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TRIBUTYRIN-ESTERASE BIOSYNTHESIS IN *Geobacillus sp.* AGP-04 A STUDY OF REGULATORY MECHANISM.

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ABSTRACT

Microbial esterases have a promising biotechnological potential to be used in various industrial purposes from the food industry to pharmaceutical industry. To accomplish appropriate function and higher quantity of the biocatalysts there is a need to govern the specific control of the enzyme synthesis. This article reports the regulation of a trybutyrin-esterase synthesis in a thermophilic bacterium, *Geobacillus sp.* AGP-04. Tributyrin alone itself induced esterase synthesis. Other triglycerides, sugar-alcohol and other carbon sources exhibited little or no enzyme induction. Lower concentration (0.01–0.15% v/v) of the enzyme catalyzed end products, i.e. glycerol and butyric acid in the presence of tributyrin induced enzyme synthesis though higher concentration (>0.15%) was inhibitory. The addition of superfluous energy rich phosphonucleotides did not enhance the enzyme synthesis. Addition of glucose (about 0.2%) to the media supplemented with tributyrin repressed tributyrin-esterase biosynthesis. cAMP alleviated the enzyme synthesis in the glucose mediated repressed cell. Potent ionophores like 2-4-dinitrophenol and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) repressed enzyme synthesis by inhibiting ATP synthesis in the cell. The study revealed that tributyrin-esterase synthesis to be an energy dependent process and it is under the control of catabolic repression.

KEY WORDS: **Tributyrin; Esterase; *Geobacillus sp.;* Enzyme regulation;Catabolic repression**

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INTRODUCTION

Esterases (EC 3.1.1.x) denote a different group of hydrolases catalyzing the cleavage and formation of ester bonds. They are broadly disseminated in animals, plants and microorganisms. A large number of them show an extensive substrate tolerance, which prompted the supposition that they have developed to empower access to carbon sources or to be included in catabolic pathways. Furthermore, esterases also show high regio- and stereo-specificity, which makes them magnetic biocatalysts for the production of optically pure compounds in fine-chemicals synthesis [1, 2, 3,4,5,6]. Esterases have pulled in significant consideration in biotechnology in view of current provisions and the viewpoint of new compound synthesis to be used in food, pharmaceutical, and chemical industries[7]. Esterases catalyzed several important reactions include enantio- and regio-selective hydrolysis, synthesis of natural and non-natural pro-drugs, detergents, polyesters, and additives, resolution of racemic mixtures by transesterification[7, 8, 32, 34]. Microbial enzyme synthesis depends upon the insufficiency of the readily utilizable organic carbon. Microbes generally produce enzymes when the required nutrients for their growth becomes depleted in the environment as a result, they express their genes for enzyme synthesis to release readily utilizable nutrients from complex organic matter, if the producer organisms get the freely utilizable necessary nutrient the enzyme synthesis becomes repressed and when the required substrate availability drops down the enzyme synthesis induced again. It is obvious that microorganisms are capable of profligate synthesis of the inducible enzymes using the repression strategy. Another crucial phenomenon is that many enzyme synthesis can be repressed by the buildup catalytic end products of the enzymes in the cell or in the growing environment of the microorganisms[9, 10, 11, 12, 13, 14, 31]. Enzyme biosynthesis depend upon the microbial extracellular and intracellular events and regulation is quite multifaceted. Enzymes are endorsed as secondary metabolites and assume to be important in the adaptation of microbes. For the adaptation to the environment, microbes follow the switch off and switch on strategy of a particular operon for their need [15, 29]. According to the literature, regulation of tributyrin-esterase are not extensively studied. Thus the aim of this work was to analyze the effect of several carbon sources as inducers or repressors of the tributyrin-esterase of *Geobacillus sp.* AGP-04 and the effect of cyclic AMP mediated catabolic regulation of the enzyme.

MATERIALS AND METHODS

The esterase producing Organism

Geobacillus sp. AGP-04, a potent esterase-producing bacterial strain was used in this study. *Geobacillus sp.* AGP-04, a thermophilic bacterium, isolated from soil sediment of a hot spring situated at Bakreshwar, West Bengal, India. The bacteria were grown in the Luria-Bertani (LB) liquid medium containing (g L^{-1} in distilled water) yeast extract 5.0, bactotryptone 10.0 and NaCl 5.0, medium pH was adjusted to 8.0 and incubated at 60^{o}C with the shaking condition (200 rev min^{-1}) for 72 h. The isolated bacteria have been explored as potent esterase producer[16, 17]. The strain was lyophilized and stored at −20 °C.

Enzyme production by the Geobacillus sp. AGP-04

1.0 ml fresh culture of *Geobacillus* sp. AGP-04 was inoculated into 10 ml LB medium containing 1.5 % tributyrin and incubated overnight at 60^{o}C with shaking (200 rev min^{-1}). The culture was then transferred into the 1000 ml Erlenmeyer flask containing 250 ml fermentation medium containing: [(g L^{-1}): yeast extract 5.0, bactotryptone 10.0, NaCl 5.0; soluble starch, 20; peptone, 20; KH_2PO_4, 5; $(NH_4)_2SO_4$ 1; $MgSO_4.7H_2O$ 1; $(NH_2)_2CO$, 1; and tributyrin at a ratio of 15 ml l^{-1}]. The pH of the media was adjusted to 8.0 (±0.2) and grown on a rotary shaker (200 rev min^{-1}) at 60°C for 96 h. Besides, other triglycerides (triacetin, tricaprylin, tricaproin, trilaurin and triolein) were also tested in place of tributyrin at the same concentration in the fermentation medium containing same ingredients stated above for enzyme production. The cell-free supernatant after centrifugation (8,000 g×10 min, at 4^{o}C) was studied for esterase activity.

Esterase assay

Esterase activity was determined by using the spectrophotometric assay described by Lee et. al[18] with some modification. For activity assay, the stock substrate solution was prepared by dissolving p-nitrophenyl butyrate (PNPB) in acetonitrile at a concentration of 10 mM. For the enzyme assay the substrate solution included stock substrate solution, ethanol and 50 mM phosphate buffer (pH 8) in ratio of 1:4:95 (v/v/v), respectively. To determine the enzymatic activity 0.3 ml of the cell free supernatant was added to 0.7 ml of the substrate solution. After the incubation of the reaction mixture at 60^{o}C for 15 min, the change in absorbance at 405 nm was monitored spectrophotometrically (UV-VIS SPECTROPHOTOMETER 118, Systronics). The amount of released p-nitrophenol (p-NP) was determined for the esterase activity. The non-enzymatic hydrolysis was subtracted by using a blank without enzyme. One unit of enzyme activity was defined as 1µmol of p-NP formed per min under assay conditions.

Investigation of the regulatory mechanism for esterase biosynthesis

The effects of a number of carbon sources were studied separately in the presence or in the absence of tributyrin for esterase production by the organism. The sugar alcohol (0.5% w/v) like Erythritol, Threitol, Arabitol, Xylitol, Ribitol, Mannitol, Sorbitol, Galactitol, Fucitol, Inositol; monosaccharides like Glucose, Fructose, Galactose and some polysaccharides viz; Cellulose, Starch and tributyrin were used as sole carbon source for enzyme production. Glycerol and Butyric acid are the two main hydrolytic end products of tributyrin. Different concentrations (0.01–1.0%) of both the end products of tributyrin were added separately in the tributyrin containing medium to study their effects on esterase production by *Geobacillus sp.* AGP-04. To study the catabolic repression, glucose [1% (v/v)] and cAMP (10 mmol) were added in the tributyrin [0.5% (v/v)] containing culture media using following groupings: (1) basal medium; (2) basal medium +1% glucose; (3) basal medium + cAMP (10 mmol) and (4) basal medium + 1% glucose + cAMP (10 mmol). Chemicals like 2-4-dinitrophenol (DNP; 0.1–0.4 mmol) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 0.1-0.4 mmol) were used in the culture broth to divulge their effect. Enzyme synthesis by the age of inoculum was also studied. To determine the esterase synthesis by the age of inoculum, various inoculum age groups (12 hours to 96 hours) in same amount were transferred to basal media to study their effect on esterase production. And to study the effect of energy rich compound on enzyme synthesis, different phosphonucleotides (1 mg ml^{-1}) like adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), uridine triphosphate (UTP), and nicotinamide adenine dinucleotide phosphate (NADP) were added to the culture medium to reveal their effect on the enzyme synthesis. Determination of the growth of the organism was carried out according to the colony forming unit. The esterase activity was determined under standard assay condition as described earlier.

RESULTS

Enzyme production by the Geobacillus sp. AGP-04 using different triglycerides

As shown in Figure 1 maximum enzyme production was observed when the fermentation medium was supplemented with tributyrin. Other triglycerides had little or hardly had any significant effect on the esterase production in the fermentation medium. The results indicated that the organism synthesized tributyrin specific esterase. Thus the enzyme regulation study was carried out with tributyrin as the main carbon source and all the further study was related to tributyrin as other triglycerides had limited role on enzyme synthesis.

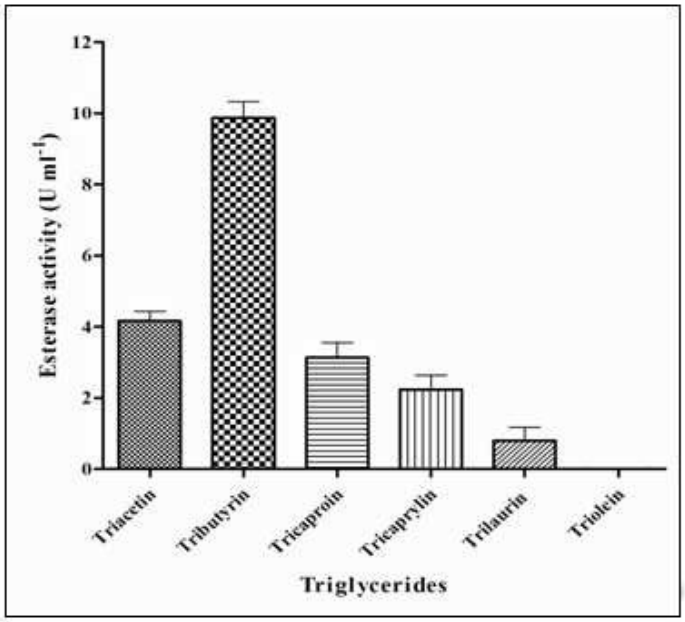

Figure 1
Synthesis of esterase using different triglycerides by Geobacillus sp. AGP-04

All the triglycerides were added separately to the fermentation medium at a concentration of 15 ml l^{-1} and fermentation was carried out at 60°C and fermentation medium pH of 8.0 under shaking (200 rev min^{-1}) condition for 96 h. Data represented were the mean of three independent assay ±SEM.

Study of the induction of esterase biosynthesis by different substrates

Among the different carbon source tested on enzyme induction, the enzyme synthesis was maximally induced in the presence of tributyrin. Each carbon source in combination with tributyrin (0.5% v/v) produced little amount of esterase. Cellulose, Starch, Galactitol, Sorbitol, Ribitol and Inositl in the presence of tributyrin showed better effect. However, all the other tested carbon sources did not have a sizable inducing impact on the enzyme synthesis (Table 1). Surprisingly, it was found that presence of glucose drastically repressed the enzyme synthesis though tributyrin was present in the medium.

Table 1
Effect of different carbon source on esterase production by Geobacillus sp. AGP-04.

Carbon sources (0.5% w/v)	Esterase activity (U ml^{-1})	
	Absence of tributyrin	Presence of tributyrin (0.5%)
Erythritol	ND	0.70±0.27
Threitol	ND	1.02±0.29
Arabitol	ND	0.90±0.12
Xylitol	ND	0.86±0.42
Ribitol	ND	2.68±0.26
Mannitol	ND	0.98±0.31
Sorbitol	ND	2.36±0.38
Galactitol	ND	2.07±0.18
Fucitol	ND	0.72±0.03
Inositol	ND	1.15±0.26
Glucose	ND	0.04±0.00
Fructose	ND	0.98±0.31
Galactose	ND	0.68±0.26
Cellulose	ND	3.62±0.24
Starch	ND	3.06±0.22
Tributyrin	ND	7.21±0.62

Fermentation was carried out at 60°C and fermentation medium pH of 8.0 under shaking condition (200 rev min^{-1}) for 96 h. Data represented the mean of three independent assay ±SEM. ND denotes not detectable.

Catabolic Repression on Esterase Production

Catabolic repression was studied by growing the organism in the fermentation medium using various concentrations of glucose. As shown in Figure 2, the enzyme synthesis did not inhibit by the glucose concentration ranging from 0.01-0.05%, whereas beyond this concentration, enzyme synthesis became inhibited. And at the 1.0% glucose concentration the enzyme synthesis became severely repressed. The study clearly indicated that glucose and cAMP being together absolved the repression and microorganism started esterase synthesis, although cAMP itself had no acceleratory impact (Figure 3). The same sorts of analyses were carried out with glycerol and butyric acid instead of glucose and no substantial recuperation of enzyme synthesis was observed (Table 2).

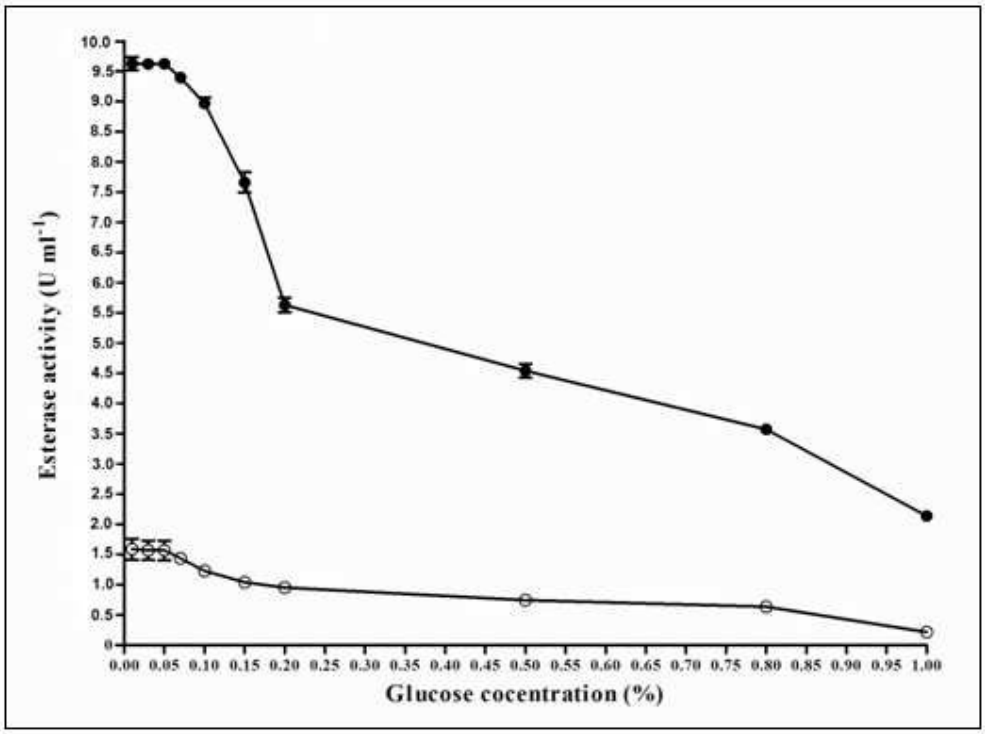

Figure 2

Effect of glucose on esterase biosynthesis by Geobacillus sp. AGP-04

(●) with 0.5% tributyrin, (○) without tributyrin. Fermentation was done at 60°C and fermentation medium pH of 8.0 under shaking (200 rev min^{-1}) condition for 96 h. Data represented were the mean of three independent assay ±SEM.

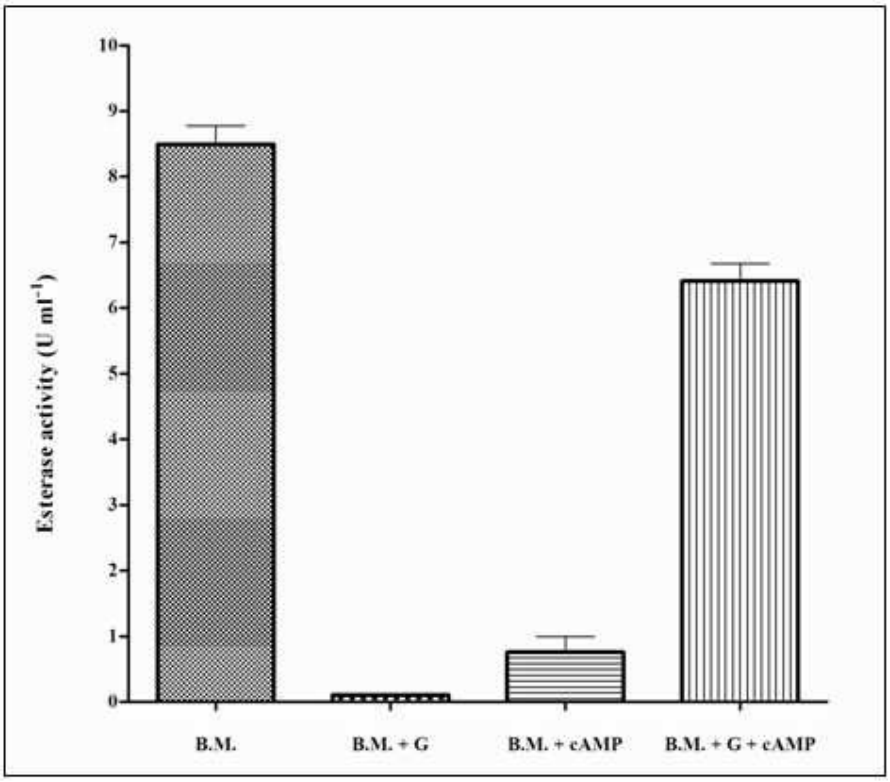

Figure 3
Effect of cAMP and glucose on the tributyrin esterase biosynthesis by Geobacillus sp. AGP-04.

(B.M. denotes basal medium, G denotes 1% glucose). 10 milimolar cAMP was used in the study. Fermentation was done at 60°C and fermentation medium pH of 8.0 under shaking (200 rev min^{-1}) condition for 96 h. Data represented were the mean of three independent assay ±SEM.

Table 2
Effect of cAMP on the recovery of catabolic repression on esterase synthesis by Geobacillus sp. AGP-04

Carbon source	Esterase activity (U ml^{-1})	
	With cAMP (10 mmol)	Without cAMP
0.5% tributyrin + 1% glycerol	0.21±0.06	ND
0.5% tributyrin + 1% butyric acid	0.14±0.02	ND
0.5% tributyrin + 1% glucose	5.42±0.03	ND

Data represented the mean of three independent assay ±SEM. ND denotes not detectable.

Study of Enzyme Inhibition by End Products on Esterase Biosynthesis

From the end product inhibition study, it was found that lower concentration of both the (0.01–0.15% v/v) enzyme catalyzed end products enhanced enzyme production but higher concentrations (above 0.15% v/v) in both circumstances are inhibitory to the enzyme synthesis (Tables 3 and 4).

Table 3
Effect of glycerol on esterase production by Geobacillus sp. AGP-04

Concentration of glycerol (%)	Esterase activity (U ml^{-1}) Presence of tributyrin (0.5%)	Absence of tributyrin
0.01	8.12±0.36	1.24±0.21
0.02	8.01±0.24	1.18±0.27
0.04	7.86±0.31	1.08±0.16
0.06	7.72±0.39	0.92±0.22
0.08	7.69±0.06	0.86±0.20
0.10	7.62±0.14	0.64±0.32
0.15	3.58±0.12	0.60±0.06
0.20	0.21±0.28	ND
0.50	ND	ND
0.80	ND	ND
1.00	ND	ND

Fermentation was carried out at 60°C and fermentation medium pH of 8.0 under shaking condition (200 rev min^{-1}) for 96 h. Data represented the mean of three independent assay ±SEM. ND denotes not detectable.

Table 4
Effect of butyric acid on esterase production by Geobacillus sp. AGP-04.

Concentration of butyric acid (%)	Esterase activity (U ml^{-1}) Presence of tributyrin (0.5%)	Absence of tributyrin
0.01	7.13±0.16	0.92±0.17
0.02	7.29±0.28	0.74±0.12
0.04	7.53±0.31	0.71±0.14
0.06	7.52±0.39	0.57±0.08
0.08	5.13±0.42	0.36±0.11
0.10	4.38±0.20	0.24±0.03
0.15	2.29±0.16	ND
0.20	0.53±0.10	ND
0.50	ND	ND
0.80	ND	ND
1.00	ND	ND

Fermentation was carried out at 60°C and fermentation medium pH of 8.0 under shaking condition (200 rev min^{-1}) for 96 h. Data represented the mean of three independent assay ±SEM. ND denotes not detectable.

Study of the Effect of 2-4-Dinitrophenol (DNP) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on Esterase Biosynthesis

Firstly only 2-4 DNP was used to study, again CCCP was used to confirm the study in the same manner. As depicted in the Figure 4, it was clearly seen from the experiment that the enzyme synthesis was serially decreased by the addition of different concentrations (0.1–0.40 mmol) of ionophore DNP and CCCP.

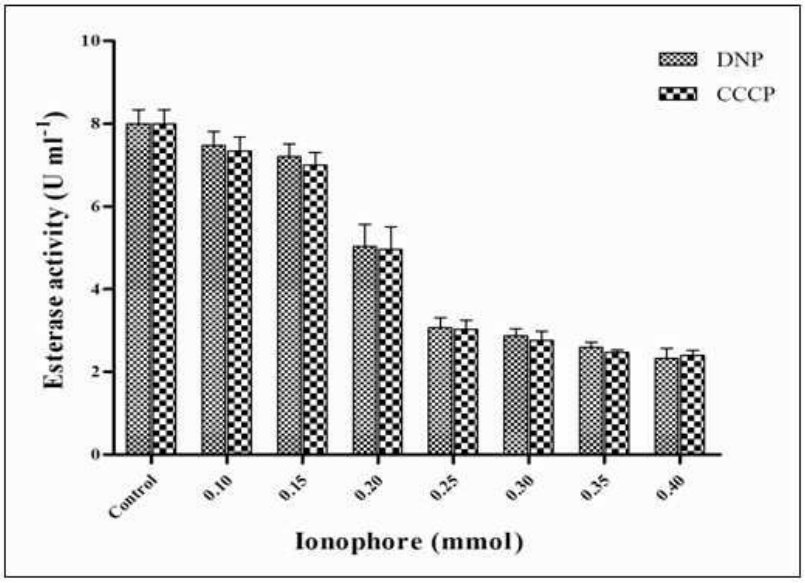

Figure 4
Effect of DNP and CCCP on esterase synthesis by Geobacillus sp. AGP-04

Bacteria were grown in fermentation media containing different concentration of DNP and CCCP in shaking condition (200 rev min^{-1}) at 60^{o}C and fermentation media pH of 8.0 for 96 h of incubation. Control did not contain DNP and CCCP. Data represented were the mean of three independent assay ±SEM.

Effect of Age of Inoculum on the Esterase synthesis

To determine the esterase synthesis by the age of inoculum, various inoculum age groups in same amount were transferred to the fermentation media to study their effect on esterase production. Maximum enzyme synthesis was accomplished by using bacterial inoculum of 84 h age (Figure 5). At the 84 h of inoculum age enzyme synthesis was reached maximally to 6.18±0.12 U ml^{-1}.

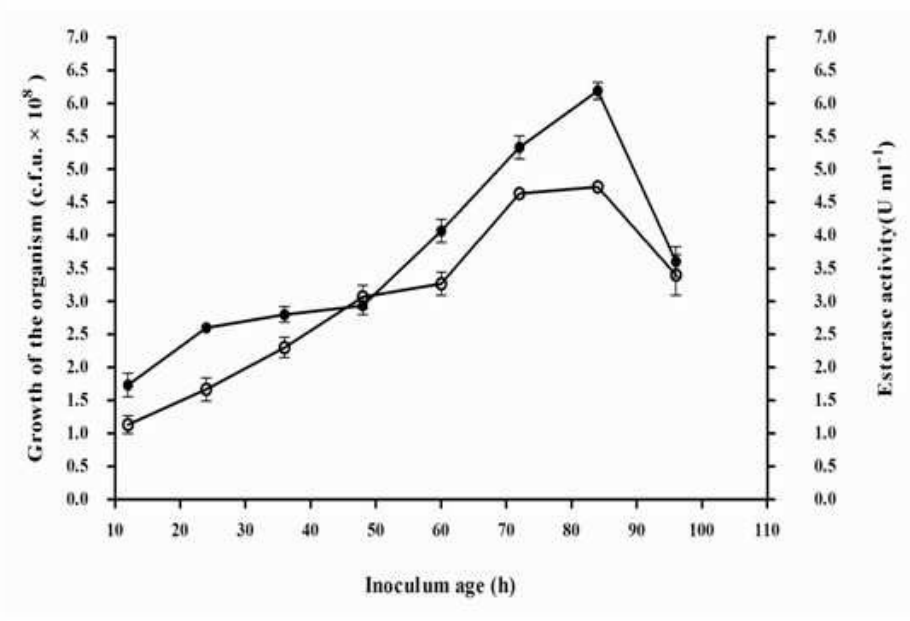

Figure 5
Effect of inoculum age on growth (○) and esterase (●) biosynthesis by Geobacillus sp. AGP-04

Different age of inoculum were added to the fermentation media in same amount and fermentation were carried out at 60^{o}C and medium pH of 8.0 under shaking (200 rev min^{-1}) condition for 96 h. Data represented were the mean of three independent assay ±SEM.

Effect of Phosphonucleotides on Esterase synthesis

Among the tested phosphonucleotides, all of the nucleotides were repressive to esterase production by *Geobacillus sp.* AGP-04 (Figure 6).

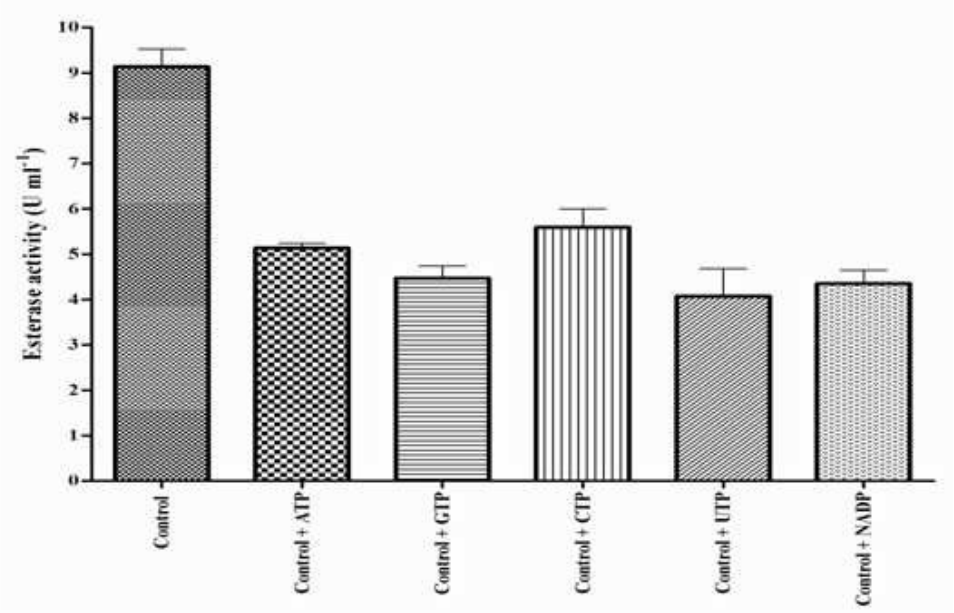

Figure 6
Effect of phosphonucleotides on the biosynthesis of tributyrin esterase by Geobacillus sp. AGP-04

Fermentation was carried out at 60°C and medium pH of 8.0 under shaking (200 rev min^{-1}) condition. Control did not contain any phosphonucleotide. Data represented were the mean of three independent assay ±SEM.

DISCUSSION

From the detailed study it was found that *Geobacillus sp*. AGP-04 synthesized substantial amount of tributyrin specific esterase in the presence of tributyrin in the fermentation medium as it was found that other triglycerides and other carbon sources did not have any significant inducing effect except some of carbon sources like sugar alcohol, polysaccharides though their effect was little with comparison to tributyrin. It appears that the *Geobacillus sp.* AGP-04 esterase was an inducible enzyme and tributyrin served as the inducer. The supplementary carbon sources had scarcely any invigorating impact on enzyme production as the organism gets needed energy by metabolizing those readily utilizable nutrients. This finding supported the verdict of Gupta et. al.[19] stated that lipase expression depends upon the carbon source as lipases are inducible enzymes and are generally produced in the presence of lipid source. In our study it was found that in the presence of glucose, esterase synthesis drastically repressed even though tributyrin was present in the medium. It may be due to that when glucose present in the medium the organism gets the necessary energy by metabolizing the glucose. This finding directed us to carry out the glucose mediated catabolic repression study. Tsuboi et. al. reported that extracellular esterase synthesis may be repressed by dextrose [30]. Triglyceride (specifically tributyrin) presumably elicited induction of the esterolytic genes, and repression of the genes relies on the concentration of the preferential carbon source. Therefore the transcription level of the structural gene was directed by the harmony between the induction and repression pathways. The present study carried out with *Geobacillus sp.* AGP-04 exhibited that glucose-interceded suppression [20] was partly absolved by the addition of cAMP in the media. In case of lac operon system, it was

proved that the level of cAMP was extremely pivotal for transcription and in case of xylanase (xylan-esterase) biosynthesis; repression by glucose seems to be a common phenomenon [21, 22, 23, 29] and this process was influenced by cAMP[24, 29]. In this study, it was found that expansion of glycerol and butyric acid (the end product of tributyrin degradation) at quite low concentrations (0.01–0.15%) stimulated enzyme synthesis, yet above this concentration they were found to be repressive. This phenomenon indicated that the concentrations of this end product were extremely critical for esterase biosynthesis. The age of inoculum plays a critical role for microbial extracellular enzyme production. In this experiment it was observed that the older inoculum synthesize more enzyme than the younger one. The highest esterase induction was accomplished by 84 h old inoculum. This finding is similar to the *Pseudomonas spp P. fragi* mediated lipase production [19]. The probable reason behind this phenomenon can be explained as the microbial cells in late log period of growth are active in esterase synthesis and transfer of such dynamic inoculum to a new culture media could easily activate the efficient synthesis of specific protein by associative preparation of new ribosomes and mRNA [33]. It was presumed that when this accrued mRNA transferred to fresh media then more enzymes synthesized. Eggert et al. [25] reported that at the immediate end of exponential phase *B. subtilis* produced maximum lipase. It was observed that esterase production was diminished by the addition of respiratory inhibitor 2-4-dinitrophenol and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in the growing culture. This phenomenon may be because of that, upon addition of DNP and CCCP, the intercellular level of ATP was ephemerally brought down by uncoupling and initiated the favored hindrance of ATP-ward biosynthetic process [26]. Thus it can be supposed that biosynthesis of esterase is an energy-dependent process. This type of energy-dependent regulation supports the report of Saito and Yamamoto in case of synthesis of alpha amylase production by *Bacillus licheniformis*[27] and Mandal et. al[29] in case of xylanase synthesis. To study the impact of distinctive phosphonucleotides on esterase synthesis by *Geobacillus sp.* AGP-04, ATP, GTP, CTP, UTP, and NADP have been added in the fermentation media. The phosphonucleotides demonstrated inhibitory impact in esterase biosynthesis. Phosphonucleotide precursors are commonly utilized in energy production and recreate the exhausted nucleotide pool for enzyme synthesis [28, 29]. In optimized culture media, bacteria could produce ample phosphonucleotides, hence, addition of superfluous energy rich compound was ineffective for the esterase biosynthesis.

CONCLUSION

From the study, it may be suggested that tributyrin-esterase from *Geobacillus sp.* AGP-04 is an inducible enzyme. The maximal enzyme induction was achieved in the presence of pure tributyrin. Glycerol, butyric acid, the hydrolyzed products of tributyrin has the ability to prompt the enzyme synthesis. However, higher concentration of the end product causes inhibition of esterase production. Moreover, the age of inoculum likewise has a significant effect on the esterase production by *Geobacillus sp.* AGP-04. In addition, higher concentration of readily utilizable carbon source like glucose repressed enzyme synthesis, though the repression could be resolved by cyclic AMP. As the organism gets the needed energy by metabolizing supplemented carbon sources, thus additional energy rich phosphonucleotides did not have any impact on enzyme synthesis. The study suggested that the enzyme production by the *Geobacillus sp.* AGP-04 is under the control of catabolic repression and enzyme synthesis is an energy dependent process. The study will explore tributyrin-esterase regulation in microbial cells. Further genomic study is needed to complete molecular study and is under process.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

Conflict of interest declared none.

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