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A Review on Microbial Lipases – Gateway to the ERA of Biodiesel Production

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Abstract—*Lipases enzyme have emerged as one of the leading biocatalysts with exceptional potential for biodegradable and nonpolluting biodiesel fuel production. Wide diversity and enormous properties of microbial lipases represent them as efficient enzymatic tool for biotechnological applications and organic industry. Optimization of fermentation conditions to achieve maximum growth of lipase producing microorganism is essential to obtain high lipase production. Microbial heterologous hosts such as Escherichia coli, Pseudomonas sp., and Bacillus sp. provide high expression level required for improvement in microbial lipase enzyme productivity to meet the need of biofuel producing industries. Advantages of microbial lipases for biodiesel production mainly include their extracellular nature, cost efficiency and high stability in the adverse environment. Key researches from many researchers on microbial Lipase with unique properties has been collected, summarized and discussed.*

1. INTRODUCTION

Biodiesel is defined as non-petroleum-based diesel fuel which consists of short chain alkyl (methyl or ethyl) esters, typically made by transesterification of vegetable oils or animal fats. This biodiesel shows positive synergic effect of biodegradation by means of co metabolism, when it is mixed with conventional petro-diesel. Biodiesel has drawn attention as "Green" renewable alternative energy source with its valuable properties such as non toxicity, biodegradability and significant reduction in emission of greenhouse gases. Enzymatic reactions that involve lipases as catalyst in transesterification reaction produces highly purified product and enables easy separation from its byproduct glycerol prove it as promising alternative to produce biodiesel [1].But high cost of enzyme remains a potential barrier for its industrial implementation. In order to overcome this advanced emerging biotechnology methods as protein engineering and recombinant DNA technology helping in considerable improvement in catalytic efficiency, reduction in downstream processing problems and increased quantities of lipases enzyme. Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) are defined as carboxylesterases that catalyze both the hydrolysis and synthesis of long-chain acylglycerols at oil-water interface. [3]

2. SOURCES OF MICROBIAL LIPASE AS BACTERIA AND FUNGI

Lipases from bacterial and fungal species are present in great diversity. Extremophilic microorganisms provide industries enzymes with outstanding stability and activity in extreme temperature, high salinity, acidity, alkalinity, which are predominant conditions in a various industrial processes. Microbial lipases from Rhizomucor miehei (RML), Burkholderia, Pseudomonas cepacia (PCL), and Thermomyceslanuginosa (TLL) are extensively studied for biodiesel production [2]. Biodiesel production from soybean oil using lipase enzyme obtained from Pseudomonas cepacia, Rhizopus oryzae and Pseudomonas has also been reported [29].

Table 1: Bacterial lipases with unique properties

Organism	Lipase characterization	Optimum (pH)	Optimum Temp.(°C)	Ref.
Bacillus cereus C71	enantioselective lipase	9	33	15
Staphylococcus epidermidis	Acidic lipase	7	22	16
Pseudomonas sp. (MSI057)	psychrophilic alkaline lipase (halophilic)	9	30	17
Pseudomonas fluorescense MP11	psychrophilic lipase	n.s.	5-20	18
Salinivibriosp. strain SA-2	Thermophilic, moderately halophilic	7.5	30	19
Streptomyces sp. CS326	Neutral lipase	7	40	20
P gessardii	Acidic lipase	5	30	21

3. INFLUENCE OF CULTIVATION CONDITIONS ON LIPASE PRODUCTION BY MICROORGANISMS

Yield of biodiesel production through enzymatic catalysis is highly affected by factors such as substrate ratio (alcohol/oil), type of alcohol, optimum reaction temperature, water content,

and purity of the triacylglycerol and most importantly properties of catalytic enzyme. Nature as well as yield of lipase obtained from microbes is highly influenced by various nutritional and physiochemical factors of culture environment such as temperature, pH, carbon sources, presence of lipids, and concentration of inorganic salts. Optimization of growth conditions and proper subcellular localization of lipase enzyme are essential for obtaining high yield. Few important physiochemical factors are detailed below.

4. MICROBIAL GROWTH CURVE AND LIPASE ACTIVITY

Optimum lipase production varies with growth curve of microbial species which can be from few hours to several days. It has been observed that Most of the microorganisms show peak for lipase production in late exponential phase or stationary phase when maximum cell mass reached such as maximum lipase activity (750 U/ml) for Pseudomonas sp.(MSI057) obtained at stationary phase and Bacillus sp. RJS1 showed maximum growth and lipase production both at 50°C [6, 17]. It has been observed that at optimum growth temperature lid domain of lipase enzyme is open and expose catalytic site for substrate binding and make it more active. It has also been observed that production of some other enzymes from same microorganism can affect lipase production. For example some microorganisms are well known protease producer which is induced by newly synthesized lipases. This protease production is responsible for inhibition of further lipase production in exponential growth phase. In this context, Leonov S L. reported that cultivation of P. fluorescens MP 11 at 15°C should be stopped after 48 hr. to minimize inhibition effect of proteases on lipase production [18].Another lipase from B.Subtilis showed high susceptibility for degradation by extra cytoplasmic proteases produced from bacterial cell wall or secreted into growth medium [30]. Zhang Y reported a novel commercially important proteolysis resistant lipase from Streptomyces fradiae [31].

5. EFFECT OF CARBON SOURCE AND LIPASE INDUCTION

Lipase in an inducible enzyme thus generally lipids used as carbon source such as oils, triglycerols, fatty acids, hydrolyzable esters, tween, glycerol, long and short chain fatty acids for lipase production. Gupta et *al*.by experimentation reported that 0.05 % Tributyrin as a substrate in production media for *Pseudomonas sp. G 6* enhanced lipase production by 2.4 fold [32] and maximum lipase activity for *P.auroginosa KM110*was obtained when 2% olive oil been used as substrate [33].Lipid substrate forms an emulsion at lipid water interfaces, when lipids are present at above their solubility limits shows dramatic enhancement in lipase activity due increase in the interfacial hydrophobicity. This promotes opening of the lid at the catalytic site and interfacial enzyme activation. Surfactant induces lipase production by increasing cell permeability which facilitates export of compounds which improve substrate availability. In this context, It has been reported that 179 U/ml alkaline lipase has been produced from *B. cenocepacia ST8* , when 1 % (v/v) Tween 80 , a detergent added with 1 % (v/v) sunflower oil and amount of lipase obtained was 1.5 fold in comparison to the basal medium [4]. Lipases with exceptional nature from *P.auroginosa and B. subtilis* also reported, which are active on both emulsified and non-emulsified substrate, some short chain substrate such as triacetin and tripropionin can activate lipase production without interfacial activation also been reported. Carbohydrates such as Sugars, sugar alcohol, polysaccharides, whey, casamino acids can also be used as carbon source for lipase production but not much effective as lipid sources. Kiran *et al.* reported that readily available carbon source such as glucose, maltose, sucrose potentially inhibit lipase production from *Pseudomonas sp.* MSI057 by catabolic repression [17] and glycerol and starch were stimulated growth of lipase producing *Salinovibrio sp. SA2*strain but inhibited lipase production [19].

6. EFFECT OF SALTS

Salinity of media helps in maintaining structure of lipase producing halophilic and halotolerant microbes. Phenomenon behind this is at high salt concentration uncharged phospholipids covert to negatively charged phospholipids and prevent microbial membrane to get ruptured. Sodium (Na) salts in media helps to improve Na^+ pump driven respiration and transport of ions in the cell membrane increases microbial growth by improved metabolism [34]. Many of the taxonomically diverse lipase producing *Bacillus* species have been isolated from different saline habitats such as *Bacillus pseudofirmus, B. cohnii, B. vedderi, B. agaradhaerens isolated* from Ethiopian Soda Lakes [35].Some halophilic and halotolerentlipase producing Bacillus strainsbeen reported such as *Bacillus* sp. SD B1,*Bacillus* sp. MO12, *B. licheniformis* strain GXN151,*B. licheniformis*CICC 10219, *Bacillus* sp. GSP63[36]. A halophilic bacillus strain, *B. vallismortis* BCCS 007 isolated from salt lake, which was able to successfully tolerate upto 15 % NaCl concentration and produced halophilic lipase with high lipase activity of 3.41 ± 0.14 U/ml [37].Study of effect of different salt concentration growth media for *Pseudomonas sp. MSI057* provided that 1.5 % NaCl showed highest lipase production, whereas no lipase production was observed in the absence of NaCl [17]. Amoozegar *et al*. reported *Salinovibrio sp. SA2* can tolerate upto 4 M NaCl whereas no lipase production has been observed in absence of NaCl salt [19]. It has been studied for *Thermosyntropha lipolytica* lipase that salts such as NaCl and Na_2So_4 improves enzyme stability by increasing melting temperature by 20°C and thereby decelerate unfolding of enzyme [9]. Salt $(NH_4)_2SO_4$ act as a chaotropic agent, thereby increases entropy in water which increases hydrophobic interactions within enzymes tertiary structure, thus Salinity becomes prominent cause of enhanced enzyme stability [38].

7. EFFECT OF METAL IONS

Interfacial activation of lipase enzyme is a key phenomenon to perform its catalysis process. Metal ions are responsible for change in solubility and behavior of ionized fatty acid at interface there by affects catalytic properties of lipase enzyme. Metal ion dependent enzymes are termed as "metalloenzymes". Yang et.al extensively studied role of Ca^{+2} binding site on lipase enzyme activation that replacement of Ca^{+2} binding residues Asp242 and Asp288 with alanine affect the activation of *Pseudomonas* sp. strain KWI-56 lipase gene and observed that activity of mutants D242A, D288A, and D242/288A were 12%, 5.3% and 2.8% in comparison with wild type respectively and conferred calcium binding site structure affect lipase enzyme activation [39]. Interaction between opposite charged metal ion and amino acid residues reduces electrostatic repulsion causes rigid enzyme structure, improved catalytic efficiency and enhanced thermo-stability of enzyme. To confer dependency of enzyme activity on presence of metal ion, reduction in enzyme activity by chelation of metal ion has been studied by Choi *et al.* They reported that chelation by 10Mm EDTA decreases lipase activity of Mn^{+2} ion dependent *Thermosyntropha lipolytica* alkaline lipase [40].

8. SUBCELLULAR LOCALIZATION OF LIPASE

Lipases may be located intracellular, extracellular or membrane bound in microbial cells. Mostly lipases are extracellular in nature and can secrete by different types of secretion system. Enhancement in extracellular secretion of lipase enzyme is beneficial for lipase enzyme industrial applications. Angkawidjajaa *et al.* reported that *Pseudomonas sp. MIS38* lipase belongs to Lipase I.3 family and secreted via type I secretion system, which contain repetitive nine-residue sequence motif that helps lipase enzyme to stabilize intracellularly. Replacement two Ca^{+2} binding aspartic acid residue that were present in II and III repetitive sequence with alanine residue after deletion of 405-543 amino acid residues of lipase enzyme, resulted in increased extracellular secretion (5 mg/l) than intracellular accumulation (< 1 mg/l) [43].Filamentous fungi allow localization of lipase on cell wall are preferred sources of extracellular lipase enzymes. Most of the lipase producing fungal species belong to the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Penicillium* and *Geotrichum* [44]. Localization of lipases is related with their molecular mass and affected by difference in amino acid sequence of whole enzyme molecule or additional peptide segment. In this context many studies have been conducted. Hama *et al.* reported that *Rhizopus oryzae* produces two lipases of different molecular mass of 34 KDa(ROL 34) which is bound to the cell wall and another 31KDa(ROL 31) which is membrane bound. They found that ROL 31 originate from limited proteolytic cleavage of ROL 34 by specific serine proteases and form large proportion of extracellular lipases [45]. Davranov *et al.* also reported that *Oosporalactis* secrete two lipases with different M.W. and observed that lipase of 43KDa allocated in the periplasmic space and can easily secreted extra cellularly but lipase of 40 KDa was tightly bound to the membrane and require detergent treatment for extracellular secretion [46]

9. EFFECT OF TEMPERATURE AND PH

Thermophiles possess structural changes to tolerate high temperatures. Forces in enzyme structure like hydrophobic interaction, Hydrogen bond, salt bridge, and di sulfide bond maintain integrity of enzyme at high temperature. Lipid composition of Cell membrane of thermophiles such as presence of saturated fatty acids creates hydrophobic environment and maintains cell integrity and Presence of lipids linked with ether on the cell wall also stabilize cell structure [47]. As we discussed extremophiles in previous sections, a novel lipase from *Thermosyntropha lipolytica* is an exceptional example of thermophilic lipase which was active at 96°C, thus highly advantageous for industries[9].whereas lipase from *P. fluorescens MP11* is a novel example of psychrophilic cold active lipases which is able to act at <5°C .Psychrophilic lipase, although very few are reported are more advantageous than mesophilic and thermophilic lipase because of their more flexible structure and their ability to function in low water conditions[18].Study of effect of high temperature on *P. auroginosa* KM110 lipase enzyme resulted in reduction in stability that confers mesophilic nature of enzyme [32].Whereas Mesophilic filamentous fungi *P. simplicissimum* showed high lipase activity in a wide temperature range of 35–60°C at acidic pH range 4.0–6.0that confers both thermophilic nature and the thermal stability of lipase enzyme [27].

pH of the cellular compartments and growth medium impact cellular transport affecting cell growth by improving physiological performance of cell. Microbial lipases are active at various pH such as alkaline, neutral and acidic. It has been reported that lipase from most prominent lipase producing species such as *Bacillus sp., Pseudomonas sp., streptomyces sp.* And *Acinetobacter sp* showed maximum activity at alkaline pH, thus proven promising catalyst for industrial applications. Only very few acidic lipases are reported such as lipase obtained from *Pseudomonas gessardii* and A.niger [21, 23].

Table 2: Alkaline thermophilic bacterial lipases

Organism	Optimum (pH)	Optimum Temp.(°C)	Ref.
Burkholderia cenocepaciaST8	9	n.s.	4
Flavobacteriumodoratum	9 – 10.5	50-65	5
Bacillus sp. RSJ-1	8 – 9	50	6
Acinetobacter species SY-01	10	50	7
Streptomyces rimosus	9 – 10	50 – 60	8
Thermosyntrophalipolytica (Lip A)	8.7	96	9
Thermosyntrophalipolytica (Lip B)	8.9	96	9
B.alcalophilus	10.6	60	10
B. licheniformis H1	9	50	11

B. stearothermophilus	7.4	68	12
B. thermocatenulatus	8.5	70	13
Pseudomonas aeruginosa	9.3	50	14

Table 3: Fungal lipases

Organism	Optimum (pH)	Optimum Temp.(°C)	Ref.
Aspergilluscarneus	9	37	22
A. niger	5.2	47.5	23
A. niger NCIM1207	8.5	50	24
Aureobasidiumpullulans	7.8	35	25
Amycolatopsismediterranei DSM 43304	8	60	26
P. simplicissimum,	4-5	50	27
Rhizopus arrhizus	8	n.s.	28
Rhizopus delemer	5.6	37	28

10. MOLECULAR CLONING OF LIPASE IN HETEROLOGOUS HOST

Increased functional expression of lipase enzyme is needed in suitable host to meet commercial requirement. Microbes as heterologous hosts are suitable for lipase production because of their extracellular nature, survive at extreme conditions and ability to grow fast with radially available substrates. Along with enhancing the heterologous protein expression balanced protein flux should be maintained properly to avoid undesired byproducts. Few widely used heterologous host bacteria species such as *Escherichia coli (E.coli), Pseudomonas sp., and Bacillus subtilis* are discussed in detail.

11. ESCHERICHIA COLI (E.COLI)

A widely studied gram-negative bacterium *E-coli* is most commonly used for heterologous lipase production because of its well characterized genetics, very well defined large number of cloning and expression vectors systems with well characterized regulated promoters and mutant strains. Some problems associated with lipase over expression in *E.coli* such as accumulation of inclusion bodies (inactive insoluble protein) in cytoplasm, protein misfolding, and reduction in disulfide bonds were reported [49]

Extracellular secretion of lipase enzyme mostly perform via type II secretary pathway. Expression of lipase in cytoplasm of host cell and secretion via type II secretion system involves periplasmic space as an intermediate where folding of protein with assistance of chaperon “lif”, lipase-specific foldase create chances of misfolding. Type I and type III *E-coli* secretion system which transport lipase directly from cytoplasm to outer membrane appear as a promising alternative approach. In this context Narayanan *et al.* reported an industrially important three disulfide bonds containing psychrophilic *Psuedozyma Antartica* lipase enzyme “Pal B” expressed in *E-coli* and extra cellularly secreted via type I system in the form of Pal B – Hyl A fusion protein via ABC transporter and via type III flagellar system in active form[50].

Lipase expression directly into periplasm is more advantageous by providing oxidizing environment required for disulfide bond formation in lipase enzyme reduces chances of misfolding and easy downstream processing with minimum contamination. Several periplasmic chaperons have been reported to improve stability of protein expression in periplasm. Xu *et al.* reported that misfolding which increases cell’s degradation capacity can be reduce by coexpression of periplasmic folding factors such as Deg P, Fkp A, Dsb A and Dsb C instead of cytoplasmic folding factors [51]. Chaperons plays very important role for production of proteins in active form.

12. PSEUDOMONAS SP

A modulator gene *liml* which is present at the downstream of lipase producing gene (*Lipl*) act as chaperon for renaturation / denaturation of heterologous protein in several *Pseudomonas sp*. like *E.coli* chaperones [60]. It has been reported that active expression of lactonizing lipase in *P. auroginosa* ADD 1976 was increased by 13 fold than the wild type in the presence of plasmid borne LipL-LimL genes under control of T-7 promoter [61].A transformant containing three LipL gene and one *LimL* gene show very high lipase activity 1070 units/ml has been observed. [62].Ability of *LimL* gene express in many other heterologous hosts also such as *E-coli, B.Subtilis* and *streptomyces lividans*is highly beneficial. Ihara *et al.* reported that inactive inclusion body of lipase in *E-coli* can be activated by presence *LimL* chaperon [63].Another chaperon ORF-2 obtained from *Pseudomonas aeruginosa* strains function as activator gene for ORF-1 and activate S5 lipase gene in E-coli. This gene constructs improved organic solvent tolerance of lipase enzyme in presence of 25 % (v/v) n-d decane and n-tetradecane [64]. A lipase variant from *Pseudomonas* species KFCC 10818 only with single amino acid exchange Pro112Gln promoted protein folding into active conformation and displayed 63% high enzymatic activity of its wild-type even in the absence of its cognate Lif protein [65].

13. BACILLUS SUBTILIS

Bacillus subtilis a gram positive bacterium established as a cell-factory for heterologous lipase production by providing high accessibility for genetic manipulations, ability to secrete protein direct extracellularly, availability of efficient mutant strains and well recognized as safe microorganism. Problem associated with *B. subtilis* for using it as host machinery is inefficient translocation across the plasma membrane and inefficient release of recombinant protein from cell surface. To overcome these problems, Pre-pro part of *staphylococcus* lipase gene added as secretion carrier in *B. subtilis* for efficient translocation in active form with preventing degradation of lipase from proteases [66].Lu *et al.* reported that recombinant *B. subtilis* WB 800 cells that were lacking extracellular proteases shown high *Protease vulgaris* lipase activity 356.8 units/ml [67].

14. ADVANTAGES OF MICROBIAL LIPASE

key properties Excellent chemoselectivity, stereoselectivity and regioselectivty of microbial lipase becomes an important reason of tremendous interest among scientists and industrialists [69].Microbial lipase are able to use wide range of biotic and abiotic substrates with various solvents such as water, organic solvents or ionic liquids to provide products of industrial importance. The reasons for of using lipases in biodiesel production includes the facts that (1) it can be produce at high yield from common bacterial and fungal species(2) Crystal structure of many lipases is well defined. (3) Broad range of substrate specificity (4) Specificity of enzymes prevent diversion into nonproductive (5) High stability in organic solvents (6) high resistance to product inhibition (7) and provide cost-effectiveness to the downstream processing. [70,71].

Thermostable biocatalyst are advantageous because higher reactivity, lower diffusional restrictions and lower viscosity. At high temperature solubility of substrates increase and helps to provide high process as well as decreases contamination problems by common mesophiles [47].Cold-adapted microorganisms are potential source of psychrophilic lipases .These lipases acquire special structural features which is a high level of flexibility particularly around the active site. Psychrophilic lipases have attracted attention due to their unusual properties such as low optimum temperature and high specific activity at very low temperatures [72].

Microbial lipase enzymes are more advantageous over lipases from plants or animals sources because absence of seasonal effects and availability of variety of catalytic activities. Microbial cells can be handled easily with rapid growth of on inexpensive media and can be genetically manipulated easily using well defined screening procedures [23].

15. CONCLUSION AND FUTURE PROSPECTS

Microbial lipases that are active under various conditions can reduce energy expenditure required to conduct reactions at elevated temperatures prevent destruction of labile reactants and products. Natural and engineered lipases improve catalytic efficiency and make overall process of biodiesel production economically more efficient. More research efforts should be focused in genetic engineering approaches to increase their operational stability and reusability to make the transesterification process economically feasible and more competent than conventional processes. Genetic engineering can also be used to engineer new crops and improve the oil levels in existing crops, to provide sufficient renewable raw materials for biodiesel production by enzymatic catalysis.

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