GENETIC IMPROVEMENT OF SOME LIPASE PRODUCING BACTERIAL STRAINS

By

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ABSTRACT

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The present investigation aims to identify and characterize lipolytic bacillus strains isolated from soil. Isolated lipolytic strains were phenotypically, physiologically, and biochemically characterized. All isolates were Gram-positive, aerobic, and motile; Oxidase -positive, rod shaped, endo spore-forming bacteria, and produced extracellular lipase enzymes. 16S rDNA sequencing identified these *Bacillus spp* as *Bacillus subtilis*, *Bacillus pumilus* and *Anoxybacillus flavithermus*.

Bacillus subtilis and *Bacillus pumilus* as higher lipolytic strains were evaluated for lipase production using broth and solid formulation of one medium (tributyrin medium) the medium contained tributyrin as the only lipid source. Two genetic protocols were applied in this study to improve lipase production, the first is mutation induction by UV irradiation and EMS and the second is protoplast fusion technique for producing new genetic recombinants. Our result revealed that the survival percentage was decreased by increasing the exposure time of UV irradiation at 254nm and increasing of EMS concentration in case of *Bacillus subtilis* and *Bacillus pumilus.* Qualitative assay of lipase in mutants of both *B. subtilis* and *B. Pumilus* was determined by colorimetric method using *PNP* caprylate. Induction of UV and EMS mutation give rise higher lipolytic mutants such as Bsm52, Bsm23, Bpm42

and Bp25 their lipase activity was 220u/ml and 202u/ml, 203u/ml and 189u/ml respectively. Fourteen auxotrophs of *B. subtilis* and twelve auxotrophs of *B. pumilus* were isolated and their nutritional requirements were determined. Intra specific Protoplast fusion was used for complementation between auxotrophs of bacillus strains and inter generic protoplast was done between higher lipolytic bacillus mutant and *Anoxybacillus flavithermus* stain.

Key Words: Lipase*, Bacillus*, 16S rDNA, SDS PAGE, mutation, UV, EMS, protoplast fusion

INTRODUCTION

The advent of enzymology represents an important breakthrough in the biotechnology industry, with the worldwide usage of enzymes being nearly U.S. $ 1.5 billion in 2000 **(Kirk *et al*., 2002).** The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. With the development of global economy; the demand for energy is increasing quickly**.** Microbial lipase provides multiple catalytic activities with high yield. It is easily modified genetically and grows rapidly on inexpensive medium. However, some of their characteristics are not suitable for industrial production and it has become a hot topic for researchers to study on how to improve microbial lipases characters through directed development. In the past 10 years, directed development has been the powerful tool for transformation of lipase function. It makes lipase with greater activity, thermostability, organic solvent tolerance in industrial condition. The short version addresses the various directed evolution methods to optimize lipase characters above **(Jing *et al.,* 2012).**

Among many ubiquitous enzymes, lipases (triacylglycerols acylhydrolases, E.C. 3.1.1.3) are of considerable physiological significance with wide industrial applications. The unique features of lipases include the functionality at aqueous and nonaqueous phase. The wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents. Among lipases of plant, animal and microbial origin, it is the microbial lipases that find immense applications. This is because microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Lipase producing as industrial wastes, vegetable oil processing factories dairies, soil contaminated with

oil, oilseeds, decaying Lipases has been used for industrial applications. The unique features of lipases development of flavors in cheese ripening, bakery products and beverages, also, lipases are used to aid removal of fat from meat and fish products. Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts **(Kumar *et al*., 2012).** Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for induction. However, there are a few reports of constitutive lipase production by bacteria. **(Gao *et al*., 2000).** Lipases are usually secreted out in the culture medium; but membrane bound lipases and intra-cellular lipases have been reported. **(Large *et al*., 1999).**

The onset of lipase production is organism specific, but, in general, it is released during late logarithmic or stationary phase **(Matselis and Roussis, 1992).** The lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* were different from other *Bacillus* lipases. They were the smallest true lipases known (approximate molecular mass 20 kDa) and shared very little sequence homology (approximately 15%) with the other lipases. Based on amino acid analysis and biochemical characteristics *bacillus* lipases were classified into two subfamilies, The subfamily I.4 included lipases with low molecular mass in the range 19-20 kDa from *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*. The lipases from *Bacillus themocatenulatus, Bacillus thermoleovorans*, and *Bacillus stearothermophilus* were included in the subfamily I.5 and they had molecular mass around 43 kDa **(Shah and Bhatt, 2011)**. Because phenotypic and physiological identification not verify the right taxonomic position especially in *Bacillus spp* **(Watabe *et al*., 2004).**

The lipolytic Bacillus strains from tributyrin agar plate **(Elwan *et al.,*1977)** were investigated by PCR amplification of 16SrRNA and sequencing. Identification of the lipolytic bacterial

isolates was performed based on their morphological, physiological, and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology **(Whitman *et al*., 2012).** Then confirmed by PCR amplification and sequencing of 16S rDNA **(Gomaa and Momtaz, 2006).** As well as electrophoretic separation of total protein (SDS-PAGE) according to the method described by **(Berber, 2004)**. In order to obtain strains showing more suitable properties, genetic manipulation methods have been used. Improvement lipase production in *Bacillus spp* than wild-type strains mostly depended on random mutation as UV irradiation **(Ramachandra *et al.,* 2012)** and EMS **(Khattab and Abdel-Aziz, 2012) and (Lotfy *et al*., 2007).** The progress in studies of bacillus strains as model organisms has depended in a large part on advanced genetic methods based on auxotrophic mutations and their respective marker genes **(Chen *et al.,* 1986).**

The use of protoplast fusion technique is necessary in order to obtain recombinant features **(Rygielska, 2004).** Protoplast fusion is considered a good tool for having new genetic recombinations. In this process, two strains are converted to protoplast with the use of lysozyme and then mixed and fused in the presence of polyethylene glycol. The fused protoplasts are then allowed to grow into vegetative cells. Recombination occurs within the transient diploid protoplast, which is followed by segregation and haploidization. It appears to be a useful method to bring about recombination among bacteria in which other mechanisms of genetic exchange are yet known**.** Moreover, the genetic manipulation *via* protoplast fusion protocol is considered as a more recent method to promote recombination. Furthermore, protoplast fusion led to produce superior enzyme production fusants from Streptomyces ***(*Khattab and Abdel-Aziz, 2012)**.

The objective of this study is to study is to improve lipase production from local isolated bacterial strains. This will be done

through mutation induction using physical and chemical mutagens, interspecific and inter generic protoplast fusion between different strains.

REVIEW OF LITERATURE

1- The importance of enzymes

The advent of enzymology represents an important breakthrough in the biotechnology industry, with the worldwide usage of enzymes being nearly U.S. $ 1.5 billion in 2000 **(Kirk *et al*., 2002).** The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases with the development of global economy. The demand for energy is increasing quickly**.** Microbial lipase provides multiple catalytic activities with high yield. It is easily genetically modified and grows rapidly on inexpensive medium. However, some of their characteristics are not suitable for industrial production and it has become a hot topic for researchers to study on how to improve microbial lipases characters. In the last 10 years, genetic modification has been the powerful tool for transformation of lipase function. It makes lipase with greater activity, thermostability, organic solvent tolerance in industrial condition. **(Jing *et al*., 2012)**

2- Definition of lipase

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) form a large series of enzymes that catalyze the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids, and glycerol at the interface between the aqueous and lipid phases **(Bajaj *et al*., 2010).** The natural substrates of lipases are long-chain triacylglycerols. Under micro-aqueous conditions, lipases possess the unique ability to carry out the reverse reaction, leading to esterification, alcoholysis and acidolysis, *i.e* besides being lipolytic, lipases also possess esterolytic activity and thus have a very diverse substrate range, although they are highly specific as chemo-, regio- and enantioselective catalysts **(Jaeger and Eggert, 2002)** Lipases are serine hydrolases which

act at the lipid–water interface. Lipase has α/β-hydrolase fold, a conserved catalytic triad (Ser, His, Asp/Glu) and usually a consensus sequence (Gly-x-Ser-x-Gly) where X may be any amino acid residue is found around the active site serine **(Gupta *et al.*, 2004).** This triad is buried completely beneath a short α-helical segment, which opens upon contact of the lipase with an interface. The enzyme's structural rearrangement during catalysis creates an electrophilic region (the oxyanion hole) around the serine residue. This stabilizes the transition state intermediate by exposing the hydrophobic residues and by burying the hydrophilic ones **(Stamatis *et al*., 1999).** The presence of features in lipases like a lid and an amphiphilic loop covering the active site differentiates them from esterase. It would not be surprising if lipases take the top position in the enzyme application area in the near future.

3- Sources of lipases

Lipases can be obtained from plants **(Bhardwaj *et al*., 2001).** Plant lipases are available but not exploited commercially because of the low yield and the processes involved. Animal lipases are mainly obtained from forestomach tissue of calves or lambs and pancreatic tissues of hogs and pigs **(Carriere *et al*., 1994).** Among the disadvantages of using animal lipases include presence of trypsin in pig pancreatic lipases, which results in bitter tasting amino acids, presence of residual animal hormones or viruses as well as their undesirable effects in the processing of vegetarian or kosher diets **(Vakhlu and Kour, 2006)**. Finally microorganisms are considered as the major source of lipases **(Olempska-Beer *et al*., 2006).** Microbial lipases are the preferred one. Thus, microbial lipases are currently receiving more attention because of their technical and economic advantages, where the organisms are cultured in medium containing appropriate nutrient composition under controlled conditions **(Srivastava, 2008).** Also,

lipase production by microorganisms varies according to the strains, the composition of the growth medium, cultivation conditions, pH, temperature, and the kind of carbon and nitrogen sources **(Gupta *et al.,* 2004; Souissi *et al.,* 2009; Treichel *et al.,* 2010).** Generally, bacteria, fungi, yeast and actinomycetes are recognized as preferred sources of extracellular lipases, facilitating the enzyme recovery from the culture broth: although *Bacillus spp* **(Jaeger *et al.,* 1999)** *Candida, Pseudomonas, Mucor, Rhizopus* and *Geotrichum sp* stand out as the major commercially viable strains **(Ertugrul *et al.,* 2007).** Several products based on bacterial lipases have been launched successfully in the market in the past few years A number of such products are from *Pseudomonas spp*, such as Lumafast and Lipomax with their major application as detergent enzymes in which the enzyme is dissolved potent sources due to several industrial potentials **(Hasan *et al.,* 2006).** Lipases display useful properties related to their stability as organic solvent-tolerant **(Rahman *et al.,* 2005)** and thermostable enzymes**.** Therefore, microbial lipases have been of recent research interests and a number of lipases have been identified, purified and characterized to date. In general, microbial lipases are 20–60 kDa molecular weight proteins, with an active Ser residue of the active site structure Ser-His-Asp. Asp may be replaced by Glu in case of *Geotrichum candidum* lipases.

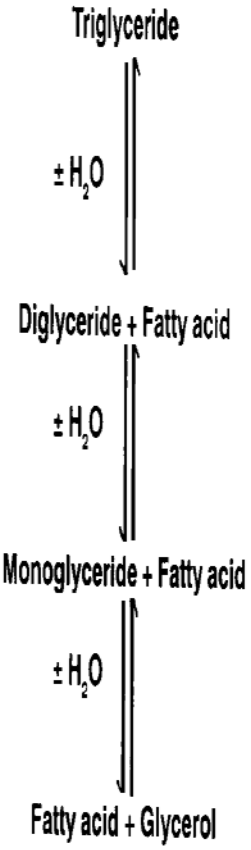
4- Properties and reactions of lipases

Besides the conventional ability of lipases to catalyze hydrolytic reactions, they can catalyze synthetic reactions such as esterification and transesterification in form of acidolysis, alcoholysis and interesterification in the presence of small amount of water. Unlike other enzymes, oil-water or air-water interfaces activate the lipases reactions into three important types

4-a- Hydrolysis: This occurs in aqueous media, when there is high amount of water, cleavage of ester bonds is the dominant reaction. This technology is currently employed in the production of fatty acids, diglycerides, monoglycerides, flavouring agents for dairy products and detergents for laundry and household uses **(Divakar and Manohar 2007).**

4-b- Esterification: This reaction occurs under low water conditions such as in nearly anhydrous solvents; a high yield of the esterified products is obtained under controlled conditions. Production of oleic acid esters of primary and secondary aliphatic and terpenic alcohols is among the commonest example. Others are the formation of geranyl and menthyl esters from butyric acid and geranol or lauric acid and menthol **(Marlot *et al.,* 1985).**

4-c-Transesterification: this reaction involves the exchange of acid moiety between two or more compounds. If the acyl donor is a free acid the reaction is called acidolysis. Whereas the reaction is called interesterification if the acyl donor is an ester. In alcoholysis, the nucleophile alcohol acts as an acyl acceptor **(Macrae, 1985).**



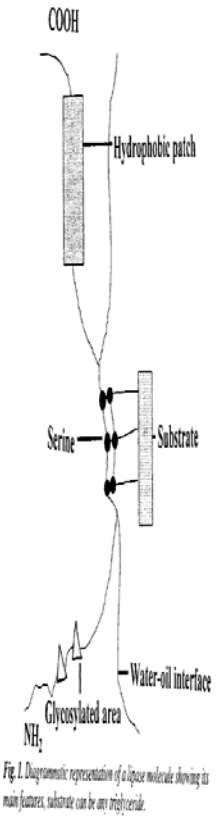
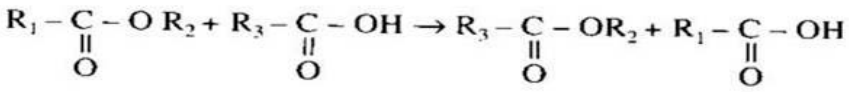


Fig. 1. Diagrammatic representation of a lipase molecule showing its main features, substrate can be any triglyceride.

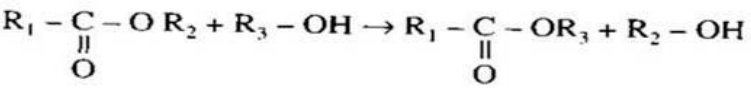
Fig (1) Hydrolytic reactions of lipase

Transesterification Reaction

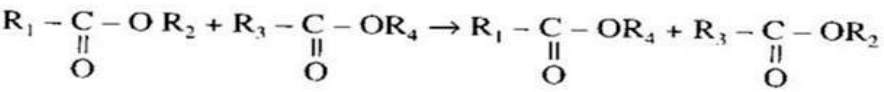
(a) Acidolysis



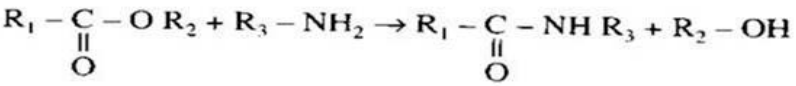
(b) Alcoholysis



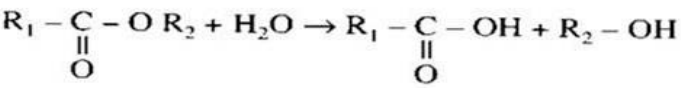
(c) Ester Exchange



(d) Aminolysis



Hydrolysis



Ester Synthesis