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Isolation and characterization of Extracellular Lipase Producing Strain of *Enterococcus durans* from Camel milk

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ABSTRACT
Present study deals with the isolation of lipolytic lactic acid bacteria from camel milk. Bacterial lipase is secreted extracellular and hydrolyses acyl glycerides into free fatty acid. Thus, the intake of bacterial lipase directly in food sample or as probiotic formulation can be helpful for those people suffering from health implications related to high serum triglyceride level. For this purpose, a lipolytic bacterial strain *Enterococcus durans* purified from camel milk employing enrichment and selective culture technique. Identification was done by sequencing of 16S rDNA/D1/D2 domain of LSU rDNA or ITS region and BLAST analysis. Strain showed significant lipase activity of 3988 U/ml. Physicochemical characterization showed that optimum lipase activity was observed between 7.2-7.5 pH, 30^{o} C – 40 ^{o} C temperature, 3.5 %- 4.5 % salinity and 4 % (v/v) inoculum's size whereas presence of peptone, calcium and tributyrin in media enhanced the lipase production. Optimization of lipase production was done using RSM model. Further purification via DEAE anion exchange leads 1.9 fold purification of lipase with retention of 81 % lipase activity. Quality assurance of this bacterial strain as a probiotic and subsequent formulation development can be useful achievement in the field of medical microbiology.

Introduction

Earth flora and fauna shows the wide distribution of lipase yet the abundance of enzyme is found to be in microbial flora comprising bacteria, fungi and yeast (Joseph et al. 2008). Lipases (triacylglycerol acyl hydrolases; EC 3.1.1.3) belong to class hydrolase (EC 3.1.1.1) which hydrolyze acyl glycerides into free fatty acid and glycerol. Lipases show the reaction preference on water insoluble substrate at water soluble and water insoluble system interface which differentiate it from esterase. Lipases generally do not require cofactor and display chemo, region and enantio-selectivity which suggest it to be industrially important biocatalysts such as agrochemical, pharmaceutical, detergent and food industries (Bouke et al. 2007).

Microbial lipases are of industrial concern due to cost effectiveness, wide variety and stability in organic solvent. The extracellular bacterial lipases are of commercial importance for their bulk production and easy handling. Microbial strains find to show prominent role as probiotic entity. The ability of lactic acid bacteria to produce probiotics (Temmerman et al. 2002) and stimulation of the immune system (Galdeano et al. 2007) render this group of microorganism's essential importance in industry. Among various lactic acid bacteria, strains of Enterococci have also been used as probiotics. The ability to withstand usual food production condition renders *Enterococcus* important constituent as fermented food microbiota especially in cheese and meat. The use of Enterococci as probiotic is because of its possible health promoting capacity as stated by Moreno et al. (2006). The virulence feature of Enterococci are strain dependent thus find place in food industry and can be implicated as probiotic.

Physical and chemical factors can greatly influence lipase biosynthesis thus optimization of physicochemical condition for lipase activity necessitates for mass production of lipase to meet industrial demands. Response Surface Methodology (RSM) is collection of mathematical and statistical design which can be used to find the effect of independent factor (Lee et al. 2003) and help to design the fermentation condition to enhance the activity of lipase (Liu et al. 2006). For efficient and successful usage enzyme is required to be purified to a certain degree though there is no need for homogeneous lipase preparations commercially. Saxena et al. (2003) stated that purified lipase preparations are needed in industries employing the enzymes for the biocatalytic production of fine chemicals, pharmaceuticals and cosmetics. Thus, the present work aims to isolation of lipolytic lactic acid bacteria from camel milk. Further purification of lipase degrading principle and optimization of physicochemical conditions for achievement of maximum lipase activity is also done. Effect of plasmid curing on lipase activity was also determined to confirm presence of lipase coding gene on genome.

Material And Methods

Isolation of Lipolytic bacteria

Camel milk samples collected from different regions nearby Udaipur (Raj) were subjected to enrichment culture technique (Vargas et al. 2004) and subsequently lipolytic activity was detected on tributyrin agar media (HiMedia). MRS agar, MRS broth, M17 agar and M17 broth (HiMedia) were used as selective media for lactic acid bacteria.

Identification of purified lipolytic bacterial isolate

Biotyping of bacterial isolate was done by studying morphological, cultural and biochemical characteristics as given in Bergey's manual (Haynes and Burkholder 1957). Molecular typing was carried out by using analytical grade chemicals and API strep (bioMérieux) and sequencing of 16S rDNA/D1/D2 domain of LSU rDNA or ITS region and BLAST analysis at MTCC Chandigarh.

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Detection of Lipolytic activity

To assess the lipolytic activity of lactic acid bacteria, agar well diffusion method (Schillinger and Luke 1989) was used. Formation of clear zone around the well confirms the lipolytic activity of the bacteria.

Purification of Lipase

Partial purification of lipase was done by ethyl alcohol precipitation (Rifaat et al. 2010) and subsequently purified by employing ion exchange chromatography using 5 ml column (1 cm interior diameter) containing DEAE Sepharose (Sigma) (Saxena et al. 2003). Stepwise elution was carried out by a pH gradient of NTM with 1 x Tris HCl buffer (7.2-8.2 pH). Fractions (0.1 ml/min) were collected. Determinations of protein concentration in the fractions were carried out by Bradford method. The activity of each fraction showing maximum absorbance was determined. The identification of lipase in the fractions showing efficient lipase activity was subjected to SDS PAGE against the commercial *Pseudomonas* lipase (29 kDa). A low molecular weight marker with sizes ranging from 2.35 to 46 kDa (Sigma) was used.

Purification fold = Specific activity of purified protein/specific activity of crude protein

Recovery = Volume of purified / volume of crude x 100

Physico-chemical characterization

For determination of effect of physical factors on lipase activity from bacterial isolate varied range of pH (3-9), temperature (15-45 ^{o}C), inoculum size (2-10 % v/v) and salinity were used. Effect of different physical factors on secretion of lipase was determined by titrimetric method (Table I).

For determination of effect of chemical factors on lipase activity from bacterial isolate different minerals, nitrogen source, carbon source and substrate were used. They were individually tested and effect on secretion of lipase was determined by titrimetric method (Table II).

$$\mu\text{mole of fatty acid} = \frac{\text{Reading of Burette x N x 1000}}{\text{Volume of sample taken x volume of sample in cell free extract}}$$

Specific activity =µ mole of fatty acid /24 x Protein content = Unit/mg

Optimization of lipase activity

On determining the effect of physical and chemical factor on lipase activity it was found pH, salinity and incubation time showed a range for the activity. Taking these three factors into consideration, a Response surface methodology using 2^3 factorial Central Composite Design (CCD) was adopted for optimization of lipase activity. The design lead to a set of 20 experimental runs. The maximum and minimum range was investigated and complete experimental plan with respect to observed and predicted value is listed in table III-V. Each experiment was conducted in triplicates and average lipase activity was taken as dependent variable or response (Y).

The data was subjected to ANNOVA

$$Y=\beta_{o+}\ \beta_1A+\ \beta_2B+\ \beta_3C+\ \beta_{11}A^2+\ \beta_{22}B^2+\ \beta_{33}C^2+\ \beta_{12}AB+\ \beta_{13}AC+\ \beta_{23}BC$$

β_o- intercept; β_1, β_2, β_3-linear coefficient; β_{11}, β_{22}, β_{33} -squared coefficient; β_{12}, β_{13}, β_{23}-interaction coefficient

Plasmid curing

Plasmid curing trials were carried out as per the method described by Trevors (1985) using ethidium bromide at their highest sublethal dose for respective isolates in LB broth.

Treated as well as control culture of respective isolates were spot inoculated on TBA and detect for the existence of lipolytic activity. Further plasmid curing was confirmed by plasmid isolation and subsequent gel electrophoresis of control and treated sample.

Results and Discussion

Isolation and Identification of Lipolytic Isolate:

Bacterial strain showing significant lipase activity was purified from camel milk. Bacterial isolate was demonstrated as gram positive cocci on Gram staining. Biotyping using analytical grade chemicals and API strep (bioMérieux) and sequencing of 16S rDNA/D1/D2 domain of LSU rDNA or ITS region and BLAST analysis at MTCC Chandigarh identified the isolate as *Enterococcus durans*. On assay of lipolytic activity by tributyrin agar well diffusion method isolate showed 14 mm of halo zone in comparison to standard (*Pseudomonas* lipase and pancreatic lipase) which showed 20 and 25 mm clear zone respectively. Quantitatively *Enterococcus durans* lipase released 3988 U/ml of fatty acids per unit time.

The probiotic potential of lactic acid bacteria attracts the attention of research towards identifying and exploiting their properties which are useful for human benefits. Extracellular secretion of majority bacterial lipases imparts it to be promising biocatalyst as degradation of lipids exterior to cell is requisite for easy adsorption of cells and is found to show water-lipid interfacial reactions. Many lactic acid bacteria have been reported to show lipolytic activity isolated from different sources. Among Lactic acid bacteria, *Streptococcus* and *Enterococcus* are found to have certain close similarities in many morphological and biochemical characteristics and it has been stated that they can be clearly distinguished on basis of DNA-DNA and DNA-rRNA hybridization and 16 S RNA sequencing (Janda and Abbot 2007). Enterococci are more lipolytic when compared with *Streptococcus* strains (Moreno et al. 2006). The most widely used substrate is tributyrin and triolein as reported by Jaeger et al. (1999). Chander et al. (1979 a,b) on examining the effect of certain fatty acids on lipase production by *Enterococci* observed that low molecular mass fatty acids enhance lipase production compared to high molecular mass fatty acids and found maximum enzyme activity in presence of tributyrin. There are various methods for identification of lipase producing bacteria. Among various methods lipase production can be detected by the formation of clear zone around the colonies grown on tributyrin containing plates (Atlas et al. 1996; Jaeger et al. 1999). The comparative advantage of the camel as a dairy animal over the other species in the same environment is difficult to quantify; however, it is widely recognized that in absolute terms, the camel produces more milk, and for a longer period of time, than any other milch animal kept under the same conditions (Farah 1996). Camel milk not only contains more nutrients compared to cow milk (Arrowal et al. 2005), but also it has therapeutic and antimicrobial agents (Agrawal et al. 2005). The presence of lipolytic lactic acid bacteria in camel milk has diverted the attention of researchers that direct consumption of this food article or development of probiotic will be helpful for reduction of serum triglyceride level in patients suffering from atherosclerosis.

Milk samples have been taken as source for lipolytic lactic acid bacteria by many researchers.

Purification of lipase degrading principal:

Chromatography allows the isolation of substance in very small amounts and in non-denaturing conditions. Saxena et al. (2003) concluded that the protocol for purification involving precipitation and chromatographic steps can be designed on the

nature of the lipase produced by the organism as well as the purity of the enzyme required for its usage, which is important from the economic viewpoint. They have reported the purification of lipase using DEAE anion exchanger chromatography.

Partially purified Lipase preparation was purified by diethylaminoethyl (DEAE) sepharose (Sigma), anion ion exchange chromatography. It was observed that 81 % of lipase was purified with 1.9 purification fold from *Enterococcus durans*. This chromatography efficiently allows the maximum purification of partially purified preparation. Purification is based on exchange of ions between stationary and mobile phase. A *Pichia burtonii* lipase was purified to homogeneity by a combination of DEAE-Sephadex A-50 ion exchange chromatography, Sephadex G-100 gel filtration, and isoelectric focusing (Sugihara et al. 1995).

Physico- chemical characterization:

Physico chemical characterization of biocatalyst is very important step to determine its specific utility. For this purpose crude lipase from *Enterococcus durans* exposed to ranges of physical and chemical factors by using single variable at a time. Optimum lipolytic activity of isolate was observed between 7.2-7.5 pH, 30 ^{o} C – 40 ^{o} C, 3.5 %- 4.5 % salinity. 4 % (v/v) inoculum's size was observed to be optimum for lipase activity whereas presence of peptone, calcium and tributyrin in media enhanced the lipase production from *Enterococcus durans*. Chander et al. (1979 a, b) observed maximum enzyme activity of lipase of *Enterococcus faecalis* at pH 7.5 and 40 ^{o}C.

Bacterial lipases influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration (Rosenau and Jaeger 2000). Temperature and pH available during enzymatic process responsible for the favorable charge of amino acids present at active sites. Appropriate temperature is also required to expose active site and easy binding of substrate. Nutrients also play important role in secretion of lipase and might be related to the growth of bacterial isolate. Presence of calcium might act as a cofactor and enhance the lipase activity. In most of the cases, it is also require for correct confirmation of enzyme. Generally, organic nitrogen source is preferred by bacteria, such as peptone and yeast extract (Gupta et al. 2004). The initial pH of the growth medium is important for lipase production. Most bacteria prefer pH around 7.0 for their best growth and lipase production. The optimum temperature for lipase production is parallel with the growth temperature of the respective microorganism. It has been reported that lipases are produced in the temperature range from 20 to 45 ^{o}C (Jaeger et al. 1999). Incubation periods change from few hours to many days until the maximum lipase production from bacteria is recorded.

Gupta et al. (2004) reported that cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity due to the formation of the calcium salts of long-chain fatty acids (Macare and Hammond 1985; Godtfredsen 1990). Calcium- stimulated lipases have been reported in the case of *Bacillus subtilis* 168 (Lesuisse et al. 1993), *Bacillus thermoleovorans* ID- 1 (Lee et al. 1999), *Pseudomonas aeruginosa* EF2 (Gilbert et al. 1991b), *Streptococcus aureus* 226 (Muraoka et al. 1982), *Streptococcus hyicus* (Van Oort et al. 1989) and *Acinetobacter* sp. RAG-1 (Snellman et al. 2002). In contrast, the lipase from *Pseudomonas aeruginosa* 10145 (Finkelstein et al. 1970) is inhibited by the presence of calcium ions.

Optimization of lipase production using RSM model:

To meet the increasing demand of lipases, feasible methods capable of their bulk and cost effective production from microorganisms need to be developed further and improved (Rathi et al. 2002). Factorial design and response surface analysis are important tools to determine the optimal process conditions. In this study, response surface model resulted in a 3.9 fold increase (12 to 46.2 in 72 hrs) in lipase production at pH 7.5, salinity 3.5 % and temperature 40 ^{o}C. (Table IV,V). the Model F-value of 5.15 implies the model is significant. Values of "Prob > F" less than 0.0500 (i.e. **Prob > F** = 0.0086) indicate model terms are significant. A negative "Pred R-Squared" (i.e. -0.3471) implies that the overall mean is a better predictor of your response than the current model. "Adeq Precision" measures the signal to noise ratio. The ratio of 9.872 indicates an adequate signal (Table IV, V and Fig 1).

Response surface methodology (RSM) is a collection of mathematical and statistical technique useful for analyzing the effects of several independent variables (Lee et al. 2003). Recently, different statistical designs for fermentation condition optimization for lipase production, including factorial experiments and RSM have been reported (Liu et al. 2006).

Using the RSM approach, Mahler et al. (2000) reported that lactic acid used as carbon source does not have any significant effect on lipase production, while gum arabic increases the yield of extracellular lipase by 2 to 5 fold and oleic acid has a negative effect on lipase production from *Acinetobacter calcoaceticus*. An overall 2.4 fold increase in lipase production and a 1.8 fold increase in specific activity were obtained from *Burkholderia cepacia* after validation of RSM in shake-flasks (Rathi et al. 2002). Abdel-Fattah (2002) reported a 4 fold increase in lipase production in shake-flask cultures from a thermophilic *Geobacillus* sp., using a Box–Behnken experimental design.

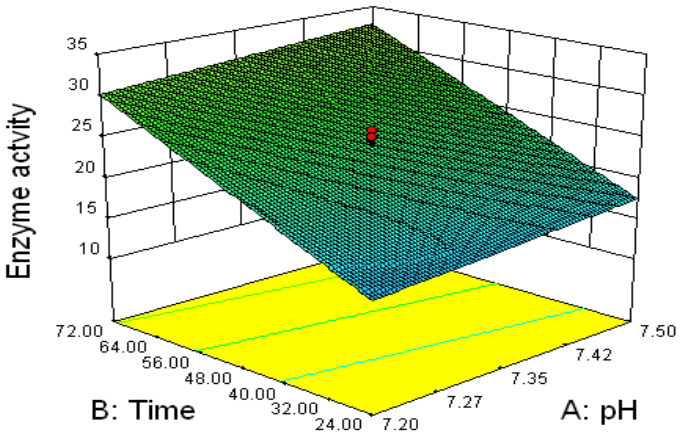

Fig 1: Response surface curve for the effect of pH and incubation time on lipase production by *Enterococcus durans*

Effect of Plasmid curing

Plasmid curing experiment was done to detect whether lipase coding gene is genome borne or plasmid borne. Results suggested that lipase coding gene is genome borne in *Enterococcus durans* as treated sample showed lipolytic activity on TBA plates. Thus, study proved that lipase coding gene stably inherited from generation to generation and improve its commercial utility.

The intercalating agent ethidium bromide was used to eliminate plasmid DNA which specifically intercalate into plasmid DNA.

Table I: Effect of physical factors (pH, temperature, Incubation time, inoculums size) on lipase activity of *Enterococcus durans*

pH	Specific lipase activity of *E. durans* (U/mg)
Control	6.144
3	5.154
4	5.865
5	7.298
6	8.731
7	13.02
8	9.01
9	4.577
10	3
Temperature (^{0}C)	
Control	6.144
15	4.144
20	7.587
25	6.577
30	8.742
35	9.154
40	5.721
50	2.711
NaCl (w/v)	
Control	6.489
2.50%	4.673
3.50%	5.666
4.50%	4.623
5.50%	4.172
6.50%	2.797
7.50%	1.031
Inoculum's size (v/v)	
2	4.217
4	6.336
6	6.032
8	4.217
10	2.858

Table II: Effect of chemical factors (nitrogen source, metals, metals+ EDTA) on Lipase activity of *Enterococcus durans*

Nitrogen source (5g/l)	**Specific lipase activity of *E. durans* (U/mg)**
Control	5.8
NH_4Cl_2	4.53
NH_4SO_2	4.38
NH_2NO_3	5.37
Urea	4.1
Peptone	6.09
Yeast extract	5.23
Tryptone	4.95
Beef extract	4.54
Metal salts (10 mM)	
Control	5.8
$HgCl_2$	4.53
$MgSO_4$	4.38
$CaCl_2$	6.09
KCl	5.23
$FeSO_4$	4.95
$BaCl_2$	5.37
$MnCl_2$	4.1
$HgCl_2$	5.8
EDTA(0.1M)+ Metal salts (10mM)	
Control	5.8
EDTA+$Hgcl_2$	3.68
EDTA+$Mgso_4$	3.68
EDTA+$Cacl_2$	4.24
EDTA+ Kcl	2.82
EDTA+$Feso_4$	4.1
EDTA+$Bacl_2$	3.95
EDTA+ $Mncl_2$	3.54

Table III: Experimental range for two isolated strains in RSM

Factor	Name	Low Level	High Level	Coding
A	pH	7.20	7.50	Actual
B	Time	24.00	72.00	Actual
C	Salinity	3.50	4.00	Actual

Table IV: Results of CCD using three independent variables showing observed and predicted responses for *Enterococcus durans*

Std	Parameters			*Enterococcus durans*	
	pH	Time	Salinity	Enzyme activity (U/ml)	Predicted Value
1	7.20	24.00	3.50	17.8	16.15
2	7.50	24.00	3.50	17.6	18.71
3	7.20	72.00	3.50	40.6	31.44
4	7.50	72.00	3.50	46.2	34.00
5	7.20	24.00	4.00	12.3	13.78
6	7.50	24.00	4.00	17.4	16.34
7	7.20	72.00	4.00	39.2	29.07
8	7.50	72.00	4.00	38.3	31.63
9	7.10	48.00	3.75	20.5	21.73
10	7.60	48.00	3.75	25.2	26.05
11	7.35	7.64	3.75	8	11.03
12	7.35	88.36	3.75	11.1	36.75
13	7.35	48.00	3.33	20.9	25.88
14	7.35	48.00	4.17	20.2	21.90
15	7.35	48.00	3.75	23.3	23.89
16	7.35	48.00	3.75	25.9	23.89
17	7.35	48.00	3.75	20.5	23.89
18	7.35	48.00	3.75	25	23.89
19	7.35	48.00	3.75	25.1	23.89
20	7.35	48.00	3.75	22.7	23.89

Table V : ANNOVA for optimization experiments of pH, incubation time and salinity for lipase production for *Enterococcus durans*

Term	Lipase yield (U/ml)
F- Value	5.15
P>F	0.0086
Mean squared	110.96
R^2	0.8225
Adjusted R^2	0.6627
Coefficient of variance	22.73%
Adequate Precision	9.872

Other mutational events associated with chromosome changes occurred at high frequency; they resulted in phenotypic changes such as loss of enzyme activity, antibiotic resistance etc. (Crameri et al. 1986).

Crameri et al. (1986) observed that these 'mutations' did not occur randomly, but appear to be limited to plasmids and to other regions of DNA showing an increased spontaneous genetic instability. (Chin Chin et al. 2005) done plasmid curing experiment in *Lactobacillus* strains isolated from the gastrointestinal tract of chicken.

Conclusion:

Results show that lipolytic bacterial strain, *E. durans* showed significant lipase activity. Further optimization of physicochemical factors leads improvement in lipase activity from this bacterial strain. Thus, can be useful for the development of probiotic formulation for reducing the increased serum triglyceride level. In addition, this lipase can be useful for those people produce inadequate pancreatic lipase.

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Reference

Abdel-Fattah YR (2002) Optimization of thermostable lipase production from a thermophilic *Geobacillus* sp. using Box–Behnken experimental design. Biotechnol Lett 24:1217–1222

Agrawal RP, Sahani MS, Tuteja FC, Ghouri SK, Sena DS, Gupta R, Kochar DK (2005) Hypoglyce,ic activity of camel milk in chemically pancreatectomized rats – An experimental study. Int J. Diab Dev Countries. 25: 75-79.

Arrowal RP, Beniwal R, Kochar DK, Tuteja FC, Ghorui SK, Sahaui MS, Sharma S (2005) Camel milk as an adjunct to insulin therapy improves long-term glycaemia control and reduction in doses of insulin in patients with type-1 diabetes A 1 year randomized controlled trial. Diabetes Res. Clin. Pract. 68:17

Atlas R. M. ed. (1996) Handbook of Microbiological Media. Boca Raton, FL:CRC, 1440.

Bouke KHL Boekema, Beselin A, Breuer M, Hauer B, Koster M, Rosenau F, Jaeger KE, Tommassen J (2007) Hexadecane and Tween 80 stimulate lipase production in *Burkholderia glumae* by different mechanisms. Appl. Environ. Microbiol. 73:3838-3844.

Chander H, Ranganathan B, Singh J (1979a) Role of some fatty acids on the growh and lipase production by the *Streptococcus faecalis*. Journal of Food Science 44:1566-1567.

Chander H, Ranganathan B, Singh J (1979b) Purification and some properties of lipase from *Streptococcus faecalis*. Journal of Food Science 44:1747-1751.

Chin Chin S, Abdullah N, Siang T W, Wan H Y (2005) Plasmid Profiling and Curing of *Lactobacillus* Strains Isolated from the Gastrointestinal Tract of Chicken. The Journal of Microbiology 43(3):251-256.

Crameri R, Davies JE, Hutter R (1986) Plasmid Curing and Generation of Mutations Induced with Ethidium Bromide in *Streptomycetes*. Journal of' General Microbiology 132(8):19-824.

Dovat A M, Reinbold G W, Hammond EG, Vedamuthu E R (1970) Lipolytic and proteolytic activity of *Enterococci* and lactic group *Streptococci* isolated from young Chedder cheese. Journal of Milk and Food Technology 33:365-372.

Farah Z (1996) Camel milk, properties and products. Laboratory of Dairy Science, Swiss Federal Institute of Technology.

Finkelstein AE, Strawich ES, Sonnino S (1970) Characterization and partial purification of a lipase from *Pseudomonas aeruginosa.* Biochim Biophys Acta 206:380–391

Galdeano CM, de LeBlanc A, de M, Vinderola G, Bonet MEB, Perdigón G (2007) Proposed Model: Mechanisms of Immunomodulation Induced by Probiotic Bacteria. Clin. Vaccine Immunol. 14:485–492

Gilbert EJ, Cornish A, Jones CW (1991b) Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. J Gen Microbiol 137:2223–2229

Godtfredsen SE, Fogarty WM, Kelly C T (1990) Microbial Enzymes and Biotechnology. Elsevier, Amsterdam, 255-273

Gupta R, Gupta N, Rathi, P (2004) Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol **64**: 763-781

Haynes W C, Burkholder W H **1957**. In Breed RS, Murray E G D and Smith N R (eds.).

Bergey's Manual of Deteriminative Bacteriology, The Williams and Wilkins Company, Baltimore.

Janda M J, Sharon L. A (2007) 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls J. Clin. Microbiol. 45 (9): 2761-2764

Jaeger K-E, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures and biotechnological applications of lipases. Annu Rev Microbiol 53:315– 351

Joseph, B., P.W. Ramtekeand and G. Thomas, 2008. 51(2): 12-16. Cold active microbial lipases: Some hot issues and recent developments. Biotech Advances, 26: 457-470.

Lee O-W, Koh Y-S, Kim K-J, Kim B-C, Choi H-J, Kim D-S, Suhartono MT, Pyun Y-R (1999) Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol Lett 179:393–400

Lee H, Song M, Hwang S (2003) Proc Biochem 38:1685-1693.

Lesuisse E, Schanck K, Colson C (1993) Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur J Biochem 216:155–160

Liu C H, Lu W B, Chang J S (2006) Pro Biochem 41:1940-1944

Macare A R, Hammond R C (1985) Biotechnol. Genet. Eng. Rev. 3:193-219

Mahler GF, Kok RG, Cordenons A, Hellingwerf KJ, Nudel BC (2000) Effects of carbon sources on extracellular lipase production and lipA transcription in *Acinetobacter calcoaceticus*. J Ind Microbiol Biotechnol 24:25–30

Moreno M R F, Sarantinopoulos P, Tsakalidou E, Vyust L De (2006) The role and application of *Enterococci* in food and health. International Journal of Food Microbiology. 106: 1-24.

Muraoka T, Ando T, Okuda H (1982). Purification and properties of a novel lipase from *Staphylococcus aureus* 226. J Biochem 92:1933–1939

Rathi P, Goswami VK, Sahai V, Gupta R (2002) Response surface methodology for improving production of hyperthermostable lipase from *Burkholderia cepacia*. J Appl Microbiol 93:930– 936

Rifaat HM, El-Mahalawy A A, El-Menofy H A, Donia SA (2010) Production, Optimization And Partial Purification Of Lipase From *Fusarium Oxysporum*. Journal of Applied Sciences in Environmental Sanitation 5 (1): 39-53.

Rosenau F, Jaeger K E (2000) Bacterial lipases from *Pseudomonas*: Regulation of gene expression and mechanisms of secretion, *Biochimie* 82: 1023−1032.

Saxena RK, Sheoran A, Giri B, Davidson S (2003) Purification strategies for microbial lipases. J Microbiol Methods 52:1–18

Schillinger U, Luke F K (1989). Antibacterial activity of *L. sake* isolated from meat. Appl Env. Microbiol., 55: 1901-1906

Snellman EA, Sullivan ER, Colwell RR (2002) Purification and properties of the extracellular lipase, Lip A, of *Acinetobacter* sp. RAG-1. Eur J Biochem 269:5771–5779.

Sugihara A, Scnoo T, Enoki A, Shimada Y, Nagao T, Tominaga Y. (1995) Purification and characterization of a lipase from *Pichia burtonii*. Appl Microbiol Biotechnol 43:277–81.

Temmerman R, Pot B, Huys G, Swings J (2002). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81: 1-10

Trevors JT (1985). Plasmid curing in bacteria Plasmid curing in bacteria. FEMS Microbiol. Lett., 32: 149-157.

Van Oort MG, Deever AMTJ, Dijkman R, Tjeenk ML, Verheij HM, Haas GH de, Wenzig

E, Gotz F (1989) Purification and substrate specificity of *Staphylococcus hyicus* lipase. Biochemistry 28:9278–9285

Vargas VA, Delgado OD, Hatti-Kaul R, Mattiasson B (2004). Lipase-producing microorganisms from a kenyan alkaline soda lake. Biotechnol. Lett. 26:81– 86.