0273-2289/02/102–103/0395/$12.75

Optimization of Fermentation Conditions for Production of Glycopeptide Antibiotic Vancomycin by *Amycolatopsis orientalis*

P. Naga Padma,[1] A. Bhaskar Rao,[2] J. S.Yadav,[2] and Gopal Reddy*,[1]

[1]Department of Microbiology, Osmania University, Hyderabad 500 007, India; and [2]Indian Institute of Chemical Technology, Hyderabad 500 007, India, E-mail: gopalred@hotmail.com

Abstract

Glycopeptides produced by *Streptomyces* species are the drugs used against β-lactam drug-resistant staphylococcal infections, and vancomycin is important among them. Increased prevalence of resistant strains increased the usage of vancomycin worldwide and also promoted attempts for indigenous production. The optimum process conditions pH, temperature, inoculum size, agitation, and aeration for vancomycin production by *Amycolatopsis orientalis* were evaluated, statistically analyzed, and the response surface curves were constructed. The optimum process conditions were a pH of 7.6, a temperature of 29°C, an inoculum size of 4.5%, an agitation of 255 rpm, and an aeration of less than 1:10 medium-to-air ratio.

Index Entries: Fermentation; antibiotic; glycopeptide; vancomycin; optimization.

Introduction

Vancomycin, the glycopeptide antibiotic, was isolated at the Lilly Research laboratories from *Amycolatopsis orientalis* and was introduced for clinical use in 1958 for the treatment of staphylococcal infections resistant to then-available antibiotics. Increased prevalence of multiple antibiotic-resistant strains of staphylococci, pneumococci, and enterococci resurrected vancomycin, and its usage has increased worldwide over the last decade. It is the drug of choice for treatment of methicillin-resistant *Staphylococcus aureus* infections *(1)*, coagulase-negative staphylococci *(2)*. It is also used to

*Author to whom all correspondence and reprint requests should be addressed.

treat penicillin-resistant strains of *Streptococcus pneumoniae (3)*; Gram-positive bacilli such as *Bacillus anthracis* and *Bacillus cereus (4)*; Corynebacteria such as *Corynebacterium diphtheriae* and *C. jeikeium (5)*; and many of the clinically important clostridial species such as *C. difficile*, *C. perfringens*, *C. botulinum*, and *C. septicum* that are sensitive to vancomycin *(6)*. Intraventricular application of vancomycin is an effective therapeutic regimen for the treatment of shunt-associated staphylococcal ventriculitis *(7)*.

Vancomycin is bactericidal for most Gram-positive organisms and acts by inhibiting one or both of the two sequential enzymatic reactions involved in cell-wall synthesis: peptidoglycan elongation or transglycosylation and crosslinking or transpeptidation *(8,9)*. Indigenously produced vancomycin is not available in India. Because it is a necessity, researchers are aiming to develop indigenous technology through studies at laboratory-scale fermentation. Productivity can be increased by manipulation of fermentation conditions and media components. The application of statistical-mathematical methods for fermentation experiments, though rare, is gaining prominence owing to its success in faster yield improvement, the target of industrial users. The present study was aimed at evaluating the effects of pH, temperature, inoculum size, rate of agitation, and aeration on vancomycin production and statistically optimizing these process variables to enhance productivity. Each parameter was tested over a range, fixed depending on prior experience and literature survey *(10–12)*. Interaction between process parameters and fermentation time was taken into consideration to get the best production in the least amount of time. The data obtained were analyzed statistically and response surface curves constructed *(13)*. From these curves, optimal physical parameters were evaluated. The predicted yields calculated using regression coefficients showed nearly a twofold increase. In these experiments, a cubic model of response surface was applied to construct curves, and a third-order equation was used in the statistical analysis to obtain predicted yields. All the analyses were done using an Indostat software package.

Materials and Methods

Microorganism and Preparation of Media

A. orientalis ATCC 43491 was grown and maintained on ISP2 media slants or yeast-malt agar slants (4 g/L of glucose, 10 g/L of malt extract, and 4 g/L of yeast extract at pH 7.2).

The inoculum for the fermentation was prepared in two stages. Sporulating culture from slant was inoculated into a shake flask of the medium and incubated at 28°C for 3 d at 220 rpm. The culture grown was used for further study of fermentation parameters.

Bioassay

The flasks were incubated for 11 d. The fermented broth was sampled and assayed every alternate day until the maximum antibiotic broth

potency had been passed. Samples were assayed from d 3 to 11 of fermentation. The samples were collected by centrifugation at 5000*g* for 15 min. The supernatant was filtered through a 0.45-µm Millipore filter. The filtrate was bioassayed using the sensitive organism, strain *B. subtilis* ATCC 11774 *(14)*. The zones of inhibition developed were measured, and the concentration of the antibiotic was determined using a graph of the standard antibiotic vancomycin. The bioassay results were compared with those of high-performance liquid chromatography and were tallied *(15)*.

Statistical Analyses

The antibiotic yields obtained for each parameter over a period of time were tabulated and the results analyzed statistically, and their regression coefficients were calculated. The data were analyzed using the Indostat statistical package, and the response surface curves and corresponding contour plots were drawn. From the contour plots, the optimum level for each parameter and its corresponding peak day of production were obtained. Using these and the regression coefficients for each parameter, the maximum yield of antibiotic was estimated. The equation used is as follows:

$$Y = a + b_1x^1 + b_2x^2 + b_3x^3 + b_4y^1 + b_5y^2 + b_6y^3 + b_7xy + b_8xy^2 + b_9x^2y + b_{10}x^2y^2 + e$$

in which a is the intercept; b_1 to b_{10} are the regression coefficients; x and y are the interactive terms, x being the days and y the parameter under study; and e is the random error.

Later an experiment was run with the optimum levels of pH, temperature, inoculum size, agitation, and aeration. The antibiotic yield was estimated over a period ranging from 3 to 11 d.

Results and Discussion

Using statistical analysis, the interactions among process parameters under study could be understood, and optimum levels of each parameter for high productivity of the desired antibiotic could be known. Table 1 gives results of the cubic model of the response surface used for analysis of various process variables in the form of analysis of variance (ANOVA). ANOVA is required to test the significance of experimental runs, and experiments were found to be significant, as indicated by the low probability values given in Table 1. The closer the value of R to 1, the better is the correlation between observed and predicted values. For all the parameters under study, both R^2, the correlation coefficient, and R^2adj, the adjusted coefficient, are nearer to 1, indicating a high degree of correlation between the observed and predicted values. This is true since a practically run experiment with all fermentation parameters at optimum levels for the same period of fermentation time gave antibiotic yields nearer to the predicted values and the peak day of production corresponded as well.

Table 1
ANOVA for Selected Cubic Method Used
to Analyze Fermentation Process Variable for Vancomycin Production

Serial no.	Parameter	R^2	R^2 adj	RMS error	F	df	Probability
1	pH	0.8600	0.8189	85.29	20.90	10,34	0.000001
2	Temperature	0.9110	0.8882	57.98	39.96	10,39	0.000001
3	Inoculum size	0.8726	0.8287	60.68	19.87	10,29	0.000001
4	Agitation	0.9304	0.9099	54.94	45.47	10,34	0.000001
5	Aeration	0.9871	0.9804	28.48	146.30	10,19	0.000001

[a] R^2, correlation coefficient; R^2 adj, adjusted R^2; RMS, root mean square; F, F-test value; df, degrees of freedom.

Table 2
Model Terms and Regression Coefficients
for Various Fermentation Process Variables

		Regression coefficients for various parameters				
Serial no.	Model term[a]	pH	Temperature	Inoculum size	Agitation	Aeration
1	Intercept	8942.5779	3036.8645	–185.2359	2994.8717	26.5176
2	x^1	–1243.6234	–1663.2818	–59.0387	–362.5770	158.2201
3	x^2	96.1009	113.2416	–2.3628	30.0226	–9.7842
4	x^3	–1.4236	–0.9167	–0.0391	–1.8883	–0.0868
5	y^1	–4034.8855	–252.8296	35.8970	–46.3416	–0.4986
6	y^2	589.7422	6.0589	–19.8122	0.2237	–0.0782
7	y^3	–27.8281	–0.0396	1.9313	–0.0003	0.0005
8	xy	361.3536	120.1027	67.1260	3.7591	1.5937
9	xy^2	25.9713	–2.0369	–6.5729	–0.0078	–0.0157
10	x^2y	–22.7160	–7.3277	–4.8592	–0.2041	–0.1043
11	x^2y^2	1.6415	0.1239	0.4649	0.0004	0.0010

[a] x = day of peak production; y = optimum level of parameter under study.

Table 2 gives the regression coefficients of different parameters under study, obtained after subjecting the experimental data to statistical analysis. x_1, x_2, and x_3 are the linear, quadratic, and cubic terms of peak day of production, respectively. y_1, y_2, and y_3 are the linear, quadratic, and cubic terms of the optimum level of the parameter under study, respectively. xy, xy^2, x^2y, and x^2y^2 are the interactive terms for the two (i.e., peak day of production and optimum level of parameter under study).

The response surface curves and their corresponding contour plots were drawn for each process variable under study taking fermentation time vs process variable into consideration. These response surface curves are three-dimensional plots in which one dimension, X_1, was represented by the variable fermentation process parameter, the second dimension, X_2, by fermentation time, and the third dimension, Y, by vancomycin production, respectively. For all the curves, the variables occupied the same

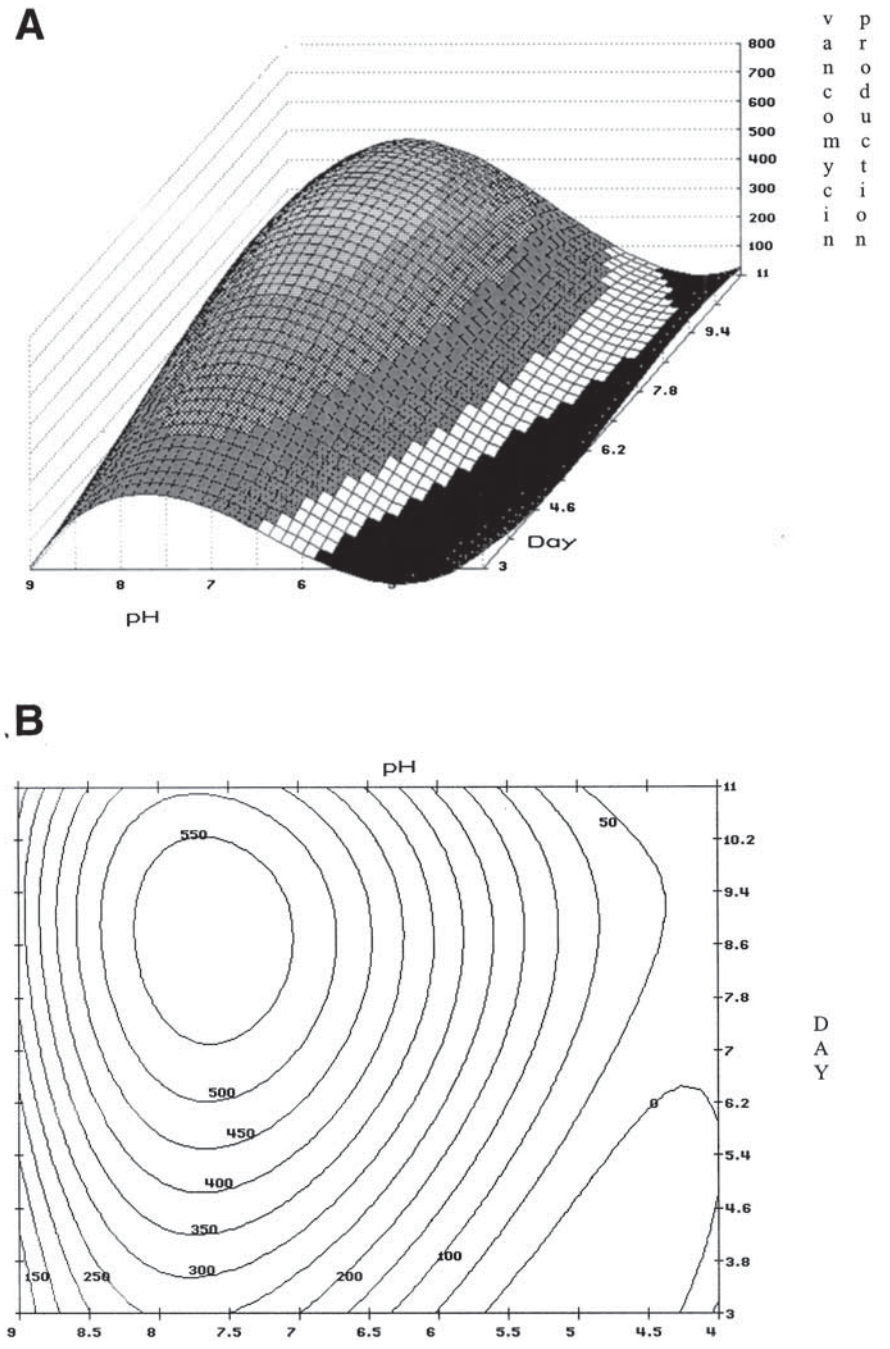

Fig. 1. **(A)** Response surface curve and **(B)** its contour plot for influence of pH on vancomycin production.

dimensions. From Figs. 1–5, the optimum level for each process variable and peak day of production could be identified as indicated by the hump in the response surface curve and the central point in the contour plot.

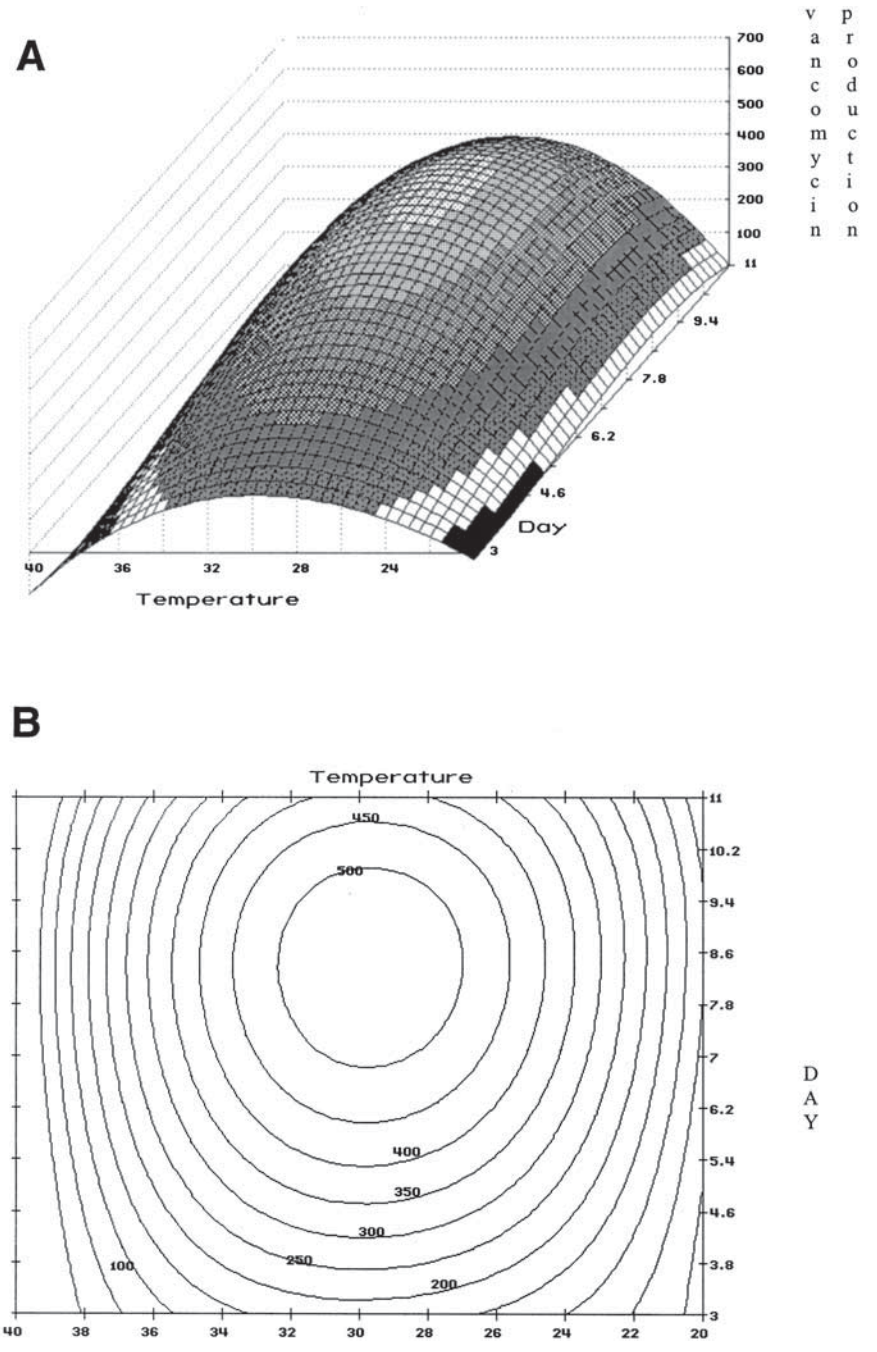

Fig. 2. **(A)** Response surface curve and **(B)** its contour plot for influence of temperature on vancomycin production.

Figure 1A gives the response surface curve and Fig. 1B the corresponding contour plot of pH. The hump in the curve and the central point in the contour plot indicate that the optimum pH is 7.6 and peak day of produc-

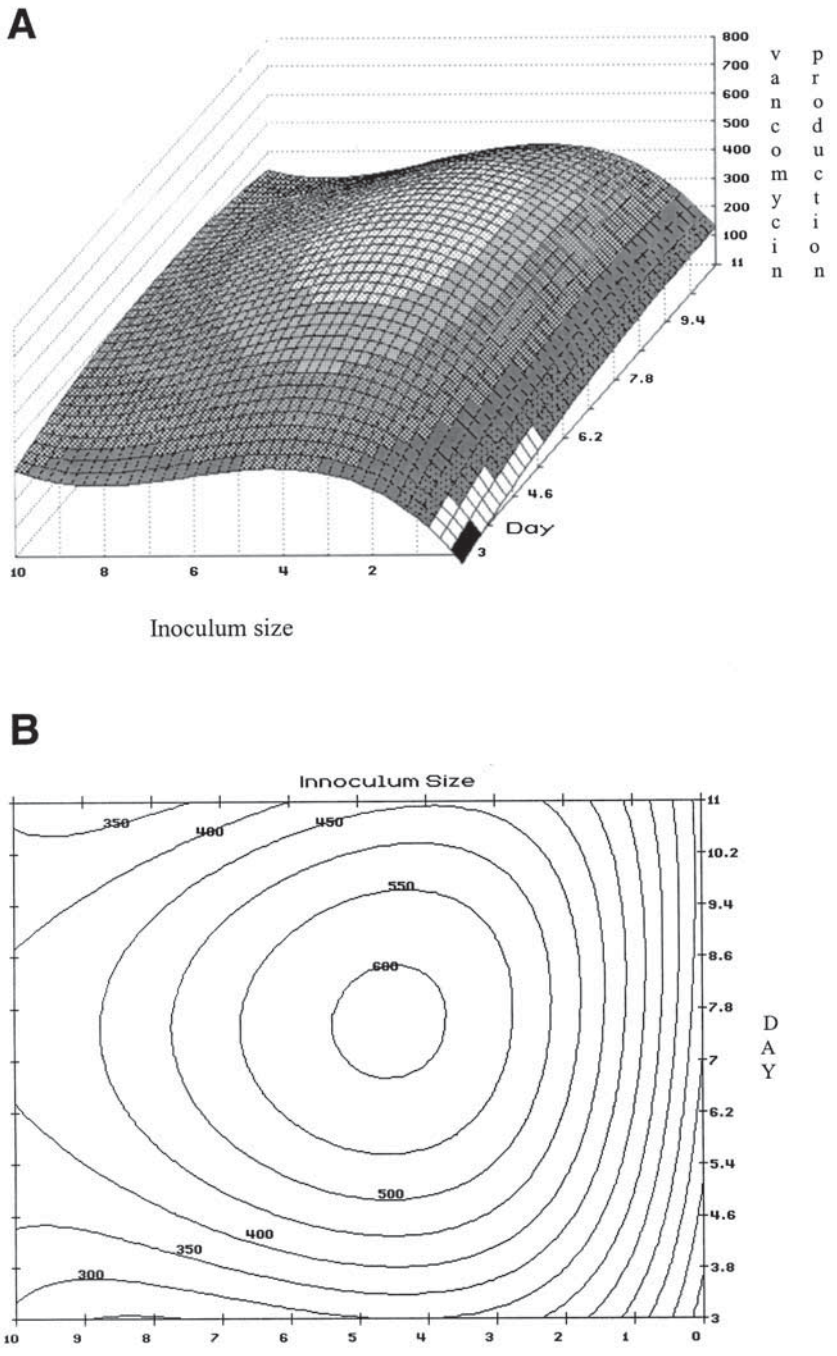

Fig. 3. **(A)** Response surface curve and **(B)** its contour plot for influence of inoculum size on vancomycin production.

tion is 8.4 d. Similarly, Figs. 2A, 3A, 4A, and 5A represent the response surface curves and Figs. 2B, 3B, 4B, and 5B their corresponding contour plots for the process variables temperature, inoculum size, agitation, and

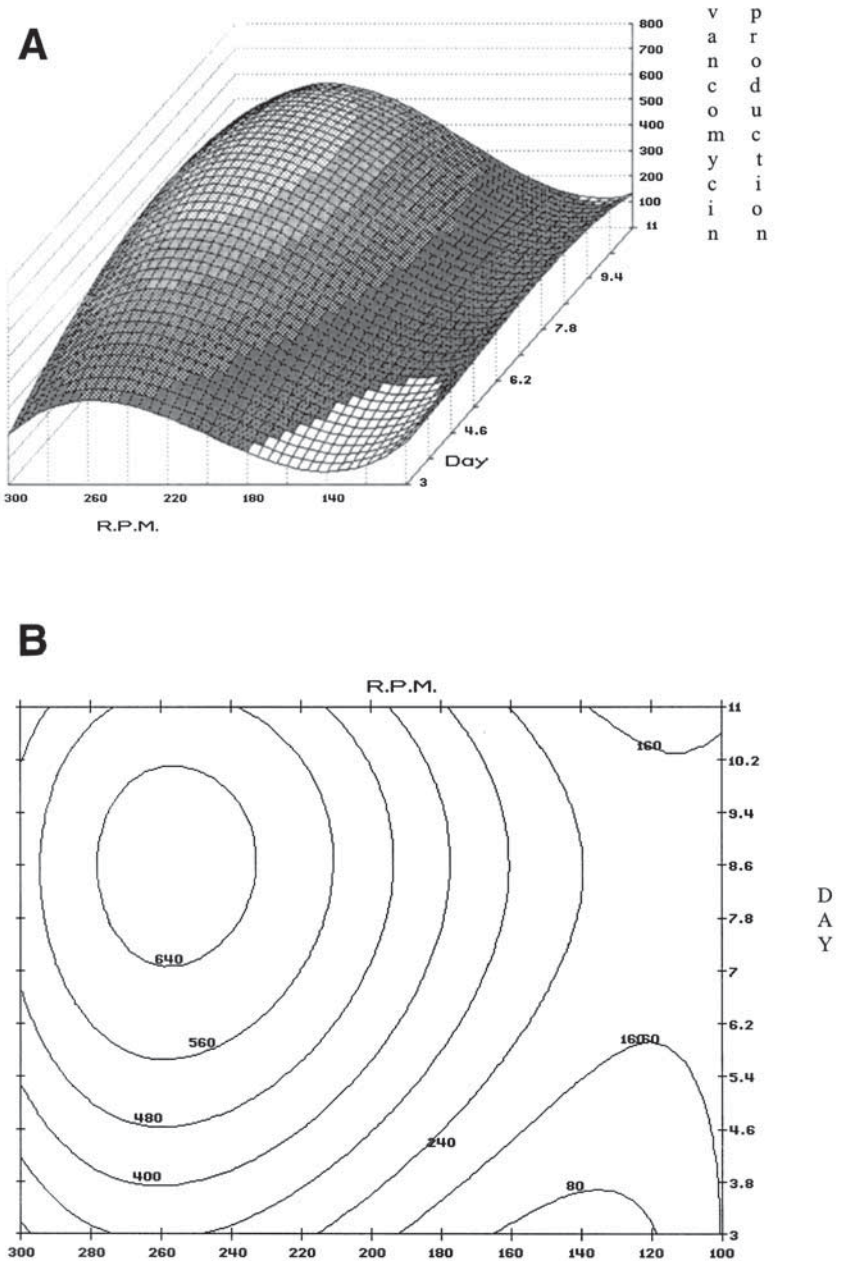

Fig. 4. **(A)** Response surface curve and **(B)** its contour plot for influence of agitation on vancomycin production.

aeration, respectively. These figures show that the optimum levels and their corresponding peak day of production for different process variables are 29°C and 8.4 d for temperature, 4.5% and 7.6 d for inoculum, 255 rpm and 8.4 d for agitation, less than 1:10 medium-to-air ratio, and nearly 7.6 d for aeration, respectively.

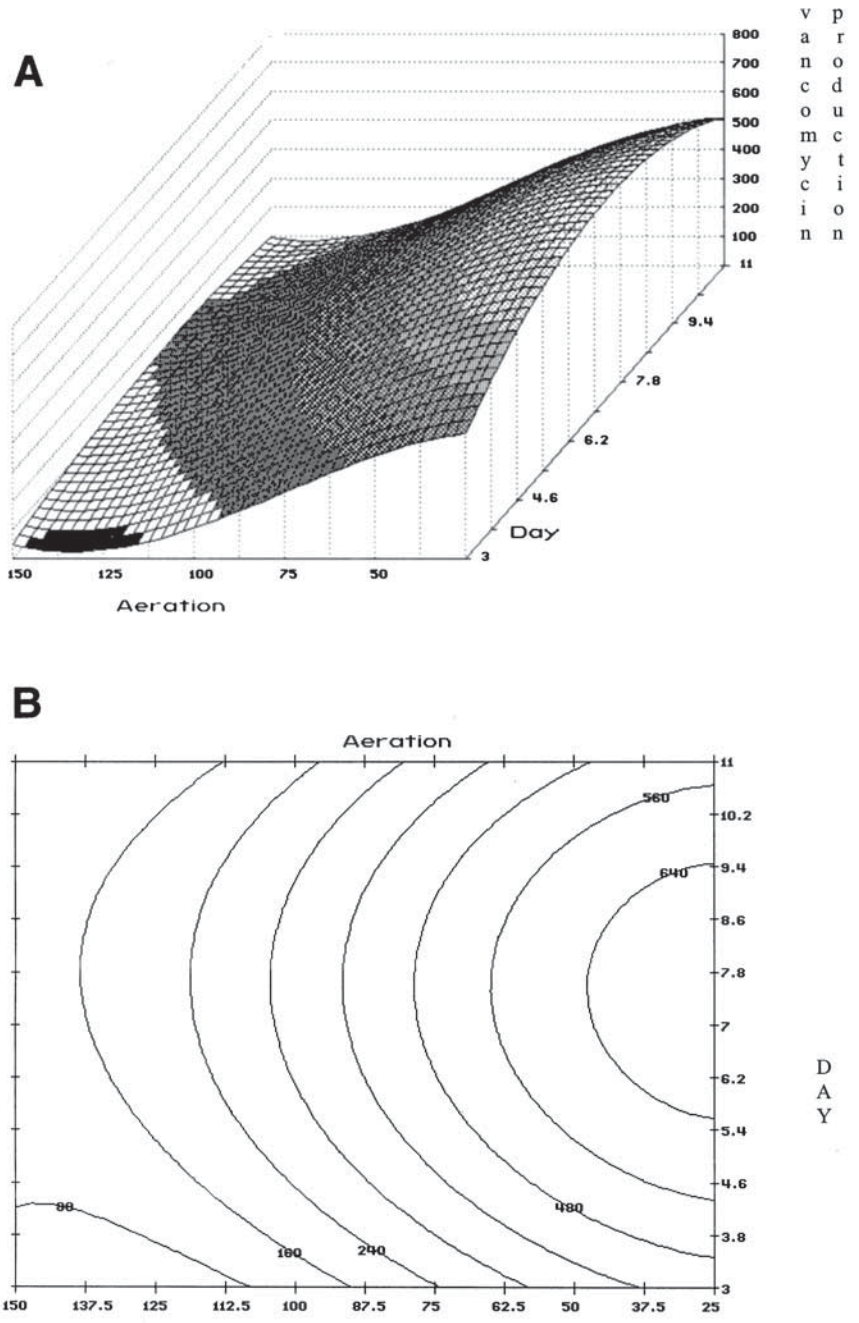

Fig. 5. **(A)** Response surface curve and **(B)** its contour plot for influence of aeration on vancomycin production.

With the equation given in Materials and Methods, antibiotic yield under a particular optimal process condition could be calculated using regression coefficients (as indicated in Table 2), peak day of production,

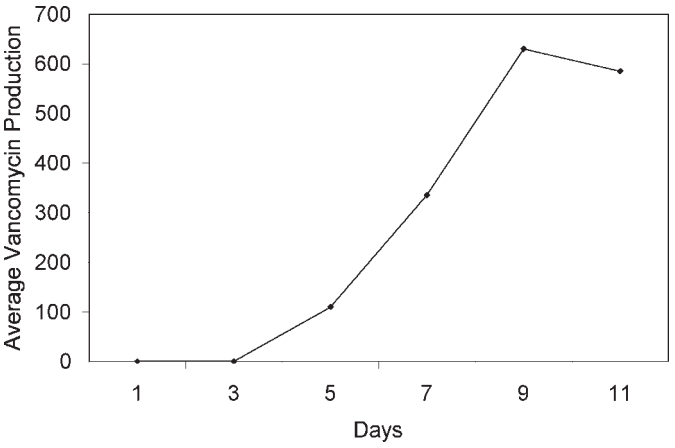

Fig. 6. Vancomycin production by *Amycolatopsis orientalis* at optimized fermentation conditions.

which is x, and optimum process variable, which is y (both obtained from the response surface curve and corresponding contour plot). Different yields of the antibiotic vancomycin for each parameter were calculated individually, and as for the process variable pH, the calculated vancomycin yield was 592 µg/mL, and for temperature the calculated yield was 565 µg/mL. Similarly, for inoculum size it was 626 µg/mL, for agitation it was 667 µg/mL, and for aeration it was 686 µg/mL. These individual yields not only indicate the vancomycin yields under the optimum process condition but also the importance of each process variable independently. From these data, it is clear that the most influential parameter was aeration, since by taking a 1:10 ratio of medium to air the yield was 686 µg/mL; however, from the Fig. 5B, which has no central point, it is clear that more aeration is necessary, and if so provided the yield might be even more. Good aeration is promoted by agitation so the second influential parameter is agitation followed by inoculum size, pH, and temperature. pH and temperature are not critical, and a wider range is tolerable by the organism under study, so comparatively their influence is less, as indicated by the data.

The mentioned yields are the predicted yields for the process variables. An experiment was run by setting up these optimum process variables, and from its results antibiotic yields were calculated using a standard graph. A linear graph was drawn taking fermentation time in days on the x-axis and vancomycin yields in micrograms/milliliter on the y-axis, as represented in Fig. 6. The actual yield of vancomycin obtained from this experiment was 630 µg/mL, and it is nearer to the average of the predicted yields obtained from each parameter, which was 629.4 µg/mL. The peak day of production was d 9 for the practically run experiment, which also corresponds with those of the various fermentation process parameters for which the peak days of production were mostly between 8 and 9 d.

The graphic representation giving the interaction between fermentation time and fermentation process parameter gave the peak day of production as 7.6 and 8.4 d but since an assay was carried out every alternate day, peak production was indicated in the linear graph (Fig. 6) after d 8 (i.e., d 9).

From the experimental results the optimum levels of the process variables obtained were a pH of 7.6, a temperature of 29°C, an inoculum size of 4.5%, an agitation of 255 rpm, and an aeration of less than 1:10 medium-to-air ratio. The peak day of production was between 8 and 9 d for almost all the variables, which corresponded with that of the experiment run at the determined optimum fermentation condition.

No reports are available in the literature on the application of a statistical method for optimization of fermentation process conditions for vancomycin production. With the help of this statistical analysis, the best optimum levels could be determined as both the process variable and its interaction with time were taken into consideration. Without this analysis, one could end up with mediocre fermentation performance even with a high potential strain.

Acknowledgment

We thank Council of Scientific and Industrial Research (CSIR), New Delhi, India, for financial support.

References

1. Sorrell, T. C., Packham, D. R., Shanker, S., et al. (1982), *Ann. Intern. Med.* **97,** 334–350.
2. Gruer, L. D., Barlett, R., and Ayliffe, G. A. J. (1984), *J. Antimicrob. Chemother.* **13,** 577–584.
3. Goldstein, F. W., Geslin, P., Acar, J. F., and the French Study Group. (1994), *Eur. J. Clin. Microbiol. Infect. Dis.* **13,** 33–34.
4. Weber, D. J., Saviteer, S. M., Rutala, W. A., and Thoman, C. A. (1988), *Antimicrob. Agents Chemother.* **32,** 642–645.
5. Jadeja, L., Fainstein, J., Le Blanc, B., and Bodey, G. P. (1983), *Antimicrob. Agents Chemother.* **24,** 145–146.
6. Watanakunakorn, C. (1984), *Antimicrob. Agents Chemother.* **14(Suppl. D),** 7–18.
7. Nagl, M., Neher, C., Hager, J., et al. (1999), *Antimicrob. Agents Chemother.* **43,** 1932–1934.
8. Williams, D. H. and Waltho, J. P. (1988), *Biochem. Pharmacol.* **37,** 133–141.
9. Nagarajan, R. (1991), *Antimicrob. Agents Chemother.* **35,** 605–609.
10. Tunac, J. B. (1989), *J. Ferment. Bioeng.* **68,** 157–159.
11. Sarada, I. (1995), PhD thesis, Osmania University, Hyderabad, India.
12. Sen, R. and Swaminathan, T. (1997), *Appl. Microbiol. Biotechnol.* **47,** 358–363.
13. Watier, D., Dubourguier, H. C., Leguerinel, I., and Hornez, J. P. (1996), *Appl. Environ. Microbiol.* **62,** 1233–1237.
14. *British Pharmacopoeia*. (1999), A 352–353.
15. Sztaricskai, F., Borda, J., Puskas, M. M., and Bognar, R. (1983), *J. Antibiot.* **36,** 1691–1698.