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Role of Amino Acids and Vitamins in the Production of Alkaline Phosphatase and Erythromycin by *Saccharopolyspora Erythraea*

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Resting cell experiments with washed mycelia of *Saccharopolyspora erythraea* were performed to study the effects of amino acids and vitamins on the biosynthesis of erythromycin and alkaline phosphatase at two pH optima. It was found that amino acids (glycine and L-leucine), and vitamins (pantothenic acid and ascorbic acid), each increased alkaline phosphatase as well as erythromycin formation by *S. erythraea*. A combination of the vitamins and the amino acids was found to give additive effect. The amino acids with the help of the vitamins exerted such additional effect on the enzyme and erythromycin biosynthesis.

Erythromycin is a secondary metabolite and its biosynthesis depends on the physiological characteristics and the cultural conditions[1,2]. Alkaline phosphatase of *Saccharopolyspora erythraea* had isoenzymes of different molecular weights with different pH optima (pH 8.4 and pH 9.2) and is reported to be related with erythromycin production[3]. Complex nutrients as source of amino acids or vitamins were also used in the fermentation and favoured erythromycin formation by *S. erythraea*[4-7]. Branched chain amino acids (L-valine, L-isoleucine and L-leucine) have been found to play a vital role in the biosynthesis of erythronolide[8] in the medium depending upon its composition. To find out the role of different amino acids and vitamins on alkaline phosphatase and erythromycin formation in the present study, washed mycelia of *S. erythraea* were used and experiments were carried out in salt-medium.

MATERIALS AND METHODS

Culture and conditions:

A parent strain[7,9] of *S. erythraea* (formerly *Streptomyces erythreus*, Parent B) was maintained in Czapek agar slants. Four to six loopfuls of spores from a well sporulated 10 d old slant culture were suspended in 10 ml water containing 50 mg yeast extract (pH 7) in a 100 ml Erlenmeyer flask. The flask was incubated on a rotary shaker (150 rpm) at 30° for 6 h with glass beads to make a homogenous suspension and to initiate germination of spores. One millilitre of the treated suspension ($1.5x10^7$cfu/ml) was used for inoculation of 50 ml fermentation medium containing 1.5 g glucose, 0.15 g $NaNO_3$, 0.02 g K_2HPO_4, 0.025 g $MgSO_4.7H_2O$, 0.25 g NaCl, 0.25 g $CaCO_3$, 0.0025 g $FeSO_4.7H_2O$, 0.0005 g $MnCl_2.4H_2O$, 0.0005 g $NiSO_4.6H_2O$, pH 7 in 250 ml Erlenmeyer flask and incubated on a rotary shaker (150rpm) at 29°.

Preparation of washed mycelia:

After 60 h incubation, the mycelia were separated by centrifugation (3000 rpm) for 15 min. The mycelia were then washed thoroughly with 0.5% KCl. The washed mycelia (~2 g wet wt) were then transferred to a 100 ml Erlenmeyer flask containing 20 ml of 0.5% NaCl in water at pH 8.

Fermentation with amino acids and vitamins by washed culture:

Different amino acids at a concentration of 0.5% (w/v) were added to the washed cell suspension of 20 ml volume (pH 8) in 100 ml flasks. Different vitamins at concentration of 5 and 10 µg/ml were then tested in the salt medium supplemented with optimum amount of amino acids. Finally, differ-

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ent combinations of the effective amino acids and vitamins were studied for production of alkaline phosphatase and erythromycin by the culture. After addition of amino acids and vitamins, the flasks were incubated on a rotary shaker (150 rpm) at 29°.

Assay of erythromycin:

The potency of erythromycin in broth of washed cell culture was determined by the conventional cup plate method[10] using *Sarcina lutea* ATCC 9341 as the test organism. The potency was expressed in terms of erythromycin base.

Enzyme activity estimation:

The activity of alkaline phosphatase at pH 8.4 and pH 9.2 was determined in a reaction mixture. Each reaction mixture in a final volume of 2 ml contained 0.1 ml enzyme source, 2.5 μmole disodium-*p*-nitrophenyl phosphate, 190 μmol buffer of required pH and 4 μmol $MgCl_2$. Tris-HCl buffer of pH 8.4 and glycine-NaOH buffer of pH 9.2 were used. The reaction mixture was incubated at 37°. After incubation for 30 min, 5.5 ml of 0.1 N NaOH was added. The level of released *p*-nitrophenol in the supernatant was measured at 410 nm in a Beckman DV64/UV spectrophotometer[11], using a blank where disodium-*p*-nitrophenyl phosphate was added after incubation and addition of 0.1N NaOH to the reaction mixture. Protein content in the cells was estimated[12] after treating with TCA following the method described by Escalante *et al*[13].

RESULTS

The effects of different amino acids were tested on the synthesis of alkaline phosphatase of two pH optima (pH 8.4 and pH 9.2) and on erythromycin formation by washed cells of *S. erythraea*. It was observed that 0.3% glycine and 0.3% L-leucine individually stimulated the biosynthesis of erythromycin as well as alkaline phosphatase without increasing the cell mass (Table 1 and Table 2).

The amino acids (0.3% glycine and 0.3% L-leucine) were added to the washed cell suspension in 0.5% NaCl and effects of different vitamins were studied as per table 3. Among different vitamins, pantothenic acid (10 μg/ml) and ascorbic acid (5 μg/ml) increased highly the yield of eryth-

TABLE 1: EFFECTS OF DIFFERENT AMINO ACIDS ON ALKALINE PHOSPHATASE AND ERYTHROMYCIN FORMATION BY *S. ERYTHRAEA*.

Amino acids (5 mg/ml)	Phosphatase activity (μmol/ml) after				Erythromycin (μg/ml) after	
	24 h		48 h		24 h	48 h
	pH 8.4	pH 9.2	pH 8.4	pH 9.2		
0	5.38	5.59	5.95	6.19	10.9	12.6
L-Arginine	4.65	5.18	5.17	6.22	12.1	12.1
B-Alanine	2.29	3.42	4.56	5.15	10.6	13.4
L-Glutamate	8.37	8.37	16.0	16.6	12.0	12.0
Glycine	23.2	24.9	25.8	27.5	31.8	36.6
L-Leucine	23.2	23.2	24.0	25.0	31.8	40.1
L-Methionine	2.23	8.37	2.23	3.42	6.71	6.71
L-Tyrosine	12.9	14.2	18.1	19.4	21.8	25.1
L-Valine	1.94	2.58	2.28	3.86	7.57	9.09
L-Isoleucine	18.3	18.7	19.4	19.9	27.5	29.2
L-Lysine	5.79	7.11	7.10	7.76	17.3	19.9

Washed cell suspension of *Saccharopolyspora erythraea* in 0.5% NaCl [cell protein: 0.427mg/ml] was used. Results are means of triplicate experiments.

romycin and phosphatase activity at both pH optima (Table 3). When the vitamins (pantothenic acid and ascorbic acid) and the amino acids (glycine and L-leucine) were added to 0.5% NaCl solution for studying their combined effects, it was found that these four compounds in combination showed additive effect on the enzyme activity and erythromycin formation by washed mycelia (Table 4). On the other hand, the results of two amino acids in combination indicate that glycine and L-leucine without the vitamins were not able to provide such additive effect.

DISCUSSION

In our previous study on alkaline phosphatase and erythromycin production by *S. erythraea* in different synthetic media, production of erythromycin was found to be related to two isoenzymes of alkaline phosphatase with different pH optima (pH 8.4 and pH 9.2), each being the isomers of different glycoprotein[3]. The role of alkaline phosphatase in erythromycin formation will be clear when phosphorylated compounds as intermediates in the biosynthetic pathway to erythromycin are reported. Glucose-6-phosphate is such an intermediate in *S. erythraea* when D-glucose is taken up inside the cells. Alkaline phosphatase of pH 8.4 releases glucose from glucose-6-phosphate at cytosolic pH and provides intact glucose for the synthesis of neutral sugars[14] in the molecule of erythromycin, which in turn concomitantly removes the repressive effect of glucose-6-phosphate[3] on the enzyme itself. In the present experiments with amino acids and vitamins in washed cell culture, alkaline phosphatase increased highly and functioned for erythromycin formation when glycine, L-leucine, pantothenic acid and ascorbic acid, all in a combination, were used (Table 4). Glycine and L-leucine in combination without vitamin are not capable of conferring their individual effect on the synthesis of alkaline phosphatase and erythromycin formation and, therefore, no additive effect was observed (Table 4). L-leucine and glycine are reported to supply methylmalonic acid and propionic acid in the formation of erythronolide[4]. The amino acids during metabolism require coenzyme A and form methylmalonyl-CoA and propionyl-CoA. Pantothenic acid is converted to coenzyme A[15] and is supposed to be involved in the formation of the erythronolide components from L-leucine and glycine. Ascorbic acid is chemically an antioxidant and creates favourable conditions for activity of alka-

TABLE 2: OPTIMUM CONCENTRATIONS OF GLYCINE AND L-LEUCINE FOR ALKALINE PHOSPHATASE AND ERYTHROMYCIN FORMATION BY *S. ERYTHRAEA*

Amino acid	Conc. of amino acid (mg/ml)	Phosphatase activity (μmol/ml) after				Erythromycin (μg/ml) after	
		24 h		48 h		24 h	48 h
		pH 8.4	pH 9.2	pH 8.4	pH 9.2		
Control	0.0	5.38	5.59	5.95	6.19	10.9	12.6
Glycine	0.5	16.6	17.8	24.6	26.3	24.3	38.0
	1.0	16.6	17.8	26.8	28.6	30.4	38.1
	2.0	17.7	19.0	31.6	33.8	30.4	38.1
	3.0	36.5	39.2	40.1	42.8	34.9	38.3
	5.0	23.2	24.9	25.8	27.5	31.8	36.6
L-leucine	0.5	25.1	26.1	29.0	30.1	25.1	31.8
	1.0	30.5	31.6	31.8	33.0	28.8	34.9
	2.0	34.0	45.1	44.2	45.9	31.6	40.0
	3.0	41.7	43.2	49.0	50.9	34.8	44.1
	5.0	23.2	23.2	24.0	25.0	31.8	40.1

Washed cell suspension of *S. erythraea* in 0.5% NaCl [cell protein: 0.417 mg/ml] was used. Results are means of triplicate experiments.

TABLE 3: EFFECT OF DIFFERENT VITAMINS ON ALKALINE PHOSPHATASE AND ERYTHROMYCIN FORMATION BY *S. ERYTHRAEA*

Vitamins	Conc. of Vitamin (μg/ml)	Phosphatase activity (μmol/ml) after				Erythromycin (μg/ml) after	
		24 h		48 h		24 h	48 h
		pH 8.4	pH 9.2	pH 8.4	pH 9.2		
-	-	21.9	28.6	48.6	60.8	32.0	41.1
Thiamine	5	27.9	36.5	45.4	56.7	60.5	76.3
hydrocloride	10	25.5	33.2	44.4	55.5	38.2	72.4
Pyridoxal	5	22.0	28.6	25.8	32.3	41.1	54.9
hydrocloride	10	26.0	33.9	18.6	23.2	37.8	61.0
Riboflavin	5	22.0	28.6	37.2	46.5	32.5	41.1
	10	26.8	34.9	43.4	54.3	44.5	49.6
Pantothenic	5	24.7	32.2	47.4	59.2	48.4	105
acid	10	32.4	42.3	65.8	82.3	48.5	117
Ascorbic	5	39.3	51.2	114	142	69.5	120
acid	10	39.3	51.2	109	136	69.5	70.7
B_{12}	5	22.9	29.9	44.8	56.0	48.0	53.1
	10	32.9	43.0	54.0	57.5	57.8	73.2
Nicotinamide	5	16.6	21.7	11.3	14.1	27.7	24.5
	10	24.7	32.2	21.5	26.9	32.0	27.3
Biotin	5	16.6	21.7	8.5	10.6	27.7	44.8
	10	21.4	27.9	14.1	17.6	35.5	48.7
Paraamino-	5	32.7	42.7	50.4	63.0	59.5	76.3
benzoic acid	10	23.0	30.0	30.8	38.5	53.2	68.3

Washed cell suspension of *S. erythraea* in salt-medium of 0.3% glycine, 0.3% L-leucine and 0.5% NaCl [cell protein: 0.43 mg/ml] was used. Results are means of triplicate experiments.

line phosphatase during fermentation. However, we can conclude that glycine, L-leucine, pantothenic acid and ascorbic acid, all in a combination, do create favourable conditions in the fermentation and increase the synthesis of alkaline phosphatase and erythromycin formation by *S. erythraea* .

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TABLE 4: EFFECT OF VITAMIN AND AMINO ACID IN COMBINATION ON ALKALINE PHOSPHATASE AND ERYTHROMYCIN FORMATION BY *S. ERYTHRAEA*

Amino acid (mg/ml) and Vitamin (μg/ml)	Phosphatase activity (μmol/ml) after				Erythromycin (μg/ml) after	
	4 h		48 h		24 h	48 h
	pH 8.4	pH 9.2	pH 8.4	pH 9.2		
0	5.45	5.60	5.80	6.29	11.0	12.6
Glycine (3.0)+ L-leucine (3.0)	27.3	35.6	41.1	51.4	32.3	40.5
Glycine(3.0)+ Pantothenic acid (10.0)+ Ascorbic acid (5.0)	59.2	77.3	127	158	110	155
Glycine(3.0)+L-leucine (3.0)+ Pantothenicacid (10.0)+ Ascorbic acid (5.0)	87.9	114	153	191	118	198

Washed cell suspension of *S. erythraea* in 0.5% NaCl [cell protein: 0.427 mg/ml] was used. Results are means of triplicate experiments.

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