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SELECTION OF MACRONUTRIENTS AND VITAMINS FOR VANCOMYCIN PRODUCTION BY *AMYCOLATOPSIS ORIENTALIS* USING PLACKETT –BURMAN DESIGN

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ABSTRACT

Screening of diverse phosphate, sulphate and vitamins for glycopeptide antibiotic vancomycin production was done using Plackett-Burman statistical design. Different sulphate and phosphate salts of ammonium, sodium and potassium and thiourea numbering 11 were screened using 12 experimental designs. Similarly different vitamins were screened by the same 12 experimental design of Plackett-Burman. The fermentation was carried out for 10 days and the vancomycin yields were subjected to statistical analysis. Significant phosphate, sulphate, and vitamins were short listed based on regression coefficients and t-values. The significant phosphate sources were disodium hydrogen phosphate, potassium dihydrogen phosphate, and sulfate sources were, sodium thiosulphate, ammonium sulphate. These were short listed for further optimization studies using second step of Plackett-Burman screening and Response Surface Methodology (RSM). Optimized production medium is a necessity for any commercial production for significant product yield with minimum inputs. This screening strategy not only short listed the efficient diverse nutrients but also improved the antibiotic yields as the combined effects of the nutrients were also considered.

KEYWORDS: *Amycolatopsis orientalis*, Vancomycin, phosphate sources, sulphate sources, vitamins, Plackett-Burman.

INTRODUCTION

Glycopeptides are a group of dalbaheptides[1] produced by Streptomyces species are highly effective against drug resistant Staphylococcal infections. Vancomycin is one among them and to date is being used as the last drug of choice for drug resistant Gram-positive bacterial infections like infective endocarditis caused by *S.aureus.*[2][3] This antibiotic is bactericidal and acts by inhibiting one or both of the two sequential enzymatic reactions involved in cell wall synthesis namely peptidoglycan elongation or transglycosylation and cross linking or transpeptidation.[4] With increased prevalence of multiple antibiotic resistant strains in the 21st century its use was resurrected after its first clinical use in 1958. It is used to treat infections caused by different bacteria like penicillin-resistant strains of *Streptococcus pneumonia,*[5] *Bacillus anthracis, Bacillus cereus,*[6] *Corynebacterium diphtheria.*[7] Intraventricular application of vancomycin is an effective therapeutic regimen for treatment of shunt associated staphylococcal ventriculitis.[8] It is also used to combat Gram-positive bacterial infections in intensive care patients.[9] Looking at the importance of the drug, its usage and increased prevalence of drug resistant infections, indigenous production is a necessity both to meet the demand and reduce the cost of drug. Development of fermentative technology for indigenous production of vancomycin suitable to Indian conditions is required and so the present study concentrated on this aspect. Different phosphate, sulphate and vitamin sources were screened using 12 experimental design of Plackett- Burman (1946) .[10] This is an efficient statistical methodology that is used to screen up to n-1 variables in just n number of experiments hence saves both time and materials.

MATERIALS AND METHODS

Microorganism, its physical and physiological conditions (Medium used)

Amycolatopsis orientalis 43491 was grown and maintained on ISP 2 medium slants or yeast-malt agar slants (4g/L of glucose, 10g/L of malt extract and 4g/L of yeast extract at pH 7.2. The inoculum for the fermentation was prepared in two stages where in the first stage sporulating culture from slant was inoculated into a shake flask of the medium and incubated at 28^{0}Cfor 3 days at 220 rpm. The culture grown was used for further study of Screening of different nutrients.

Bioassay

The flasks were incubated for 12 days. The fermented broth was collected and assayed every alternate day (2nd, 4th, 6th, 8th, 10th and 12th day) until the maximum broth potency had been

passed. The samples were collected aseptically, centrifuged at 5000g for 15 min. The supernatant was filtered through a 0.45µm Millipore filter. Bioassay plates were prepared by pouring 25ml of pre-seeded agar having 1 ml of sensitive culture containing 10^5cells/ml. After the agar was set wells were made with a 6mm diameter cork borer. The filtrate was bioassayed using the sensitive organism, strain *Bacillus subtilis* ATCC 11774[11]. 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.[12]

Statistical Analysis

Different sulphate phosphate sources were together tested by a 12 experimental design taking care of their concentrations and status (ingredients added as such to prevent any precipitation). The vitamins were also tested using the same design. While fixing levels for all nutrients care was taken to see that their combined concentrations were not inhibitory to either growth or production. In each case the nutrients were added according to the pattern of the design. The fermentation was carried out for a period of 12 days with sample collection from 4th to 10th day. The broth samples collected were bioassayed, antibiotic yields determined and results analysed statistically using 'Indostat' software package. The most important nutrients under different categories were selected after statistical analysis based on regression coefficients and highest t-values were ranked first and so on. Those with probabilities less than 0.005 were considered to be significant. The significant nutrients were shortlisted for further optimization studies.

RESULTS

Different phosphate and sulphate sources which were salts of ammonium, sodium and potassium and thiourea numbering 11 were screened using 12 experimental design of Plackett-Burman.[11] Similarly different vitamins of B-complex group and other vitamins too were screened using 12 experimental designs. The antibiotic yields varied in different flasks on different days ranging from a minimum of 100µg/ml to a maximum of 1140 µg/ml. The antibiotic bioassay data when subjected to statistical analysis yielded regression coefficients and t-values. The probability of the experiment was 0.00001 and thus the experiment was highly significant. Nutrients with highest positive regression coefficients and their corresponding t-values were ranked first and so on, on different days (Table 1). The ranking of ingredients on different days for sulphate sources was sodium thiosulphate as 1st

followed by ammonium sulphate as 2nd (Fig 1). Among phosphate sources di-sodium hydrogen phosphate ranked 1st and potassium dihydrogen phosphate ranked 2nd (Fig 2).

Similarly different vitamins were screened using the same experimental design. The effect of different vitamins varied on different days as indicated by the regression coefficients and their corresponding t-values (Table 2). While considering the ranking of vitamins on different days three vitamins were considered as there was variation indicated in (Fig 3) it was observed that riboflavin ranked 1st on 4th, 8th and 10th days while nicotinic acid ranked 1st on 6th day. The second position was occupied by nicotinic acid for 4th and 8th days, riboflavin for 6th day and thiamine hydrochloride was third for all the days. As riboflavin ranked 1st most of the time (on 4th, 8th and 10th days) it was selected for second step screening as a vitamin source.

Table 1: Regression coefficients and t-values calculated from vancomycin yields of *A.orientalis* in different sulphate and phosphate sources.

S. No.	Ingredient	4th Day		6th Day		8th Day		10th Day	
		Reg. Coeff	t-value	Reg. Coeff	t-value	Reg. Coeff	t-value	Reg. Coeff	t-value
1	Intercept	333.33	38.83	558.33	46.26	714.17	45.93	707.50	58.21
2	Ammonium sulphate $(NH_4)_2SO_4$	-33.75	12.43	-46.25	12.12	-64.17	13.05	-62.50	16.26
3	Ammonium per sulphate $(NH_4)_2S_2O_8$	-68.75	25.32	-75.42	19.76	-78.33	15.93	-46.60	12.14
4	Sodium thiosulphate $(Na_2S_2O_3)$	157.08	57.86	219.58	57.53	242.50	49.32	213.33	55.50
5	Sodium sulphate (Na_2SO_4)	-46.25	17.04	-95.42	24.99	-86.67	17.63	-92.50	24.07
6	Potassium sulphate $K_2SO_4)$	-54.58	20.11	-87.92	23.03	-50.00	10.17	-48.33	12.58
7	Thiourea CH_4N_2S	-58.75	21.64	-107.08	28.06	-110.00	22.37	-70.00	18.21
8	Ammonium dihygrogen phosphate	-37.95	13.97	-56.25	14.74	-57.50	11.69	-66.67	17.35
9	Disodium hydrogen phosphate Na_2HPO_4	-36.25	13.35	-60.42	15.83	-85.00	17.29	-120.83	31.44
10	Sodium dihydrogen phosphate NaH_2PO_4	-40.42	14.89	-25.42	6.66	-80.00	16.27	-77.50	20.16
11	Dipotassium hydrogen phosphate K_2HPO_4	-34.58	12.74	-77.08	20.02	-25.83	5.25	-20.00	5.20
12	Potassium dihydrogen phosphate KH_2PO_4	-18.75	6.91	-23.75	6.22	-18.33	3.73	-20.83	5.42

Probabilty -0.0001: Highly significant

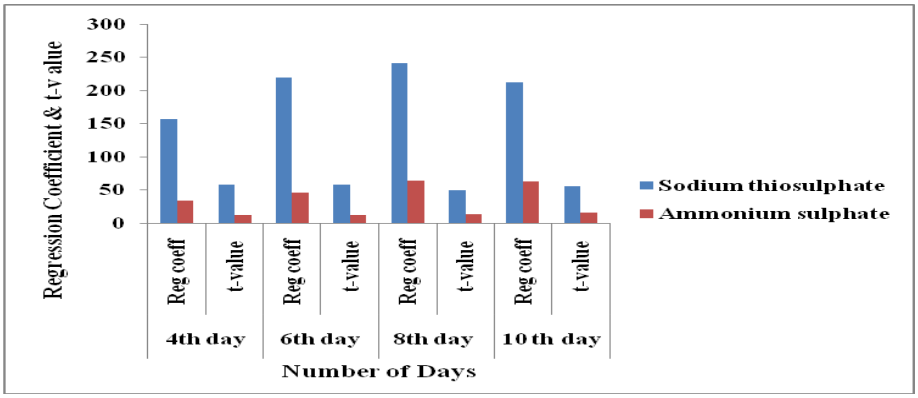

Figure 1: Variation in the Regression coefficients and t-Values of the Selected and Short Listed Sulphate Sources for Vancomycin Production on 4th, 6th, 8th and 10th Day

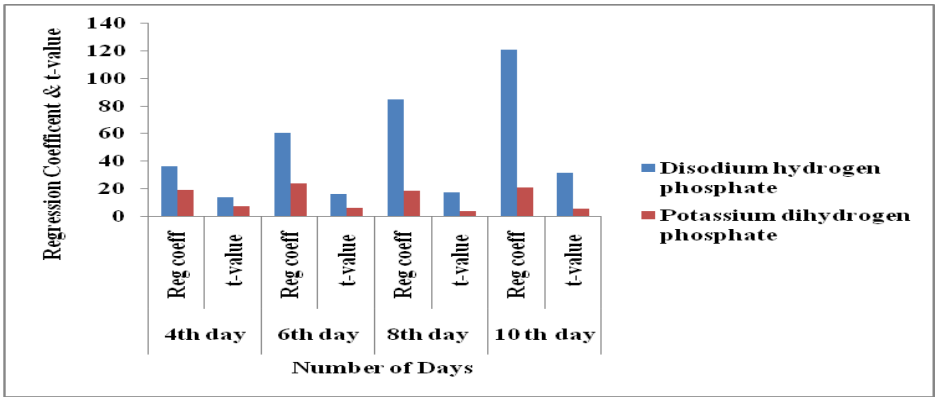

Figure 2: Variation in the Regression coefficients and t-Values of the Selected and Short Listed Phosphate Sources for Vancomycin Production on 4th, 6th, 8th and 10th Day

Table 2: Regression coefficients and t-values calculated from vancomycin yields of *A.orientalis in* different vitamins using Plackett-Burman design.

S.no	Ingredient	4th day		6th day		8th day		10th day	
		Reg coeff	t-value	Reg coeff	t-value	Reg coeff	t-value	Reg coeff	t-value
1.	Intercept	490.83	30.29	857.50	108.60	1178.33	50.75	1225.83	43.42
2.	Ascorbic acid	-0.83	0.16	-12.92	5.17	18.33	2.49	-7.92	0.89
3.	Biotin	-20.00	3.90	-27.08	10.85	-22.50	3.06	8.75	0.98
4.	Cynacobalmin	8.33	1.63	-35.42	14.18	23.33	3.18	22.08	2.47
5.	Folic acid	-2.50	0.49	-27.08	10.85	-20.03	2.84	-38.75	4.34
6.	Nicotinic acid	10.83	2.11	83.75	33.54	44.17	6.02	-15.42	1.73
7.	Riboflavin	29.17	5.69	69.58	27.87	60.00	8.17	67.92	7.61
8.	Pantothenic acid	2.50	0.49	42.08	16.85	25.83	3.52	48.75	5.46
9.	Pyridoxine hydrochloride	5.00	0.98	-7.92	3.17	0.00	0.00	24.58	2.75
10.	P-amino benzoic acid	-17.50	3.14	-27.08	10.85	10.83	1.48	34.58	3.87
11.	Thiamine hydrochloride	8.33	1.63	14.58	5.84	30.83	4.20	15.42	1.73
12.	Tocopherol	-42.50	8.29	-96.25	38.55	-156.67	21.34	-165.42	18.53

Probability- 0.00011: Highly significant

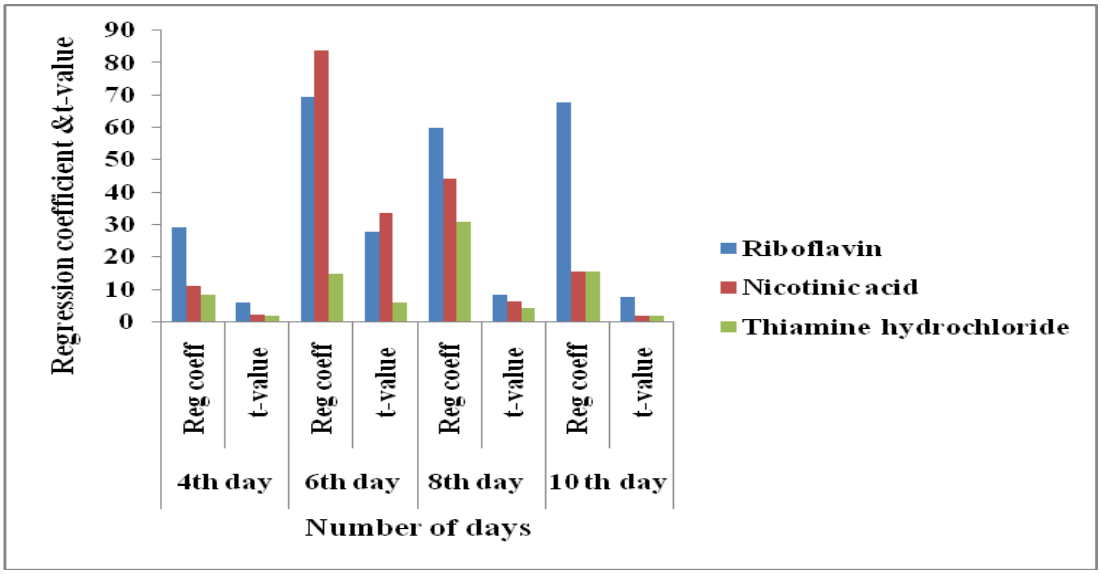

Figure 3: Variation in the Regression coefficients and t-Values of the Selected and Short Listed Vitamins for Vancomycin Production on 4th, 6th, 8th and 10th Day.

DISCUSSION

An optimized production medium for microbial culture is a necessity as it ensures that the required nutrients are present in appropriate forms and at non-inhibitory optimum concentrations.[13] Considering this important aspect diverse macronutrients and vitamins were screened using statistical method like Plackett-Burman design.[14] Mertz and Doolin.[15] Working with *S.orientalis* (now *A.orientalis*) found that there is a correlation between phosphate concentration and vancomycin production. Their study revealed that increased phosphate concentrations did not inhibit growth but markedly inhibited both the initiation and the amount of antibiotic synthesized. From their studies it is clear that phosphate is necessary for general cellular activities of the organism under study but higher concentrations were inhibitory for synthesis of desired antibiotic, vancomycin. Taking these facts into consideration different phosphate sources were screened though they were less significant compared to sulphate sources. Comparatively more significant phosphate source identified was disodium hydrogen phosphate (Fig 1). There are no reports on influence of sulphate or sulfur sources on vancomycin production, but as sulfur is necessary for formation of sulfur containing amino acids and generally for forming disulfide bonds in most secondary structure formations of proteins, therefore sulfur sources too were screened. Among the different sulfur sources sodium thiosulphate was found to be significant and almost ranked 1st on all days (Table 2). Compared to phosphate sources, sulfur sources were found to be more significant.

Vitamins act as cofactors and coenzymes in different enzymatic reactions in cell metabolism.[16] These are mostly present in the media ingredients in the required minor quantities. Some organisms may require vitamins in some additional quantities. Riboflavin is present in flavoproteins which contain the prosthetic groups FMN (Flavin mononucleotide) or FAD (Flavin adenine dinucleotide). Flavoproteins have a role in electron transport. Thus riboflavin has an important role in oxidative metabolism like respiration [17] and so it appeared significant in first step screening. For efficient productivity of the organism under study, which is highly aerobic, efficient respiration appears to be very important and so the need of flavoproteins and thus the vitamin riboflavin. Taking these facts into consideration riboflavin which appeared significant after second step screening was included in the second step screening for optimized medium.

CONCLUSION

Optimized production medium is a necessity for any commercial production for significant product yield with minimum inputs. The significant phosphate sources were disodium hydrogen phosphate ranked and potassium dihydrogen phosphate, while the sulfur source sodium thiosulphate was found to be significant and almost ranked 1st on all days. Vitamins like riboflavin, nicotinic acid and thiamine hydrochloride were found to have significant effect. Thus, This statistical screening strategy not only short listed the efficient diverse nutrients but also improved the antibiotic yields as the combined effects of the nutrients were also considered.

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