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ROLE OF ALKALINE PHOSPHATASE ENZYME IN THE FERMENTATIVE PRODUCTION OF ERYTHROMYCIN ANTIBIOTIC AND ITS INCUBATION PERIOD

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ABSTRACT

Erythromycin is prepared from fresh strains of *Saccharopolyspora erythraea* which was maintained and improved in our laboratory. The spores of *S.erythraea* from czapeck slant were taken and two loops full were transferred into the seed medium (20 ml) and placed on a rotary shaker for 48hours. The seed (1 ml) was aseptically transferred into 50 ml of fermentation medium and kept for incubation on a rotary shaker for 5th, 6th, 7th and 8th days to obtain the optimum period of incubation. With the extended period of fermentation it was seen that the concentration of erythromycin, enzyme (alkaline phosphatase) activity and its protein content started decreasing from the 7thday (168 Hours) of fermentation. So it can be said that at the 6thday (144 hours) of incubation of the product of antibiotic attained its peak as well as the enzyme activity and the protein content.

KEY WORDS: Fermentation, Alkaline Phosphatase, Czapek slant, Nutrient Broth.

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INTRODUCTION

Alkaline Phosphatases(AP) from various mammalian tissues and bacteria have been studied extensively[1] which provided evidences that alkaline phosphatase dephosphorylates proteins containing phosphotyrosine and is therefore involved in the regulation of phosphorylation- dephosphorylation reactions. A role in bone calcification has also been suggested [2]. In the mammary gland myo-epithelial cells contain most of the alkaline phosphatases[3] which might suggest a function related to the milk ejection process. Several isozymes of AP have been recognized, the intestinal enzyme, the human placental enzyme and the isoenzyme found in bone, liver and kidney and in the most other tissues. Histochemical evidences suggest that AP in Secondary cells is associated in an inactive form with the interfacial layer of milk lipid droplets. In mammals, they are linked with transport mechanisms. Many APs have been characterized since the 1960s. The *E.coli* AP has been widely studied in terms of biosynthesis[6,7]structure and catalytic properties[8]. Numerous mammalian AP cDNAs have been characterised[9,10,11]. Alignments of the deduced protein sequences have shown a strong conservation of the catalytic site, which involves a serine residue and three metal ions per monomer, two Zn(II) and one Mg(II)[12]. However, mammalian APs differ from their *E.Coli* counterpart in terms of Mg(II)secondary ligand[13], membrane anchoring[14], glycosylation etc. APs represent a large research field as they are good to study metal for ion dependent catalysis and are used in several application fields of molecular biology[15] and immunodetection[16] . Although Archaea have been studied for a few decades relating few archaeal APs have been described. All of them are from halophilic species. All have been isolated and characterized from three species of the genus halobacterium[17,18,19]. In 1990 a study described an AP from halophilic archaeon *Haloarcula marismortui* [20]. So far no APs from hyperthermophilic archaeons have been isolated and characterized. As studying thermostable enzymes appear interesting for the understanding of life at high temperatures as well as for industrial processes, new APs exhibiting this property have been investigated. Thermostable APs from the following thermophilic bacteria have been described *Thermotoga neapolitana*[21], *Thermus caldophilus*[22], *Thermus thermophilus*[23] and *Bacillus stearothermophilus*[24].

MATERIALS AND METHODS

Preparations of various media (Czapek, fermentation, seed etc) were done by various laboratory grade materials of sucrose, $NaNO_3$, KH_2PO_4, $Mg(SO_4)_2.7H_2O$, NaCl, yeast extract, glucose etc. Laboratory grade chemicals like TCA (trichloro acetic acid) ammonium molybdate solution, ANSA (amino napthol sulfonic acid), TRIS-HCl (0.1M, pH-8.4), Glycin-NaOH buffer (0.1M, pH-9.2), sodium bisulfite, sodium metabisulfite, sodium sulphite, Na_2CO_3, sodium potassium tartarate, $CuSO_4.5H_2O$ solution, alkaline copper sulphate solution etc were used. Rotary shaker, colorimeter, laboratory grade were used in the experiment.

Preparation of standard curve of erythromycin

Microbiological assay was done from the standard stock solution of Erythromycin of 0.64,0.8,1.0,1.25and1.56µg/ml using *Micrococcus lutea* [25] ATCC 9341 as the test organism.The standard curve was prepared.

Preparation of standard curve of phosphate[26]

A solution of potassium dihydrogen phosphate was prepared with 10% trichloro acetic acid (TCA), 0.5% ammonium molybdate and ANSA (amino napthol sulfonic acid). The blue color formed was measured at 660 nm.

Estimation of activity of Alkaline phosphatase in broth

P-nitrophenyl phosphate disodium reacts with alkaline phosphatase produced from the broth to yield p-nitrophenol (yellow coloured) and inorganic phosphate (Pi) which reacts with ammonium molybdate to produce phosphomolybdic acid which reacts with ANSA(amino napthol sulfonic acid) to produce blue colour which is measured by colorimeter at 660nm.

Procedure for the assay of alkaline phosphatase

Alkaline phosphatase was assayed in broth control and sample. Tris buffer pH-8.4 (0.1M tris HCl) and glycin buffer pH 9.2(0.1M glycin NaOH) were used. In the broth control 1 ml buffer, 4 µmole $MgCl_2$, 0.5 ml substrate (2.5 µmole) in buffer and 0.5 ml broth was taken. Incubation of the broth control and the sample at 37^0C for 30 min was done. 4 ml of10% TCA (Tri cloro acetic acid) and 0.5ml broth was added and centrifuged at 3000 rpm for 10 mins. In supernatant 2 ml 5% ammonium molybdate and 0.5 ml ANSA (amino napthol sulfonic acid) were added. Incubation at room temperature for 30 min for both broth control and sample was done. The O.D. was measured at 660 nm in colorimeter.

Estimation of Protein in broth[27]

0.1ml of sample (blank with 0.1ml G.D.W) was treated with 5ml of alkaline copper sulphate solution. Incubation was done at 37^oC for 10 mins. Then 0.5ml of folin-phenol reagent was added and incubated for 45 mins at 37^oC. Blue color was formed and O.D. was measured at 660 nm. Composition of suitable complex and synthetic medium for estimation of optimum incubation period.

1. Glucose (3%w/v), $NaNO_3$ (0.3%w/v), $Mg(SO_4)_2.7H_2O$ (0.05%w/v), NaCl (0.5%w/v), K_2HPO_4 (0.01%w/v), yeast extract (0.5%w/v), $CaCO_3$ (0.1%w/v), distilled water was adjusted to q.s. and pH was adjusted to 7.
2. Glucose (3%w/v), $NaNO_3$ (0.3%w/v), $Mg(SO_4)_2.7H_2O$ (0.05%w/v), NaCl (0.5%w/v), K_2HPO_4 (0.01%w/v), $CaCO_3$ (0.1%w/v), distilled water was adjusted to q.s. and pH was adjusted to 7.

RESULTS AND DISCUSSION

Preparation of standard curve of Erythromycin

Standard curve of erythromycin is shown in diagram with different concentration of standard erythromycin with zone diameter.

Table 1
Standard curve of Erythromycin (Log Conc vs. Zone diameter)

ERYTHROMYCIN CONCENTRATION(µg/ml)	LOG (Conc.×100)	ZONE DIAMETER(mm)
0.64	1.806	13
0.80	1.903	15.6
1.0	2.0	17.6
1.25	2.09	19.6
1.56	2.19	21.6

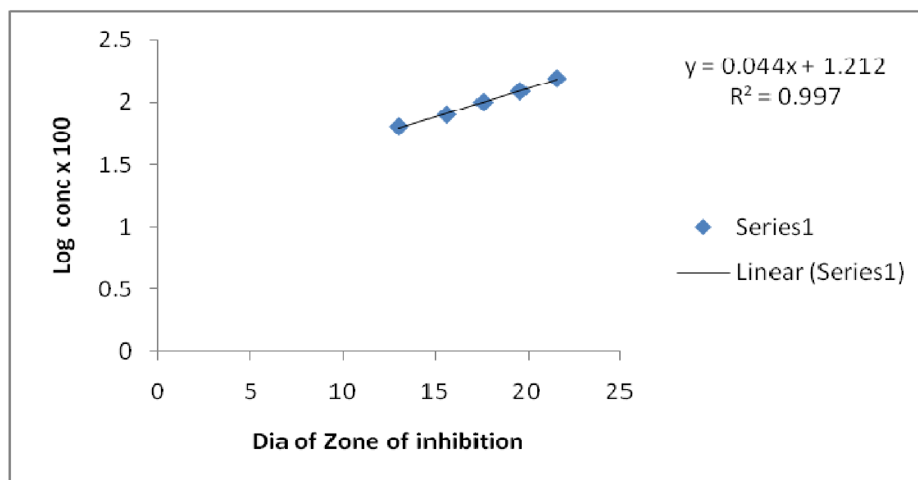

Figure 1
Standard curve of Erythromycin (Log Conc Vs. Zone diameter)
Preparation of standard curve of phosphate

Table 2
Standard curve of phosphate

KH_2PO_4 Conc(µg/ml)	O.D at 660nm Reaction volume	
	7.5ml	15ml
20	0.38	0.21
30	0.52	0.28
40	0.70	0.38
50	0.91	0.48

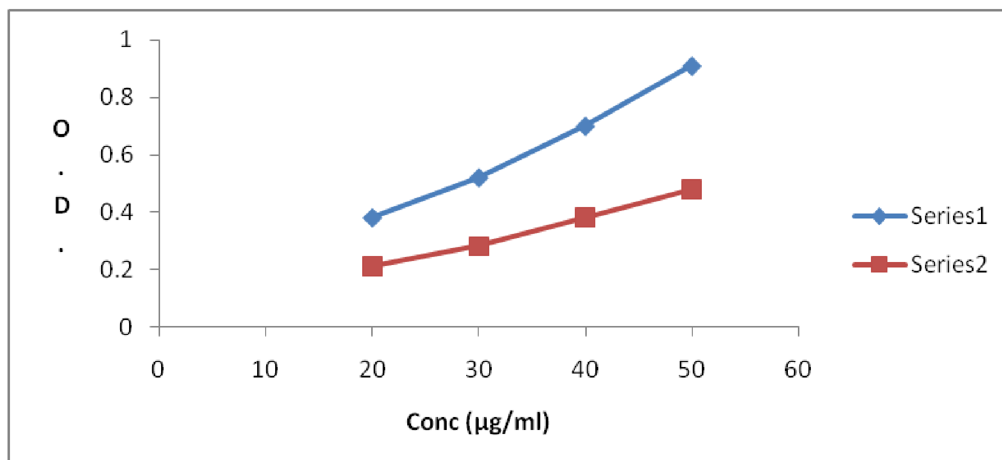

Figure 2
Standard curve of phosphate.Key:Series1:7.5ml and Series2:15ml

Erythromycin, Alkaline Phosphatase and Protein in the broth of S.erythraea after fermentation in complex media

Table 3
Erythromycin, Alkaline Phosphatase and Protein in the broth of S.erythraea after fermentation in complex media at an incubation temp of 28-30°C

Period of incubation (Days)	Erythromycin in Broth (µg/ml)	AlkalinePhosphatase activity in broth (µmole/Pi/min/ml)	Protein content in broth (µg/ml)
4	46.615	$2.786x10^{-3}$	18.33
5	49.30	$3.1x10^{-3}$	40.47
6	51.425	$3.8x10^{-3}$	80.95

Table 4
Erythromycin, Enzyme activities and Protein in the broths of S.erythraea after fermentation in complex media at an incubation temp of 28-30°C

Period of incubation (Days)	Erythromycin in Broth (µg/ml)	AlkalinePhosphatase activity in broth (µmole/Pi/min/ml)	Protein content in broth (µg/ml)
Fermentation Medium 1			
5	60.11	$3.4119x10^{-3}$	66.67
6	62.94	$4.96x10^{-3}$	69.04
7	56.10	$3.72x10^{-3}$	40.47
8	52.35	$2.729x10^{-3}$	23.80
Fermentation Medium 2			
5	67.74	$4.03x10^{-3}$	97.6
6	70.6	$4.96x10^{-3}$	116.67
7	65.912	$3.72x10^{-3}$	107.142

Erythromycin is produced during the idliophase (Stationary phase) just after tropophase (when rapid growth occurs). The production of the erythromycin in the fermentation broth depends on various factors like the composition of fermentation medium, temperature of incubation, periods of incubation, bacterial or fungal contamination etc. Period of incubation is an important parameter in the fermentation process. A complex fermentation medium was prepared to study the effect of increasing incubation period on the production of erythromycin and alkaline phosphatase activity. It contains glucose (3 %w/v), $NaN0_3$ (0.3%w/v), NaCl (0.5%w/v), yeast extract (0.5%w/v), $Mg(SO_4)_2.7H_20$ (0.05%w/v) distilled water added to q.s and pH 7. It was found that with the increase of incubation period the production of antibiotic and activity of alkaline phosphatase was also increased. So another one experiment was made for incubation for the production of erythromycin and the activity of alkaline phosphatase. One complex and synthetic medium was used. The composition of the complex medium was that of used in the first experiment. The composition of the synthetic medium glucose (3%w/v), $NaN0_3$ (0.3%w/v), Mg $(S0_4)_2.7H_20$ (0.05%w/v), NaCl (0.5%w/v) distilled water to q.s and pH was adjusted to 7. It was observed that the production of erythromycin and the activity of alkaline phosphatase was increased up to the 6th day (144hr.) of incubation and then it started decreasing.

CONCLUSION

The effects of incubation period on alkaline phosphatase and erythromycin production was studied in the fermentative production of Erythromycin by shake-flask fermentation method from *S.erythraea.* A suitable basal fermentation medium was used which contains glucose (3%w/v), $NaNO_3$ (0.3%w/v), $Mg(SO_4)_2.7H_2O$ (0.05%w/v), NaCl (0.5%w/v), K_2HPO_4 (0.01%w/v), yeast extract (0.5%w/v), $CaCO_3$ (0.1%w/v), distilled water was adjusted to q.s. and pH was adjusted to 7. The optimum period of incubation for the production of erythromycin and the activity of alkaline phosphatase was studied in a suitable complex and synthetic medium. The optimum period of incubation was found to be 144 hrs when the production of antibiotic was maximum and the activity of alkaline phosphatase was also maximum.

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