IDENTIFICATION AND CHARACTERIZATION OF ESTERASE PRODUCING MICROBES FROM DAIRY SLUDGE THROUGH METAGENOMIC APPROACH

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by

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(2009-09-104)

THESIS

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DEPARTMENT OF PLANT BIOTECHNOLOGY

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KERALA, INDIA

2014

DECLARATION

I hereby declare that the thesis entitled **"IDENTIFICATION AND CHARACTERIZATION OF ESTERASE PRODUCING MICROBES FROM DAIRY SLUDGE THROUGH METAGENOMIC APPROACH"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"IDENTIFICATION AND CHARACTERIZATION OF ESTERASE PRODUCING MICROBES FROM DAIRY SLUDGE THROUGH METAGENOMIC APPROACH"** is a record of research work done independently by Mr. ABEESH P. (2009-09-104) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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ABEESH P.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
BSA	Bovine serum albumin
bp	Base pair
BLAST	Basic local alignment search tool
$CaCl_2$	calcium chloride
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribose nucleic acid
dNTPs	Deoxyribose nucleotide tri phosphates
et al.	And others
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
g	gram
h	Hour
kg	Kilogram
kb	Kilo base pair
M	Molar
mg	milligram
min	Minute
ml	Millilitre
mM	Millimolar
nBLAST	nucleotide blast
NaCl	Sodium chloride

NCBI	National center for biotechnology information
ng	Nanogram
nm	Nanometre
^{o}C	Degree Celsius
OD	Optical density
PCR	Polymerase chain reaction
pNP	Para Nitrophenol
pNPA	Para Nitrophenol acetate
R	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
s	Second
SDS	Sodium dodecyl sulfate
sp.	Species
TAE	Tris- glacial acetic acid EDTA buffer
TBA	Tributyrin agar
TE	Tris-EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (Hydroxy Methyl) aminomethane
hydrochloride	
U	Enzyme unit
V	Volt
Viz.	Namely
v/v	volume/volume
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

Enzymes are the most favorable catalysts for the development of environmentally benign industrial processes. Most of the industrial enzymes are of microbial origin. Microbial enzymes are found to be more efficient and ecofriendly. Industries always demand for efficient, effective and dynamic enzymes for higher yield.

Esterases (EC 3.1.1.1, carboxyl ester hydrolases) are a diverse group of enzymes that catalyze the hydrolysis of ester bonds in triacylglycerides to glycerol and fatty acids. They are widely distributed in animals, plants and microorganisms. Esterases are distinguished from lipases in that their action is generally restricted to short chain fatty acids. These enzymes have significant biotechnological importance because of their ability to catalyze regio and stereo selective organic reactions. Esterases have no requirements for cofactors, are stable in organic solvents and have broad substrate specificity (Couto *et al*., 2010). Esterases play a major role in the degradation of natural materials, industrial pollutants and other toxic chemicals. They have a wide range of industrial applications including production of cosmetics, paper, detergents and animal feed. They are used for the synthesis of optically pure compounds, biosurfactants, perfumes, and antioxidants. Esterases have been isolated from many microbial sources. But the reasons of limiting industrial usage of known esterases are their limited thermostability at high temperatures and pH and instability in the organic solvent in operating industrial conditions. Therefore, the exploration for new microbial enzyme sources is vital for the identification of new thermostable and organic solvent resistant esterases suitable for different industrial purposes.

In the present study metagenomic approach is used for identifying esterase producing genes of microbial origin from dairy sludge. Metagenomics is a means of systematically investigating, classifying and manipulating the entire genetic material isolated from environmental samples (Zeyaullah *et al*., 2009). It is a functional genomic approach which acts as an alternative to the conventional

microbial screening (Uchiyama and Miyazaki, 2009). In this approach the environmental DNA or metagenome is directly cloned into a surrogate host and screened for the discovery of new microbial genes. By this method novel enzymes of unique activities have been identified (Jeon *et al*., 2011).

Application of metagenomics largely depends on the construction of genomic DNA libraries and subsequent sequencing or library screening. Two major strategies are used for the recovery of novel biomolecules from metagenomic libraries i.e. function based and sequence based screenings. Function based screening (functional metagenomics) involves screening metagenomic libraries for a particular phenotype, e.g. salt tolerance, antibiotic production or enzyme activity, and then identifying the phylogenetic origin of the cloned DNA (Dinsdale *et al*., 2008). Sequence based approaches involve screening clones for the highly conserved 16S rRNA genes for identification purposes and then sequencing the entire clone to identify other genes of interest.

Using conventional screening method we get only one percent of organisms from a sample, but metagenomic approach provides genes even from those microbes in the environment which are not culturable. Several novel lipases/esterases have been discovered with the use of metagenomic approach, (Lee *et al*., 2006; Rhee *et al*., 2005; Tirawongsaroj *et al*., 2008; Kim *et al*., 2009; Couto *et al*., 2010). Litthauer *et al*. (2010) constructed a metagenomic library using a South African deep mine biofilm sample for lipolytic activity. Abbai *et al.* (2011) used deep gold mine metagenome as a source of novel esterases. The screening of library provided two esterolytic clones, which differed from their known counterparts. Berlemont *et al*. (2013) isolated novel cold adapted esterase from an Antarctic soil metagenome. The biochemical characterization of this enzyme showed its adaptation to cold temperature.

In this study metagenomic approach is adopted to construct a genomic library of microbes from dairy sludge using a BAC (bacterial artificial chromosome) cloning vector in *E coli* host strain for identifying esterase encoding

genes. Dairy sludge is the final waste material from milk processing unit after wastewater treatment. It contains higher amounts of lipids that can act as a source for esterase producing microbes. Therefore it is anticipated that the metagenomic library of dairy sludge can provide novel esterases having industrial applications.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Lipolytic enzymes are one of the most essential groups of biocatalysts for biotechnological practices. Their stability under the harsh conditions, broad substrate specificity, regioselectivity and chemoselectivity of catalyzed reactions make them attractive biocatalysts in many industrial processes.

Lipolytic enzymes of microbial origin are widely used in detergent manufacturing, food ingredient production, paper processing, pharmaceutical production, and fine chemicals synthesis (Jaeger and Reetz, 1998; Gupta *et al*., 2004). In the detergent industry, enzymes are widely used to remove clothing stains from various types of fabric. These lipases are active in the presence of surfactants (Gupta *et al*., 2004). Lipolytic enzymes are also used to modify properties of fats or oils to make them more useful in food industries (Hasan *et al*., 2006). Oil from soybean is hydrolyzed by lipase in making Koji, a traditional Asian food and soybean fermented food Tempeh utilizes lipase from *Rhizopus oligosporous*. In leather industries, lipolytic enzymes are employed in bating, soaking and degreasing stages (Sangeetha *et al*., 2011). Both esterases and lipases are biocatalysts useful in different industrial applications.

The knowledge of bacterial lipases and esterases has increased significantly in the last decade and every year many novel lipolytic enzymes are being discovered and characterized. Many of them have been successfully utilized in industrial processes (Chen *et al*., 2011).

2.1 ESTERASE

Esterases belong to hydrolases, which catalyze the cleavage and formation of ester bonds. The enzyme commission number assigned to esterase is E.C. 3.1.1.x where x depends on the substrate (Bornscheuer, 2002). Esterases hydrolyze short chain carboxylic acids ($C \leq 12$) while lipases hydrolyze insoluble long chain ($C \geq 12$) triglycerides and secondary alcohols (Meghji *et al.,* 1990; Kim *et al.,* 2005; Faiz *et al.,* 2007). Esterases can catalyze three types of reactions: esterification,

interesterification and transesterification reactions with very good chemo-, regio- and/or enantioselectivity (Gupta *et al.,* 2012). Although esterases are responsible for hydrolysis reactions in the presence of water, they also catalyze several types of biotransformations in anhydrous solvents (Vieille and Zeikus, 2001). Esterases do not require cofactors and this property makes them attractive biocatalysts (Godinho *et al.,* 2011). Esterases from thermophilic microorganisms have been extensively employed in commercial applications due to their inherent stability (Demirjian *et al*., 2001).

2.1.1 Source of esterase

Esterases are widely distributed in animals, plants and microorganisms. Microbial enzymes are attractive because the cost of production is less and they are easy to manipulate. They can be engineered for production of esterase with desirable properties for industrial need. All classes of microorganisms like bacteria, fungi, and actinomycetes produce esterases, either constitutively or with inducers. Organisms isolated from cheese surface (Gandolfi *et al.,* 2005), oil contaminated area of city garbage (Gupta *et al.,* 2012) and marine squid (Ranjitha *et al.,* 2009) have been used for esterase production.

The bacterial sources reported to produce esterase are *Bacillus licheniformis* (Torres *et al*., 2005), *Bacillus megaterium* (Jung *et al*., 2003), *Bacillus* sp (Bakir Ateslier and Metin, 2006), *Sulfolobus tokodaii* (Suzuki *et al*., 2004), *Streptococcus thermophilus* (Liu *et al*., 2001), *Fervidobacterium nodosum* (Yu *et al*., 2010), *Geobacillus* sp. (Ayna *et al*., 2013; Ghati *et al*., 2013***)*** and *Pseudomonas* sp. (Kim *et al*., 2002).

The fungal species include *Trichoderma* sp. (Maeda *et al*., 2008), *Ophistoma* sp. (Calero-Rueda *et al*., 2002), *Pencillium* sp. (Horne *et al*., 2002), *Melanocarpus albomyces* (Kontkanen *et al*., 2006), *Aureobasidium pullulans* (Kudanga *et al*., 2007), *lactobacillus brevis* (kim *et al*., 2013) etc.

Esterase producing yeast include *Saccharomyces* sp. (Lomolino *et al*., 2003), *Candida* sp. (Ghosh *et al*., 1991) and *Kluyveromyces marxianus* (Monti *et al*., 2008). The actinomycetes like *Streptomyces coelicolor* (Soror *et al*., 2007) and *Streptomyces* sp. (Nishimura and Inouye, 2000) are known to produce esterase.

A considerable number of microbial carboxyl esterases is known, however, only a few of them have been used for biotechnological purposes. The major reason for this are their limited commercial availability and their frequently observed moderate enantioselectivity (Bornscheuer and Poh, 2001).

Eggert *et al*. (2002) reported the cloning, expression, purification and biochemical characterization of esterolytic enzymes from *Bacillus* species. Ren *et al*. (2006) produced recombinant hyperthermophilic esterase from agricultural waste such as corn steep liquor. This study showed that corn steep liquor was an efficient complex medium for recombinant enzyme production, compared with costly traditional medium containing yeast extract and peptone.

2.1.2 Applications of microbial esterases

Microbial esterases are currently receiving increased attention because of their potential applications in synthetic chemistry, biotechnology and biodegradation of industrial wastewater and agricultural pesticides. They have a wide range of industrial applications including production of cosmetics, food processing, paper, oil manufacture, detergents and animal feed (Panda and Gowrishankar, 2005; Li *et al*., 2008; Wheelock *et al*., 2008).

Quax and Broekhuizen (1994) reported the pharmaceutical use of esterase. The carboxyl esterase originated from *Bacillus subtilis* has been used in the synthesis of anti-inflammatory drugs. Esterases of extracellular origin are extensively used in the dairy industry in determining the final characteristics and attributes of products such as cheese (Smacchi *et al*., 1999). Esterases would help the detoxification of organophosphorous compounds found in insecticides in agricultural industries (Horne *et al*., 2002). Carboxyl esterases are also important

in the hydrolysis and subsequent detoxification of pyrethoid (Casida *et al*., 1983) and carbamate (Pohlenz *et al*., 1992; Gupta and Dettbarn, 1993).

Esterases are also found to be useful in the synthesis of major flavour compounds such as vanillin (Lesage-Meessen *et al*., 1996). Vanillin is produced as a result of the activity of carboxyl esterases on plant cell wall polysaccharides such as pectin and xylan. The carboxyl esterases produce ferulic acids from the polysaccharides which is then enzymatically converted to vanillin. Esterases from lactic acid bacteria (LAB) are involved in the development of fruity flavours and the improvement of quality in dairy and meat products like cheese, cured bacon and fermented sausages (Gobbetti *et al*., 1997). Torres *et al*. (2009) developed an important flavor compound isoamyl acetate from *Bacillus licheniformis.*

Feruloyl esterases have been isolated and characterized from a number of organisms. (Donaghy and Mckay, 1997; Christov and Prior, 1999; De Vries and Visser, 1999). The synthesis of flavour esters for food industry, modification of triglycerides for fat and oil industry, resolution of racemic mixtures used for the synthesis of fine chemicals for the pharmaceutical industry can also be performed (Molinari *et al*., 1996). Esterases are involved in formation of ester bonds. Gandolfi and workers carried out the ester synthesis using the esterases of non-starter bacteria which were isolated from cheese surface (Gandolfi *et al.,* 2000).

Deinking is the process of separation and removal of ink from the printed material. Different enzymes like cellulases, xylanases, esterases, lipases and lignolytic enzymes are used for enzymatic deinking process (Bolan and Bolan, 2004). Esterase is also used in paper, textile, leather and baking industries (Panda and Gowrishankar, 2005).

Microbial esterases are resistant to organic solvents, which makes them important catalysts in organic syntheses, especially enantioselective and steroselective hydrolysis of esters (Choi *et al*., 2003). Other useful reactions performed by microbial esterases are the synthesis of short chain esters, alcohol,

lactones and phenolic compounds, which contribute to the flavour of food (Holland *et al*., 2005).

2.2 METAGENOMICS

Microbial populations occur in every part of earth and many of them are metabolially useful. Ninety nine per cent of all microorganisms in almost every environment on earth cannot be cultured using conventional methods (Couto *et al*., 2010) and hence they remain as untapped reservoir of novel enzymes and metabolic capabilities. Isolation of novel metabolite from microorganisms based on culture based techniques yield limited amount of success when considering the number of microbes available (Sleator *et al*., 2008). This limitation led to the development of metagenomic approach, which avoids culturing of microbes, for understanding and accessing microbial genomes and their functions (Hardeman and Sjoling, 2007).

Metagenomics is a means of systematically investigating, classifying and manipulating the entire genetic material isolated from environmental samples (Zeyaullah *et al*., 2009). It is a functional genomic approach which acts as an alternative to the conventional microbial screening (Uchiyama and Miyazaki, 2009). First metagenomic process was proposed by Pace *et al*. (1991). Using this approach picoplankton DNA was cloned in to phage vector for subsequent 16S rRNA gene sequence analyses. The field of metagenomics became mainstream in the late 1990s. Healy *et al.* (1995) reported first successful function driven screening of metagenomic libraries, termed zoolibraries.

The uncultured microbial community's metagenomes are rich sources for novel enzymes. By this method a lot of novel biocatalysts are identified and characterized. The advantage of this technique is that significantly diverse genes can be isolated directly from the environmental sources (Park *et al*., 2007), and several novel lipases/esterases have been discovered with the use of metagenomic approach, (Rhee *et al*., 2005; Lee *et al*., 2006; Tirawongsaroj *et al*., 2008; Kim *et al*., 2009; Couto *et al*., 2010). Thus, it is becoming one of the best approaches to

mine novel enzymes from environment (Kennedy *et al*., 2008). Recently, there has been an increase in the number of metagenomic enzymes being reported.

Elend *et al*. (2006) isolated two novel metagenome derived esterases, derived from a soil metagenome and from a drinking water metagenome. These metagenomes were characterized to determine their applicability for industrial processes. Abbai *et al*. (2011) reported that screening of metagenome library from the deep mine biofilm provided two esterolytic clones, which differed from their known counterparts.

Jeon *et al*. (2011) identified novel lipolytic enzymes from metagenomic library of deep sea sediment using a combination of functional metagenomic approach and protein expression technology.

Couto *et al*. (2010) isolated a gene encoding a new lipolytic enzyme by functional screening of a metagenomic library derived from mangrove sediment sample. Sequence analyses revealed limited identities with other lipolytic enzymes from cultivated microorganisms, and phylogenetic analysis suggests that lipolytic enzyme with uncultured bacterium lipase is a member of a new subfamily of bacterial lipases within family I.

Chu *et al*. (2008) identified and characterized two novel esterases from a metagenomic expression library created with surface water microbes from the South China Sea. They also carried out amino acid sequence comparison and phylogenetic analysis for identifying family.

Metagenomics allows the assessment and exploitation of the taxonomic as well as the metabolic diversity in microbial communities in a highly extended fashion compared to other methods.

2.2.1 Metagenomics approach for esterase identification

Metagenomic approach has been found useful for expanding our knowledge of enzyme diversity, especially for bacterial esterases (Zhang *et al*., 2009). These involve directly assessing the genomes of organisms in the environment (Handelsman *et al*., 1998). Following studies are some examples of metagenomic esterase identification.

Elend *et al*. (2006) isolated two novel esterases EstA3 and EstCE1 derived from soil metagenome and drinking water metagenome respectively. Chu *et al*. (2008) isolated two novel esterases from a marine microbial metagenomic library derived from South China Sea. Zhang *et al*. (2009) identified a new esterase, EstAS, belonging to family III lipases from activated sludge metagenomic library. Abbai *et al*. (2011) used deep gold mine metagenome as a source of novel esterases. The screening of library provided two esterolytic clones, which differed from their known counterparts.

Berlemont *et al.* (2013) isolated novel cold adapted esterase from an Antarctic soil metagenome. The biochemical characterization of this enzyme showed its adaptation to cold temperature. Fang *et al*. (2014) identified novel esterase from a marine metagenomic library, named as est9x. The biochemical characterization of this enzyme exhibited its salt tolerance ability.

2.2.2 Metagenomic nucleic acid extraction

Nucleic acid extraction from the environmental sample is the most important step in metagenomics. As it involves direct cloning of metagenome for a specific activity, recovery of large sized DNA is also important. Metagenomic DNA extraction approach remains similar to that used in the extraction of DNA from pure cultures. Cell lysis, separation of the DNA from cell debris, and DNA recovery and purification are included in this process. Many factors hinder the successful extraction of metagenome. These include incomplete cell lysis, co-extraction of enzymatic inhibitors from sample, as well as the loss, degradation and

damage of DNA (Rochelle *et al*., 1992; More *et al*., 1994; Frostegard *et al*., 1999). Despite these limitations two principle strategies exist for the recovery of microbial DNA i.e. the cell extraction (Holben *et al*., 1988) and direct lysis methods (Ogram *et al.,* 1987; Robe *et al.,* 2003).

Schmitz *et al*. (2008) developed a rapid protocol for constructing plasmid libraries from small quantities of genomic/ metagenomic DNA. Pang *et al*. (2008) developed a modified protocol to extract DNA from forest topsoil that was suitable for construction of large insert soil metagenomic library with insert size of between 23.1 kb - 40 kb.

2.2.2.1 Direct lysis method

To achieve direct cell lysis, combinations of enzymatic treatment, high temperatures and detergent treatments have been used. In addition, several methods use mechanical disruption steps such as bead beating, freeze thawing or grinding of samples to lyse cells (Gabor *et al*., 2003). In addition to the DNA that is recovered from lysed prokaryotes, extracellular DNA and eukaryotic DNA are also recovered (Treusch *et al*., 2004).

2.2.2.2 Cell separation method

DNA extraction methods based on cell separation, although less efficient in terms of the amount of DNA recovered, are less harsh than direct lysis methods. The separation of microorganisms from the sample is achieved by mild mechanical forces or chemical procedures such as blending, rotating pestle homogenization or the addition of cation exchange resins, followed by density gradient or differential centrifugation (Treusch *et al*., 2004). The advantage of this method is that many naturally occurring contaminants which may hamper the subsequent molecular manipulation of the recovered DNA can be removed. The DNA obtained is almost entirely prokaryotic. DNA recovered by this method seems to be less contaminated with matrix compounds, including humic substances. In addition, the average size of the isolated DNA is larger than that typically obtained by the direct lysis

approach and is therefore more suitable for the generation of large insert libraries (Daniel, 2005).

2.2.3 Metagenomic libraries

Traditionally, genomic DNA libraries were constructed to represent the genome of a single organism (Kanoh *et al*., 1998; Song *et al*., 1999), but metagenomic library includes genome of many organisms. This technique provides the method whereby an entire genome can be stored stably for further investigation. Library construction involves several basic steps: the generation of suitably sized DNA fragments, cloning of these fragments into an appropriate vector and screening for the desired gene from metagenomic library.

Depending on the desired insert size, metagenomic libraries have been constructed using plasmids (up to 15 kb), fosmids, cosmids (both up to 40 kb), or bacterial artificial chromosomes (>40 kb) as vectors. The choice of the vector system depends on the DNA quality, targeted genes, and screening strategy. Small insert libraries can be employed for the identification of novel biocatalysts encoded by a single gene or a small operon, whereas large insert libraries are required to recover large gene clusters, which code for complex pathways (Daniel, 2005). Many studies have reported the recovery of entire metabolic pathways through the construction of large insert libraries. Such studies use bacterial artificial chromosomes (BAC) as vector (Courtois *et al.*, 2003; Velazquez- Sepulveda *et al*., 2012; Leon *et al*., 2013).

The approach of screening metagenomic libraries by functional gene expression for the presence of a specific enzyme activity has been employed with success. There have been a number of reports describing both the construction of metagenomic DNA libraries from diverse environments, and the subsequent recovery of novel genes from these libraries. Novel genes discovered this way include chitinase, 4-hydroxybutyrate dehydrogenase, lipase (Cieslinski *et al*., 2009), esterase (Abbai *et al*., 2011), cellulases (Duan *et al*., 2009), chitinases (Hjort

et al., 2010), DNA polymerases (Simon *et al*., 2009), proteases (Waschkowitz *et al*., 2009), and antibiotics (Riesenfeld *et al*., 2004) and genes encoding Na^+/H^+ antiporter activity (Henne *et al.*, 2000; Majernik *et al.*, 2001).

2.2.4 Analysis of metagenomic library

Techniques to recover novel biomolecules from environmental samples use two main approaches: a function driven approach, in which metagenomic libraries are initially screened for an expressed trait, and a sequence driven approach, in which libraries are initially screened for particular DNA sequences (Gabor *et al*., 2007).

2.2.4.1 Sequenced based screening

By screening the metagenome of a given environmental sample, a more complete picture of the true microbial biodiversity can be obtained as compared to culturing alone. The application of sequence based approaches involves the design of DNA probes or primers which are derived from conserved regions of already known genes or protein families (Daniel, 2005).

The most common approach is based on the amplification of the 16S rRNA gene, unique to prokaryotes, using a set of universal primers that is specific to a group of organisms; *i.e.*, bacteria or archaea. The amplified products are subsequently cloned to generate rRNA gene library and such clones can be sequenced for further phylogentic analysis. This approach has been very widely used in the study of bacterial and archaeal diversity (Dunbar *et al*., 1999).

By this approach only novel variants of known functional classes of proteins can be identified. Nevertheless, this strategy has led to the successful identification of genes encoding novel enzymes, such as dimethyl- sulfonio- propionate degrading enzymes (Varaljay *et al*., 2010), dioxygenases (Zaprasis *et al*.,2010), nitrite reductases (Bartossek *et al*., 2010), $[Fe^-Fe]^-$ hydrogenases (Schmidt *et al*., 2010), [NiFe] hydrogenases (Maroti, G., *et al*. 2009), hydrazine oxidoreductases (Li *et al*.,

2010), chitinases (Hjort *et al*., 2010), and glycerol dehydratases (Knietsch *et al*., 2003).

2.2.4.2 Function based screening

The function driven analysis is initiated by identification of clones that express a desired trait, followed by characterization of the active clones by sequence and biochemical analysis. This approach quickly identifies clones that have potential applications in medicine, agriculture or industry by focusing on natural products or proteins that have useful activities. A powerful yet challenging approach to metagenomic analysis is to identify clones that express a function. Success requires faithful transcription and translation of the gene or genes of interest and secretion of the gene product, if the screen or assay requires it to be extracellular. Although analysis has identified novel antibiotics (Gillespie *et al.,* 2002; Courtois *et al.,*2003; Venter *et al.,* 2004) antibiotic resistance genes (Riesenfeld *et al.,* 2004), Na^+ (Li^+)/ H^+ transporters (Majernik *et al.,* 2001), and degradative enzymes (Henne, *et al.,* 1999; Henne *et al*., 2000).

Most of the screens for the isolation of genes encoding novel biomolecules are based on the metabolic activities of metagenomic library containing clones. The power of the approach is that, it does not requires sequence information of the genes of interest, thereby making it the only approach to metagenomics having the potential to identify entirely novel classes of genes encoding known or novel functions (Ferrer *et al*., 2009; Gloux *et al*., 2010).

The function based screening is also called enzyme activity based or phenotype based screening (Streit and Schmitz, 2004). To detect the expression of a certain function in a clone, three different function driven approaches have been used to recover novel biomolecules: phenotypical detection of the desired activity (Liaw *et al*., 2010), heterologous complementation of host strains or mutants (Wang *et al*., 2006; Chen *et al*., 2010), and induced gene expression (Uchiyama *et al*., 2009).

2.2.4.2.1 Phenotypical detection of the desired activity

Specific substrates or indicator dyes which can interact with the desired gene product are incorporated into the growth medium. Clones that express the gene product can then be detected in a screen due to a color change in the growth medium of the individual clones (Handelsman, 2004; Ferrer *et al*., 2009).

Example of such an activity driven screen for the targeted genes encoding bacterial β- D- glucuronidases, which are part of the human intestinal Microbiome. A metagenomic library comprising 4,600 clones derived from bacterial DNA extracted from pools of feces was screened using an *E. coli* strain which is deficient in β- D- glucuronidase activity. In this way, 19 positive clones, of which one exhibited strong β- D- glucuronidase activity after cloning of the corresponding gene into an expression vector, were detected (Gloux *et al*., 2010).

2.2.4.2.2 Heterologous complementation of host strains or mutants

In this approach, mutants are used as host organisms. These mutants do not grow under normal conditions, they require certain selective conditions. If a foreign gene compensates the inactive gene of the mutant, the mutant can also grow under normal conditions. This technique allows a simple and fast screening of complex metagenomic libraries comprising millions of clones. Since almost no false positives occur, this approach is highly selective for the targeted genes (Simon *et al*., 2009). Examples for screens employing heterologous complementation include the identification of genes encoding lysine racemases (Chen *et al*., 2010), antibiotic resistance (Denef *et al*., 2009), enzymes involved in poly-3-hydroxybutyrate metabolism (Wang *et al*., 2006), DNA polymerases (Simon *et al*., 2009), and Na^-/H^-antiporters (Majernik *et al*., 2001).

2.2.4.2.3 Substrate and metabolite induced gene expression screening methods

In 2005, Uchiyama *et al*. introduced a third type of activity driven screen, which was termed substrate-induced gene expression screening. This is a method

for the detection of catabolic genes. This high throughput screening approach employs an operon trap *gfp* expression vector in combination with fluorescence activated cell sorting. The screen is based on the fact that catabolic gene expression is induced mainly by specific substrates and is often controlled by regulatory elements located close to catabolic genes. In this way, Uchiyama *et al*. (2005) isolated aromatic hydrocarbon induced genes from a metagenomic library derived from groundwater. One drawback of this approach is the possible activation of transcriptional regulators by effectors other than the specific substrates (Galvao, 2005).

A similar type of screen, designated metabolite regulated expression (METREX), has been published by Williamson *et al*. (2005). Williamson *et al*. (2005) developed a biosensor that detects small diffusible signal molecules that induce quorum sensing is inside the same cell as the vector harboring a metagenomic DNA fragment. Guan e*t al*. (2007) has identified a new structural class of quorum sensing inducers from the mid gut microbiota of gypsy moth larvae by employing METREX. In 2010, Uchiyama and Miyazaki (2010) introduced another screen based on induced gene expression, termed product induced gene expression (PIGEX).

2.2.5 Next generation sequencing and metagenomics

The introduction of next generation sequencing platforms, such as the Roche 454 sequencer (Margulies *et al*., 2005), the Solid system of Applied Biosystems (Bentley, 2006), and the Genome Analyzer of Illumina, had a big impact on metagenomic research (Bentley, 2006). The advances in throughput and cost reduction have increased the number and size of metagenomic sequencing projects, such as the Sorcerer II Global Ocean Sampling project (Rusch *et al*., 2007; Biers *et al*., 2009) and the metagenomic comparison of 45 distinct microbiomes and 42 viromes (Dinsdale *et al*., 2008). The analysis of the resulting large data sets allowed the exploration of the taxonomic and functional biodiversity and of the system biology of diverse ecosystems (Sjoling and Cowan, 2008).

2.2.6 Bioinformatics for metagenome processing

Metagenomics is an applied science that deals with the throughput genomic analysis of environmental isolates. Advances in genomic sequencing technologies, such as parallel sequencing and sequence analysis methods have contributed to the very foundation of this field. Metagenomics techniques have broad prospects in the field of environmental biotechnology. Bioinformatic tools, software and databases are used for metagenome processing (Patake and Patake, 2011).

Micro and macro environmental samples are collected and then high throughput sequencing is done to obtain metagenomic reads. Then all metagenomes are assembled separately using computational algorithms. The array of assembled genome is then subjected to analysis.

2.2.6.1 Sequence Processing

Processing of both, the genomic and metagenomic sequence data, follow common steps like preprocessing the sequence reads, assembly, Gene Prediction and Annotation. However, the main difference between genomes and metagenomes is that the former has a fixed end point like one or more completed chromosomes. However, in the case of metagenomes, we just get draft assemblies and may be sometimes almost complete genome of dominant populations (Chen and Pachter, 2005; Kunin *et al*., 2008).

2.2.6.1.1 Preprocessing the sequence reads

This is a very important step in metagenome processing. It involves base calling of raw data, removal of low complexity reads, removal of contaminant sequences, and removal of outliers, i.e, reads with very short length. Base calling involves identifying DNA bases from the DNA sequencing trace files. The most commonly used base calling tool is Phred (Ewing and Green, 1998). The other tool which is used in many other researches is Prinseq (Schmieder and Edwards, 2011). Prinseq is a web as well as a standalone tool that allows to filter, trim and reformat

the metagenome data. It removes low quality reads based on quality scores obtained from Phred to avoid complications in assemblies and downstream analysis. It trims poly-A/T tails, repeats of A's and T's at the end of the sequence because it can result in false positives during similarity searches, since they have a good alignment with low complexity regions or sequences with tails. It removes sequences with a lot of ambiguous bases (Morgulis *et al*., 2006).

2.2.6.1.2 Assembly

Assembly is the process of combining reads based on similarity to obtain contiguous DNA segments called contigs. There are challenges in assembling metagenomes as there could be problems like coassembly of reads coming from different species because of non uniform species distribution. This can happen if there is high sequence similarity between reads coming from closely related species. There are many publicly available assembly programs like Phrap, Celera Assembler and Newbler but these were all designed for assembling genomes from isolates and not for metagenomes, which comprise of multiple species with read coverage that is non uniform. Therefore, their performances vary significantly. To mitigate these problems for *de novo* assembly, we need to pass our data through more than one assembler so that it helps solving misassembly of the largest contigs. To further strengthen our assembly, we can perform multiple assemblies by tweaking parameters for a particular assembler. To be absolutely sure of our assembly so that problems do not percolate to further downstream analysis, we can perform manual inspection using scaffolding programs like ScaffViz or visualization programs like Consed (Gordon *et al*., 1998).

Comparative assemblies are easier to work with; where a reference genome or fully sequenced genome is passed to assembler along with the metagenome. AMOS is an assembler that performs comparative assembly.

2.2.6.1.3 Gene Prediction and Annotation

The process of identifying protein coding genes and RNA sequences is known as gene prediction. There are two ways of performing gene calling: one is evidenced-based and the other is ab initio gene prediction. The evidenced based method is based on BLAST similarity search to find homologs against a database of previously found genes. The *Ab initio* gene prediction method allows gene identification based on intrinsic features of the DNA sequence to differentiate between coding regions of a sequence from non-coding regions. This method is useful to identify those genes that do not have homologs to existing database sequences, and to find novel genes. For the ab initio method, there are many gene prediction tools, some of which requires training data set (fgenes) while some are self trained on the target sequence (MetaGene, Glimmer, Genemark).

MetaGene is the prokaryotic gene prediction tool developed specifically for metagenomes. The program does not require training data set and it estimates di-codon frequency from the GC content of a given sequence (Noguchi *et al*., 2006). In case of complete genomes, both the ways of gene prediction are employed and the hits to genes in the database act as training sets. In case of unassembled pyrosequencing reads and high complexity metagenomes, evidence based gene prediction is the only method used because of the fragmented nature and short read lengths of these data sets; as pointed out by Mavromatis (Kunin *et al*., 2008). Even in case of less complex communities, it is better to perform gene prediction on both reads and contigs because reads from less abundant organism remains unassembled and these reads may contain important functionality.

The most commonly used tool to predict RNA genes like tRNA and rRNA is tRNAscan (Lowe and Eddy, 1997). Finally, to assign protein function to metagenome data, protein sequences are compared to the database of protein families sequences like TIGRFAM, Pfam, and COGs (Kunin *et al*., 2008).

2.2.6.2 Data Analysis

Depending on the metagenome, there are different data analysis methods. The most common analysis methods are composition analysis on contigs, reclassification of reads after preprocessing, and binning.

2.2.6.2.1 Binning

Binning is a crucial step in the taxonomic analysis of large metagenomic data sets. Within this step, the sequences derived from a mixture of different organisms are assigned to phylogenetic groups according to their taxonomic origins. Depending on the quality of the metagenomic data set and the read length of the DNA fragments, the phylogenetic resolution can range from the kingdom to the genus level (Yang *et al*., 2010). Currently, two broad categories of binning methods can be distinguished: similarity-based and composition based approaches. The similarity based approaches classify DNA fragments based on sequence homology, which is determined by searching reference databases using tools like the Basic Local Alignment Search Tool (BLAST) (Huson *et al*., 2007; Meyer *et al*., 2008). Examples of bioinformatic tools employing similarity based binning are the Metagenome Analyzer (MEGAN) (Huson *et al*., 2007), CARMA (Krause *et al*., 2008), or the sequence ortholog-based approach for binning and improved taxonomic estimation of metagenomic sequences (SortITEMS) (Haque *et al*., 2009). CARMA assigns environmental sequences to taxonomic categories based on similarities to protein families and domains included in the protein family database (Pfam) (Finn e*t al*., 2010), whereas MEGAN and Sort-ITEMS classify sequences by performing comparisons against the NCBI nonredundant and NCBI nucleotide databases (Sayers *et al*., 2009).

Recently, Web based metagenomic annotation platforms, such as the metagenomics RAST (mg-RAST) server (Meyer *et al*., 2008), theIMG/M server (Markowitz *et al*., 2008), or JCVI Metagenomics Reports (METAREP) (Goll *et al*., 2010) have been designed to analyze metagenomic data sets. Via generic

interfaces, the uploaded environmental data sets can be compared to both protein and nucleotide databases, such as the Gene Ontology (GO) database (Ashburner e*t al*., 2000), the Clusters of Orthologous Groups (COG) database (Tatusov *et al*., 2001), and the Pfam (Fierer and Jackson, 2006), NCBI (Sayers *et al*., 2009), SEED (Overbeek *et al*., 2005), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa e*t al*., 2008) databases.

In this way, multiple metagenomic data sets derived from various environments can be compared at various functional and taxonomic levels (Goll *et al*., 2010).

2.3 ESTERASE SCREENING METHODS

Esterase producing organisms are selected and screened using two different screening methods such as plate assay method and UV fluorescence method.

2.3.1 Plate assay method

The most widely used plate assays for carboxl esterases assays contain triolein, olive oil or tributyrin which are emulsified mechanically in various growth media and poured into petri dishes (Jeager e*t al*., 1999). Lipolytic activity is observed by the formation of clear halos around the colonies growing on tributyrin containing agar plates (Gobbetti *et* al., 1997). This is the most commonly used assay where different substrates can be used in Luria Bertani agar. The different substrates used are tributyrin, ethyl acetate, sodium lactate, tween-20, tween-80, rhodamine olive oil, and α- naphthyl acetate (Zhang *et al*., 2009).

Esterase producing organisms produce clear zones around the colony after 24-48 hrs of incubation at 37°C. Some additional chemicals can be used to detect the clearance zones. Fluorescent substances like rhodamine B which can be detected under UV radiations, or chemicals like Fast Blue R R can be used to produce a brown coloured product. Even after incubation, plates can be exposed to

chemicals like Lugol s iodine solution which helps enhance the clear zones (Faiz *et al*., 2007; Kim *et al*., 2005; Gupta *et al*., 2012).

2.3.2 UV fluorescence method

This method for screening esterase producing microbial colonies within a mixed culture was reported in 1971. The protocol included overlaying the culture with sterile fibre filter saturated with 4- MUB (ester of 7-hydroxy-4-methylcoumarin) and incubating it at room temperature for 3 min. After incubation, the glass fibre filter is placed in another Petri plate with same orientation. It is then photographed with UV light (360-365 nm). The highly fluorescent 4-MU (7-hydroxy- 4-umbelliferone) spots are obtained by colonies producing extracellular esterase (Pancholy and Lynd, 1971).

2.4 ESTERASE ASSAY

The most commonly used method is the use of p-Nitrophenol esters (p-nitrophenyl acetate or p-nitrophenyl butyrate). The reaction mixture contains sodium phosphate buffer, p-NP ester, distilled water and enzyme. It is incubated for 30 min at 30°C followed by taking absorbance at 405 nm. One unit of esterase activity is defined as the amount of enzyme producing 1 mol of p-nitrophenol per min at 30°C (Faiz *et al.,* 2007; Meghji *et al.,* 1990). Esterase activity was mainly determined using two different method such as spectrophotometric assay and titrimetry.

2.4.1 Titrimetry

Esterase enzyme can be quantified using titrimetry method. In this 1 per cent substrate is titrated against 10 mM NaOH. Esterase activity can be calculated using the formula

$$\text{Esterase activity} = \frac{\text{Volume of NaOH consumed (ml)} \times \text{Molarity of NaOH}}{\text{Volume of enzyme (ml)} \times \text{Reaction time (min)}}$$

One unit of esterase activity can be defined as the amount of enzyme that liberates 1 mol of fatty acid per min at 30°C at pH 7 under the assay conditions (Gupta *et al.,* 2012).

2.4.2 Spectrophotometric assay

A spectrophotometric assay for the quantitative determination of esterases was developed by Mastihuba and coworkers. They worked on estimation of feruloyl esterase where 4-nitrophenyl ferulate releases 4-nitrophenol. Advantages of using this method are that it is easy, rapid and accurate. Enzyme activity can be calculated from the slope of the plot absorbance v/s time (Mastihuba *et al.,* 2002).

Li *et al*. (2012) determined esterase activity by using p-nitrophenyl butyrate (p-NPB) as substrate and the specific activity expressed in the units of enzyme activity per milligram of protein.

Lopes *et al*. (2011) used olive oil and p-nitrophenyl butyrate (pNPB) for determining esterase activity. One unit of esterase activity was defined as the amount of esterase required to release 1 μmol of p-nitrophenol in one min, under the specified conditions. Kumar *et al*. (2012) used tributyrin as substrate and esterase activity measured by titrimetric method.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Identification and characterization of esterase producing microbes from dairy sludge through metagenomic approach" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2013-2014. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 SAMPLE COLLECTION

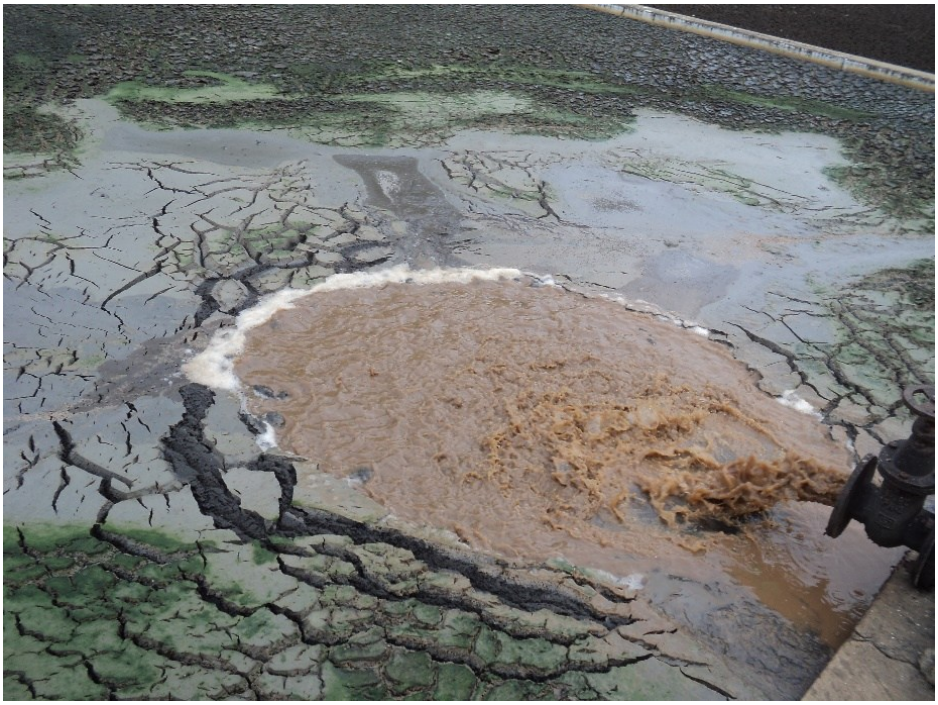
Dairy sludge samples (Plate 1) were collected from outlet of wastewater treatment plant of MILMA (Kerala Co-operative Milk Marketing Federation) unit, Thiruvallam, Thiruvananthapuram. Samples were stored at -20°C until the DNA extraction was performed. Sterile glass bottles were used for storing the samples.

3.2 METAGENOMIC DNA ISOLATION

C-TAB method of DNA extraction (Singka *et al*., 2012) with slight modifications was used for metagenomic DNA isolation.

A 5 ml of sludge sample (2 g wet weight) was taken a 25 ml centrifuge tube and centrifuged at 10,000 rpm for 10 min. Sludge pellet was lysed by adding 5 ml of hexadecyltrimethyl ammonium bromide (CTAB) extraction buffer. CTAB Extraction buffer (Appendix I) contains equal volume of 10% CTAB in 0.7 M Nacl and 240 mM potassium phosphate buffer (Appendix I). The suspension was mixed by inverting tube for 1 to 2 min and 5 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture. The resulting suspension was mixed by inverting for 1 min and frozen on ice for 1 min. This step was carried out 3 or more times to release individual cells from the flocs. This step is important for effective lysis, keeping the mixture on ice can help prevent DNA degradation. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a new centrifuge tube. Phenol extraction was repeated with 500 µl of

Plate 1. Dairy sludge



phenol: chloroform: isoamyl alcohol (25:24:1). The supernatant was transferred to a new centrifuge tube and phenol removed by mixed with 500 µl of chloroform: isoamyl alcohol (24:1), followed by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA was precipitated with 0.1 volume of 3 M sodium acetate (NaOAc), pH 5.5 and 2 volume of ethanol at -20°C for 30 min. DNA pellet was washed with 70% (v/v) ice cold ethanol, air dried and resuspended in 40 µl of TE buffer (Appendix I).

3.2.1 Agarose gel electrophoresis

The most common method to assess the integrity of genomic DNA is to run an aliquot of the DNA sample on agarose gel. The gel was run using horizontal gel electrophoresis unit (BIORAD, USA). Aliquot of DNA sample (5 µl) was loaded on agarose gel (1%) made of 1 X TAE buffer (Appendix I). The gel was run at 5 Vcm^{-1} until the dyes migrated $3/4^{th}$ of the distance through the gel. The gel was visualized under the gel documentation system (BIORAD, USA) using 'Quantity One Software'.

3.2.2 Spectrophotometer analysis

The absorbance of the DNA samples was recorded to determine the quantity and quality of DNA. T60 UV- Visible Spectrophotometer (Oasis Scientific, USA) was used for measuring optical density (O.D.) of the sample. Spectrophotometer was calibrated to blank (zero absorbance) at 260 nm and 280 nm wavelength with 3 ml TE buffer and absorbance of 1 µl DNA sample dissolved in 3 ml of TE buffer at respective wavelengths were recorded.

Since an absorbance value of 1.0 at 260 nm indicates the presence of 50 $ng\mu l^{-1}$ of double stranded DNA, the concentration of DNA in the extracted sample was estimated by employing the following formula:

Amount of DNA ($ng\mu l^{-1}$) = A_{260} x 50 x dilution factor

(Where A_{260} is absorbance reading at 260 nm)

The DNA quality could be judged from the ratio OD values recorded at 260 and 280 nm. The A_{260}/A_{280} values between 1.6 and 1.8 indicate the good quality of DNA.

3.3 METAGENOMIC LIBRARY CONSTRUCTION

Metagenomic library constriction consists of three different steps such as restriction digestion of metagenomic DNA, cloning of the digests and transformation.

3.3.1 Digestion of metagenomic DNA with Hpa I

The metagenomic DNA was partially digested by using restriction enzyme Hpa I (*Haemophilus parainfluenzae* I). In this study partial digestion was done used because larger fragments were required for cloning into BAC vector. Larger fragments may contain operon sometimes, which is functionally significant. Digestion was carried out in a 20 µl reaction mixture containing DNA sample (1µg), restriction enzyme (1unit), buffer and distilled water. The mixture was incubated at the 37^0C for 1, 2, 3 and 4 h. The reaction was stopped by adding 0.5 M EDTA (pH 8) to final concentration of 10mM.

The size of Digested DNA was determined on 1% (w/v) agarose gel in 1× TAE buffer, using 1 kb DNA ladder (Genei) as molecular weight marker and electrophoresed at 75 V for 1 h. The gel was stained with 0.5 µg/ml ethidium bromide (EtBr) and visualized under the gel documentation system (BIORAD, USA) using 'Quantity One Software'.

3.3.2 Cloning of the fragments

Copy right pEZ™ BAC cloning kit of Lucigen (USA) was used for cloning of metagenomic DNA fragments. Ligation was carried out using pEZ BAC vector, clone smart ligase and Hpa I digested DNA fragments. The Hpa I digested fragments were ligated into the linearized pEZ BAC vector. BAC library construction reaction was carried out in a volume of 100 µl.

The ligation was performed as follows:

The following components were taken in a 1.5 ml tube and mixed using a large bore pipette tip.

12 µl Insert DNA (100 ng)
75 µl H_2O
10.0 µl 10X Clone direct ligation buffer
1.0 µl pEZ BAC Vector (25 ng/µl)
2.0 µl clone smart ligase
100.0 µl total reaction volume

The solution was mixed by stirring and incubated at room temperature for 4 h. After that ligation reaction was stopped by heating the reaction at 65^0C for 15 min. Then mixture was cooled to room temperature for 15 seconds followed by 0-4^0C for 15 seconds to condense water vapor inside the tube. Finally mixture was spinned briefly in a microcentrifuge. After this steps the ligated product was ready for transformation.

3.3.3 Preparation of competent *E.coli* using calcium chloride

A single colony of bacteria was inoculated in a 100 ml of LB broth (Appendix II) and incubated for 12-16 hrs at 37^0C with vigorous shaking. The broth was transferred to sterile, disposable, ice cold 50 ml polypropylene tubes. The cultures were cooled to 0^0C by storing the tubes on ice for 10 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4^0C. The tubes were kept on a stand in an inverted position for 1 min, to decant the media from the cell pellets. Each pellet was resuspended in 10 ml of ice cold 0.1 M $CaCl_2$ and stored on ice. The cells recovered by centrifugation at 4000 rpm for 10 minutes at 40^0C. The tubes were kept in a stand in an inverted position for 1 min, to decant the media from the cell pellets. Each pellet was resuspended in 2 ml of ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture.

3.3.4 Transformation of clones into competitive *E. coli*

Using a chilled, sterile pipette tip, 200 µl of competent cell suspension was transferred to a sterile microfuge tube. Then ligated product (not more than 50 ng in a volume of 10 µl or less) was added to each tube. The resulting solution was mixed by swirling gently. The mixture was stored on ice for 30 min and transferred the tubes to a rack placed in a circulating water bath that has been preheated to 42^0C. The tubes were left in the rack for exactly 90 seconds without disturbing. Then the tubes were rapidly transferred to an ice bath, allowed the cells to chill for 1-2 min.

800 µl of LB broth was added to each tube. The culture was incubated for 45 min in a water bath set at 37^0C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by vector. The transformed cells (up to 100 µl per plate) were transferred on to YT agar medium (Appendix III) containing X- gal (5-bromo 4-chloro 3-indolyl β-D-galactopyranoside), IPTG (Isopropyl β-D-1-thiogalactopyranoside) and chloramphenicol. Then plates were left at room temperature until liquid has been absorbed. The plates were inverted and incubated for 20 to 24 h at 37°C. Transformed clones were picked out and inoculated into TB medium (Appendix IV) and stored at -80 ^{0}C.

3.4 SCREENING OF THE METAGENOMIC LIBRARY FOR ESTERASE ACTIVITY

3.4.1 Tributyrin agar plate assay

Esterolytic activity was assessed after three days of incubation at 37°C using tributyrin plate assay (Ro *et al*., 2004). Esterase producing micro-organisms produced a zone of clearance (hydrolysis) when their appropriate dilutions were spread on the TBA medium (Appendix V). The clear zone size was measured after 12, 24, 36 and 48 h of incubation at 37°C. Clones displaying large zones of clearance after the second round of screening were selected for sequencing and for further analysis.

3.4.2 Determination of esterase activity using the p-nitrophenyl acetate (pNPA) assay

Positive clones were inoculated in to 10 ml LB broth containing chloramphenicol and incubated for 12-16 hrs at 37^0C with vigorous shaking. The broth was transferred in to microfuge tubes and centrifuged for 5 min at 10000 rpm. The supernatant were taken as enzyme extract. In this assay, the enzyme hydrolyzed the acetate ester with the help of water. The products were acetic acid and p-nitrophenol (pNP), the latter showing an absorption maximum at about 405 nm. The esterase activity of the crude cell extract was determined spectrophotometrically in a 50 mM phosphate buffer (pH 7.5) solution (Appendix VI). One unit of esterase activity was defined as the amount of enzyme that released 1 mmol of p-nitrophenol per min or 1 mmol of acetic acid per min under assay conditions. First a 10 mM stock solution of the substrate p-nitrophenyl acetate (pNPA) was prepared in dimethyl sulfoxide (DMSO). In each microtitre plate well 190 µl phosphate buffer (50 mM, pH 7.4) and 10 µl enzyme preparation was pipetted and 40 µl of substrate (solved in DMSO) was added at different concentrations. The final volume per well was 240 µl. The enzymatic reaction was started after adding the substrate. The absorbance was measured at 405 nm for every second for 90 seconds at 37°C. The blank represented the auto hydrolysis of pNPA without enzyme. In addition, a pNPA free negative control (NC) was run that consists only of buffer and enzyme.

3.4.3 Total protein estimation by Lowry's method

Total protein content of the crude enzyme extracts was estimated by using procedure described by Lowry (1951). Bovine Serum Albumin (BSA, 1 mg/ ml) was used as working standard. Different volumes of BSA were pipetted into test tubes and the volume in each test tube was made up to 5 ml with water before starting the reaction. Concentration of BSA ranged from 0.05 to 1 mg/ ml. From the cell extracts prepared, 0.2 ml each was pipetted to different test tubes and added 2 ml of alkaline copper sulphate reagent (Appendix VII). The contents were mixed

well and incubated at room temperature for 10 mins. To each tube 0.2 ml of Folin Ciocalteau reagent was added and incubated for 30 min at room temperature. The spectrophotometer was calibrated to zero absorbance at 660 nm wavelength with 3 ml of blank solution. The absorbance of different working standard solutions of BSA and the samples of unknown protein concentrations were recorded at 660 nm against blank. The concentration of standard protein samples was plotted against the corresponding absorbance resulting in a standard curve that was used to determine the protein content in samples.

3.5 SEQUENCING OF POSITIVE CLONES

The vector DNA of the positive clones were isolated and given to M/S Genei, Bangalore for sequencing.

3.5.1 Isolation of vector DNA by alkali lysis method

Vector DNA was isolated using alkali lysis method (Sambrook *et al*., 1989). A single colony of bacteria was inoculated in a 2 ml of LB medium containing appropriate chloramphenicol and Incubated for 12-16 h at 37^0C with vigorous shaking. The broth was transferred in to microfuge tubes and centrifuged for 5 min at 10000 rpm. The supernatant was discarded and the tubes were inverted on a paper and blotted. 100 µl of ice cold solution I (Appendix VIII) was added and the cells were resuspended in it by vigorous vortexing. This was followed by the addition of 200 µl of freshly prepared solution II (Appendix VIII) and the contents were mixed by inverting it four times. The tubes were then incubated for 5 min in ice. To this mixture 150 µl of ice cold solution III (Appendix VIII) was added and incubated at room temperature for 5 min. The bacterial lysate was centrifuged at 12000 rpm for 5 min. The cleared lysate was transferred to an eppendorf tube. Equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and the contents were mixed well. The contents were centrifuged at 12000 rpm for

5 min to separate the aqueous layer. To the aqueous layer equal volume of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed gently and centrifuged at 12000 rpm for 5 min. The aqueous layer was separated and two volumes of 100 per cent ethanol at room temperature were added to precipitate the DNA. The contents were mixed well and allowed to stand for 2 min at room temperature. Then it was washed with 70 per cent ethanol, air dried and dissolved in 50 µl 1X TE and stored at -20^{0}C.

3.5.2 PCR amplification of vector DNA

The vector DNA of esterase positive clones were amplified using BEZ-F1 primer and BEZ-R1 primer provided in the copy right cloning kit for the amplification of inserts. The components of the mixture were optimized as listed below:

Water	:	12.9 µl
10x Taq buffer A	:	2 µl
(Tris with 15 mM MgCl2)		
dNTPS (2.5 m*M* each)	:	1.6 µl
BEZ-F1 Forward primer (10 µM)	:	1 µl
BEZ-R1 Reverse primer (10 µM)	:	1 µl
Template DNA (50 ngµl^{-1})	:	1 µl
Taq polymerase (3 Uµl^{-1})	:	0.5 µl
Total volume	:	20 µl

PCR was carried out in a thermal cycler (Bio-Rad, USA). PCR programme was set with initial denaturation at 94^{o}C for 4 min followed by 35 cycles of denaturation at 94^{o}C for 45 s, annealing at 50^{o}C for 30 s and extension at 72^{o}C for 45 s. Final extension was done at 72^{o}C for 5 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The amplified products along with Quantum PCR Marker (low range) from 'Genei,

Bangalore' were separated on agarose gel (1.5%). The gel was viewed under gel documentation system (BIORAD, USA).

3.6 SEQUENCE ANALYSIS AND CHARACTERIZATION

The resultant sequence of the positive clones were analyzed using bioinformatic tools like nBLAST and tblastx in order to identify the sequence and dendrogram was constructed NJ plot software following multiple sequence analysis using Clustal X.

RESULTS

4. RESULTS

The results of the study entitled "Identification and characterization of esterase producing microbes from dairy sludge through metagenomic approach" carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani, during 2013-2014 are presented in this chapter.

4.1 METAGENOMIC DNA ISOLATION

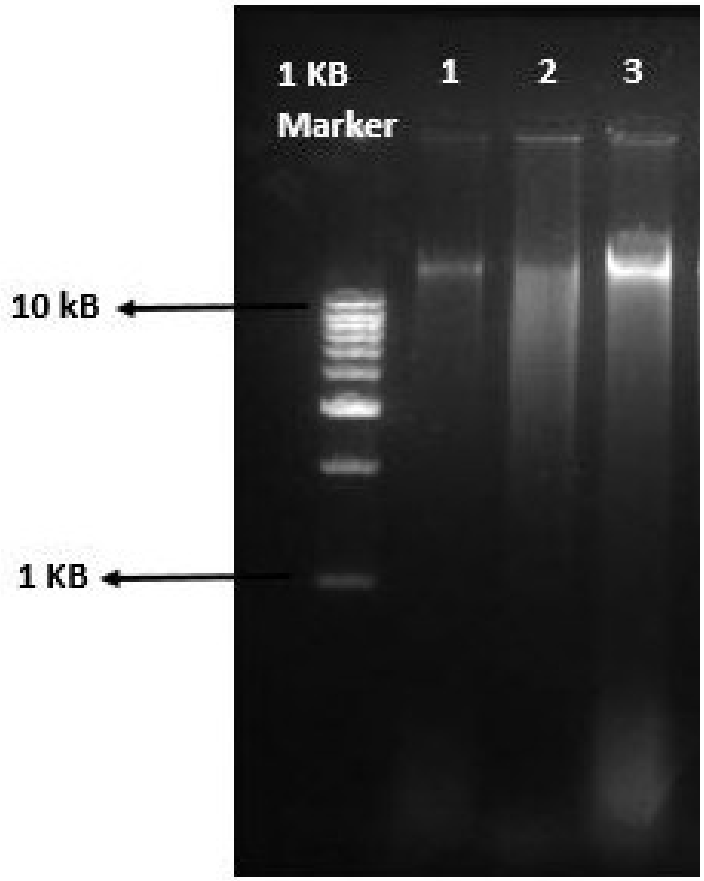
The Dairy sludge sample collected from MILMA (Kerala Co-operative Milk Marketing Federation Ltd.) unit was used in this study. As DNA extraction methods from dairy sludge were not available, three extraction methods reported for other environmental samples were tried. A comparison of the procedures and chemical differences in each DNA extraction method is summarized in Table1.

The method (M1) of Guobin *et al*. (2007) used freeze/thaw in liquid nitrogen combined with TENP buffer and reaction took 5 h to complete. In (M2 (Singh *et al.*, 2013) CTAB and SDS were used for cell lysis and extraction process was more time consuming (18 h) compared to others. Method (M3) reported by Singka *et* al. (2011) used vortexing with glass bead, followed by chemical cell lysis and the protocol took only 3.30 h for DNA extraction. The agarose gel electrophoresis (1%) of the extracted genomic DNA using three different protocols showed the presence of intact DNA bands on the gel (Plate 2). Among these protocols the good quality DNA was obtained (Plate 2) with the protocol of Singka *et al.* (2012).

DNA extraction method (M3) of Singka *et al.* (2012) was simple using a combination of mechanical and chemical lysis. This method showed good quantity of DNA (12.6 µg/g sample) compared to others. The methods M1 and M2 yielded 10 and 9.4 µg DNA/g of sample (Table 2).

The DNA quality could be judged from the A_{260}/A_{280} values recorded. The A_{260}/A_{280} value of DNA obtained with M3 method was comparatively better (1.66) than others. M1 and M2 yielded A_{260}/A_{280} values 1.53 and 1.577 respectively

Plate 2. Metagenomic DNA isolated from dairy sludge
Using three different protocols



Lane 1- Protocol of Guobin *et al.* (2007)

Lane 2- Protocol of Singh *et al.* (2013)

Lane 3- Protocol of Singka *et al.* (2012)

(Table 2). The ratio between A_{260} and A_{230} was also recorded, which ranged from 1.16 to 1.36, indicating contamination with polysaccharides.

The protocol (M3) of Singka *et al*. (2011) was modified by increasing the concentration of NaCl, to optimize it for dairy sludge, which contained polysaccharides and humic acids as contaminants. Use of glass beads were avoided in the cell lysis step.

Agarose gel (1%) profile of the genomic DNA extracted using four different protocols is shown in Plate 4. Among these protocols the good quality DNA was obtained with the modified protocol (M4). Increased concentration of NaCl (2 M) in this method increased the quality of DNA (Plate 3). It was reflected in the A_{260}/A_{230} value, which was increased from 1.36 to 1.54 (Table 3). The intensity of DNA band was high and the size was larger with less shearing.

Absorbance values (Table 2) of the extracted genomic DNA using spectrophotometric method showed a higher A_{260}/A_{280} value (1.784). This method also yielded good quantity of DNA, 11.4 µg/g.

4.2 CONSTRUCTION OF METAGENOMIC LIBRARY

Metagenomic library constriction consisted of three different stages such as restriction digestion, cloning and transformation.

4.2.1 Restriction digestion of metagenomic DNA

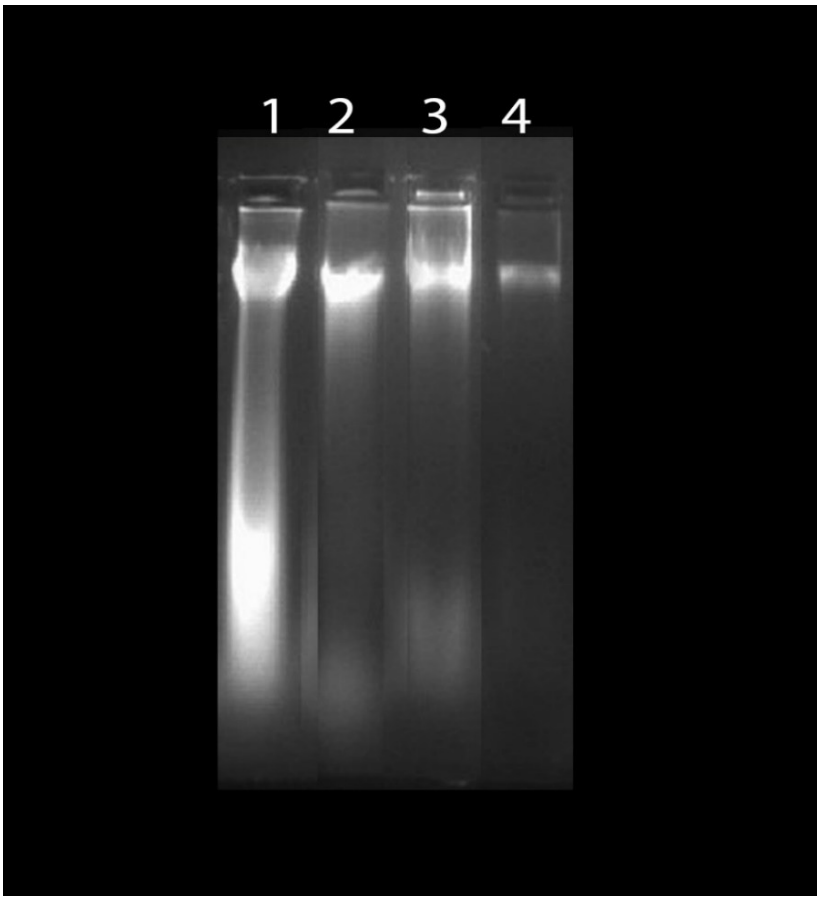
Metagenomic DNA was digested with the restriction enzyme Hpa I (*Haemophilus parainfluenzae I*). Digestion was carried out for three time intervals, 1h, 2h, and overnight. The agarose gel electrophoresis (1%) of the digested metagenomic DNA showed partial digestion (Plate 5). Among these overnight reaction has shown higher level of digestion compared to other reactions. Since the BAC vector used in this study could carry larger fragments, digests obtained after 1 and 2 h were taken for further steps. **Table 1.** Comparison of protocols used for metagenomic DNA isolation

Sl No.	Method	Sample preparation	Cell lysis	Protein removal	Time taken
M1	Guobin *et al* (2007)	TENP buffer (Tris-base, EDTA, NaCl, PVPP)	Lysozyme and SDS	P: C: I	5 h
M2	Singh *et al* (2013)	TENS buffer (tris HCL, EDTA, Nacl and SDS)	CTAB and SDS	P: C: I	18 h
M3	Singka *et al* (2012)	CTAB extraction buffer	CTAB and Glass beads	P: C: I	3.30 h
M4	Modified protocol	CTAB extraction buffer	CTAB	P: C: I	3. 30 h

Table 2. Quality and quantity of metagenomic DNA isolated by different protocols

Method	Absorbance (260 nm)	Absorbance (280 nm)	A 260/280	DNA Yield (µg/g)
M1	0.1567	0.1024	1.5302	9.4
M2	0.1689	0.1071	1.5770	10
M3	0.2108	0.1265	1.664	12.6
M4	0.1957	0.1097	1.7839	11.4

Plate 3. Effect of NaCl on Metagenomic DNA isolation using Singka *et al.* (2012) protocol



Lane 1- 0.75 M NaCl

Lane 2- 1 M NaCl

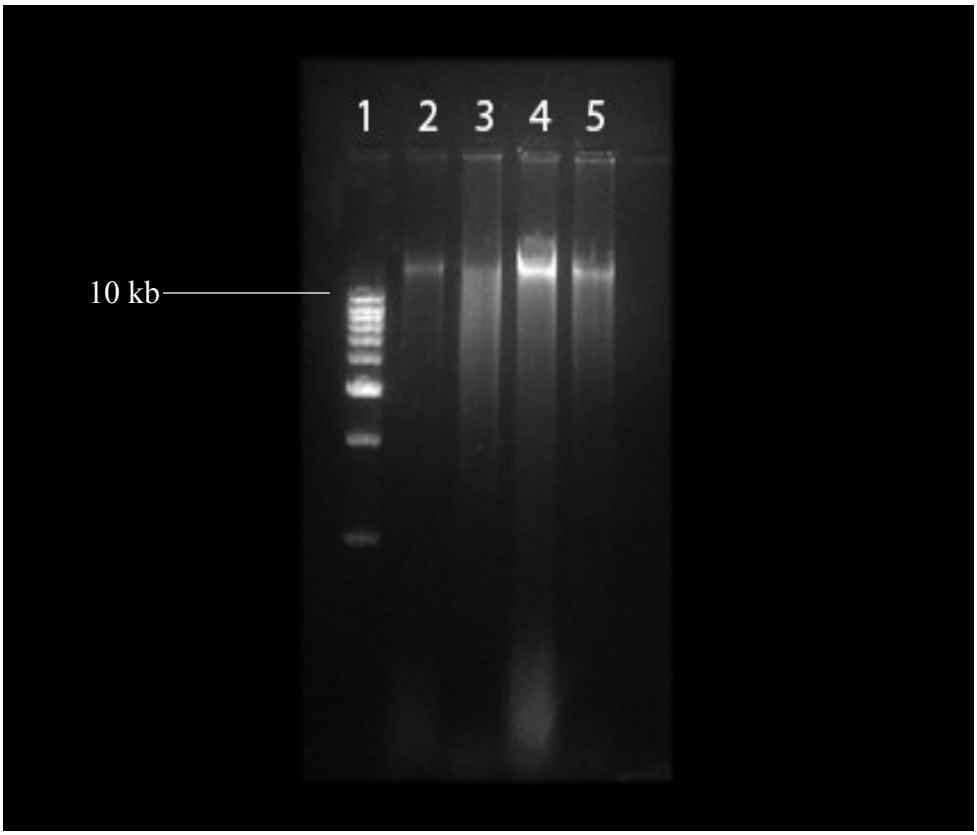
Lane 3- 1.5 M NaCl

Lane 4- 2 M NaCl

Table 3. Comparison of the quality of metagenomic DNA isolated by different methods

Method	**Absorbance (260nm)**	**Absorbance (230 nm)**	**A 260/230**
M1	0.1567	0.1346	1.16
M2	0.1689	0.1385	1.21
M3	0.2108	0.1571	1.36
M4	0.1957	0.1265	1.54

Plate 4. Comparison of protocols used for the Metagenomic DNA isolation from dairy sludge



Lane 1- Marker 1 kb

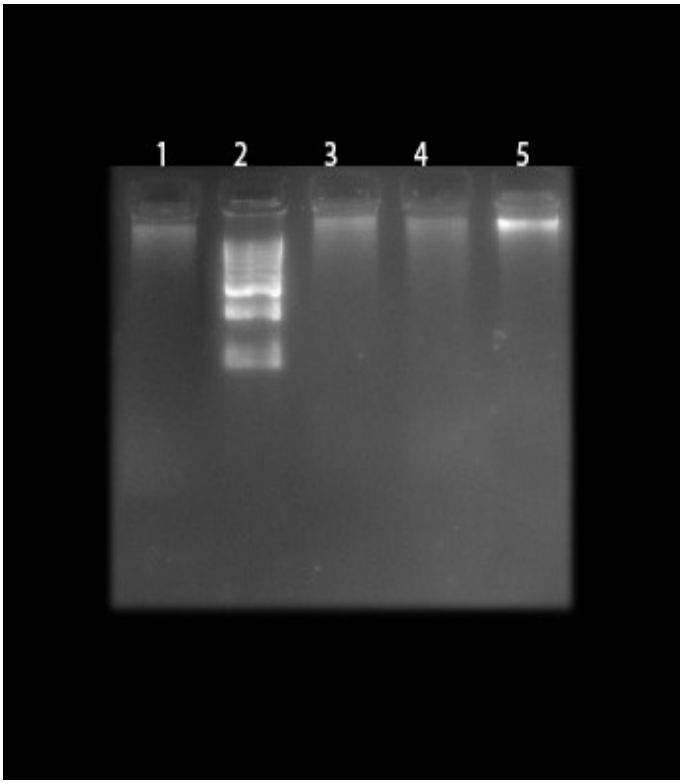
Lane 2- Protocol of Guobin *et al.* (2007)

Lane 3- Protocol of Singh *et al.* (2013)

Lane 4- Protocol of Singka *et al.* (2012)

Lane 5- Modified protocol

Plate 5. Metagenomic DNA digested with Hpa I



Lane 1- 3 h digestion

Lane 2- Marker 1 kb

Lane 3- 1 h digestion

Lane 4- 2 h digestion

Lane 5- Undigested DNA

4.2.2 Cloning of genome fragments

Hpa I digested DNA fragments were cloned in to pEZ BAC vector using clone smart ligase of Lucigen (USA). After ligation reaction the recombinant vectors were used for the transformation of DH5α.

4.2.3 Transformation

The transformed DH5α colonies were selected on chloramphenicol (25 μg/ml) containing plates. The Blue white screening system combined with antibiotic was also used for the selection. Both blue and white coloured colonies were obtained on LB plate with X- gal, IPTG and chloramphenicol (Plate 6). More than fifty per cent of colonies were white coloured, indicating the transformed cells having metagenome insert. Blue coloured colony indicated non transformed cells without insert.

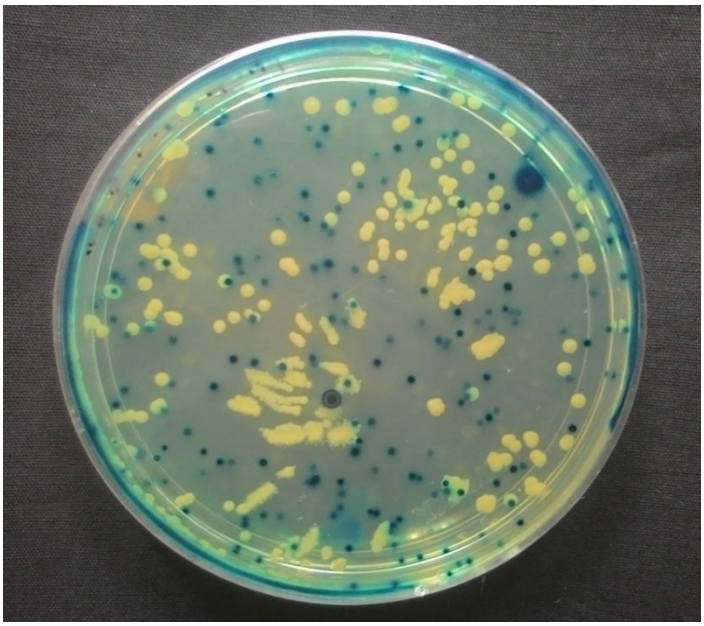
When incubation time was increased from 45 min to overnight, there was an increase in the number of transformed colonies (Plate 6). Transformed colonies were stored at -80^{0}C in TB medium containing chloramphenicol (25 μg/ml) and glycerol.

4.3 SCREENING OF THE METAGENOMIC LIBRARY FOR ESTERASE ACTIVITY

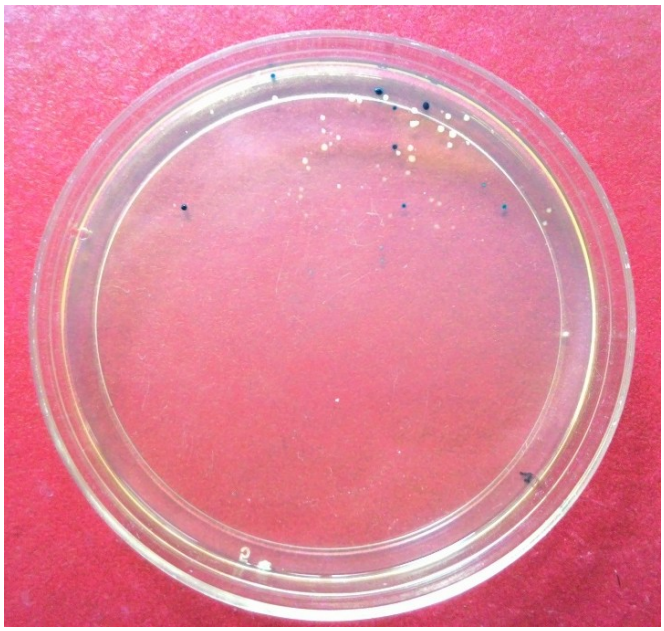
4.3.1 Tributyrin agar plate assay

The transformed clones selected on chloramphenicol (25 μg/ml) were subjected to qualitative screening for esterase production on tributyrin agar (TBA) plates containing the corresponding concentration of chloramphenicol. Twenty nine per cent of clones showed clear zones indicating the presence of esterase activity (Plate 7). DH5α without vector, kept as control, didn't show any clear zone in TBA plates with chloramphenicol. Also vector without insert did not show esterase activity in chloramphenicol containing TBA plates. Esterase positive clones were selected for further analysis.

Plate 6. Transformed *E.coli* on LB plate containing X-GAL, IPTG and chloramphenicol

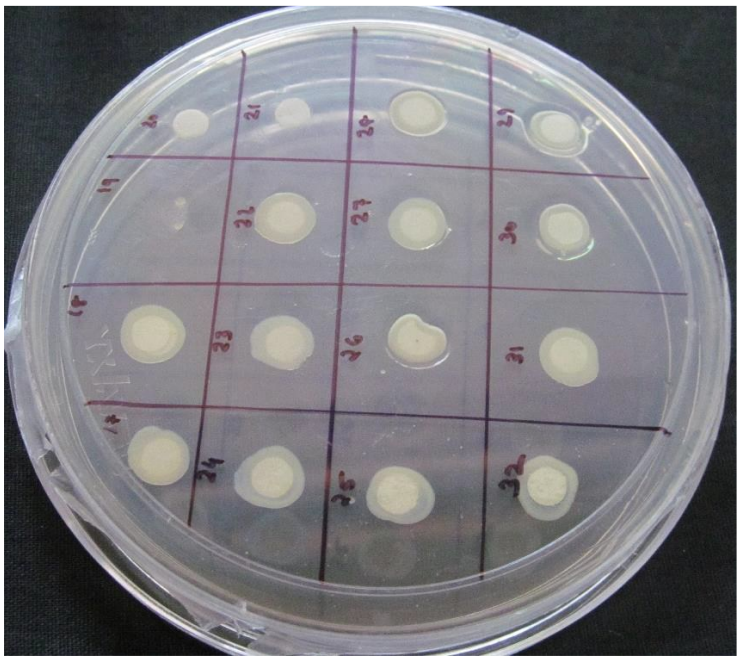


Transformed cells after overnight incubation



Transformed cells after 45 min incubation

Plate 7. Screening of transformed clones for esterase activity on TBA plates



Colonies with clear zones on TBA plates

4.4 ENZYME ASSAY

All the esterase positive clones (24 nos.) were taken for enzyme assay. Spectrophotometric readings of the products formed by the enzyme action of crude extracts of clones at wavelength 405 nm ranged from 1.031 to 3.285 (Table 4). The absorbance of ten clones (Figure. 1) showing higher enzyme activity ranged from 2.372 to 3.285 (Table 5), which were selected for further study.

Protein content in the crude extracts was estimated by Lowry's method. The absorbance of the crude cell extracts of the ten esterase positive clones at wavelength 660 nm ranged from 0.765 to 1.398 (Table 6). The concentration of protein (Figure. 2) present in the extracts, calculated from standard curve prepared using protein standard, ranged from 0.527 to 0.94 $\mu g \mu l^{-1}$ (Table 6).

The enzyme activity of the clones were determined, one unit of esterase activity is defined as the amount of enzyme producing 1 mol of p-nitrophenol per minute at 30°C. Esterase activity of ten positive clones ranged from 3.929 to 8.942 U/mg protein (Table 7). The maximum activity was shown (Figure. 3) by clone C19 (8.942 U/mg protein).

4.5 SEQUENCING OF POSITIVE CLONES

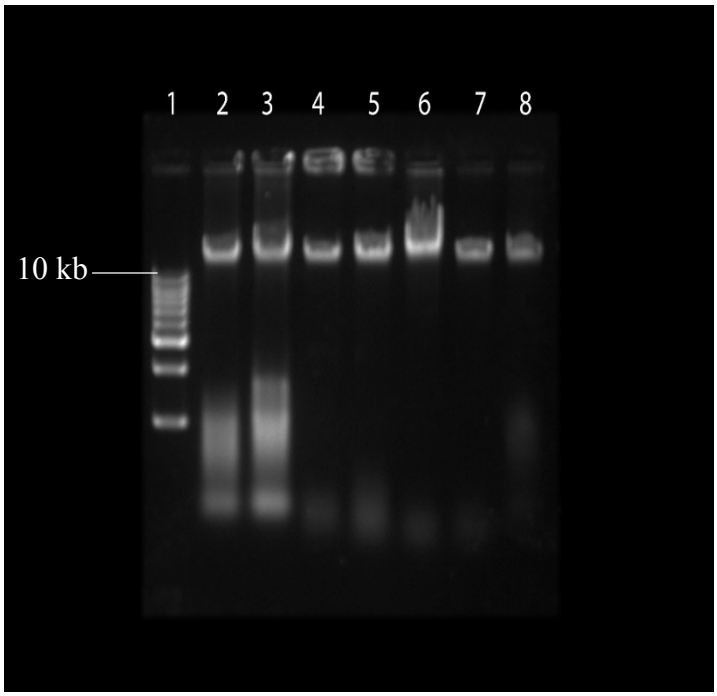
4.4.1. Isolation of vector DNA by alkali lysis method

The plasmid DNA separated on agarose gel (1%) showed good quality (Plate 8). The pEZ BAC vector used in this study was of size 7.2 kb. The vector DNA obtained was larger than 10 kb in size which indicated the presence of insert.

4.4.2 Amplification of metagenome insert from esterase positive clones by PCR

The inserts in the recombinant vector was amplified from esterase positive clones by PCR using primers specific for vector's cloning sites, the sequences which are shown in Table 8.

Plate 8. Vector DNA isolated from esterase positive clones



Lane 1- Marker 1 kb

Lane 2- clone 19

Lane 3- clone 42

Lane 4- clone 60

Lane 5- clone 44

Lane 6- clone 33

Lane 7- clone 16

Table 4. Esterase assay- Spectrophotometric readings of crude enzyme extracts from different clones

Clone no	Absorbance (405 nm)	Clone no	Absorbance (405 nm)
C1	1.045	C18	1.424
C2	1.167	C19	3.213
C3	1.118	C20	2.492
C4	1.299	C20	2.492
C5	1.480	C21	2.721
C6	1.485	C22	1.663
C7	1.712	C23	2.766
C8	2.008	C24	1.210
C9	1.456	C25	2.138
C10	1.369	C26	1.621
C11	1.408	C27	2.044
C12	1.104	C28	2.302
C13	1.337	C29	1.031
C14	2.661	C30	2.265
C15	1.508	C31	2.927
C16	1.550	C32	1.723
C17	1.663	C33	1.803

Table 4. Continued

Clone no	Absorbance (405 nm)	Clone no	Absorbance (405 nm)
C34	2.275	C50	1.936
C35	1.339	C51	3.100
C36	1.453	C52	2.498
C37	1.962	C53	2.816
C38	1.328	C54	1.186
C39	1.463	C55	1.789
C40	1.322	C56	1.642
C41	1.658	C57	1.615
C42	1.433	C58	1.285
C43	1.791	C59	2.109
C44	1.782	C60	3.285
C45	1.276	C61	2.372
C46	1.498	C62	2.117
C47	1.523	C63	1.501
C48	1.604	C64	1.530
C49	2.491	C65	1.437

Table 4. Continued

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Clone no	Absorbance (405 nm)	Clone no	Absorbance (405 nm)
C67	1.480	C75	1.045
C68	1.485	C76	1.167
C69	1.712	C77	1.118
C70	2.008	C78	1.299
C71	1.276	C79	1.186
C72	1.498	C80	1.789
C73	1.523	C81	1.642
C74	1.604	C82	1.615

Table 5. Higher absorbance showing esterase positive clones at 405 nm

Sl No.	Clones	Absorbance (A_{405} nm)
1	C60	3.285
2	C16	3.213
3	C24	3.100
4	C33	2.927
5	C42	2.816
6	C40	2.766
7	C26	2.721
8	C44	2.661
9	C19	2.492
10	C51	2.372

Figure 1. Esterase activity of 10 selected clones

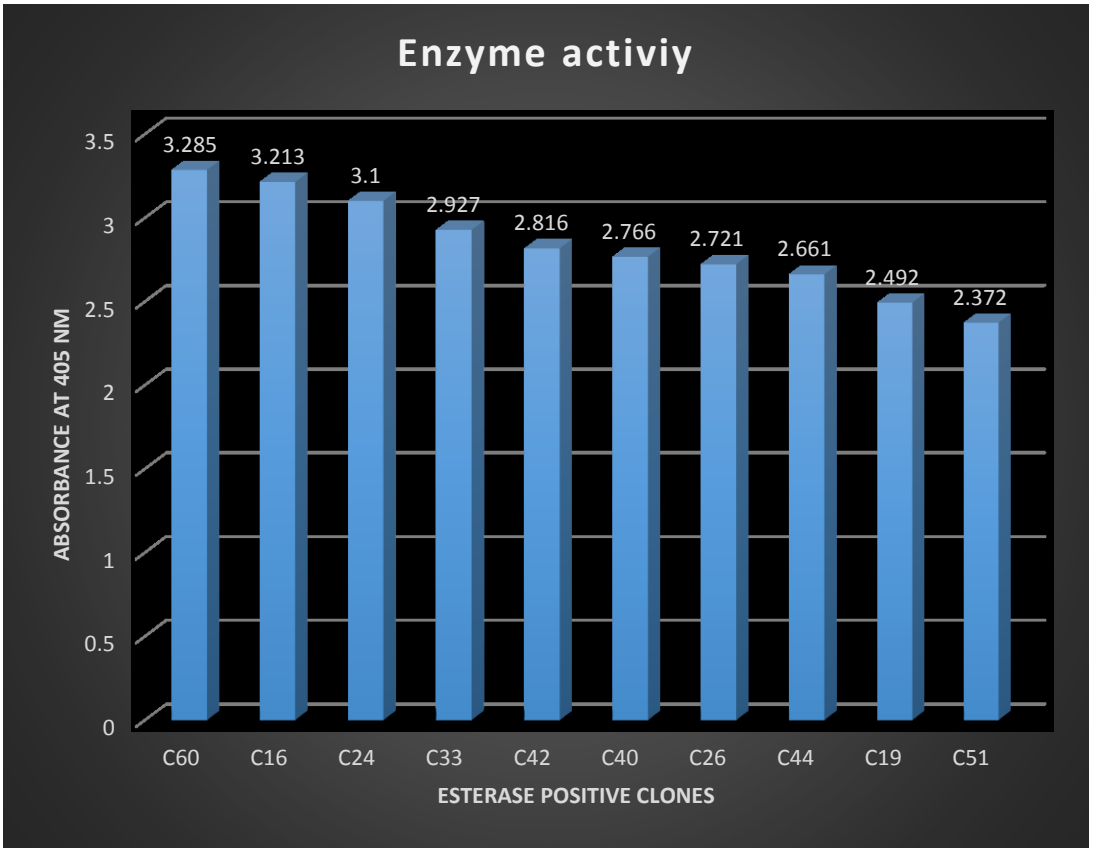
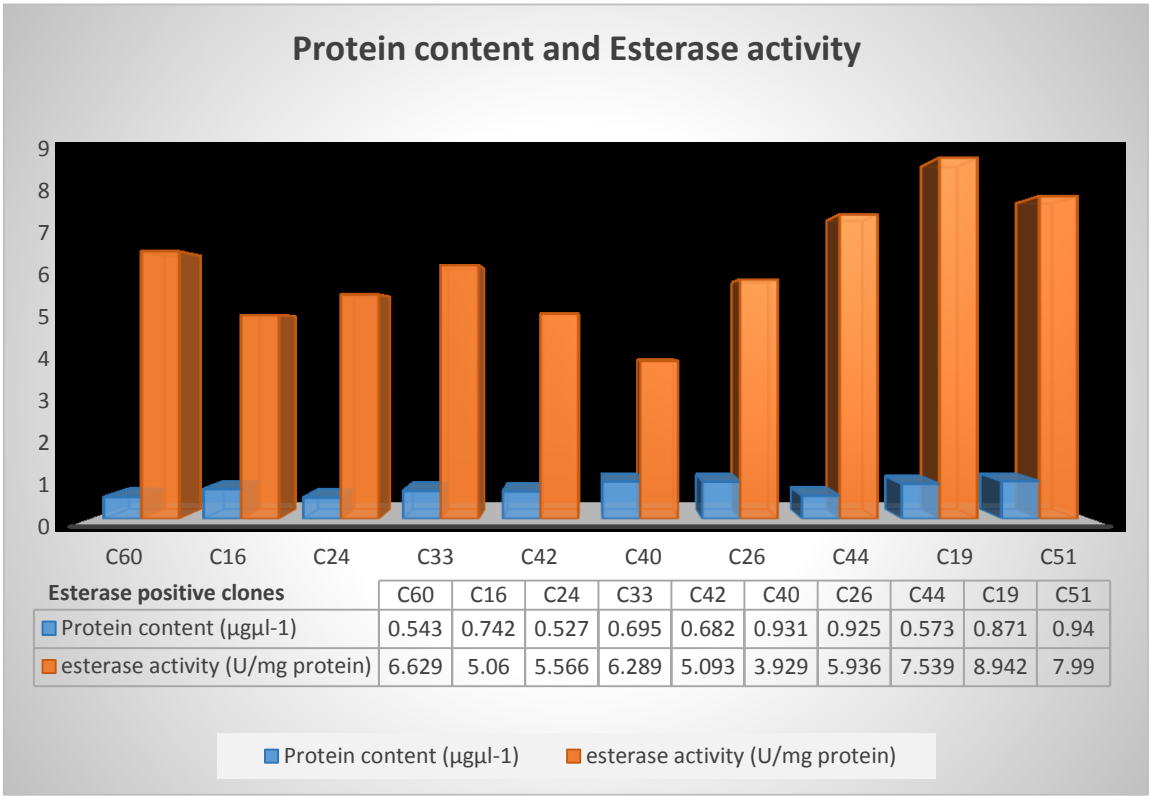


Table 6. Protein content of the esterase positive clones

Sl. No.	Sample	Absorbance (A_{660} nm)	Conc. of Protein ($\mu g \mu l^{-1}$)
1	C60	0.768	0.543
2	C16	1.095	0.742
3	C24	0.765	0.527
4	C33	1.155	0.695
5	C42	1.034	0.682
6	C40	1.372	0.931
7	C26	1.365	0.925
8	C44	0.824	0.573
9	C19	1.266	0.871
10	C51	1.398	0.94

Figure 2. Comparison of protein content and esterase activity of clones



Esterase positive clones	C60	C16	C24	C33	C42	C40	C26	C44	C19	C51
Protein content (µgµl-1)	0.543	0.742	0.527	0.695	0.682	0.931	0.925	0.573	0.871	0.94
esterase activity (U/mg protein)	6.629	5.06	5.566	6.289	5.093	3.929	5.936	7.539	8.942	7.99

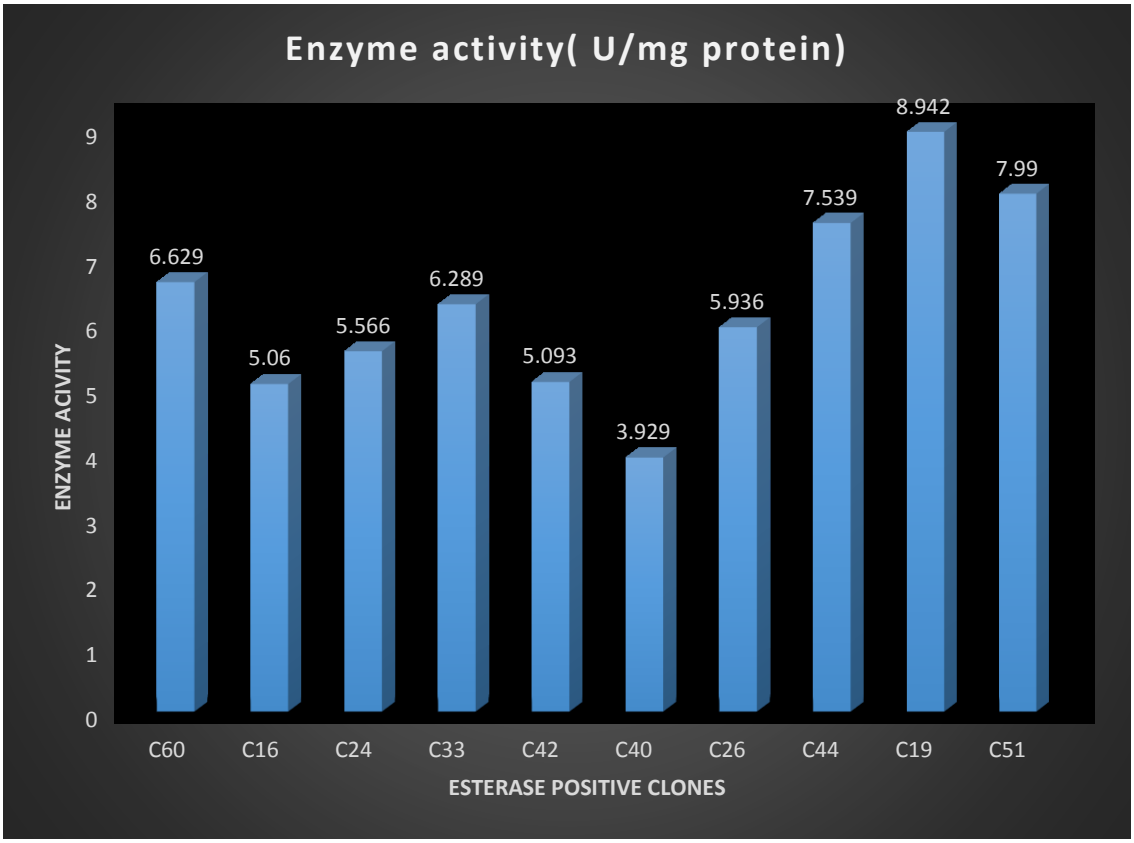
Table 7. Selected clones having higher esterase activity

Sl No.	Clones	Protein content From std graph (mg/ml)	Enzyme activity (U/mg protein)
1	C60	0.543	6.629
2	C16	0.742	5.06
3	C24	0.527	5.566
4	C33	0.695	6.289
5	C42	0.682	5.093
6	C40	0.931	3.929
7	C26	0.925	5.936
8	C44	0.573	7.539
9	C19	0.871	8.942
10	C51	0.94	7.99

Table 8. Sequences of primers used for PCR amplification

Primer name	Primer sequence (5' to 3')	No. of bases	GC content (%)	Tm (^{0}C)
BEZ-F1	CAC TTT ATG CTT CCG GCT CGT ATG	24	50	**57.4**
BEZ-R1	GGG ATG TGC TGC AAG GCG ATT AAG	24	54.2	**59.1**

Figure 3. Enzyme activity of esterase positive clones

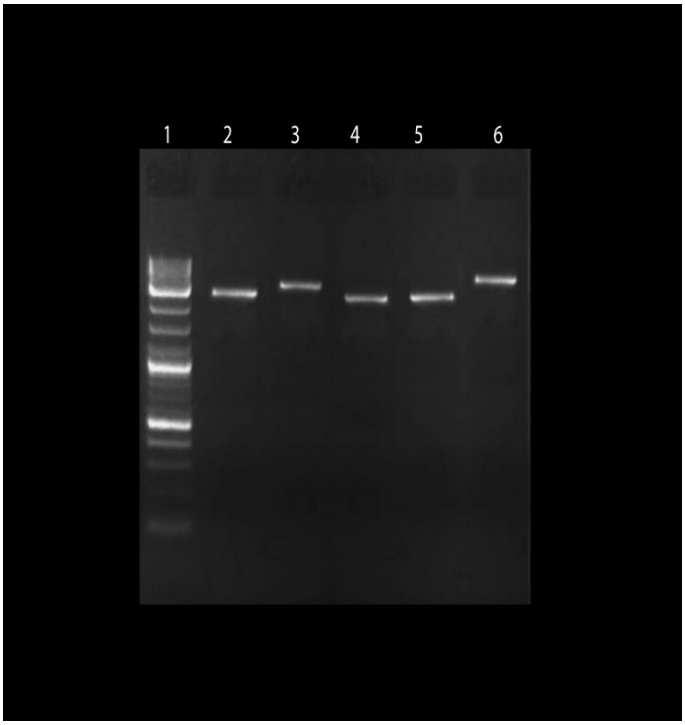


The amplified products from five clones are shown in Plate 9. All the amplicons were larger than 3 Kb, as compared with the marker DNA.

4.4.3 Sequencing of the inserts

Sequencing of the inserts were carried out at Genei, Bangalore under M/S Merck India. The sequencing reaction was carried out with BAC vector specific primers. The sequencing results were not of good quality.

Plate 9. PCR amplification of insert from esterase positive clones



Lane 1- Marker 3 kb
Lane 2- Insert of clone 19
Lane 3- Insert of clone 42
Lane 4- Insert of clone 51
Lane 5- Insert of clone 44
Lane 6- Insert of clone 60

DISCUSSION

5. DISCUSSION

The present study, 'Identification and characterization of esterase producing microbes from dairy sludge through metagenomic approach', was carried out during 2013-2014 at the Department of Plant Biotechnology, College of Agriculture, Vellayani. The results obtained are discussed in this chapter.

5.1 METAGENOMIC DNA ISOLATION

Isolation of quality environmental DNA in appreciable quantity from the preferred sample is one of the key factors responsible for the success of metagenomics. The dairy sludge contains higher amounts of lipids which act as a source for esterase producing microbes. The dairy sludge was highly viscous and deep brown in color. A protocol that results in high molecular DNA which is free from contaminants and represents maximum detectable diversity is mostly preferred for metagenomic DNA isolation. The currently available DNA extraction methods used for activated sludge are complicated and time consuming (Roh *et al*., 2006). Also DNA extraction methods used for dairy sludge were not available, so three extraction methods used for other environmental samples were tried in this study.

Protocols developed by Globin *et al.* (2007), Singka *et al.* (2012) and Singh *et al.* (2013) were used for DNA extraction from dairy sludge. The protocol of Globin *et al*. (2007) required more time for extraction compared to other methods, also the quantity and quality of DNA was comparatively less. The protocol of Singh *et al*. (2013) yielded higher quantity of DNA, but it required 18 h to complete the process. Among these three isolation methods protocols of Singka *et al.* (2012) showed comparatively better result in terms of time, quantity and quality. The extraction buffer used in this procedure contains CTAB, which helped the removal of complex humic acid compounds and polysaccharides in the sludge. It took 3.30 h to complete the procedure in fewer steps compared to other methods. Still the polysaccharide contamination was high, as evidenced from A_{230} value. So an attempt was made to optimize a protocol for dairy sludge. For this

the protocol of Singka *et al.* (2011) was modified by changing the concentration of NaCl buffer.

Modified DNA extraction method was simple using only chemical lysis. Dairy sludge samples are rich in organic materials. In this study good quality DNA could be obtained when the NaCl concentration was increased to 2 M. Sahu *et al*. (2012) reported that increasing the concentration of NaCl in the extraction buffer help the effective removal of protein and polysaccharides. Roose-Amsaleg *et al*. (2001) reported that CTAB improved the purity of the DNA by precipitation of complex humic compounds in sludge.

The absorbance ratio (A_{260}/A_{280}) in the range of 1.8 to 2.0 indicates a high level of purity of DNA. Among the four protocols tried, only the modified protocol (M4) yielded DNA with an A_{260}/A_{280} value greater than 1.7. This method also yielded higher quantity of DNA (11.4 µg/g). The DNA band was intact and showed high fluorescence intensity. The modified method could be completed within 3 h and 30 min.

5.2 METAGENOMIC LIBRARY CONSTRACTION

The construction of metagenomic libraries from different environments has shown a great potential to identify new or improved genetic determinants (Van Elsas, 2008). This study aimed the use of dairy sludge to develop a gene pool for esterase enzyme. Metagenomic library construction consisted of three different steps such as restriction digestion, cloning and transformation.

Metagenomic DNA was digested using restriction enzyme *Hpa I* (*Haemophilus parainfluenzae I*). This study used only the partial digests obtained after 1 and 2 h, so incubation for getting larger fragments. Partial digested DNA is recommended for the construction of libraries based on BACs (Daniel, 2005; Leon *et al*. 2012). Larger DNA fragments may have the potential to carry the entire gene cluster for a metabolic function. Genomic libraries carrying such large inserts are

particularly suited for sequence homology based screening approaches. The fragments used in this study were larger than 40 Kb in size.

The pEZ™ BAC Blunt Copy Right cloning kit of Lucigen (USA) was used for metagenomic library construction. These vectors can be used for cloning fragments of any size, from <1 kb up to 100 kb. The pEZ™ BAC Blunt vector contains chloramphenicol resistance gene, lacZ gene, and cloning site of Hpa I.

BAC cloning vector was used for the preparation of genomic library. Earlier reports (Daniel, 2005) suggest that the construction of metagenomic libraries of large inserts has several advantages for detecting genes with desirable functions, such as requiring a small number of clones for screening, and finding positive outcomes. Several studies have been reported on the preparation of metagenomic library using BAC vectors or identification of genes specific metabolic functions. Chu *et al*. (2008) reported the construction of marine metagenomic library using BAC cloning vector and identified two novel esterases. Leon *et al*. (2012) constructed an *E. coli* metagenomic library based on BAC clones with large genomic inserts from metagenomic DNA from the rhizosphere of wheat plants.

For cloning chemically competent DH5α cells were used and the cells were transformed by heat shock method. Calcium chloride was used as chemical in transformation reaction (Sambrook *et al*., 1989). In this study heat shock method was used for transformation. This method is less expensive than electroporation based transformation. The transformed cells were plated onto LB solid medium with chloramphenicol, X-gal and IPTG.

The lac Z based blue/white screening system was used along with chloramphenicol as selective agent, for the selection of transformed cells. Using this combination the vectors ligated without any inserts could be identified easily. In this study more than fifty per cent of colonies were white coloured, indicating the transformed cells having metagenome insert. Blue coloured colony indicated non transformed cells without insert. An increase in the incubation time, from 45 min to overnight, also increased number of transformed colonies. Transformed

colonies were selected and stored at -80^{0}C in TB medium containing chloramphenicol and glycerol (20%). Rondon *et al*. (2000) used 20% glycerol and antibiotic for storing transformed colonies. Leon *et al*. (2012) reported that lac Z based blue/white screening system and chloramphenicol resistance for the selection of transformed BAC clones.

5.3 SCREENING OF THE METAGENOMIC LIBRARY FOR ESTERASE ACTIVITY

Metagenomics is a powerful approach for the discovery of new enzymes. Functional screens, in contrast to sequence homology search, enable us to select enzymes based on their activity. Activity-based metagenomics also provides an opportunity to circumvent culturing.

In this study tributyrin agar plate assay was used for screening esterase positive clones. From the 82 transformed clones selected for the esterase activity screening on tributyrin agar plates with chloramphenicol, 24 clones showed esterase activity, by producing clear zones on TBA plates. Many methods are reported for the screening for esterase activity. Kumar *et al*. (2012) reported use of tributyrin as substrate for the screening of esterase producing organisms. Spirit blue agar with the tributyrin as substrate has been used by several workers (Bruni *et al*., 1982; Ranjitha *et al*., 2009). Lopes *et al*. (2011) used olive oil and p-nitrophenyl butyrate (pNPB) for determining esterase activity.

DH5α without vector, kept as control, didn't show any clear zone in TBA plates with chloramphenicol. Also vector without insert did not show esterase activity in chloramphenicol containing TBA plates. The experiments clearly showed the presence of esterase coding sequences in the cloned metagenomic fragments.

5.4 ESTERASE ASSAY

The functional significance of the clones can be determined by comparing the quantity of the enzymes produced. Esterase activity can be determined using two different methods such as spectrophotometric assay and titrimetry. Esterase shows high activity towards short-chain fatty acids (p-NP acetate, p-NP butyrate and p-NP hexanoate), while much lower towards long chain fatty acids (Zhang *et al*., 2009). Gupta *et al*., (2002) reported the routine estimation of esterolytic activity of microorganisms by employing the para-nitrophenyl palmitate (pNPP) assay. Berlemont *et al*. (2013) reported on the esterase activity assays which were carried out on p-nitrophenyl-esters (pNP-Acetate, pNP-Butyrate, pNP-Caprilate, and pNP-Laurate) by spectrophotometric methods.

In this study spectrophotometric assay was used for esterase assay by using p-nitrophenol acetate as substrate for esterase activity analysis. All the 24 esterase positive clones were taken for enzyme assay. The crude extracts of clones were used for assay. Spectrophotometric readings of the crude extracts of clones at wavelength 405 nm ranged from 1.031 to 3.285.

Esterase activity of ten positive clones showing better activity was compared, which ranged from 3.929 to 8.942 U/mg protein (Table 7). The maximum activity was shown by clone C19 (8.942 U/mg protein).

5.5 SEQUENCING OF POSITIVE CLONES

5.5.1 Amplification of metagenome insert from esterase positive clones by PCR

Sequence-based metagenomics is used to collect genomic information from microbes without culturing them. In contrast to functional screening, this approach relies on sequence analysis to provide the basis for predictions about function. In this study both function based and sequence approaches were used for the identification of esterase encoding genes.

A good quality vector DNA could be obtained by using alkali lysis method. The pEZ BAC vector used in this study was of size 7.2 kb. The agarose gel electrophoresis showed that DNA bands from all the clones selected were larger than 10 kb in size which indicated the presence of metagenome insert in those clones.

The insert in the recombinant vector was amplified by PCR using primers specific for vector's cloning sites. The amplified products from five clones used for PCR showed size larger than 3 Kb, as compared with the marker DNA.

5.5.2 Sequencing the inserts

Sequencing of the inserts were carries out at Genei, Bangalore under M/S Merck India. The sequencing reaction was carried out with the BAC vector specific primers. The sequencing results were not of good quality.

In the present study PCR analysis was conducted only in five esterase positive clones. The other clones need to be analyzed for obtaining larger fragments. The sequencing reaction need to be repeated for identifying esterase encoding sequences.

The study indicated that metagenomic approach could be used for discovering genes from environmental sources which contains many unknown and unculturable microbes. The sequence homology studies can also provide functional assignment of many proteins in the database.

The present study was successful in developing a protocol suitable for isolating considerable quantity of good quality metagenomic DNA from dairy sludge. Ten clones showing higher esterase activity could be identified from the metagenomic library. The library prepared from dairy sludge in this study can be utilized for the identification of genomic sequences coding for many other industrially important enzymes.

SUMMARY

6. SUMMARY

The study entitled "Identification and characterization of esterase producing microbes from dairy sludge through metagenomic approach" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2013-2014. The objective of the study was to construct and characterize metagenomic library of esterase producing microbes from dairy sludge. The dairy sludge samples were collected from the outlet of wastewater treatment plant of MILMA unit, Ambalathara, Thiruvananthapuram. Samples were stored at -20°C until the DNA extraction was performed.

Three protocols were tried for the isolation, out of which the protocol of Singka *et al.* (2012), which combines mechanical and chemical lysis, was found to be the best. This protocol was modified by increasing the concentration of NaCl, to optimize it for dairy sludge, which contained polysaccharides and humic acids as contaminants. The modified protocol yielded good DNA in terms of quality (A_{260}/A_{280} of 1.784) and quantity (11.4 µg/g) in 3.30 h.

Metagenomic library constriction consisted of three different stages such as restriction digestion, cloning and transformation. Metagenomic DNA was digested using restriction enzyme Hpa I (*Haemophilus parainfluenzae I*). Since BAC cloning vector was used in this study, larger fragments were preferred. Hence partially digested fragments obtained after 1 and 2 h were taken for cloning reaction.

Hpa I digested DNA fragments were cloned in to pEZ BAC vector using clone smart ligase of Lucigen (USA). The Blue white screening system, along with selection based on chloramphenicol resistance, yielded eighty two white colonies, indicating the presence of metagenome inserts in the transformed colonies.

The metagenomic library was subjected to qualitative screening for identification of esterase producing clones on tributyrin agar plates with chloramphenicol. Twenty nine per cent of clones were showing the esterase

activity. DH5α without vector, kept as control, didn't show any clear zone in TBA plates with chloramphenicol.

All the esterase positive clones (24 nos.) were taken for enzyme assay. Esterase enzyme present in the crude extracts of the overnight grown cultures of the clones were estimated. The protein content of the crude extracts, estimated by Lowry's method, ranged from 0.527 to 0.94 $\mu g \mu l^{-1}$. The absorbance of para nitrophenol produced during the reaction was measures at 405nm. The value of shown by the best ten clones ranged from 2.372 to 3.285. Esterase activity of ten positive clones ranged from 3.929 to 8.942 U/mg protein. The maximum activity was shown by clone C19 (8.942 U/mg protein).

Agarose gel electrophoresis of the vector DNA isolated from ten esterase positive clones showed DNA of size more than 10Kb, which was larger than the BAC vector used (7.2 Kb), showing the presence metagenome inserts in the clones. PCR amplification of five esterase positive clones using BEZ-F1 forward and BEZ-R1 reverse primers, specific for the vector, yielded fragments of size more than 3 Kb.

The present study was successful in developing a protocol suitable for isolating considerable quantity of good quality metagenomic DNA from dairy sludge. Ten clones showing higher esterase activity could be identified from the metagenomic library. The library prepared from dairy sludge in this study can be utilized for the identification of genomic sequences coding for many other industrially important enzymes.

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7. REFERENCES

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APPENDICES

APPENDIX I

Chemicals for isolation of metagenomic DNA from dairy sludge

CTAB Extraction Buffer

C-TAB	10 %
NaCl	0.7 M
Potassium phosphate buffer	240 mM

Potassium phosphate buffer (pH 8.0)

K_2HPO_4 (1 M)	94ml
KH_2PO_4 (1 M)	6ml

TE buffer

Tris- HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

50 X TAE buffer (pH 8.0)

Tris base	242 g
Glacial acetic acid	5.71 ml
0.5 M EDTA (pH 8.0)	100 ml

APPENDIX II

LB broth (1L)

Yeast extract	5g
NaCl	10g
Peptone	10g

APPENDIX III

YT agar medium (1L)

Bacto-tyrptone	8g
Yeast extract	5g
NaCl	5 g
Agar agar	15g

APPENDIX IV

TB medium (1L)

Bacto-tyrptone	11.8 g
Yeast extract	23.6 g
K_2HPO_4	9.4 g
KH_2PO_4	2.2 g
Glycerol	0.4 %

APPENDIX V

TBA medium (1L)

Peptone	5 g
Beef extract	3 g
Tributyrin	10 ml
Agar agar	20 g

APPENDIX VI

Phosphate buffer (pH 7.5) 1 M

K_2HPO_4 (1 M)	80.2 ml
KH_2PO_4 (1 M)	19.8 ml

APPENDIX VII

Copper sulphate reagent

Sodium carbonate	2 %
NaOH	0.1 N
Copper sulphate	1.56 %
Sodium potassium tartarate	2.37 %

APPENDIX VIII

Chemicals for isolation of vector DNA from esterase positive clones

Solution I

20% glucose	2.25 ml
0.5 M EDTA (pH 8.0)	1 ml
1 M Tris (pH 8.0)	1.25 ml
Sterile distilled water	45.50 ml

Solution II

10 N NaOH	0.4 ml
20% SDS	1 ml
Sterile distilled water	18.6 ml

Solution III

5 M Sodium acetate	60 ml
Glacial acetic acid	11.5 ml
Sterile distilled water	28.5 ml

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF ESTERASE PRODUCING MICROBES FROM DAIRY SLUDGE THROUGH METAGENOMIC APPROACH

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ABSTRACT

The study entitled "Identification and characterization of esterase producing microbes from dairy sludge through metagenomic approach" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2013-2014. The objective of the study was to construct and characterize metagenomic library for esterase producing microbes from dairy sludge.

The dairy sludge samples were collected from the outlet of waste water treatment plant of MILMA (Kerala Co-operative Milk Marketing Federation) unit, Ambalathara, Thiruvananthapuram. Three protocols were tried for the isolation, out of which the protocol of Singka *et al.* (2012), which combines mechanical and chemical lysis, was found to be the best. This protocol was modified by increasing the concentration of NaCl, to optimize it for dairy sludge, which contained polysaccharides and humic acids as contaminants. The modified protocol yielded good DNA in terms of quality (A_{260}/A_{280} of 1.784) and quantity (11.4 µg/g).

For metagenomic library constriction, the DNA was digested using restriction enzyme Hpa I (*Haemophilus parainfluenzae I*) and the fragments obtained after 1 and 2 h were cloned in to pEZ BAC vector using clone smart ligase of Lucigen (USA). The Blue white screening system, along with selection based on chloramphenicol resistance, yielded eighty two transformed colonies.

Functional screening of the library on tributyrin agar plates could identify 24 esterase positive clones. Estimation of enzyme activity of ten selected clones showed the maximum activity of 8.942 U/mg protein.

Agarose gel electrophoresis of the vector DNA isolated from ten selected esterase positive clones showed the presence of metagenome insert. Inserts of size more than 3 Kb could be amplified from the five esterase positive clones.