

A Comparative Study of Kinetics of Itaconic Acid Production Using Four Species of the Genus *Aspergillus*

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Abstract

Itaconic acid, used as a co-monomer at a level of 1-5% for certain polymer products, is an important constituent in the fabrication of synthetic fibres, coatings, adhesives, thickeners and binders. As there is no indigenous commercial production of itaconic acid, hitherto, in India, the present study was attempted to explore the kinetics for optimal production of itaconic acid by indigenous process from the four fungal sources viz., *Aspergillus niger*, *A. terreus*, *A. nidulans* and *A. flavus*. Our experimental observations in batch cultures of the above four species revealed that the specific growth rate (μ_{max}), the yield factor ($Y_{x/s}$) for the cell mass and the yield factor for product ($Y_{p/x}$) were maximum for *A. terreus* and were found to be 0.04199 hr^{-1} , 0.4976 g/g and 0.4387 g/g respectively. Also, the doubling time for *A. terreus* was found to be minimum, 14.84 hr as compared to other three species of the genus *Aspergillus*.

Keywords: Itaconic acid, *Aspergillus* sps., Specific growth rate, Doubling time, Yield factor, Immobilization etc.

Introduction

Molasses, a by-product of sugar industry is a very convenient raw material for itaconic acid production. *Aspergillus terreus* is one of the patented micro-organisms

reported to utilize molasses as a carbon source (Kane et al., 1945^[1]). Several studies have been conducted on the influence and regulation by this substance during the production process (Lockwood and Reeves, 1945^[2]; Batti and Schweiger, 1963^[3]; Roehr and Kubicek, 1996^[4]). One of the most productive processes reported is the one by the Pfizer Company (Nubel and Ratajak, 1964^[5]) which involved submerged fermentation process using suspended *A. terreus* biomass inoculated as spores on pretreated molasses. During the initial growth phase the original pH (5.0) was reported to drop down to less than 3. The second phase is characterized by phosphate-limited growth and increased production of itaconic acid, which is substantially free from other organic acids. The cane molasses and cane molasses stillage are rich in aconitic and itaconic acids. This fermentation process, regardless of the sugar source, produces a number of additional co-products such as carbon dioxide, glycerin, succinic acid and their production rate can be manipulated by the choice of the strain and operating conditions. In addition, both beet and cane molasses are a rich source of potassium sulfate which is the main component of molasses ash. The technology is now available for commercialization to recover these compounds.

Many theories were proposed regarding the biosynthesis of itaconic acid using fungi (Kinoshita, 1932^[6]; Eimhjellen and Larsen, 1955^[7]; Shimi et al., 1962^[8]; Jakubowska, 1977^[9]). However, according to Kinoshita (1932)^[6], the main route of production is via glycolysis and tricarboxylic acid cycle (Bentley and Thiessen, 1957a^[10], 1957b^[11]; Winskill, 1983^[12]; Bonnarme et al., 1995^[13]). Thus citric acid and aconitic acid are the intermediates of the process and itaconic acid is formed from the latter by enzymatic decarboxylation (Ducrocq et al., 1995^[14]). As kinetic studies are essential for production of any industrially important compounds on commercial scale, current investigation which included determination of specific growth rate, yield factors for the biomass and the product, time required for optimal yield, was undertaken.

Materials & Methods

The four fungal species used in the present study viz., *A. niger* (MTCC, 872), *A. terreus* (MTCC, 479), *A. nidulans* (MTCC, 818) and *A. flavus* (MTCC, 871) were procured from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. Czpek Dox medium was used for culturing and maintaining all the four species of the genus *Aspergillus*. The production medium contained Molasses (10%, v/v), NH₄Cl (0.25% w/v), MgSO₄ (0.095%, w/v), KH₂PO₄ (0.0088%, w/v), CuSO₄ (0.0004%, w/v) and the pH was adjusted to 5.0. Sucrose was estimated by dinitrosalicylic acid method (Miller, 1959^[15]). Fermentation was done using shake flask bioreactor (100 ml) and New Brunswick Scientific Fermenter (2L) of the model BioFlo 2000 (USA) was used for the production of itaconic acid.

Results & Discussion

The rate of product formation based on rate of substrate composition and biomass

estimation was studied for all the four species of *Aspergillus*. After 120 hours incubation, the rate of substrate consumption and product formation were analysed and found that the product formation is inversely related to substrate consumption and maximum concentration of itaconic acid of 27 g/L was seen at 5.5 g/L substrate concentration with *A. terreus* followed by 24, 18 and 17 g/L with *A.niger*, *A. flavus* and *A.nidulans* respectively (Fig. 1 to 3) .

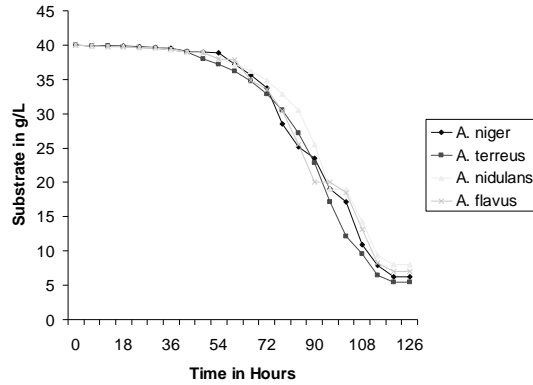


Figure 1 : Substrate Consumption in different species of *Aspergillus*

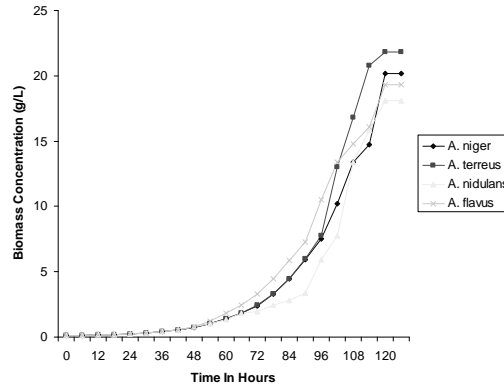


Figure 2: Biomass Concentration in different species of *Aspergillus*.

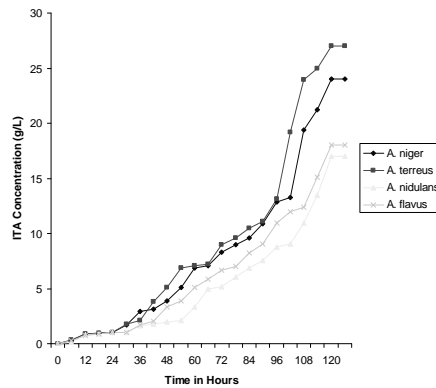


Figure 3: Itaconic acid (Product) concentration in different species of *Aspergillus*.

The effect of specific growth rate (μ_{\max}) on itaconic acid production

The specific growth rate was investigated for the above four species and noticed that with increase in time of fermentation, there is a decrease in substrate concentration and increase in biomass concentration. To calculate the specific growth rate, the data points relating to the reciprocal of substrate utilization and biomass concentration were taken. The maximum specific growth rate for *A. niger*, *A. terreus*, *A. nidulans* and *A. flavus* are 0.04119, 0.04199, 0.03308 and 0.0393 h^{-1} respectively. The maximum growth rate was observed in *A. terreus*. The results are shown in figures 4 to 7.

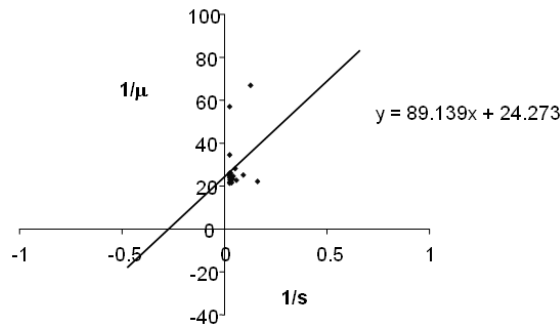


Figure 4: LB for plot determining specific growth rate in *A. niger*.

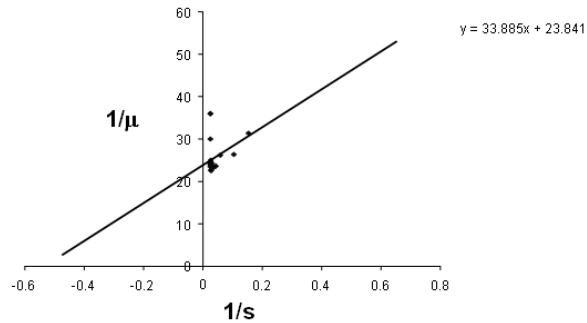


Figure 5: LB plot for determining specific growth rate in *A. terreus*.

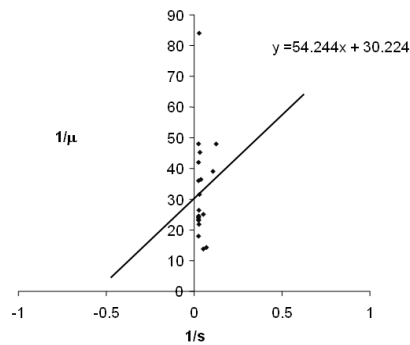


Figure 6: LB plot for determining specific growth rate in *A. nidulans*.

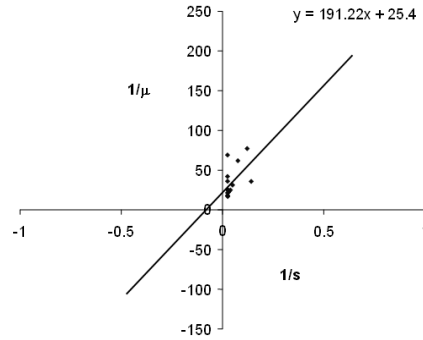


Figure 7: LB plot for determining specific growth rate in *A. flavus*.

The effect of yield factor of cell mass ($Y_{x/s}$) on itaconic acid production

The yield factor ($Y_{x/s}$), a ratio of amount of biomass formed per amount of substrate consumed, is investigated for all the four species. This was determined from the slope of the plot of r_s/X Vs. r_x/X . The 6 hours of incubation time interval was selected for determining yield factor ($Y_{x/s}$) for *Aspergillus* sps. The yield factor ($Y_{x/s}$) for *A. niger*, *A. terreus*, *A.nidulans* and *A. flavus* are 0.4776, 0.4976, 0.4629 and 0.4670 g/L respectively. The maximum yield factor ($Y_{x/s}$) was observed in *A. terreus* (Figs. 8 to 11).

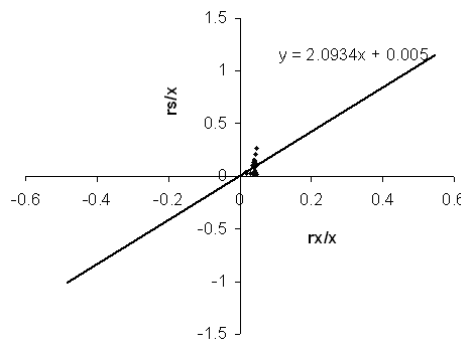


Figure 8: Determination of Yield factor ($Y_{x/s}$) of cell mass of *A. niger*.

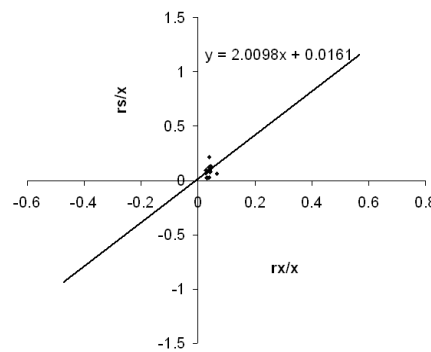


Figure 9: Determination of Yield factor ($Y_{x/s}$) of cell mass of *A. terreus*.

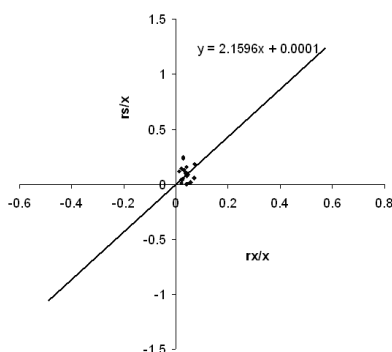


Figure 10: Determination of Yield factor ($Y_{x/s}$) of cell mass of *A. nidulans*.

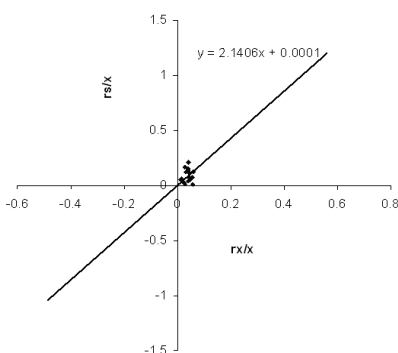


Figure 11: Determination of Yield factor ($Y_{x/s}$) of cell mass of *A. flavus*.

The effect of Cell doubling time on itaconic acid production

The time required for the microbial mass to double is called doubling time. The cell doubling time was investigated for the four selected species. It was determined during the exponential growth of the microbe, characterized by straight line on a semi-log plot of $\ln x$ Vs. time. The cell doubling time for *A. niger*, *A. terreus*, *A. nidulans* and *A. flavus* are 15.36, 14.84, 16.08 and 15.54 h respectively. The minimum cell doubling time was observed in *A. terreus* (Figs. 12 to 15).

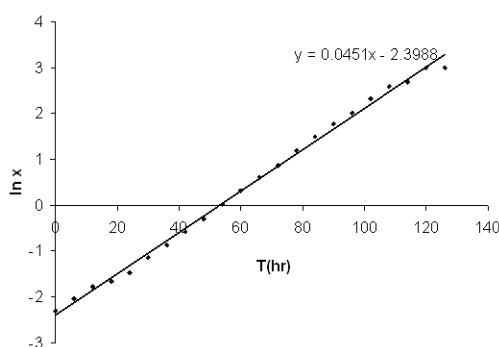


Figure 12: Determination of Doubling time of *A. niger*.

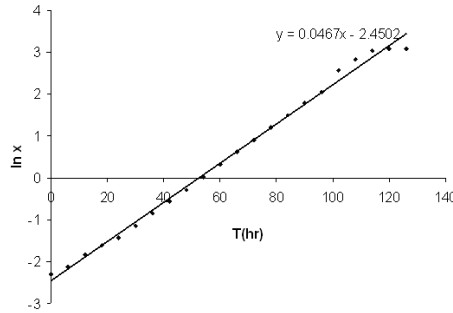


Figure 13: Determination of Doubling time of *A. terreus*.

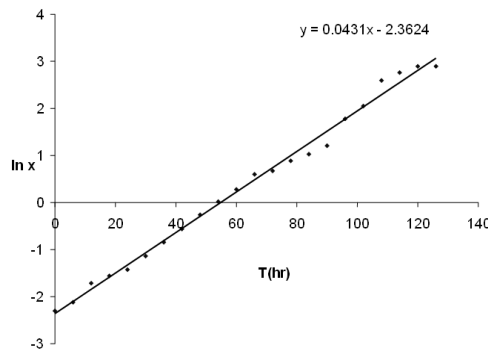


Figure 14: Determination of Doubling time of *A. nidulans*.

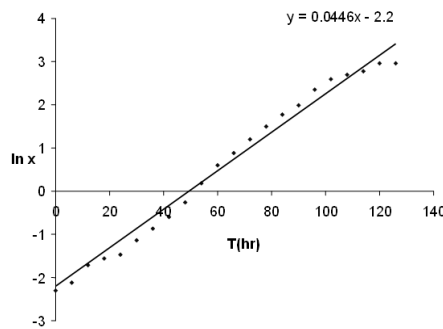


Figure 15: Determination of Doubling time of *A. flavus*.

The effect of yield factor of the product ($Y_{p/x}$) on itaconic acid production

The yield factor ($Y_{p/x}$) is the ratio of amount of product formed per amount of biomass formed. The yield factor ($Y_{p/x}$) was investigated for four selected species. It was determined from the graph of r_p/x Vs. r_x/x . The slope of the graph gives yield factor ($Y_{p/x}$). The 6 hours of incubation time interval was selected for determining yield factor ($Y_{p/x}$) for *Aspergillus sp.* The yield factor ($Y_{p/x}$) for *A. niger*, *A. terreus*, *A.nidulans* and *A. flavus* are 0.4360, 0.4387, 0.4028 and 0.4216 g/g respectively (Figs. 16 to 19).

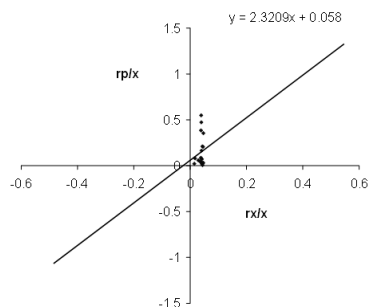


Figure 16: Determination of Yield factor ($Y_{p/x}$) of product of *A. niger*.

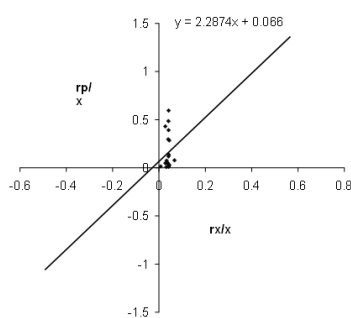


Figure 17: Determination of Yield factor ($Y_{p/x}$) of product of *A. terreus*.

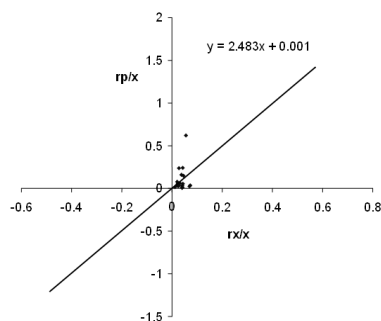


Figure 18: Determination of Yield factor ($Y_{p/x}$) of product of *A. nidulans*.

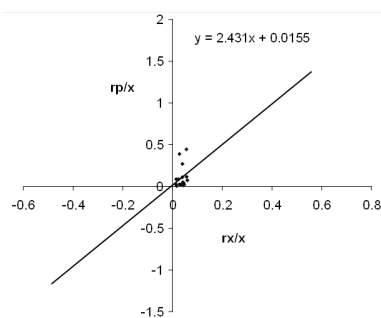


Figure 19: Determination of Yield factor ($Y_{p/x}$) of product of *A. flavus*.

These experimental results suggest *A. terreus* to have the best kinetic parameters with highest specific growth rate, yield factor of biomass as well as yield factor of the product. The doubling time was also found to be less for *A. terreus* when compared to other organisms studied. The studies on the kinetics of growth were in concurrence with the production of itaconic acid by *A. terreus* giving the insight that the organism has the potential to be commercially exploited for the fermentative production of itaconic acid.

The predictive regression equation for the itaconic acid concentration (Y, g/L) as a function of time (X_1 , hr), Substrate concentration (X_2 , g/L) and biomass concentration (X_3 , g/L) is given in the equation:

$$Y = 214 - 1.92 X_1 - 8.09 X_2 - 4.71 X_3 + 0.0474 X_1 X_2 + 0.0529 X_1 X_3 + 0.029 X_2 X_3 + 0.00215 X_1^2 + 0.0687 X_2^2 - 0.0386 X_3^2$$

$$S = 0.4186 \quad R^2 = 99.8\% \quad R^2(\text{adj}) = 99.7\%$$

The calculated results obtained from the model obtained agreed well with the obtained experimental results in the present study.

Regression Analysis

The regression equation

$$Y = 214 - 1.92 X_1 - 8.09 X_2 - 4.71 X_3 + 0.0474 X_1 X_2 + 0.0529 X_1 X_3 + 0.029 X_2 X_3 + 0.00215 X_1^2 + 0.0687 X_2^2 - 0.0386 X_3^2$$

Predictor	Coef	StDev	T	P
Constant	214.2	128.7	1.66	0.122
X1	-1.9151	0.9468	-2.02	0.066
X2	-8.086	5.226	-1.55	0.148
X3	-4.712	6.350	-0.74	0.472
X1X2	0.04743	0.02353	2.02	0.067
X1X3	0.05288	0.04204	1.26	0.232
X2X3	0.0294	0.1224	0.24	0.814
X1 ²	0.0021529	0.0004677	4.60	0.000
X2 ²	0.06867	0.05127	1.34	0.205
X3 ²	-0.03858	0.06691	-0.58	0.575

$$S = 0.4186 \quad R^2 = 99.8\% \quad R^2(\text{adj}) = 99.7\%$$

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	9	1261.05	140.12	799.77	0.000
Error	12	2.10	0.18		
Total	21	1263.16			

Source	DF	Seq SS
X1	1	1140.79
X2	1	86.07
X3	1	22.74
X1X2	1	0.74
X1X3	1	0.08
X2X3	1	4.99
X1 ²	1	4.88
X2 ²	1	0.71
X3 ²	1	0.06

Unusual Observations

Obs	X1	Y	Fit St	Dev Fit	Residual	St Resid
18	102	13.2400	13.7549	0.3588	-0.5149	-2.39R

R denotes an observation with a large standardized residual

Conclusion

Our study indicates that *A. terreus* is a good producer of the itaconic acid among the different *Aspergillus* *sps.* studied and its kinetic parameters are most dependable for the commercial exploitation. Genetic improvements like identification and upregulation of the necessary genes and appropriate design of fermenters for production are anticipated to further enhance the production of itaconic acid.

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