recovery of the product validates that the process can be explored for commercialization after sufficient scale up studies. The present data are compared with some previous data of solid state fermentation and are shown in Table 2. Further, researches on separation of other organic acids produced during fermentation and scale up studies for the development of suitable bioreactor are needed for its commercialization.

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