Dairy Wastewater Analysis and Isolation of Lipase Producing Microbe for its Microbial and Enzymatic Treatment

Dissertation Submitted in Partial Fulfillment of the Requirement
For the Degree of
Master of Science in Environment Management

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Declaration

I, hereby declare that the dissertation entitled, **"Dairy Wastewater Analysis and Isolation of Lipase Producing Microbe for its Microbial and Enzymatic Treatment"** is authentic work carried out by me under the supervision of **Dr. Anshu Gupta** for the fulfillment of the award of the Degree of Master of Science in Environment Management. The matter embodied in this Dissertation has not been submitted anywhere else for the award of any other Degree/ Diploma.

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This is to certify that dissertation entitled, **“Dairy Wastewater Analysis and Isolation of Lipase Producing Microbe for its Microbial and Enzymatic Treatment”** which is submitted by **BAYAN A. MAHDI**, Registration Number **(0271634708)** in partial fulfillment of the requirement for the Degree of Master of Science in Environment Management, Guru Gobind Singh Indraprastha University, Kashmere Gate, Delhi is a record of the work carried out by the candidate under my supervision.

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Abstract

Wastewater from dairy industries contains various organic rich substances produced during processing of milk and its products. If not treated, it causes pollution of land and water with high oil and grease content, Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). Thus it is desired to develop suitable treatment process to meet the regulatory limits. Various Physico-chemical methods are routinely used to treat the wastewater, but due to their limitations, biological methods are preferred.

The project, aimed to characterize the dairy wastewater and investigate suitable biological processes to reduce the organic load by using potential strain or enzyme. Physico-chemical and biological characterization of dairy wastewater showed that the effluent had a high organic load with COD, 1200 mg/L; BOD_3, 850 mg/L; TSS, 425 mg/L, oil and grease, 219 mg/L); total nitrogen, 20.2 mg/L, total phosphorus, 2.4 mg/L and a high probability of total coliforms (7500 MPN/100 ml of waste water). To investigate effective treatment of dairy wastewater, screening and isolation of lipase producing microorganisms was carried out. Among the eight purified strains, the strain S1 was selected on the basis of maximum lipase production. In order to have better enzyme yields lipase production from strain S1 was further optimized by varying the culture conditions, viz. different substrates, varying pH, and different NaCl concentrations. Under optimized conditions, 195 U/ml/min of lipase activity was obtained in 24 hr of fermentation by using Sal DOC as substrate. The enzymatic properties of the lipase revealed, pH and temperature optimum of 10.0 and 55°C respectively. The enzyme exhibited moderate stability at 4°C retaining 65% of its initial activity after 144 hr of storage. These properties made it attractive for use in dairy wastewater treatment. The treatment studies showed that the lipase enzyme could successfully reduce COD by 86% in 12 hr while there was 45% and 75% reduction of TSS, and Oil & grease content respectively after 96 hr of enzymatic treatment. In another experiment, when microbe (strain S1) was used in the treatment, it was found to reduce 93%, 47%, and 75% of COD, TSS, and O&G respectively in 96 hr of treatment. These preliminary treatment results demonstrated that both microbe (S1) and its lipolytic enzyme might be useful for effective treatment of dairy wastewater.

CHAPTER ONE
INTRODUCTION

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CHAPTER ONE

INTRODUCTION

1.1. Dairy Industry

The dairy industry characterized by the multitude of products and therefore production lines and is considered to be the largest source of food processing wastewater in many countries (Alturkmani, 2007). It is associated with processing raw milk into various useful products such as consumer milk, butter, cheese, yogurt, condensed milk, dried milk (milk powder) and ice cream etc. using a number of processes such as chilling, pasteurization and homogenization. The wastewater generated from dairy industry due to various activities contain large amount of effluents with a high organic load (Briao et al., 2007). This load is basically constituted by milk (raw material and dairy products), along with fat, milk proteins, lactose, lactic acid, minerals, and detergents, reflecting an effluent with high levels of COD, BOD, oil and grease, nitrogen and phosphorus (Briao et al., 2007).

1.2. Characteristics of dairy waste water

In general, liquid wastes in the dairy industry present the following characteristics (RAC/CP, 2002):

- ✓ A high organic content: Due to the presence of milk components such as butter, cheese and whey. The average COD and BOD of wastewater from a dairy firm is about 3,800-8631 mg/L and 1,634-4953 mg/L respectively.
- ✓ Oils and fats: due to the milk fat and other dairy products, such as in the water used for rinsing the butter milk;
- ✓ High levels of nitrogen and phosphorous: mainly because of the use of cleaning and disinfection;
- ✓ Large variations in pH: waste of acidic and alkaline solutions, primarily from cleaning operations, with a pH of 2-11;
- ✓ High conductivity (especially for cheese producers because of sodium chloride waste from the salting of the cheese);

- ✓ Variations in temperature (caused by water used for refrigeration, pasteurization and sterilization).
- ✓ The wastewater may contain pathogens from contaminated material or production processes. A dairy often generates odors and, in some cases, dust, which need to be controlled.

1.3. Environmental Problems associated with dairy industries

Although the dairy industry is not commonly associated with severe environmental problems as it is not toxic, it is still important to consider its environmental impact, particularly as dairy pollutants are mainly of organic origin. All steps in the dairy chain, including production, processing, packaging, storage, transportation, distribution, and marketing, impact the environment.

Owing to the highly diversified nature of this industry, various product processing, handling, and packaging operations create wastes of different quality and quantity, which, if not treated, could lead to increased disposal and severe pollution problems (Alturkmani, 2007):

a) Odor generation.
b) Ground water contamination.
c) Surface water pollution (runoff – Heavy rain).
d) Land wastage.
e) No treated wastewater for flushing water reuse.
f) Labor intensive.

Above environmental problems, necessitates designing of "specialty" treatment so as to prevent or minimize potential environmental hazard of dairy effluent. Different strategies have been employed for the removal of organic load from industrial wastewaters; physico-chemical and/or biological. These different methods have their own advantages and limitations. Biological treatment methods are now-a-days preferred over physico-chemical methods because of later's further environmental impacts (Rajeshkumar et al., 2004). Like in physico-chemical methods strong and contaminating reagents are used for desorption, resulting in toxic sludge and secondary environmental pollution. These disadvantages even become more potent and further

maximize the process cost. The problems caused by physico-chemical treatment can easily minimized by employing the various biological methods of treatment, involving microbe(s) and enzymes.

1.4. Biological Treatment of Dairy Wastewater

As stated above, wastes from the dairy processing industry contain high concentrations of organic material (bio-macromolecules) such as lipids, carbohydrates, and proteins, high concentrations of suspended solids, or high BOD and COD, high nitrogen concentrations, high suspended oil and/or grease contents, and large variations in pH. Owing to the presence of this much high fraction of readily biodegradable organic compounds, most dairy plant wastes respond well to the biologic treatment approach. These biological treatment methods can be aerobic (activated sludge process, the rotating biological contactors, the conventional trickling filters,etc.), anaerobic (anaerobic digesters, up flow anaerobic sludge blanket reactor etc.) or enzymatic (Alturkmani, 2007). However, the highly variable nature of dairy wastewaters makes the choice of an effective wastewater treatment regime difficult and about 50% of the dairy units have yet to attain satisfactory performance levels regarding installation and operation of effluent treatment plants (Rajeshkumar et al., 2004). Hence, it is relevant to explore new possibilities of treating dairy wastewater.

Another problem associated with dairy effluent treatment is the presence of high levels of fats and proteins that present low biodegradability. Fats and proteins are present in greater quantity in dairy wastewater. Problems caused by these bio- macromolecules include a reduction in the cell aqueous phase transfer rates, a sedimentation hindrance due to the development of filamentous microorganisms, development and flotation of sludge with poor activity, clogging and the emergence of unpleasant odors (RAC/CP, 2002).

To cope with these negative effects on the water stream treatment, several strategies for removing these bio-macromolecules residues at the head of the plant are proposed. However thus far, physico-chemical pretreatment is mainly used for removal of these bio-molecules from wastewater (Rajeshkumar et al., 2004). The treatment of effluents with high organic load by using microbes/ enzymes is a new and promising approach.

Hydrolytic enzymes such as lipase, recently, potentially gained more attention because of stringent environmental regulations and clean and friendly application of enzymes (Gandhi, 1997; Mendes et al., 2005). Lipases (Triacylglycerol acylhydrolases, E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids at the water–lipid interface and the reverse reaction in non-aqueous media (Castro et al., 2004). These enzymes showed potential applications in degrading oil and fats in wastewater generated by dairy industries, slaughterhouses, edible oils, fat refineries and others (Mendes et al., 2005; Pereira, 2004). Interest in these enzymes has increased markedly over the last decades, in view of their diverse applications in medicines (digestive enzymes), food additives (flavor- modifying enzymes), clinical reagents (glyceride – hydrolyzing enzymes) and cleaners (detergent additives) and for synthesis of biopolymers and biodiesel (Sugiura, 1984; Pandey, 1999).

Because of these attributes, lipases find a wide range of applications in industry and fine chemicals (Sharma, 2001). Besides, however, like in many applications that demand high enzyme yields, lipase production depends on the cost reduction so as to be economically viable. Microbes provides a viable source to produce industrial enzymes at lower costs, (Castilho et al., 2000) with the advantage of using cheaper media as growth substrates and employing considerably less sophisticated equipment.

In the present thesis, physico-chemical and biological analysis of dairy wastewater was carried out. Some new microorganisms from dairy industry samples (soil/water/sludge) have also been isolated. These microbes were used for lipase production and dairy wastewater treatment. Optimization of lipase production by using selected microbial strain was also attempted keeping in view that the enzyme might be useful for effective removal of COD and oil and grease from dairy wastewater along with other applications.

1.5 Objectives of the study

1. Collection of sample (dairy waste water/ soil/ sludge) from dairy industry.
2. Analysis of various Physico-Chemical and Biological parameters related to dairy industry waste water treatment.
3. Isolation and purification of microbe(s) secreting extracellular product (Lipase) from dairy waste.
4. Optimization of culture conditions to increase lipase production by using selected strain.
5. Biological treatment of dairy waste water (Microbial and enzymatic).

CHAPTER TWO
REVIEW OF LITERATURE

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CHAPTER TWO

REVIEW OF LITERATURE

2.1. Dairy Industry

The dairy industry involves processing raw milk into products such as consumer milk, butter, cheese, yogurt, condensed milk, dried milk (milk powder), and ice cream, using processes such as chilling, pasteurization, and homogenization. Typical by-products include buttermilk, whey, and their derivatives (http://www.ifc.org/ifcext/....)

2.2. The main production processes used in the dairy industry and related environmental effects.

The most representative production processes for the main groups of dairy products and Subsidiary operations:

- Fresh milk;
- Products obtained from milk fat (cream and butter);
- Fermented milks (yogurt);
- Cheeses (ripened and soft);
- Secondary operations.

For each of these production processes and subsidiary operations, the main related environmental effects are described and ranked semi-qualitatively in function of their relative importance in overall production. The ranking used has three levels: primary, secondary and insignificant as shown in following table.

PRIMARY	An important effect with regard to the overall impact of the activity
SECONDARY	Effect with regard to the overall impact of the activity
INSIGNIFICANT	An insignificant effect with regard to the overall impact of the activity

Table1: Criteria for ranking environmental effects. Source: (RAC/CP, 2002).

- In this review, out of all the type products mentioned above, only fresh milk and cheese processing processes have been explained in detailed.

2.2.1. Processing of fresh milk

Milk used for human consumption can be divided into two categories (Korsström et al., 2001; RAC/CP, 2002):

- Raw milk. Raw milk without any processing has been consumed as a natural product since antiquity, and in many countries it continues to be a very frequent form for consuming milk.
- Heat-treated milk (pasteurized or sterilized). The ease with which milk can rapidly deteriorate and be altered by all types of pollution makes it necessary to submit milk to a specific treatment to increase its conservation and eliminate possible pollution before consumption. In many countries, this treatment is required by law.

2.2.1.1. Processing operations and environmental considerations

2.2.1.1.1. Reception

The raw milk arrives at the dairy in insulated road tankers, where it is kept chilled and free from air. At reception the raw milk is measured either by volume or by weight. As its temperature normally rises to slightly above +4 ºC during transportation, the milk is usually cooled to a temperature below +4 ºC before being stored in a silo waiting processing. Ice water is normally used as cooling media (Korsström et al., 2001).

2.2.1.1.2. Filtering and clarification

Next, any organic and inorganic particles of dirt in the milk that entered during the milking of the cows or transportation are eliminated. The agglomerates of proteins (coagulates) that are formed in the milk are also eliminated. The degree of impurities in the milk can vary in function of the milking techniques used and handling on the farms and during transportation. Purification is an unavoidable step in the industrial processing of milk. This operation generates the so-called “clarification sludge”, which is semi-soft waste formed by particles of dirt,

blood components, germs and other substances, primarily proteins. If they are dumped with the final effluent, they can produce large increases in pollution in the sewage system creating problems for the environment. There is also a loss of milk that can be carried away with wastewater into the sewage system. Filtering produces waste composed of the filters used in this stage. In both filtering and clarification, electricity is consumed (Korsström et al., 2001).

2.2.1.1.3. Skimming and standardization

During the skimming process, the milk fat (cream) is separated from the rest of the milk components (skim milk). This is usually done using centrifuges that separate the cream, with approximately 40 % of milk fat, from the milk. The milk fat content of the milk is then standardized, through the addition of cream to the skim milk in different proportions in function of whether the desired product is whole, low-fat or skim milk. The remaining cream is used in the production of other products such as cream or butter. The centrifuges used for skimming can simultaneously clarify and skim the milk and, as a result, are widely used. These centrifuges can also contain equipment for homogenization of the milk's milk fat content. The process of separating cream by centrifugation creates sludge with a lower content of blood components and bacteria than in the case of raw milk. Nonetheless, emptying of the sludge directly into the final effluent produces a considerable increase in the organic load of that waste. During this stage, electricity is also consumed by the skimming centrifuges (RAC/CP, 2002).

2.2.1.1.4 Heat treatment

Heat treatment almost completely destroys the microorganisms contained in the milk. An additional effect is partial inactivation of milk enzymes. In function of the characteristics of the combination of temperature and time used in the heat treatment, we can differentiate between pasteurization and sterilization (Abdulrzzak .2007).

- Pasteurization. This is heat treatment capable of destroying different type of microbes with time-temperature values that range between 15-30 seconds at 72-85 ºC. Pasteurization does not guarantee destruction of all germs in the milk; it must be kept refrigerated for conservation until consumption.
- Sterilization. There are two types of sterilization:

- ✓ Sterilization strictly speaking is heat treatment capable of destroying all the pathogenic microorganisms and inactivating enzymes. It is carried out at 100-120 ºC for 20 minutes.
- ✓ UHT treatment (ultra-pasteurization or sterilization at ultra-high temperatures) is based on the application of a very high temperature (135-150 ºC) for a short period of time (2.5 seconds) this produces a very high germicide effect.

2.2.1.1.5. Homogenization

Homogenization takes place either before or after heat treatment. This process reduces the size of fat globules thus favoring uniform distribution of the milk fat. At the same time, separation of the cream is avoided. During homogenization milk is passed at high pressure through slots narrower than the fat globules in order to reduce the diameter of the fat globules, thus keeping them in suspension. In this operation, electricity is consumed because of the functioning of the homogenization equipment (RAC/CP, 2002).

2.2.1.1.6. Refrigerated storage

Once treated, milk is stored in refrigerated tanks until packaging. This refrigerated storage makes it possible to control the quality refrigerated of the milk before packaging and makes packaging independent from the rest of the processing. The main environmental effects produced during this stage are the consumption of energy necessary to maintain the milk refrigerated and possible losses of milk that can happen during storage in tanks (Korsström et al., 2001).

2.2.1.1.7. Packaging

Packaging is the final stage in the process and consists of filling packaging with the product. The indispensable condition for conservation of the product during a long period is to maintain aseptic conditions during the packaging. At the time of choosing a certain kind of packaging, both aspects related to the conservation of the product as well as economic and environmental effects should be taken into account. The most common types of packaging for milk are glass, plastic and cardboard (RAC/CP, 2002).

- Glass bottles are important because they are reusable, although they have the inconvenience of their heavy weight and fragility. They also present problems for the conservation of long-life milk because sunlight can lead to degradation of the fat and milk proteins.
- Plastic bags, usually from polythene, have the inconvenience that they are difficult to handle because of their instability and once opened for consumption they require a recipient for handling.
- Plastic bottles use materials such as polystyrene and polythene of high and low density. This packaging is used most of all in the packaging of sterilized milk.

- Cardboard boxes, such as Tetra Brik with a laminated cardboard or paper base and often covered with plastic, paraffin or aluminum, are used above all for UHT milk.

In the packaging process, machinery consumes large amounts of energy, and waste is generated because of manufacturing defects or problems during packaging.

2.2.1.2. Environmental effects of the processing of heat-treatment milk

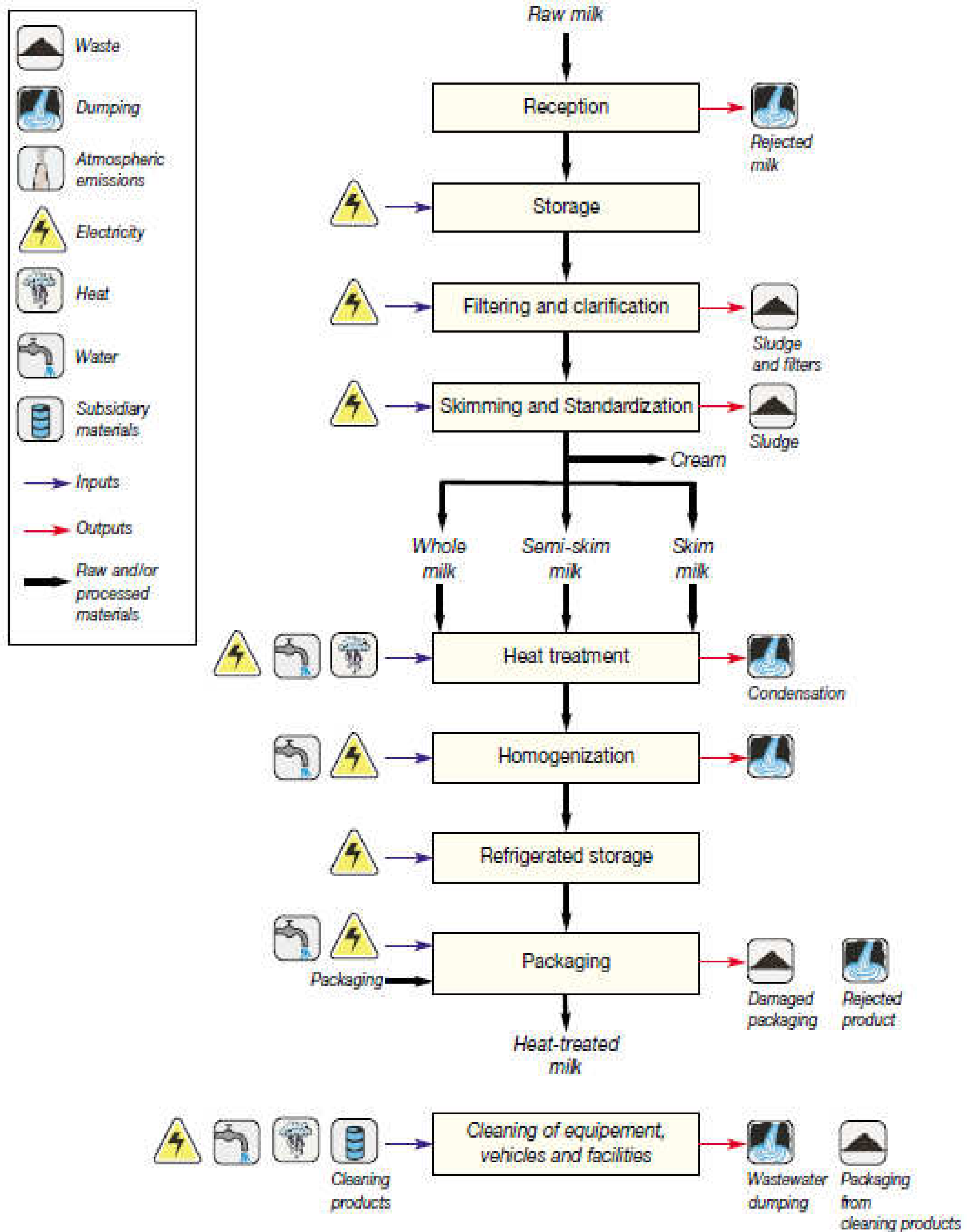


Fig1: Environmental effects of the processing of heat-treatment milk. Source: (RAC/CP, 2002).

2.2.2. Processing of Cheese

The preparation of cheese is one of the oldest forms for processing milk, and it is carried out almost traditionally within each family, village or district. Cheese is a product that is prepared from whole milk, cream, skim milk or buttermilk or with combinations of these products. In general, cheese is produced by the coagulation of milk proteins, using lactic ferments and/or rennet. The addition of enzymes, acidification or heat can accelerate this process. After coagulation, it is molded, salted, pressed and, for some types of cheese, it is treated with fungi or bacterial cultures. In some cases, special colouring or other non-lactic ingredients are also added. It is consumed as soft cheese or as cheese with different degrees of curing. There are more than 2,000 different types of cheeses throughout the world with very distinct characteristics that require a series of more or less distinct procedures for preparation. Several classification criteria can be used (RAC/CP, 2002):

- In accordance with the origin of the milk with which it has been prepared (cow's, goat's or sheep's milk);
- Depending on the characteristics of the final product (salted cheeses, melted cheeses, etc.);
- Depending on the curing process (cured cheeses, soft cheeses).

2.2.2.1. Production operations and environmental considerations

2.2.2.1.2. Coagulation

Coagulation causes an alteration in the casein and its precipitation, creating a gelatinous mass that covers all the components of the milk. The nature of the gel that forms upon co- agulation of the casein strongly influences the later processes for producing the cheese (rinsing, ripening and formation of "eyes").

Coagulation is carried out in vats where the curds are formed. These tubs must remain stationary and not be moved during the coagulation in order to avoid alteration of the coagulation processes with the resulting loss of casein with the wastewater.

There are three types of coagulation (RAC/CP, 2002):

• **Acidic coagulation.**

• **Enzymatic coagulation.**

• **Mixed coagulation.**

Acidic coagulation is produced by acids, usually lactic bacterial action, transforming the lactose into lactic acid. The formation of lactic acid reduces the pH of the milk, reaches to 4.6 producing changes in the casein-calcium compound, gradually releasing calcium from this compound. The remaining casein is precipitated into a liquid state (whey), which contains the dissolved calcium from the casein. The gel resulting from this process is formed by more or less polymerised protein chains in a network without rigidity or compactness (Ebing, 2006).

Enzymatic coagulation is the system of coagulation most widely used for the preparation of cheese and is produced by the addition of protein enzymes. The rennet obtained from the dry stomachs of lactating calves contains these enzymes and have traditionally been used in cheese production. Enzymatic coagulation transforms the casein-calcium compound in colloidal dissolution into a network of casein calcium, forming the gel or coagulate that covers the rest of the cheese components. An important part of the liquid state (whey) is thus retained in this structure. The amount of enzyme coagulant to add to the milk depends on its pH, the coagulating strength of the enzyme and the concentration and characteristics of the milk to be coagulated. Another important factor to take into account in coagulation is the temperature of the rennet or coagulant enzymes to be added. The time required for coagulation depends on the temperature of the added rennet. Normally, temperature is between 28 and 34 ºC, (Ebing, 2006).

Mixed coagulation is the result of the joint action of rennet and lactic acidification. A mixed gel can be obtained by adding rennet to acidic milk or by acidifying an enzymatic gel. During this stage, losses or spills of milk can occur through handling (RAC/CP, 2002).

2.2.2.1.3. Cutting and draining

The gel formed during coagulation, whichever method is used, is in an unstable physical state. Depending on the conditions, the liquid state or the whey that impregnates it separates more or less quickly. This phenomenon is known as draining of the whey.

There are two main methods for draining the whey. During the draining of the whey in vats, a coagulate is divided into cubes, which remain floating in the whey that they exude. During the draining of whey in moulds, the cut-up coagulate is maintained in a mass from which the

whey is separated as it forms. For some types of very acidic and demine realized coagulates, the whey is separated by centrifugation. Separation of the whey from curds left at rest is weak and slow and in most cases the cheese does not have the desired final composition. For this reason, other operations are carried out that facilitate the draining of the curds. There are two types of treatment: heat and mechanical. For the preparation of certain types of cheeses with a very high dry extract, heat treatment is used during which a rise in temperature produces an increase in the degree of draining of the cheese. Mechanical treatments applied to the curds can be cutting, agitation, molding or pressing. Depending on the type of cheese, one or several of these treatments are used (Korsström et al., 2001).

- The main environmental effect produced by the preparation of cheese is whey. Depending on the type of coagulation used, various types of whey will be obtained (RAC/CP, 2002):
  - ✓ Sweet buttermilk. This is produced during the enzymatic coagulation of milk. It usually contains between 0.6-0.9 % of soluble protein, approximately 0.3 % fat and a large amount of lactose (more than 5 %). In this type of whey, the presence of lactic acid is practically nil.
  - ✓ Acidic buttermilk. This is generated when acidic coagulation is used to coagulate the milk. This type of whey contains approximately the same proportion of soluble protein as the sweet whey but a lower proportion of fat and slightly less lactose (4.5 %), while there is up to 0.8 % lactic acid.

    Generically, the liquid obtained during cheese production is called whey. The lactose and protein content of the whey causes an especially high increase in the degree of pollution of wastewater, (reaching more than 60,000 mg COD/litre of whey). For this reason, dumping the whey together with the rest of the wastewater should be avoided.

2.2.2.1.4. Moulding and pressing

Moulding consists in pouring pieces of curd into moulds prepared for this. The moulds are usually plastic (PVC), although sometimes metal or wooden moulds are used. The moulds give the finished cheese the required dimensions and weight. Cheeses can be pressed by the weight of the cheese or by applying additional force. Like in the previous stage, consumption

of electricity depends on the degree of automation of production. During pressing, whey is also separated from the mass of the cheese, although the amount of whey produced during this stage is less than during the draining of the whey (RAC/CP, 2002).

2.2.2.1.5. Salting

Salt is added to cheese as a condiment, but it also serves as a retarding agent for starter and other bacteriological activity. The salt content of cheese is usually 0.5-2%. The method used for salting depends on the cheese type, the alternatives being (Korsström et al., 2001):

- **Dry salting**, where dry salt is applied either manually or mechanically to the curd (e.g. Cheddar, Mozzarella),
- **Brine salting**, where the cheese is placed in a container with brine (e.g. Emmental, Tilsit).

2.2.2.1.6. Curing

Cheeses, once salted and dried, are taken to storage rooms for aging, in which the temperature and humidity are controlled. The curing of cheese includes physical, microbiological and enzymatic processes, creating a finished product with certain characteristics of aroma, taste and texture (Ebing, 2006).

2.2.2.2. Environmental effects of cheese production

The main environmental effects caused by cheese production are the following.

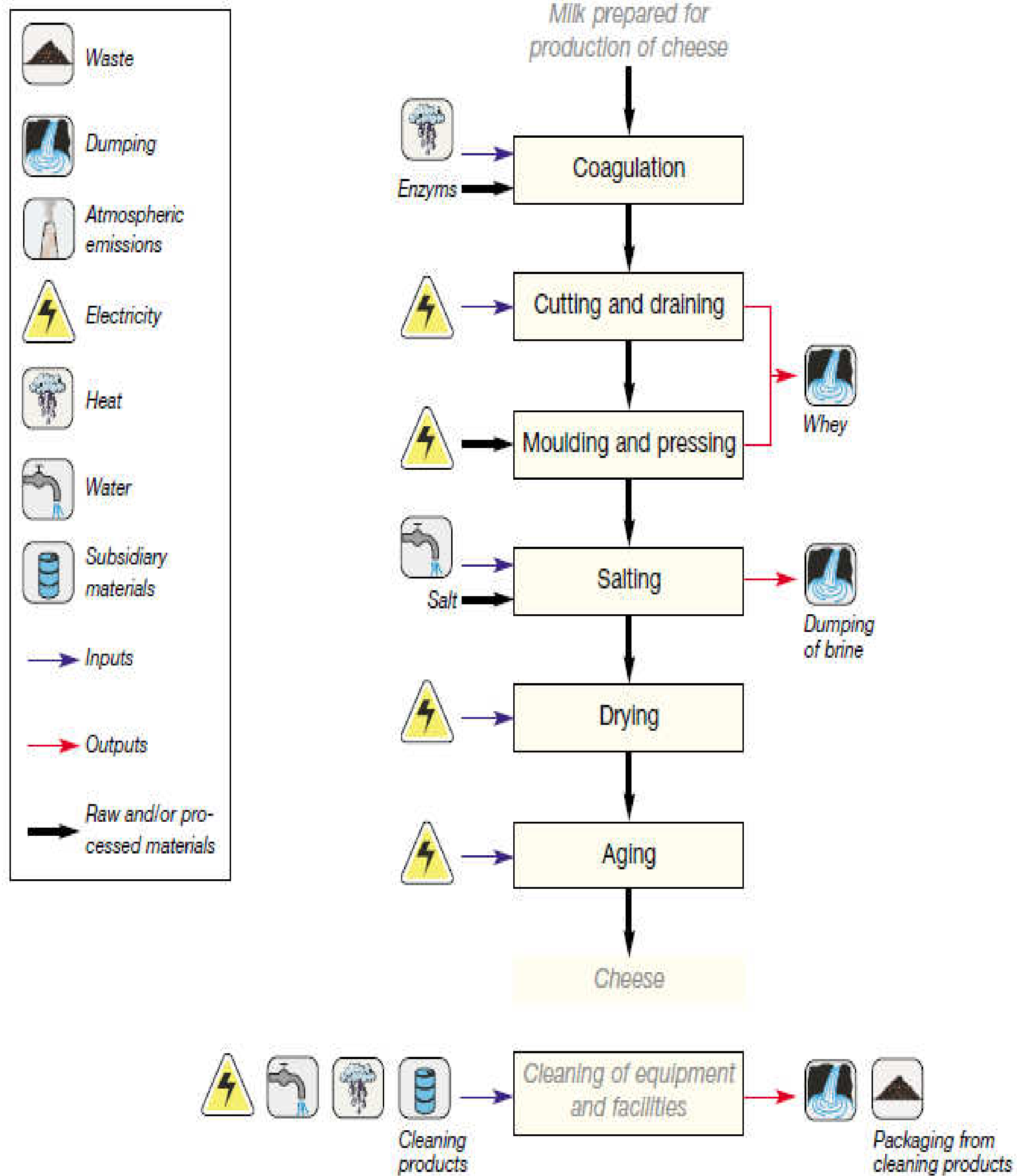


Fig 2: Environmental effects of cheese production. Source: (RAC/CP, 2002).

2.2.3. Secondary operations in the dairy industry

In this section are described the secondary operations common to all processes in the dairy industry. Environmental effects associated with these operations are evaluated at the end of this section.

2.2.3.1. Cleaning and disinfection operations

Because of the characteristics of the raw material used and the products produced, the hygienic conditions of the equipment and installations of the dairy firms should guarantee the quality of the products produced. Maintenance of the hygienic conditions in a dairy firm require continuously carrying out cleaning operations and disinfection, up to one fourth of the total time worked. These operations use most of the water, energy and chemicals consumed by the plant and produce a considerable amount of wastewater.

Cleaning means total elimination of all traces of milk or its components and other visible impurities. Disinfection means eliminating all pathogenic microorganisms and most of those that are nonpathogenic that can affect the quality of the final product. In all cases, cleaning operations and disinfection require the following:

• Water, which fulfils several functions including softening and dissolving dirt that has adhered to surfaces, the formation of detergent solutions and the elimination of the remains of cleaning solutions;

• Heat for maintaining the optimum temperature for the process and electricity for circulating cleaning solutions through the equipment and pipes (CIP systems);

• Chemicals (detergents and disinfectants);

• Personnel for carrying out the cleaning operations.

Disinfectants also contain acids or alkalis, inhibitors of corrosion and substances that form compounds in order to improve their industrial application.

Depending on the type of dirt existing on the equipment, surface or installation, the appropriate cleaning and disinfection protocol is applied. In the dairy industry, dirt is due primarily to the components of milk, mostly fats and proteins that are deposited on pipes and equipment. As a result of the cleaning operations, the wastewater generated from cleaning and the chemical products, plus the organic load from the washing away. Cleaning removes particles of sand and dust, which reach the industry from many sources, but the most common waste that

is eliminated is the remains of organic components of milk (fat, proteins and mineral salts). The use of cleaning products applied in most cases as aqueous solutions produces a greater amount of water to be treated. As has already been commented, wastewater from the dairy industry can have very high levels of COD, primarily because of the milk components, and the contribution of the detergents from the cleaning operations is minor in relation to that of dirt. In general, there is a wide range in the contribution of the organic load of each detergent (30-1,200) mg /litre wing to the various chemical compositions of these products. Thus, we can find alkaline products without surfactants in the low range and surfactant products with foam-forming detergents in the upper range of the values indicated (RAC/CP, 2002; Arnau, 1995).

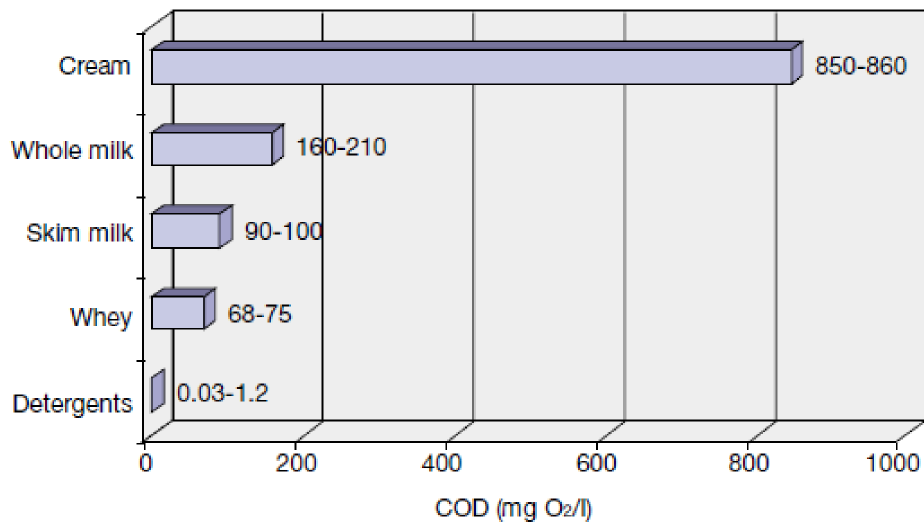


Fig 3: COD of various dairy products and detergents. Source: (RAC/CP, 2002; Arnau, 1995)

Another important aspect of the use of detergent products is their phosphate and nitrate content, which makes an important contribution to eutrophication of water. Traditional detergents based on phosphoric acid that are used in cleaning operations contain 10 to 20% phosphorous, and their contribution to the wastewater should, therefore, be kept in mind.

2.2.3.2. Steam generation

The heat needed by dairy firms is produced primarily steam or hot water depending on the needs of the operation and the process being used. Steam is produced in boilers and is then distributed through pipes to where it is used in the plant. The water used for supplying the boilers does not require special hygienic conditions but it should have a low content in carbonates and sulphates otherwise incrustations of salts are formed in the boilers and distribution pipes, rendering difficult the exchange of heat. As a result, chemicals are frequently used in order to avoid incrustations and salt deposits. Water that is produced as a result of condensation of the steam during distribution can be reused to supply the boilers or as heating water in the process, resulting in savings in water consumption.

Combustion in boilers produces atmospheric emissions of gases whose composition and quantity vary primarily in function of the type of fuel used and the conditions under which the boiler operates. The combustion of fuel oil (which is the fuel most frequently used in the dairy industry) produces and emits primarily carbon dioxide (CO_2), sulphur dioxide (SO_2) and nitrogen oxides (NO_x). Depending on the functioning of the boiler, unburned elements are emitted in the form of solid particles. Water consumption can be optimized by repairing leaks in the equipment and pipes and reusing condensation that is generated. The generation of chemical packaging wastes is significant since it is a dangerous waste that should be properly managed (Korsström et al., 2001).

2.2.3.3. Refrigeration

In dairy firms, refrigeration is produced primarily for two uses: for refrigeration of storage rooms or for the cooling of liquids. The refrigeration equipment most frequently used in the dairy industry is compression refrigeration machines with ammoniac or compounds based on chlorofluorocarbons (CFCs) as refrigerant. The consumption of electricity and water for the generation of cold is the main environmental effect of this operation. However, refrigerant gases can be emitted to the atmosphere as a result of leaks in the cold circuits.

In the case of the use of CFCs (prohibited in many countries), it is recommended that they be substituted by other refrigerant fluids, like brine or glycol water in light of the CFC's contribution to the destruction of the ozone layer (Korsström et al., 2001).

2.2.3.4. Water supply

The quality of water used in the dairy firm should be that of water for domestic use, especially if the water enters into direct contact with the product, such as water used in heat treating milk, in the rinsing of the buttermilk of butter or in the brines used in salting cheese. When the quality of the water entering the dairy firm is inadequate, it should be treated in order to eliminate possible causes of contamination of the final product. Treatment can consist in eliminating suspended solids, dissolved substances and microorganisms. The consumption of electricity is the main environmental effect produced by this operation. Electricity is used both for pumping water and for treating water. In function of the type of treatment used, wastewater with high conductivity or extreme levels of pH is created. Other environmental effects are consumption of chemicals, for example when chlorine is added to the water and the generation of waste packaging of these products (Korsström et al., 2001).

2.3. Environmental effects of the dairy industry

The main environmental effects of the dairy industry are related to the high consumption of water and energy and generation of wastewater with a high organic content. Of minor importance are emissions of gases and particles into the atmosphere and noise. It is important to point out that quantification of these aspects can vary from one installation to another in function of factors such as the size and age of the installation, equipment, use, cleaning programmes, awareness of the employees, etc.

2.3.1. Water consumption

Like most of the other firms in the food and agriculture sector, dairy industries consume daily large quantities of water in their processes, reaching to several time the vel. of milk produced.

PRODUCTIVE PROCESSES	LEVEL OF CONSUMPT.	OPERATIONS WITH HIGHEST WATER CONSUMPTION	OBSERVATIONS
Milk	Low	Heat treatment Packaging	
Cream and butter	Low	Pasteurization of cream Churning	Rinsing of buttermilk before churning
Yogurt	Low	—	Mainly in secondary operations
Cheese	Medium	Salting	Salting using brine
Secondary operations	High	Cleaning and disinfection Generation of steam Refrigeration	Consumption of water is the greatest during these operations

Table 2: Water consumption in the dairy industry Source: (RAC/CP, 2002).

Consumption is usually 1.3-3.2 litres of water/kilo of milk received, but can reach as much as 10 litres of water/kilo of milk received. Nonetheless, it is possible to optimize this consumption at 0.8-1.0 litre of water/kilo milk received using advanced equipment and proper management (UNEP, 2000). As indicated in table 9, the greatest consumption of water occurs during secondary operations, particularly in the cleaning and disinfection where 25-40 % of the total is consumed.

2.3.2. Energy consumption

Energy is consumed to ensure the quality of dairy products, especially those submitted to heat treatment, refrigeration and storage.

ENERGY	MOST FREQUENT USES	EQUIPMENT
Heat	Generation of steam and hot water, cleaning	Pasteurizers/sterilizers, CIP cleaning systems
Electricity	Refrigeration, lighting, ventilation, operation of equipment	Electrical equipment (pumps, mixers, etc.), lights

Table 3: Most frequent uses of energy by dairy firms. Source: (RAC/CP, 2002).

Approximately 80 % of total energy consumption in a dairy firm is heat obtained from the combustion of fossil fuel (fuel oil, gas, etc.) and the remaining 20 % is electricity.

PRODUCTIVE PROCESSES	LEVEL OF CONSUMP.	OPERATIONS WITH HIGH CONSUMPTION OF ENERGY	OBSERVATIONS
Milk	High	Filtration/Clarification Skimming/Standardization Heat treatment Homogenization Packaging	Mainly consumption of heat for treatment of milk
Cream and butter	Medium	Pasteurization Deodorization Aging Churning Packaging	Mainly consumption of electricity for operation of machines
Yogurt	Low	Incubation Packaging	Electricity for the operation of machines and heat for incubation
Cheese	Medium	Coagulation Cutting – draining Moulding – Pressing Drying Aging	
Secondary operations	High	Cleaning and disinfection Refrigeration	Heat is consumed in the cleaning operations while electricity is more greatly consumed in refrigeration

Table 4: Energy consumption in the dairy industry. Source: (RAC/CP, 2002).

Operations with a greater consumption of heat such as pasteurization/sterilization of milk and CIP cleaning can consume up to 80 per cent of the total heat used in the plant. Inadequate consumption of energy leads to reduction of limited natural resources such as fossil fuel and an increase in atmospheric pollution due to the emission of gases produced by the generation of energy. The emission of these gases contributes to the greenhouse effect (RAC/CP, 2002). Energy-efficient equipment and heat recovery systems are the keys to reduced energy consumption, in combination with good housekeeping practices, such as proper insulation. The use of regenerative heat exchange in the pasteurization process, and of multiple effect evaporators with mechanical vapor recompression for evaporation, can be mentioned as examples of energy saving techniques (Korsström et al., 2001).

2.3.3. Wastewater

Waste water from dairy processing contains predominantly milk and product residues from the process, as well as caustic and acidic cleaning chemicals and detergents. The dairy effluent is

characterized by a high organic load, fluctuations in pH and temperature, and by a high content of nitrogen and phosphorus (Korsström et al., 2001). A dairy firm generates a volume of wastewater between 2 and 6 litres/litre of milk processed (RAC/CP, 2002).

MAIN ACTIVITY	VOLUME OF WASTEWATER*
Production of butter	1 - 3
Production of cheese	2 - 4
Processing of fresh milk (pasteurization and sterilization)	2.5 - 9

Table 5: Volume of wastewater created by different Processes. Source: (RAC/CP, 2002.)

* Litres of wastewater/litre of milk.

The wastewater generated by a dairy firm can be classified in terms of their source: processes and cleaning or refrigeration.

ORIGIN	DESCRIPTION	CHARACTERISTICS	VOLUME*
Cleaning and Processing	Cleaning of surfaces, pipes, tanks and equipment. Loss of product, whey, brine, ferments, etc.	Extreme pH, high organic content (BOD and COD), oils and fats, suspended solids	0.8 - 1.5
Refrigeration	Water from cooling towers, condensation, etc.	Variations in temperature, conductivity	2 - 4

Table 6: Wastewater created by a dairy firm. Source: (Spreer, 1991).

* Volume expressed in litres of wastewater/litres of processed milk

PRODUCTIVE PROCESSES	LEVEL OF WASTE	OPERATIONS PRODUCING HIGH AMOUNTS OF WASTEWATER	OBSERVATIONS
Milk	Medium	Heat treatment Packaging	Waste decreases if water used for heat treatment is re-circulated
Cream and Butter	Medium	Pasteurization Churning Packaging	Water used for cleaning the buttermilk has a high fat content
Yogurt	Low	—	Mainly secondary operations
Cheese	High	Cutting – Draining Moulding – Pressing Salting	Dumping of whey leads to a high high amount of pollution. Regeneration of brine produces periodic dumping of highly conductive waste
Secondary Operations	High	Cleaning and Disinfection Refrigeration	The amounts and pollutant load of cleaning water depend on their management by the firm. Wastewater from refrigeration depends on the degree of their re-use

Table 7: Wastewater in the dairy industry. Source: (RAC/CP, 2002.)

It is estimated that 90 % of the COD of wastewater from a dairy industry is attributable to milk components and only 10 % to outside dirt.

In the composition of milk in addition to water there are fats, proteins (in solution and suspended), sugars and mineral salts. In addition to milk components, dairy products can contain sugar, salt, colorants, stabilizers, etc., depending on the type of product and the production technology used. All these components appear in wastewater to a greater or lesser degree, either through dissolution or their being carried away with cleaning water.

In general, liquid waste in the dairy industry presents the following characteristics (RAC/CP, 2002).

• **A high organic content**, due to the presence of milk components. The average COD of wastewater from a dairy firm is between 1,000-6,000 mg BOD/litre;

• **Oils and fats**, due to the milk fat and other dairy products, such as in the water used for rinsing the buttermilk;

• **High levels of nitrogen and phosphorous**, mainly because of the use of cleaning and disinfection;

• **Large variations in pH** waste of acidic and alkaline solutions, primarily from cleaning operations, with a pH of 2-11;

• **High conductivity** (especially for cheese producers because of sodium chloride waste from the salting of the cheese);

• **Variations in temperature** (caused by water used for refrigeration).

Loss of milk, which can be reach between 0.5-2.5 % of the amount of milk received or in the most unfavorable cases up to 3-4 %, is an important contribution to pollutant load of the final effluent (UNEP, 2000).

PROCESS	SOURCE OF LOST MILK
Production of milk for direct consumption	Spills from storage tanks. - Overflowing of tanks. - Spills and leaks in pipes and hoses. - Deposits on the surface of equipment. - Elimination of sludge from filtering/clarification. - Spills because of damaged packaging. - Defects in the packaging process. - Cleaning operations.
Production of butter and cream	- Spills during storage. - Spills and leaks in pipes and hoses. - Overflowing of tanks. - Cleaning operations.
Production of yogurt	- Leaks and spills from storage tanks. - Spills from incubation tanks. - Defects in the packaging process. - Cleaning operations.
Production of cheese	- Leaks and spills from storage tanks. - Losses in the curd vats. - Spilling from moulds. - Incorrect separation of the whey from the cheese. - Cleaning operations.

Table 8: Losses of milk to wastewater. Source: (RAC/CP, 2002).

In the process of preparing cheese, a great deal of whey is generated. The amount of whey generated by the production of cheese is approximately nine times the amount of milk treated, with a very high organic content (COD of approximately 60,000 mg/litre) and as a result the dumping of it together with wastewater considerably increases pollution of final waste.

2.3.4. Wastes

Most of the waste generated by a dairy firm is inorganic: primarily packaging waste from both raw and secondary materials as well as the final product. Other wastes related to the maintenance activities, cleaning or laboratory and repair work are also produced (Korsström et al., 2001).

GROUP		WASTE	PLACE OF GENERATION	CUSTOMARY USES
Organic wastes		Rejected product (raw material, semi-finished product, final product)	Process	Recycling (animal feed)
Similar to domestic waste		Bits of food, paper	Offices	Composting or storage at domestic waste a dumping site
Packag-ing And packing	Emp-ty	Removable film, wooden pallets Heavy paper bags, Plastic, glass, cardboard, paper packaging	Reception	Reuse or recycling
	Full	Plastic, glass, cardboard, paper packaging	Packaging Storage Returns	Dumping or separation of the packaging from the product and separate management
Waste from Maintenance operations		Electric cables, scrap iron	Workshops Maintenance areas	Recycling or storage at dumps
Hazardous waste		Used oils, batteries, packaging from hazardous waste	Laboratory Storage Workshop Cleaning areas	Transport, treatment and elimination or storage at hazardous dump sites

Table 9: Main waste produced by a dairy firm. Source: (RAC/CP, 2002).

The recycling and treatment of waste generated in a dairy firm begins with separation, which avoids their being discarded with liquid waste and mixing together that would prevent adequate treatment of each type of waste.

2.3.5. Atmospheric emissions

The main emissions of gases from the dairy industry are produced in boilers for producing steam or hot water required for production and cleaning operations. The pollutants in the combustion gases are CO, SO_2, NO_x and particles. The level of emission of these pollutants varies in function of the type and quality of the fuel used the state of the installations and the efficiency and control of the combustion process. The fuels most frequently used in the boi-

lers are solid fuel (coal or wood), liquid (fuel or gas oil) or gas (natural gas). (www.fao.org/WARDOCS/).

Solid fuels are characterized by a high content of sulphur and ash. In addition, they usually have traces of volatile and toxic products, such as lead and arsenic. Like solid fuels, liquid fuels have high sulphur content and the possibility of producing soot and particles through incomplete combustion. Natural gas, in spite of representing only 10 per cent of the world's energy reserves, represents a fuel that is more and more widely used given its advantages. Natural gas is free from sulphur and other impurities and does not emit these pollutants. Preventive measures against emission of polluting gases are based on adequate maintenance and cleaning of burners, self-control of emissions and, wherever necessary, implementation of corrective measures. Another source of pollution to be considered in atmospheric emissions is emission of refrigerant gases used in refrigeration systems. Losses or leaks of these gases have an important environmental impact given their repercussion on the destruction of the ozone layer.

2.3.6. Noise

Noise is mainly generated by the substantial traffic load caused by the regular milk deliveries, but also by evaporators, spray dryers and cooling compressors. Problems regarding odour and dust are also occurring, but not to a significant extent (www.fao.org /WARDOCS/).

2.3.7. Solid wastes

The packaging material is the main form of solid waste deriving from the dairy. It consists of beverage carton, cardboard, plastic materials, metallic barrels and wooden boxes and pallets. Solid waste is generated mainly by filling and packaging, where packaging mistakes are almost unavoidable. Many ingredients are also delivered in packages that have to be disposed of (Korsström et al., 2001).

2.4. Pollution Prevention and Control

In the following a summary is given of suggestions for the prevention of dairy waste. At the same time they are indicative of what is to be understood when speaking about good management of waste control (EPA, 1971):

1. Instruction to plant personnel concerning the proper operation and handling of dairy processing equipment. Major losses are due to poorly maintained equipment and to negligence by inadequately trained and insufficiently supervised personnel.

2. The carrying out of a study of the plant and the development of a material balance to determine where losses occur. Where improper maintenance is the cause of losses, a specific maintenance programmed should be set up.

3. The use of adequate equipment for receiving, cooling, storing and processing of milk, so as to take care of the maximum volume of flush production and of special products. Modification and replacement of ill-functioning equipment.

4. Accurate temperature control on plate, tubular and surface coolers to prevent freeze-on, which may result in loss of products.

5. Elimination of valves on the outlet sides of internal tubular or plate heaters and coolers and maintenance of plates and gaskets in good repair so as to eliminate waste due to blown or broken gaskets

6. Installation of suitable liquid level controls with automatic pump stops, alarms, and other devices at all points where overflows could occur (storage tanks, processing tanks, filler bowls etc).

7. Keeping in good order of vats, tanks and pipelines so as to eliminate and reduce to a minimum the number of leaky joints, gaskets, packing glands and rotary seals.

8. Proper design and installation of vats and tanks at a level high enough above the floor for easy drainage and rinsing if hand cleaned. Tanks should be pitched to insure draining.

9. Correct connections on plate type heat exchangers so as to avoid milk being pumped into the water side of the exchanger or water being pumped into the milk side.

10. Provision and use of proper drip shields on surface coolers and fillers so as to avoid those products reach the floor. Avoidance of cheese vats, vat processors or cooling tanks being overfilled so that no spillage occurs during product agitation. The liquid level in cheese vats should be at least three inches below the top-edge of the vat.

11. Avoidance of foaming of fluid dairy products, since foam readily runs over processing vats and other supply bowls and contains large amounts of solids and BOD. The use of air tight separators, proper seals on pumps and proper line connections to prevent inflow of air when lines are under partial vacuum, will avoid foam production.

12. Turning off of water hoses when not in use. Use should be made of hoses equipped with automatic shut-off valves so as to avoid excessive water usage.

2.5. General characteristic of dairy processing effluent

While effluent problems vary widely between fresh milk and dairy product manufacturing plants neither of the plant creates toxic effluent.

Effluent contains milk and milk products from tanker washes down, equipment and pipeline cleaning, product changeover, spillage, as well as nutrients, detergents and sanitizers, dissolved solids including sodium chloride, and small amounts of lubricants. Effluents containing discarded milk or milk product can have particularly high COD and BOD concentrations and oil &grease. Dissolved solids may derive from either chemicals used in the manufacturing process, or the primary water source for the operation.

The large volumes of whey produced from cheese or casein (milk protein) manufacture may be further processed into commercially valuable protein products and baking agents thus reducing the BOD level in the effluent stream. While the subsequent processing results in reduced volume, the concentration of dissolved solids in separate effluent streams may increase from regeneration of dairy industry processing effluent management, as shown in Fig. 4.

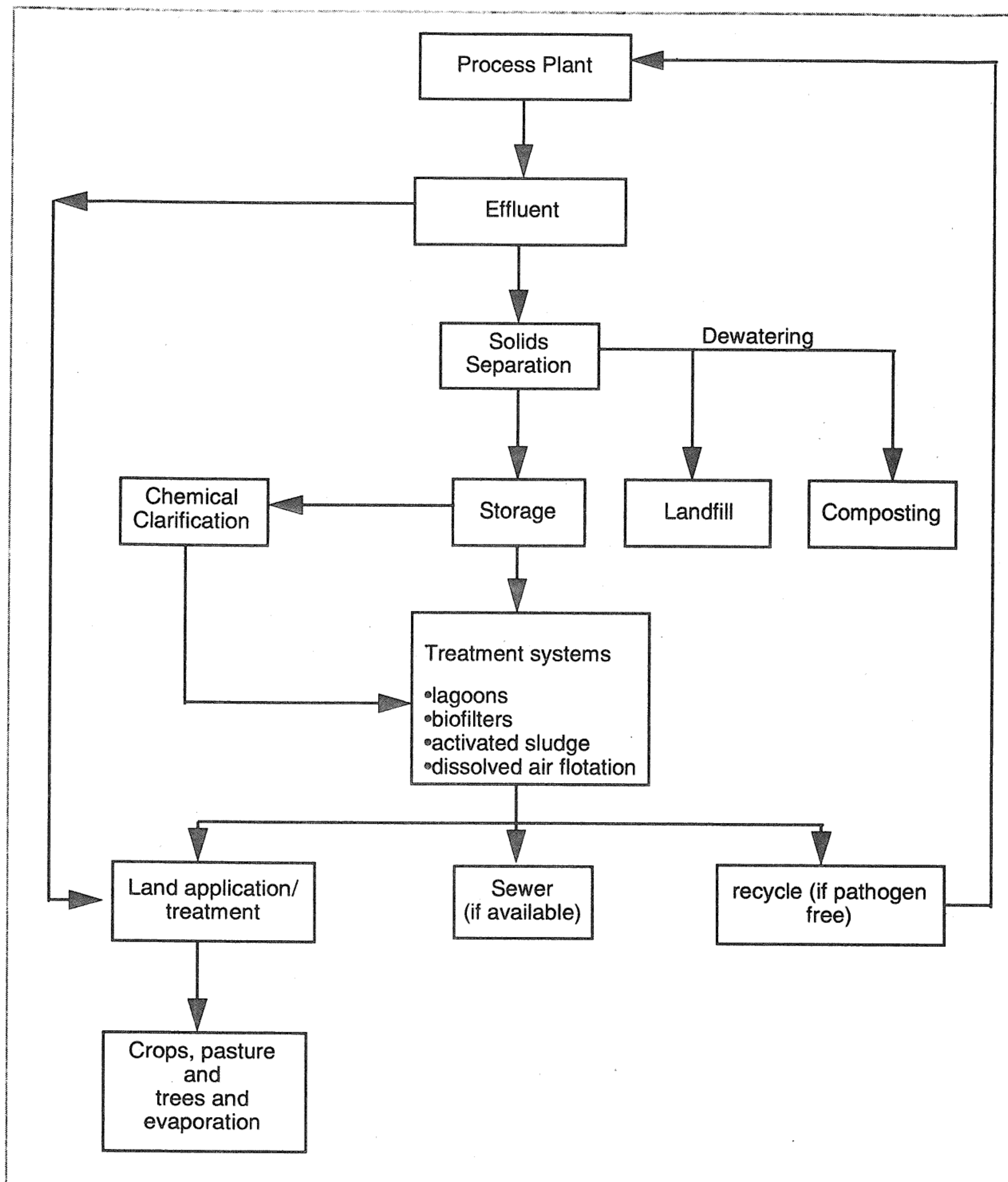


Fig 4: Dairy processing Effluent Treatment and use and discharge options. Source: (ARM-CANZ/ANZECE, 1999)

2.6. Reuse of treated wastewater for irrigation

Some wastewater streams from dairy processing plants in regional areas are used for irrigation. The suitability of wastewater for irrigation can vary according to (UNEP, 2004):

• The concentration of dissolved salts in the water, measured as electrical conductivity (EC)
• The concentrations of specific salts such as sodium, phosphate and nitrates
• Soil type (e.g. permeability and how well it drains)
• Crop type (e.g. salt tolerance of particular species)
• The climate (e.g. amount of leaching due to rainfall)
• Method of irrigation (e.g. whether from overhead sprinklers, because wastewater with high salt levels may cause leaf burn).

2.7. Dairy wastewater treatment technologies

Wastewater treatment processes can be divided into three classes; physical, chemical and biological. Biological treatment processes, in turn, can be classified as either aerobic or anaerobic and microbial and enzymatic treatment. From an enzymatic point of view, aerobic and anaerobic processes are each characterized by groups of micro-organisms and their associated enzymes (Burgess et al., 2008). Within the last decade, various technologies have been applied to treat dairy and dairy products wastewater. Among these, biological treatment is a preferred choice (Ozturk et al., 1993; Kasapgil et al., 1994; Perle et al., 1995; Gavala et al., 1996; Donkin et al., 1997; Zeemam et al., 1997). Selection of an appropriate treatment technology basically depends on demand and supply of inhabitants, geographical situation, meteorological conditions, economic welfare, industrialization, development, and finally on the discharge limits of the country. The degree of treatment necessary to treat wastewater from a dairy processing plant is determined by the end use and criteria set by regulatory authorities that is, whether the wastewater is to be discharged to sewer, reused on or off the site, discharged to surface water or used for irrigation. Processes used to treat wastewater fall into three main categories (UNEP, 2004):
• Physico-chemical (for primary treatment)
• Biological (for secondary treatment)
• Disinfection (some forms of tertiary treatment)

2.7.1. Primary treatment

Primary treatments commonly used by the dairy industry are screening, equalization, neutralization, and dissolved or induced air flotation (DAF or IAF) to remove fats and suspended

solids. Other primary treatments that are being trilled at some factories use ‘hydro cyclones’ which also remove fat and can be used in combination with air flotation units.

2.7.2. Secondary treatment

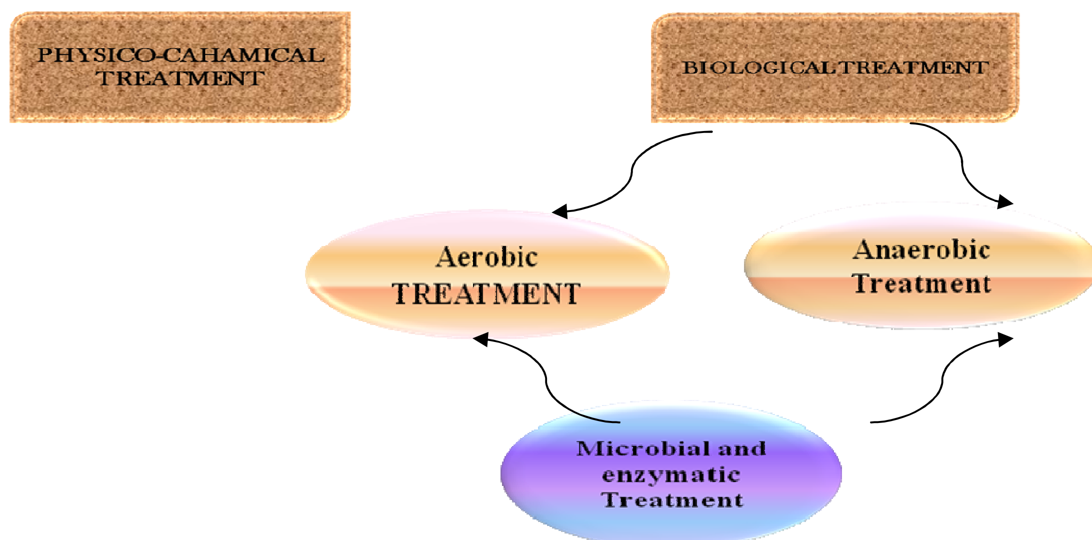
Secondary treatment may incorporate the removal of organic matter and in some cases nutrients such as nitrogen and phosphorus. It typically uses a series of anaerobic and aerobic biological treatment processes. Secondary treatment relies on micro-organisms consuming and converting organic material in the wastewater into either carbon dioxide or methane (biogas), or into more cell matter (sludge) which can be removed and usually dewatered, stabilized and removed offsite.

2.7.3. Tertiary treatment

Tertiary treatments use biological and/or physical and/or chemical separation processes to remove organic and inorganic substances that resist primary and secondary treatment, they produce very high-quality effluent.

Type of dairy industry wastewater

Treatment plant



2.7.4. Physical treatment

Solid and suspended matter can be separated from the effluent stream of dairy industry by use of equipment and separation methods such as dissolved air flotation, centrifugation and micro-filtration. This type of treatment not only reduce the sludge build up in lagoons and wear on pumps, but also should be a rapid way of reducing the BOD concentration in effluent prior to disposal or reuse (ARMCANZ/ANZECE, 1999).

2.7.5. Chemical treatment

Chemicals can be used to enhance treatment characteristics, such as settling of solids by pH correction, and to improve treatment performance or suitability for land application. Care should be taken to ensure that concentrations of any trace elements such as copper or cadmium, which may be present as impurities, do not have adverse residual impact on organisms in the treatment and disposal systems and in the general environment (ARMCANZ/ANZECE, 1999). Chemical treatment of dairy wastewater originating from a dairy and dairy products plant at Istanbul was investigated on the basis of chemical oxygen demand (COD) parameter as a pre-treatment alternative. $FeCl_3$, $FeSO_4$ and alum were used as coagulants in the jar-test experiments of four sets of daily composite wastewater samples taken once every month. The effect of acid cracking has also been searched through acid addition and pH adjustment (Tanik et al., 2002). Probably the most commonly used chemical process is chlorination. Chlorine, a strong oxidizing chemical, bacterial kill is achieved when vital biological processes are affected by the chlorine is used to kill pathogenic microbes and to slow down the rate of decomposition of the wastewater. Another strong oxidizing agent that has also been used as an oxidizing disinfectant is ozone.

2.7.6. Biological treatment

Biological degradation is one of the most promising options for the removal of organic material from dairy wastewaters. Use microorganisms, mostly bacteria, in the decomposition of wastewaters to stable end products (Anon, 1994; Wakelin et al., 1997). More microorganisms, or sludge, are formed and a portion of the waste is converted to carbon dioxide, water and other end products. Generally, biological treatment methods can be divided into aerobic and anaerobic methods, based on availability of dissolved oxygen. Microbial products in the

form of enzymes, surfactants etc. can also be applied as treatment agents. Due to their high specificity to individual species or classes of compounds, enzymatic processes can be designed to specifically target selected compounds that are detrimental to the environment. In the liquid state, these substances become more accessible to microorganisms and their lipolytic enzymes. Both diffusion coefficients and the solubility of fatty acids in aqueous media increase significantly with rising temperatures, allowing a better mass transfer (Thomas, 1987).

2.7.6.1. Microbial treatment

There are research reports and patents that have described the use of microorganisms and/or enzymes pools developed in the laboratory for the biological treatment of effluents with high fat and oil concentrations. (Mendes et al., 2005) cite various patent documents for the application of hydrolytic enzymes, especially lipases in the wastewater treatment. The patent developed by Tsochocke et al., (1990) describes a procedure for lipid degradation in grease-traps that involves the addition of lipase-producing bacteria. These bacteria are immobilized on no-biodegradable support material and immersed in the surface of grease-traps, and are reported to achieve hydrolysis rates greater than 90%. Gardon et al., (1991) proposed the use of a bioadditive that consists of various microorganisms (*Aerobacter aerogenes, Bacillus subtilis, Cellulomonas biazotea, Nitrosomonas* sp., *Nitrobacter* winogradskyi, *Pseudomonas* denitrificans, *P. stutzeri* and *Rhodopseudomonas* palustris) for the treatment of wastewater with high lipid content.

2.7.6.2. Enzymatic treatment

In the recent year, use of enzymes in the biological treatment of wastewater has received great attention. These enzymes have been used to increase degradation efficiency during or prior to other treatment processes (Aoki et al., 1991; Cail et al., 1986; De Felice et al., 2004; Lgerkwist et al., 1993; Lanciotti et al., 2005; Rintala et al., 1994). For the dairy or oil rich wastewater such pre- or co treatments methods generally consist of the cultivation of lipase-producing microbial strains in the effluents. Cail et al., (1986) tested an enzymatic mixture containing protease, amylase, cellulose and lipase and *Bacillus subtilis* spores on wool scouring wastewater with high lipid content; this mixture increased the COD reduction from 59%

in the control to 78%, increased grease removal from 47% to over 70%, and improved solids reduction from 34% to over 70%. The Enzymatic treatment can also be used as a pretreatment step to remove one or more compounds that can interfere with subsequent downstream treatment processes. For example, if inhibitory or toxic compounds can be removed selectively, the bulk of the organic material could be treated biologically, thereby minimizing the cost of treatment. Due to the susceptibility of enzymes to inactivation by the presence of other chemicals, it is likely that enzymatic treatment will be most effective in those streams that have the highest concentration of the target contaminant and the lowest concentration of other contaminants that may tend to interfere with enzymatic treatment. Aitken, (1993) suggested the following situations where the use of enzymes might be most beneficial:

- ✓ Removal of specific chemicals from dilute mixtures, for which conventional mixed culture biological treatment might not be feasible;
- ✓ Polishing of a treated wastewater or groundwater to meet limitations on specific pollutants or to meet whole effluent toxicity criteria;
- ✓ Treatment of waste generated infrequently or in isolated locations, including spill sites and abandoned waste disposal sites;
- ✓ Treatment of low volume, high concentration wastewater at the point of generation in a manufacturing facility to permit re-use of the treated process wastewater ,to facilitate recovery of soluble products, or to remove pollutants known to cause problems downstream when mixed with other wastes from the plant.

Enzymes like lipases are reported to be very useful in the treatment of dairy wastewater, because of the presence of high content of organic load in the form of fats and oil in the dairy effluent (Mendes et al., 2005; Pereira, 2004).

2.8. Lipase Enzyme

Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these water-insoluble compounds. Lipolytic enzymes are involved in the breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another (Beisson, 2000).

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids at the water–lipid interface and the reverse reaction in non-aqueous media (Castro et al., 2004). These enzymes showed potential applications in degrading oil and fats in wastewater generated by dairy industries, slaughterhouses, edible oils, fat refineries and others (Mendes et al., 2005; Pereira, 2004). Emulsifying agents have been systematically used in measurements of lipase activity, mainly to increase the lipid–water interfacial area, which, in turn, enhances the observed rates of lipase catalyzed reaction (Rocha, 1999). Lipids are an important component in wastewater that causes severe environment pollution. It can form oil films on water surfaces, preventing the diffusion of oxygen from air into water, leading to the death of many forms of aquatic life.
Aggregates formed by oil droplets and other particles present in wastewater can also block water drainage lines. The application of anaerobic reactors to treat lipids-rich wastewater can cause granular sludge flotation and wash-out, inhibition of methanogenic activity and decrease the concentration of adenosine triphosphate (ATP) (Perle et al., 1995; Mendes, 2004). To cope with these negative effects on the water stream treatment, several strategies for removing these lipid residues at the head of the plant are proposed. Among these, the alternative of using specific enzymes (lipases) has, recently, potentially gained more attention because of stringent environmental regulations and clean and friendly application of enzymes (Gandhi, 1997; Mendes et al., 2005).

2.8.1. Historical background

The presence of lipases on microorganisms has been observed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* (Eijkman, 1901). Lipases have traditionally been obtained from animal pancreas and are used as a digestive aid for human consumption either in crude mixture with other hydrolases (pancreatin) or as a purified grade. Initial interest in microbial lipases was generated because of a shortage of pancreas and difficulties in collecting available material. Lipases differ greatly as regards both their origins (which can be ba terial, fungal, mammalian, etc.) and their properties, and they can catalyses the hydrolysis, or synthesis, of a wide range of different carboxylic esters and liberate organic acids and glycerol. They all show high specific activity towards glyceridic substrates.

2.8.2. Microorganisms producing lipases

Lipases are produced by many microorganisms and higher eukaryotes. Most commercially useful lipases are of microbial origin. Some of the lipase-producing microorganisms are listed in Table 10.

Microorganism	Source	Reference
Rhizopus chinensis	Fungal	(Teng et al., 2009; Wang et al., 2008; Teng et al., 2008)
Rhizopus homothallicus	Fungal	(Diaz et al., 2006)
Penicillium verrucosum	Fungal	(Pinheiro et al., 2008 ;Kempka et al., 2008)
Geotrichum sp.	Fungal	(Yan et al., 2008; Burkert et al., 2004)
Geotrichum candidum	Fungal	(Burkert et al., 2005)
Aspergillus carneus	Fungal	(Kaushik et al., 2006)
Rhodotorula mucilaginosa	Yeast	(Potumarthi et al., 2008)
Yarrowia lipolytica	Yeast	(Lopes et al., 2009; Alonso et al., 2005; Kar et al., 2008)
Pseudomonas sp.	Bacterial	(Kiran et al., 2008)
Pseudomonas aeruginosa	Bacterial	(Ruchi et al., 2008; Mahanta et al., 2008)
Bacillus stearothermophilus	Bacterial	(Abada , 2008)
Burkholderia cepacia	Bacterial	(Fernandes et al., 2007)
Burkholderia multivorans	Bacterial	(Gupta et al., 2007)
Staphylococcus caseolyticus	Bacterial	(Volpato et al., 2008)
Serratia rubidaea	Bacterial	(Immanuel et al., 2008)
Bacillus sp.	Bacterial	(Ertugrul et al., 2007; Shariff et al. 2007)
Bacillus coagulans	Bacterial	(Alkan et al., 2007)
Bacillus subtilis	Bacterial	(Takaç et al., 2008)

Table 10: some of lipase-producing microorganisms

2.8.3. Applications of lipases

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Rubin et al., 1997a,b; Kazlauskas et al., 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse et al., 2001) and polyurethane (Takamoto et al., 2001). Major applications of lipases are summarized in Table 11.

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats
Production of biopolymers	Biosynthesis	Used as biocatalyst

Tea processing	Breakdown of membrane lipids	Formation and release of volatile flavouring agents
Oil biodegradation	As indicator	Lipase activity is a valuable indicator of diesel oil biodegradation
Oleochemical industry	Interesterification	Saves energy and minimizes thermal degradation
Ester synthesis	As catalyst for synthesis of esters	Flavoring agents in food industry
Textile industry	Assist	Removal of size lubricants and improved levelness in dyeing

Table 11: Industrial applications of microbial lipases. Source: (Vulfson, 1994).

Along with these uses, the recent application of lipases is in dairy industry, where it is finding its way for degradation of fats and oils present in the wastewater.

CHAPTER THREE
MATERIALS AND METHOD

3

CHAPTER THREE

MATERIALS AND METHODS

3.1. Chemicals

The media components were obtained from Hi Media Laboratories (Mumbai, India). The *p*-nitrophenyl palmitate (*p*NPP), substrate for lipase was obtained from Sigma Chemical Co., USA. All other chemicals used were of analytical grade.

3.2. Collection of dairy wastewater sample

Fresh inlet dairy wastewater (raw) was collected from Delhi Milk Scheme plant located at New Delhi, India. pH and temperature of the sample was analyzed on the spot. Water samples for BOD determination were collected in BOD bottles (non-reactive borosilicate glass bottles of 300 ml capacity). Analysis of BOD and COD was carried out on the same day, after bringing the sample to the laboratory. Analysis of other physico-chemical and biological parameters was started as soon as possible after collection to avoid unpredictable changes in water sample according to standard APHA method (APHA, 1995). The rest of the sample was stored in cold room (4 °C) for further studies.

3.3. Collection of samples for isolation of microorganisms

For isolation of microorganisms, used for dairy wastewater treatment and lipase production, the samples were collected from same dairy industry only. These samples were taken from sludge of oil and grease chamber, effluent and soil enriched with dairy wastewater from proximity of dairy wastewater treatment plant.

3.4. Analysis of various physico-chemical and biological characteristics of wastewater

3.4.1. pH and temperature

pH and temperature of the raw wastewater was analyzed at the sample collection site by using digital pH meter (Qickcheck, Orion) (Fig.5) and thermometer respectively.

Fig. 5: Digital pH Meter

3.4.2. Biochemical Oxygen Demand (BOD)

Biochemical Oxygen Demand (BOD) test is an empirical test used to measure the molecular oxygen utilized during specified incubation duration for the biochemical degradation of organic material (biodegradation). In biodegradation, small organisms that exist naturally in the environment particularly bacteria; decompose organic substances by using them as a food source. BOD test consists of 3 or 5 day period in which a sample is placed in an airtight bottle under controlled conditions (temperature – 27 °C or 20 °C ± 1 °C). Detailed procedure of experiment is provided in appendix No. I.

To carry out the experiment in the laboratory, BOD bottles were taken in two rows. Distilled water was added in 1st bottle in each column and taken as blank and rest bottles were used for samples. In the first row of bottles, DO was observed on the first day according to Wrinkler's method and the readings were noted as initial readings and the second row of bottles were kept in incubator at 27 °C for three days. The DO was recorded after three days and readings were taken as final readings. BOD was then calculated as the difference between initial and final DO measurements.

Fig. 6: BOD incubator

3.4.3. Chemical Oxygen Demand (COD)

The COD test also measures the amount of organic material in a water sample by measuring the oxygen demand. However, chemicals added to the sample, instead of bacteria, are responsible for breaking down, or oxidizing, the organic material. Detailed procedure of experiment is provided in appendix No. II. Briefly, pinch of $HgSO_4$ was taken in a reflux or digester flask and 10 ml of wastewater sample; 20 ml of conc. $K_2Cr_2O_7$ containing sulphuric acid was added and mixed thoroughly. The lower half portion of the digestion flask was kept immersed in water and 30 ml of H_2SO_4 containing $AgSO_4$ was added to it. Then the samples were digested for 2 to 3 hr (Fig. 7). After the completion of the digestion, the digested samples were titrated with ferrous ammonium sulphate as the titrant in the presence of ferrion as an indicator.

Fig. 7: COD digester

3.4.4. Total suspended Solids (TSS)

For the determination of total suspended solids (TSS) in dairy wastewater, 100 ml of wastewater sample was passed through a clean filter. The solids left on the filter were taken as the measure of suspended solids. The filter was weighed before and after passing the water to determine the weight of the total suspended solids in the sample. The filter was dried before the final weighing so that the moisture in the filter is not included in the weight of the solids. Detailed procedure of experiment is provided in appendix (No. III).

3.4.5. Total Nitrogen (Total Kjeldahl Nitrogen)

To carry out total nitrogen estimation, the wastewater sample was diluted two times and digested for 15 minutes with 10 ml H_2SO_4 and 1 ml $CuSO_4$, in Kjaldahl flask, till the solutions became clear. After cooling, the contents of the flask were transferred into a distillation flask and the volume was made up to 300 ml with distilled water. The sample was then titrated with 50% NaOH, using 4-5 drops of phenolphthalein indicator. After attaining the end point (violet colour), distillation of the sample was carried out. During distillation,

the tip of the condenser was immersed in 50 ml of 2% boric acid solution in a conical flask (250 ml). About 200 ml of the distillate was collected. The resulting solution was then again titrated with 0.02 N H_2SO_4, using methyl red indicator till the end point (appearance of red colour from yellow). Detailed procedure of experiment is provided in appendix (No.V).

3.4.6. Total phosphorus and Phosphate

Standard stannous chloride method was used to estimate total phosphate in wastewater (APHA, 1995). The sample was first filtered. Fifty ml of filtrate was taken in a Nessler tube. To this, 2ml of ammonium molybdate solution and five drops of stannous chloride was added and the solution mixed well. Absorbance of the sample was then measured at 690 nm, using UV- VIS spectrophotometer (Fig. 8) after 10 minutes incubation in the dark. Before, estimation of total phosphate in wastewater, calibration curve with known concentration of phosphate was prepared and treated in the same way. Detailed procedure of experiment is provided in appendix No.VI.

Fig. 8: UV-Visible Spectrophotometer (HACH, USA)

3.4.7. Oil and Grease (O&G)

Since the sample used in the present study is wastewater from dairy industry, it must be having a high amount of oil and grease. For testing it, 250 ml of wastewater sample was taken and its oil and grease content was extracted with 15 ml petroleum ether and 1-2 ml of 50% H_2SO_4, in a 500 ml separating funnel (Fig. 9). The contents in the funnel were mixed by vigorous shaking followed by leaving undisturbed for 10 minutes, till two layers were formed. The lower aqueous phase was drawn in a clear container and upper phase containing oil and grease was collected in preweighed evaporating disk (W1). The extraction was repeated twice. The disk having layer of ether with oil and grease content was then kept in oven at 100°C for 1hr. After cooling in desiccator, the weight of the disk was again recorded (W2) in analytical balance (ADAM, AAA 250LE). Similarly blank (250 ml distilled water) was treated in the same way. Detailed procedure of experiment is provided in appendix (No.IV).

Fig. 9: Separating funnel

3.4.8. MPN (Most Probable Number)

MPN method is used for examining water for fecal contamination specifically for detection of coliform bacteria. The test is carried out in three separate steps. The “presumptive” step involves testing for gas production from lactose fermentation in selective media. Microbes which are capable of using lactose as carbon source, their detection were facilitated by use of this medium. It also contains a surface tension depressants, bile salts used to suppress the growth of organism other then coliform bacteria. This is followed by the “confirmed” step where positive samples from the presumptive test are transferred to a more selective media and re-tested for gas production. Finally, the test is “completed” by isolating single colonies with a positive reaction on EMB (Eosine Methylene Blue) agar or Endo agar. The test was conducted following the APHA standard method for MPN. Aliquots of dairy wastewater to be tested were added to lactose broth containing an inverted gas vial. Three tubes with lactose medium were inoculated with 10 ml, 1 ml, and 0.1 ml aliquots of the water sample. The series consisted of three groups, each group then inoculated with the designated volume and dilution of wastewater sample. Nine tubes were incubated at 37 °C for 48 hr and examined for the presence of gas in the inverted tubes. Development of gas in tubes was presumptive evidence for the presence of coliform bacteria in the sample. The presumptive test also enabled to determine the statistical range of the number of coliform organisms present in the wastewater by referring to a MPN (Table. I appendix). The MPN was determined by the number of tubes in each group that showed the gas production following the incubation period. In second stage, gas producing samples from lactose broth culture were inoculated onto Endo agar for confirmation of coliform bacteria (Bhattacharya.,B.C & Banerjee., R. Environmental biotechnology, 2007, Oxford University press). Detailed procedure of MPN is provided in appendix No.VII.

3.5. Screening, isolation and purification of microbes

For isolation of microorganisms , used for dairy wastewater treatment and lipase production, sludge samples (1gm) collected from oil and grease chamber of the treatment plant, and soil sample (1gm) from the same dairy wastewater treatment plant was suspended in 10 ml sterile distilled water. The suspension was then spread on nutrient agar plate, with the help of spreader. The composition of nutrient broth and nutrient agar medium is given in Table 12.

The plates were incubated for 24 hr at 30 °C. The growth thus observed was then subjected for purification of microbes by repeated streaking on nutrient agar plates. A total of four microbial strains could successfully be isolated and purified from sludge samples while four strains were purified from soil sample.

3.6. Lipase Production

3.6.1. Lipase assay

Lipase activity in the cell free supernatant was determined as described by (Kilcawley et al., 2002). 1.8 ml of solution containing 0.15 M NaCl and 0.5% Triton X-100 in 0.1 M Tris–HCl buffer (pH 8.0) was preincubated at 37 °C with 200 µl of suitable dilution of cell free culture supernatant (crude lipase). Twenty microliters of substrate (50 mM *p*NPP in acetonitrile) was added to the reaction mixture and incubated at 37 °C for 30 min. The amount of liberated *p*-nitrophenol (*p*NP) was recorded at 400 nm (UV-Visible spectrophotometer, HACH) figure 8. One unit is defined as the amount of enzyme liberating 1 nmol of *p*NP under standard assay conditions.

3.6.2. Selection of lipase enzyme producers among isolated microbes

All the eight isolated strains were checked for lipase production. The strains were first spotted individually on tributyrin agar plates (substrate agar plates used for lipase production testing, composition given in Table 12. Production of clear halo zone around the colony confirmed the lipase production by the microbes. The strains which showed positive test on tributyrin plates were then checked for lipase production in liquid broth. A total of four strains were found to produce extracellular lipase in liquid media. The best lipase producing strain (S1) was selected for further use. S1 was originally isolated from sludge sample collected from oil and grease chamber of dairy wastewater treatment plant located at Delhi Milk Scheme, New Delhi, India.

All the four lipase producing strains were maintained on nutrient agar slants and repeatedly sub cultured after every 20 days time interval.

NO.	Individual composition	Quantity (g / l)
1.	Peptone	5
2.	Yeast extract	2
3.	Beef extract	1
4.	NaCl	5
5.	Agar	15*
6.	Tributyrin	0.1%**

Table 12: Composition of media

* - in case nutrient agar media.

** - in case of tributyrin agar media.

3.6.3. Culture conditions for lipase production

All the seven isolated strains were tested for lipase production. Mother culture was prepared by inoculating a loopful of stock cultures of strains (S1, S6, S7, S8) in the nutrient broth individually, followed by incubation at 30 °C and 200 rpm in an orbital shaker (New Brunswick Scientific, USA) (Fig.10). The overnight grown cultures were used as inoculum for further studies. Two percent of this mother culture was used to inoculate 100 ml of lipase production medium in 250 ml Erlenmeyer flask. The lipase production medium (pH 7.0) was consisted of (g/l): Peptone: 5; Yeast extract: 2; Beef extract: 1; NaCl: 5. Incubation was carried out at 30 °C with constant shaking at 200 rpm in an orbital shaker. Aliquots of various samples were withdrawn at different time intervals and centrifuged (Remi, India), (Fig.11) at 12000 rpm for 10 minutes to pellet down the cell mass. The supernatants thus obtained were used for lipase assay.

Fig. 10: Incubator shaker

Fig. 11: Centrifuge machine

3.6.4. Optimization of lipase production in selected strain

Strain S1 showing maximum lipase production among all the strains tested, was used for further optimization studies. In order to see the effects of various nutritional and physical factors on lipase production from strain S1, nutrient media was used as the basic media.

Mother culture was prepared by inoculating a loopful of stock culture of strain S1 in the nutrient broth, followed by incubation at 30 °C and 200 rpm in an orbital shaker. Hundred ml of the media was inoculated with 2% inoculum and incubated for 7 days at 30 °C with constant shaking at 200 rpm. Various factors were varied, one at time, in the above media and their individual effect was monitored on lipase production. After growth, cultures were harvested by centrifugation at 12000 rpm for 10 minutes at 4 ºC. Lipase activity was determined in cell free supernatants.

(i) Effect of pH on lipase production

In order to see the effect of pH on lipase production by strain S1, pH of the culture medium was varied as 5, 7, 8, 9, 10, 11, and 12 before inoculation with mother culture. Other culture conditions were kept same as above.

(ii) Effect of NaCl on lipase production

To check the effect of NaCl concentration, varying concentrations of NaCl, viz. 0%, 3% and 10 % (w/v) were added to the production medium keeping the other conditions same.

(iii) Effect of various substrates on lipase production

Carbon source is known as an important factor affecting enzyme production. Substrate, tributyrin, was replaced with cheaper natural substrates like Mahua (*Madhuca* sp.) flower extract and Sal (*Shorea robusta*) seed deoiled cake (DOC) to see their effects on lipase production by strain S1.

Preparation of Mahua and Sal DOC extract (Substrates)

To 10 gm of grounded Mahua flower and Sal DOC, 100 ml of distilled water was added, and boiled for 10 minutes. After boiling, the slurry was filtered through muslin cloth and filtrates thus obtained were used as substrate's extracts. Three percent of each extract was added to nutrient broth, in case of studying the effects of different substrates on lipase production.

(iv) Determination of pH during fermentation

The cultured samples were withdrawn at various time intervals during fermentation and their pH was determined using pH meter (DELUX pH meter, India), (Fig.12).

Fig. 12: pH Meter

3.6.5. Lipase production by using Solid-state fermentation (SSF)

In view of the reports stating dairy wastewater treatment by using lipase produced through solid state fermentation (so that lipase can be produced at low-cost), the SSF of S1 was attempted in the present study also. Sal DOC, Mahua flower, raw Sunflower seeds and Sesames seeds were used as cheaper natural substrates for SSF. The fermentation was carried out by inoculating 200 µl of inoculum to different flasks having five gram of above autoclaved substrates, moistened with 2ml distilled water. To the autoclaved preparation, 2ml of sterile distilled water was added again (For providing enough moisture) followed by incubation at 30 °C. The samples aseptically withdrawn at various time intervals were used for enzyme extraction.

Enzymes extraction in case of SSF

Enzymes extraction was carried out by the method of (Ramachandran et al., 2004). Crude enzymes were extracted by mixing a known quantity of fermented substrate with 1.0 ml of 0.1 M Tris–HCl buffer, pH 8.0, and then shaking the mixture in an orbital shaker at 200 rpm. The suspension was then centrifuged at 12,000 rpm for 10 min and the supernatant used for lipase assay.

3.7. Identification of selected microbe (S1)

Isolated and purified potential lipase producing microbe (strains S1) has been submitted for identification to Microbial Type Culture Collection Facility, Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.8. Enzymatic characteristics

Lipase was characterized for following parameters:

3.8.1. Enzyme stability

In order to check the stability of lipase, the crude enzymatic preparation was stored at 4°C for one week. The aliquots of the samples were taken at time intervals and the residual lipase activity determined under assay conditions.

3.8.2. Determination of pH optimum

Effect of pH on lipase was studied by assaying the enzyme at different pH values in the range of pH 5.0–12.0, using PNPP as the substrate.

3.8.3. Determination of temperature optimum and thermal stability

To determine the optimum temperature, the activity of lipase was measured at various temperatures (25–75) °C using PNPP as substrate. The thermal stability was studied by incubating the enzyme at 55 °C. Aliquots were withdrawn at different time intervals and the residual activities determined at assay temperature.

3.9. Treatment of Dairy waste water under laboratory conditions

3.9.1. Microbial treatment

1) **Preparation of mother culture:** The loopful of microbe (S1) was suspended in 250 ml Erlenmeyer flask containing 100 ml of nutrient broth. The flask was then incubated overnight at 30 °C and 200 rpm in orbital shaker. This overnight grown culture was used for dairy wastewater treatment.
2) **pH adjustment**: Before inoculation, pH of raw waste water was adjusted to 9.0, using 0.1N NaOH.

3) **Inoculation of S1 (Microbe) to raw waste water:** In a one liter Erlenmeyer flask containing 500 ml of raw waste water, 5% of above mother culture was added. After inoculation, the flask was incubated at 37 °C at 200 rpm for four days. Samples were withdrawn periodically including zero day sample for estimation of COD, O&G and TSS changes. A similar flask without microbial (S1) inoculation was also kept in similar condition as a control.

3.9.2. Enzymatic treatment

1) **Lipase production by S1**

Lipase production by S1 was done under above optimized conditions, in nutrient broth containing, 3% Sal DOC and with initial pH of 8. The sample was withdrawn after 24 hr of fermentation and centrifuged at 12000 rpm, and 4 °C. This cell free crude enzymatic preparation in the form of supernatant was used for dairy wastewater treatment.

2) **Dairy wastewater treatment**

pH of raw waste water was adjusted to 9.0, using 0.1N NaOH before treatment. Five percent of above mentioned crude lipase preparation (cell free) was added to 500 ml of raw waste water (in a one liter Erlenmeyer flask). The treatment was carried out at 37 °C and 200 rpm for four days. Samples were withdrawn periodically including zero day sample for estimation of COD, O&G and TSS changes. A similar flask without enzyme preparation was also kept under similar condition as control.

CHAPTER FOUR
RESULTS AND DISCUSSION

4

August 2010

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Characterization of collected dairy waste water sample

Samples of raw effluent from a milk-processing unit were collected from the Delhi Milk Scheme, New Delhi. These samples were taken from inlet tank of Dairy wastewater treatment plant. The physico-chemical and biological analysis of the dairy wastewater is presented in Table 13.

Parameter	Value	Effluent standard for Dairy wastewater according to CPCB
pH	10.0	6.5-8.5
Temperature (°C)	20	-
COD (mg/l)	1200	250
BOD_3 27°C (mg/l)	850	30
TSS (mg/l)	425	100
Oil and grease (mg/l)	219	10
Total nitrogen (mg/l)	20.2	10
Total phosphorus (mg/l)	2.4	2
Coliform bacteria(MPN/100 ml)	7500	400

Table 13: Physico-chemical and biological parameters of the dairy wastewater

4.1.1. pH

pH of the dairy wastewater sample was observed on the spot only and it was found to be 10.0. There is reported to be large variations in pH in dairy wastewater samples mainly because of different production processes, primarily from cleaning operations. The pH from

different dairy industry can vary in the range of 2-11 (RAC/CP, 2002). The pH of a sample of raw effluent from Manjoormilk Milk Plant, Manjoor, Kottayam, Kerala, is reported as 6.4 i.e. in acidic range (Rajeshkumar et al., 2004). The alkaline pH of the studied dairy wasterwater (10.0) might be attributed due to the alkaline cleanings of the system. The alkaline cleanings aim at general fat saponification and removal of organic material (Briao et al., 2007).

4.1.2. <u>Temperature</u>

Temperature is a critical water quality and environmental parameter because it governs the kinds and types of aquatic life, regulates the maximum dissolved oxygen concentration of the water, and influences the rate of chemical and biological reactions. The organisms within the ecosystem have preferred temperature regimes that change as a function of season, organism age or life stage, and other environmental factors.

In dairy industries, water is used for refrigeration, pasteurization, sterilization and cleaning operations, resulting in variation in effluent's temperature. It is recommended that the effluent should result in a temperature increase of no more than 3 °C at the edge of the zone where initial mixing and dilution take place (http://www.ifc.org/ifcext/environ.nsf/).

The dairy wastewater sample used in the present study was collected in the month of February and its temperature was recorded to be 20 °C.

4.1.3. <u>COD and BOD</u>

Without free dissolved oxygen, there will be no survival of aquatic life forms in any water body. Streams and lakes therefore, simply become inhabitable to most aquatic life without free dissolved oxygen. At normal temperature water is said to be saturated with oxygen at 9.0 mg/L. This saturation value decreases rapidly with increasing water temperature (Adeoye et al., 2009). In environmental chemistry, the chemical oxygen demand (COD) test is commonly used to indirectly measure the amount of organic compounds in water while BOD determines the biodegradable organic matter. It is normally expressed in milligrams per liter (mg/L), which indicates the mass of oxygen consumed per liter of solution (Pooja, 2008).

As shown in Table 13, the COD and BOD of the wastewater sample were found to be 1200 and 850 mg/L respectively. This value of COD was observed to be almost 5 times higher than the standard value (Table 13). Similarly, BOD was also found to very high (almost 30

times) as compared to standard values. The high organic load mainly owes to the dissolved milk protein and sugars (due to butter, cheese and whey etc) present in the waste water. Rajeshkumar et al., (2003) have reported COD value of 5095 mg/L in the sample of Manjoormilk Milk Plant, Manjoor, Kottayam, Kerala. While a BOD value of 1112.0 mg/L in sample from Cuddalore district Co-operative Dairy Industry Ltd., Sethiyathope, TN, India has been reported by Dhanam, (2009).

4.1.4. Oil and grease (O&G)

Dairy wastewaters also have high O&G content, due to the milk fat, butter, ice cream and other dairy products, such as in the water used for rinsing the butter milk. This creates a number of problems in dairy waste water treatment. In aerobic bioreactors, O&G block the gas transfer required for the biological degradation. Specifically they reduce the oxygen transfer rates to the biological microbial consortium by the formation of a lipid coat around the floc (Chao et al., 1981; Grulois et al., 1993; Lemmer et al., 1988). O&G cause problems in the pumping and aeration systems that arise due to the development of filamentous microorganisms (*Sphaerotilus natans, Thiothrix, Beggiatoa, Nocardia and Microthrix genuses)*. These microorganisms are involved in the formation of scum and stable foams on the surface of the aeration tank. The scum and foams ultimately hinder the biomass flocculation and sedimentation, a phenomenon known as bulking (Jenkins et al., 1993). High O&G contents also generate agglomerates or pellets inside the secondary sludge flocs, which hinders sedimentation, generating unpleasant odors and reducing the efficiency of the treatment station (Eckenfelder, 2000).
O&G content of the studied dairy wastewater sample was found to be 219 g/L as presented in Table 13. It is clear from Table 13, that this value is also very high as the value for the effluent to be discharged should not be more than 10.0 (Briao et al., 2007) have also reported 286.8 mg/L of O&G in raw wastewater from some other dairy industry.

4.1.5. Total suspended solids (TSS)

TSS was found to be 425mg/L compared to standard value of 100mg/L. Selmer-Oslen et al., (1996) and Khojare et al., (2005) reported TSS of 290-500mg/L and 650 mg/L respectively of dairy wastewater.

4.1.6. Total nitrogen and Total phosphorus

The dairy wastewater sample was also characterized for its nitrogen and phosphorus contents. As shown in Table 13, the nitrogen and phosphorus content were estimated to be 20.2 and 2.4 mg/L respectively. The nitrogen content was two times the standard value. The high nitrogen in dairy wastewater is mainly because of biological nitrogen source (Proteins, amino acids) already present in milk and their products and also because of the use of cleaning and disinfection. Selmer-Oslen et al., (1996), got value of 11.5-27.4mg/l of total phosphorus and total nitrogen of 38-53 mg/l from dairy wastewater of dairy at Oslo.

4.1.7. Total coliforms (MPN)

MPN is an estimate of microbial density per unit volume of water sample, based on probability theory. There are three tests presumptive, confirmed & completed. The tests are performed sequentially on each sample under analysis. It is basic test to detect the presence of coliform bacteria coloiform bacteria (indicator of fecal contamination); the gram negative, non spore forming bacilli that ferment lactose with the production of acid and gas that is detectable following 24 hr incubation period at 37 ºC .

Because of high organic content in the waste water of the food production industries, total coliforms found to be higher than the standard value. A value of > 1100 for total coliforms was found by Rajeshkumar et al., (2004) from collected dairy wastewater. Many times the wastewater from the food industries mixed up with the sewage water, and then treatment is done. On mixing with sewage, the wastewater becomes laden with organic content, which subsequently increases the total microbial count. The sample of the evaluated dairy wastewater was also found to have high MPN i.e. 7500 MPN/100 ml showing high amount of coliform bacteria present in the dairy wastewater (Table 13). The positive culture samples from presumptive test were checked for their growth on endo-agar and EMB agar. There was growth of pink colonies on endo-agar confirming the presence of coliform bacteria in the sample (Fig. 13, 14 and 15). The presence of green colonies with metallic fluorescent sheen on EMB agar confirmed the occurrence of *E. coli* in the dairy wastewater sample.

	Water sample			Dilution1:100			Dilution1:10,000		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
A Double strength	+	+	+	+	+	+	+	-	-
B Single strength	+	+	-	-	-	+	-	-	-
C Single strength	+	-	-	-	-	+	-	-	-

Table 14: MPN number determination from multiple tube test.

(+): gas production;

(-): No gas production.

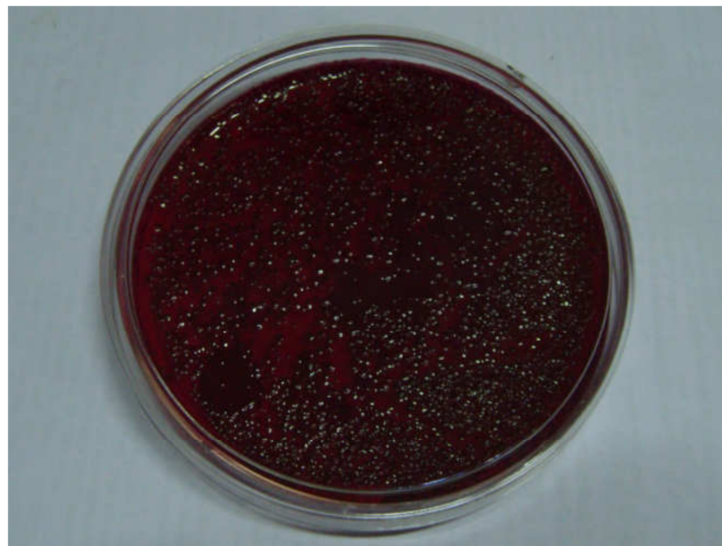


Fig.13: Growth of coliforms on endo-agar (A, dilution10^{-2})

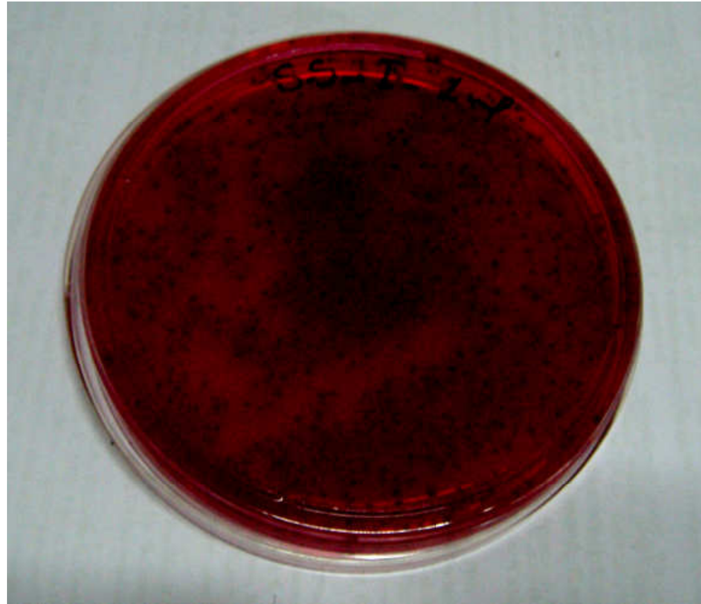


Fig.14: Growth of coliforms on endo-agar (B, dilution 10^{-2})

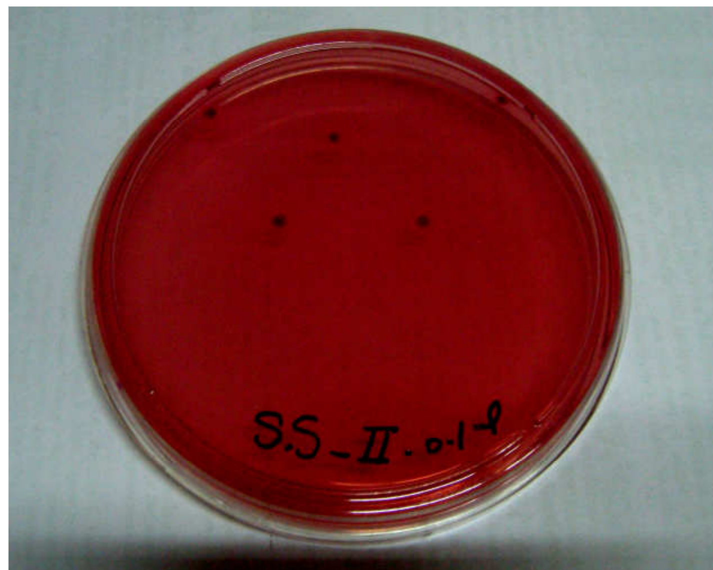


Fig. 15: Growth of coliforms on endo-agar (C, dilution 10^{-2}).

The physicochemical and bacteriologic analysis of the dairy wastewater samples are presented in Table 13. The results clearly indicate the need to treat dairy wastewater before discharging into waterways**.** Because of the presence of high fraction of readily biodegradable organic compounds in dairy wastewater, there is a high scope for the

application of biologic treatment methods. In view of this, isolation of microorganisms that could be potential for dairy wastewater treatment was carried out further.

4.2. Isolation and purification of microbial strains

For the isolation of potential microbial strains that could be used for the treatment of dairy wastewater, the samples were collected from the dairy industry only. Four microbial strains could successfully be isolated and purified from sludge samples (S1, S2, S3 and S4) while four strains were purified from soil samples (S5, S6, S7 and S8). Thus a total of eight microbial strains were isolated and used for further studies (Fig. 16, 17,18,19,20 and 21).

Since biological treatment can be done in two ways; either by using microbe(s) or by using enzyme(s). Hence, the strains selected after primary screening were subjected to further screening by testing their ability to grow and produce specific enzyme so that both (strains and their specific enzyme) can help in treatment of dairy wastewater. The microbes able to produce lipase enzyme might be useful to remove the fats and oils, which is an important prerequisite process especially in the treatment of dairy wastewater. The use of lipase producing microbes in wastewater treatment system so as to degrade fats and oils and eliminate pretreatment process is an interesting strategy, gaining attentions now days (Bhumibhaman et al., 2002).

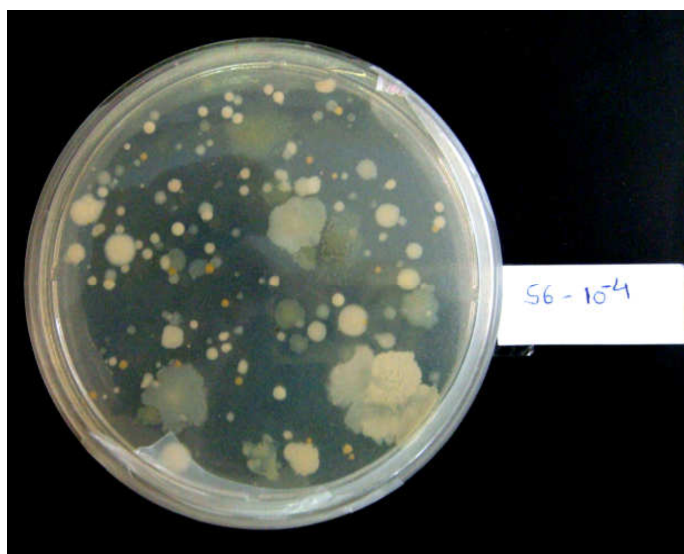


Fig. 16: Mixed culture sample

Fig. 17: Isolated and purified colonies (strain S1, S3, S4, and S7)

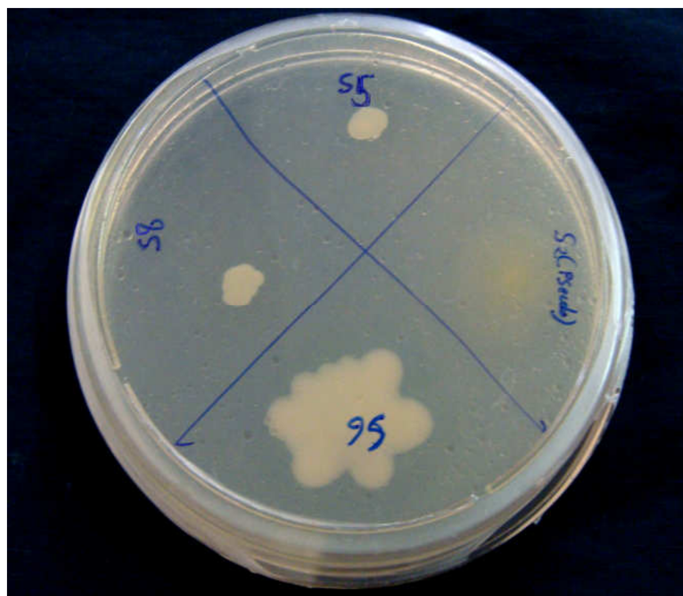


Fig. 18: Isolated and purified colonies (strain S2, S5, S6, and S8)

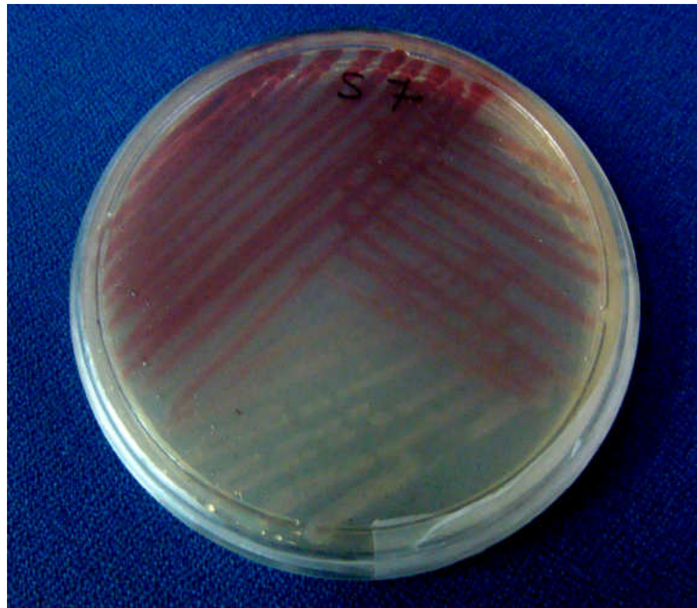


Fig. 19: Strain S7 (Streak plate)

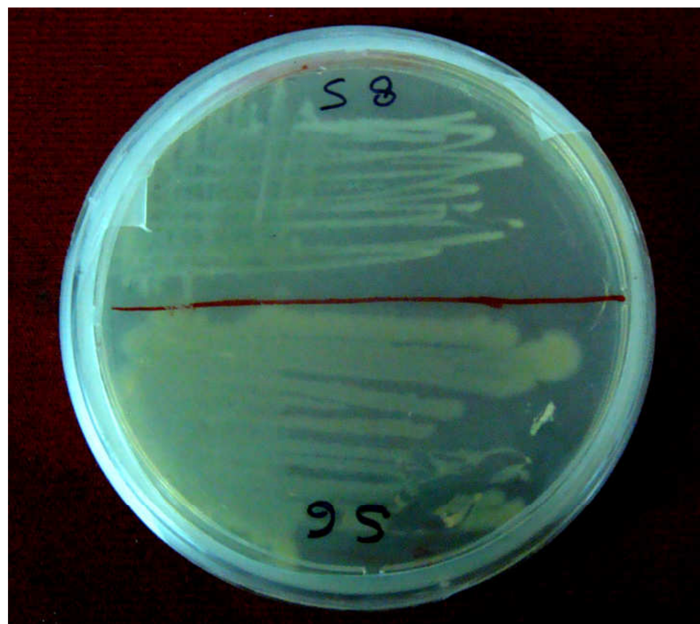


Fig. 20: Strain S6 and S8 (Streak plate)

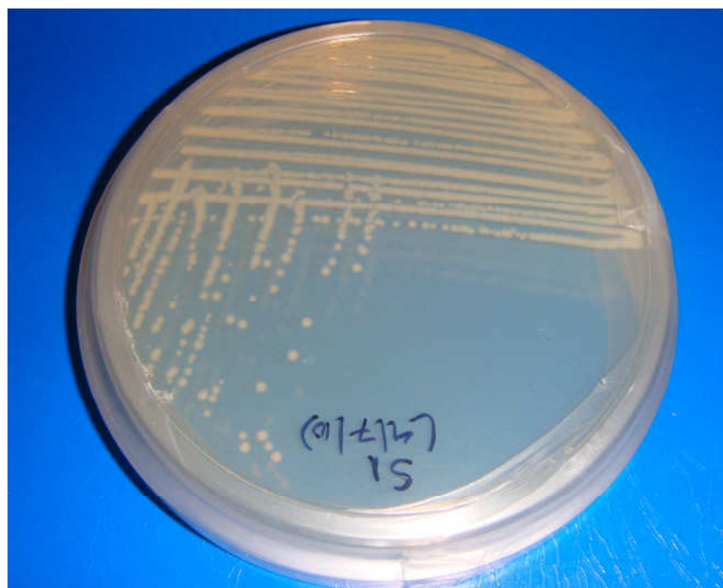


Fig. 21: Isolated and purified colonies of strain S1 (Streak plate)

4.3. Selection of lipase enzyme producers among isolated microbes

All the eight purified microbes were screened for lipase production. The strains were first spotted individually on tributyrin agar plates. Production of clear halo zone around the colony confirmed the lipase production by the microbes. The strains which showed positive test on tributyrin plates were then checked for lipase production in nutrient broth containing 0.1 % (v/v) tributyrin. A total of four strains (S1, S6, S7 and S8) were found to produce extracellular lipase in liquid media. The strain that showed maximum lipase production during the same set of conditions was selected as the potential strain for further studies.

4.3.1. Standard curve for *p*-nitrophenol

Standard curve of p-nitrophenol, product of PNPP hydrolysis was prepared for calculating lipase activity (Fig. 22).

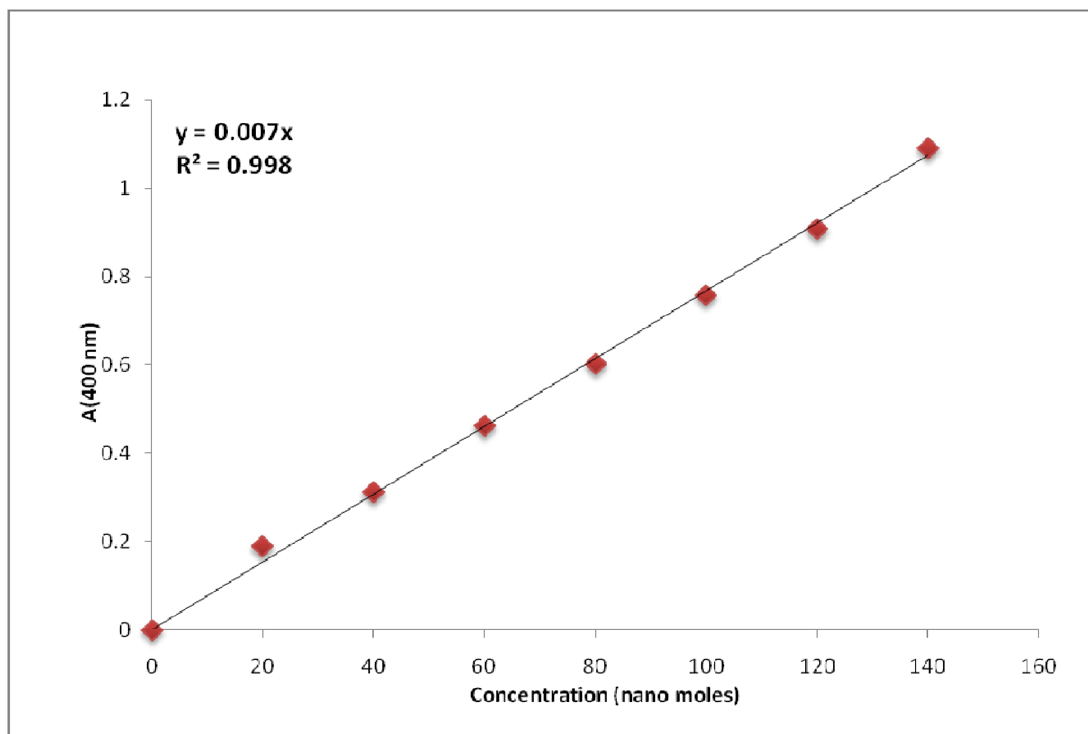


Fig. 22: Standard curve of para nitrophenol

4.3.2. Lipase production by different strains in liquid broth under unoptimized conditions

In order to select the potential extracellular lipase producing strain, all the four strains (S1, S6, S7 and S8) were tested for lipase production in liquid broth. The culture conditions were kept same as described in methods section. The time course of lipase production from all the strains is given in Fig. 23. Although all the strains tested showed enzyme production but maximum lipase activity was observed in case of strain S1.

As shown in Fig. 23, the lipase activity was found to be maximum (16.8 U/ml/min) by S1, on third day of fermentation. Thereafter, the enzyme production started decreasing. While the second highest enzyme producing strain was found to be S6, showing 4.2 U/ml/min of enzymatic activity on third day of fermentation. Strains S7 and S8 were found to show minimum lipase activity of less than 4 U/ml/min.

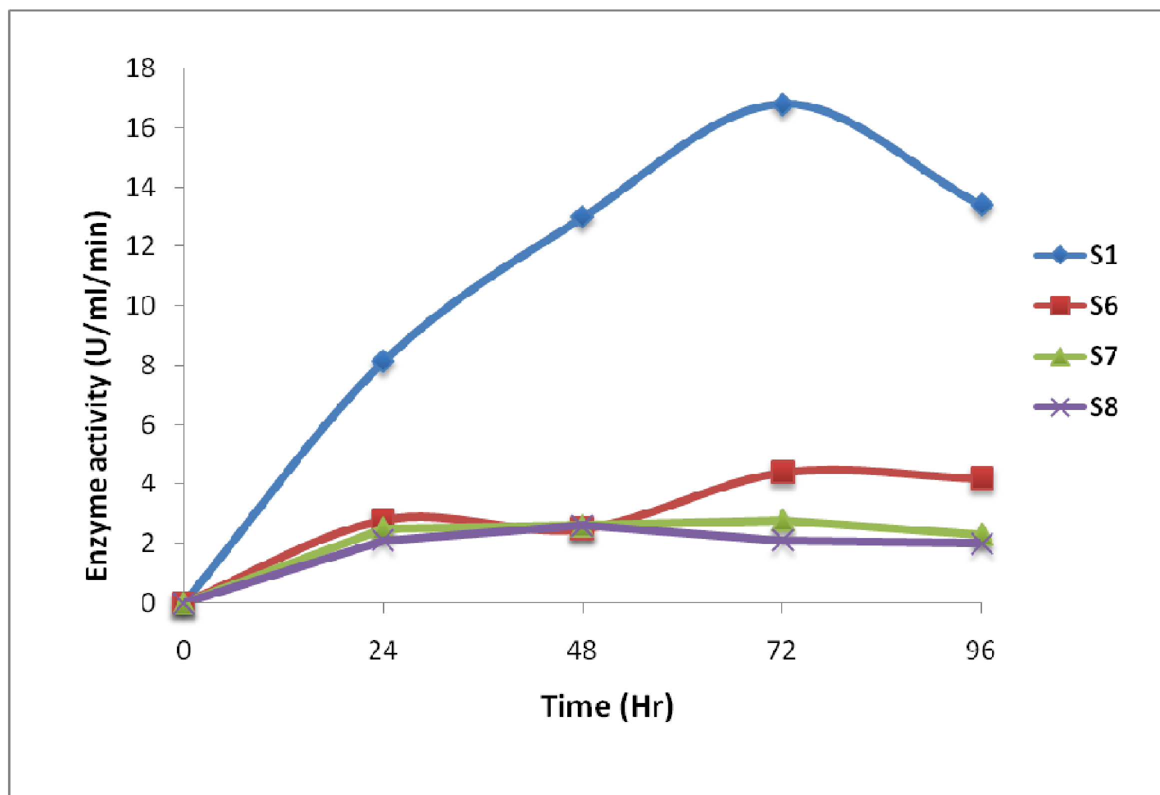


Fig. 23: Time course of lipase production by different isolated strain in nutrient broth containing tributyrin at 200 rpm and 30 º C temperatures.

Because of maximum lipase production by strain S1, it was selected for further studies. This strain has also been submitted for identification to Microbial Type Culture Collection Facility, Institute of Microbial Technology (IMTECH), Chandigarh, India.

4.3.3. Optimization of lipase production by using S1 strain

4.3.3.1. Effect of initial pH of the medium on lipase production.

pH is an important factor which affects microbial growth and enzyme production during fermentation. The media (nutrient broth containing 0.1% tributyrin) was adjusted to different pH values prior to inoculation. Fig. 24 shows that a good lipase production was observed at pH 7.0, 8.0 and 9.0. Maximum lipase production was observed at pH 9.0 (100%). While at pH 7.0 and 8.0, enzyme activity was found to be 75 % and 90 % respectively. Sharma et al., (2009) and Satish et al., (2009) have also reported maximum lipase production at pH 9.0 by

using *Arthrobacter* sp. and *Bacillus* sp. respectively. No lipase activity was observed at pH 12.0 whereas very low activity found at pH 11.0 and pH 5.0 (Fig. 24).

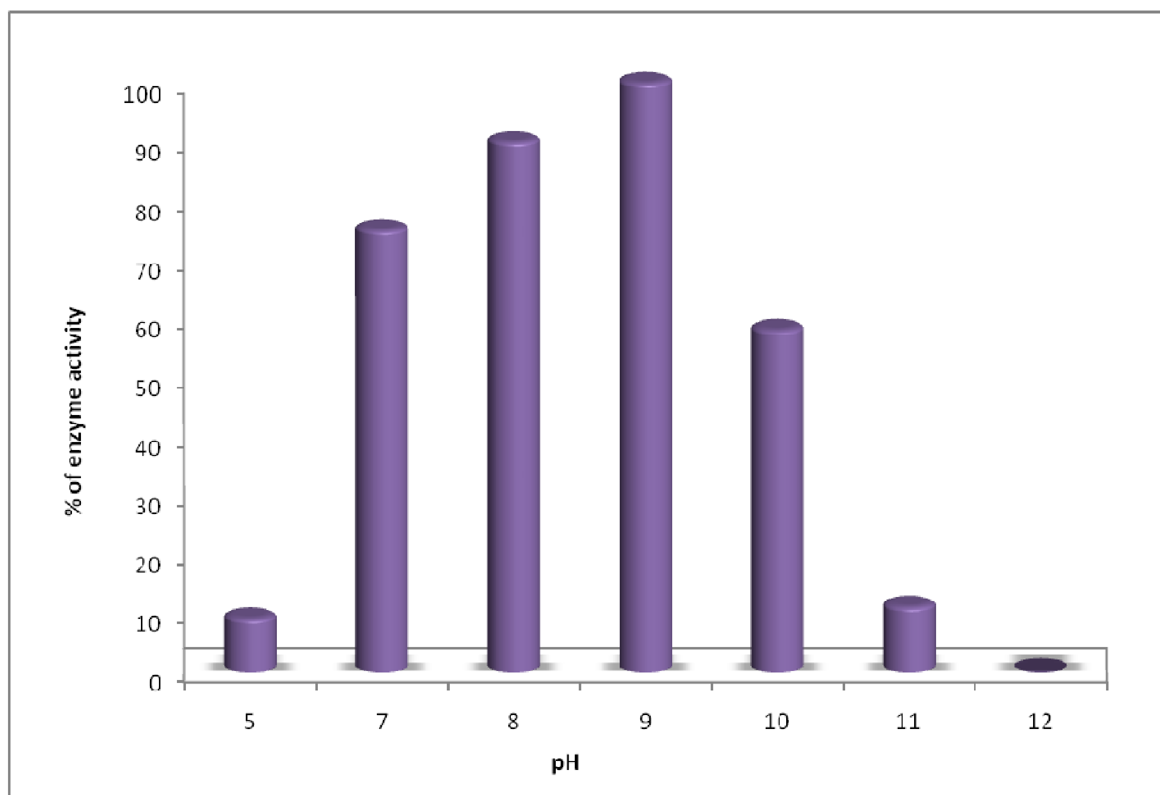


Fig. 24: Effect of different pH on lipase production by the strain S1 in nutrient broth containing 0.1 % tributyrin.

4.3.3.2. Effect of NaCl on lipase production

As shown in Fig. 25, enzyme production was found to be higher (21.0 U/ml/min) in the presence of 3% of NaCl tested, compared to media without any additional supplementation of NaCl. But at higher concentration of NaCl (10%), the microbe was not able to grow, and thus there was no lipase production. This shows that low concentration of NaCl stimulates lipase production, or acts as an inducer but at higher concentrations it acts as inhibitor for S1.

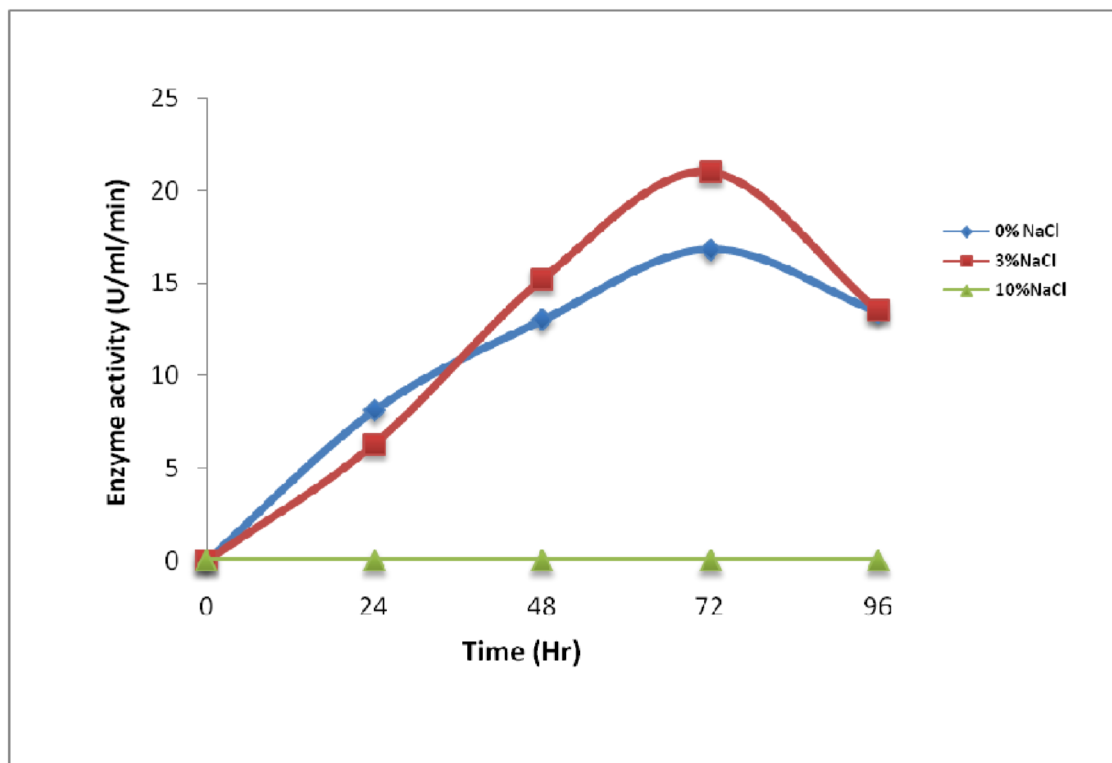


Fig. 25: Effect of NaCl on lipase production by strain S1 in nutrient broth containing 0.1% tributyrin at 200 rpm and 30ºC temperature.

4.3.3.3. <u>Effect of various substrates on lipase production</u>

In view of the reports stating dairy wastewater treatment by using lipase produced through solid state fermentation (so that lipase can be produced at low-cost), the SSF of S1 was attempted in the present study also by using available cheaper natural biomaterials, as substrates (Leal et al., 2006; Jung et al., 2002). Sal DOC, Mahua flower, raw Sunflower seeds and Sesames seeds were used as cheaper natural substrates for SSF (Pandey et al., 2000a; Pandey et al., 2000b; Mahanta et al., 2008). Since no microbial growth and lipase production was observed in preliminary studies, SSF was not carried forward.

Instead of directly using above natural materials as substrates, addition of 3% of Mahua flowers extract or Sal DOC extract as additional cheaper substrates in place of tributyrin was attempted. Similar kinds of substrates are also being used by other researchers for different enzyme productions. For instance, plant extract in the form of sugarcane bagassee hydrolysate was used for production of α amylase by *Bacillus subtilis* in liquid broth by

Rajagopalan et al., (2008). In another case, Silva et al., (2005) found maximum production of lipase by *Metarhizium anisophliae* using olive as supplemented substrates.

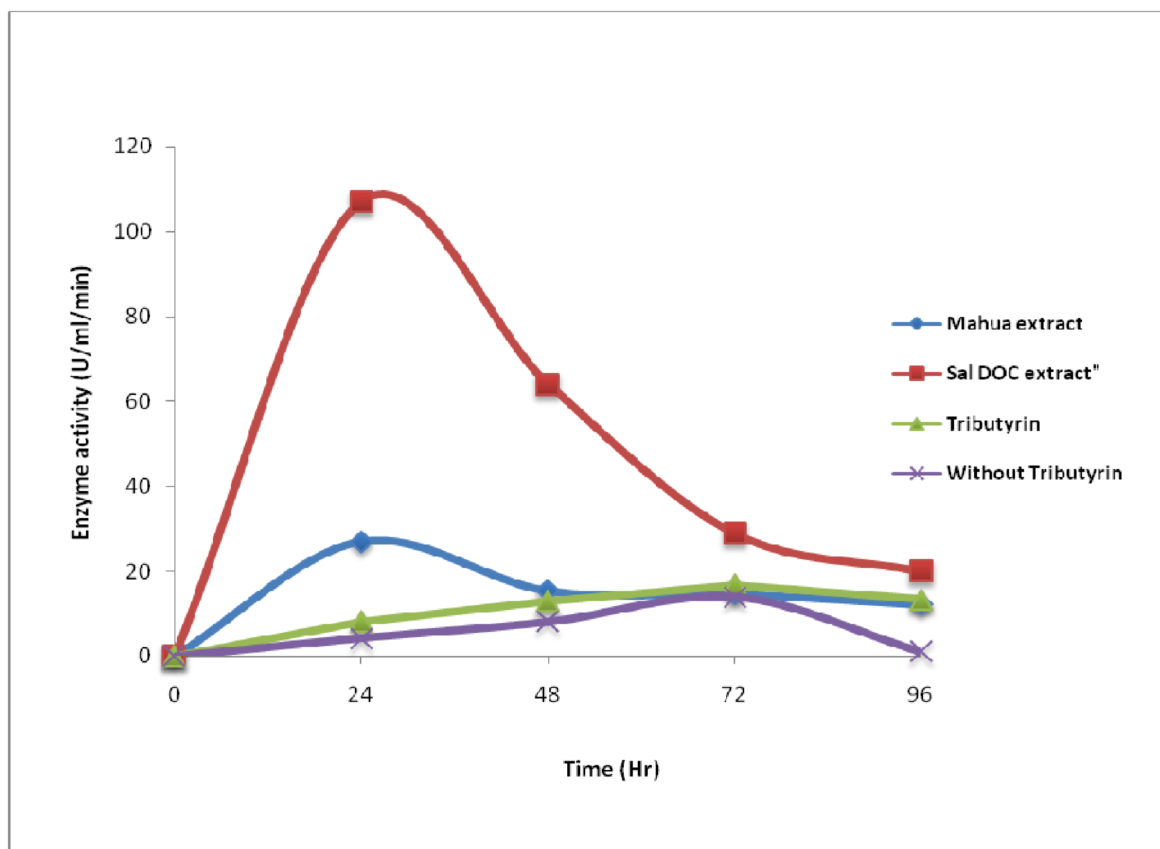


Fig. 26: Effect of different substrates on lipase production by strain S1.

The time course of lipase production from strain S1 by using different substrates is presented in Fig. 26. The results show that lipase activity was found to be the maximum in case of Sal DOC as substrate. The enzyme production was highest (107 U/ml/min) at 24 hr of fermentation and decreased thereafter. Mahua flower as extract was also found to produce good enzyme production in 24 hr (27 U/ml/min), but less compared to Sal DOC (Fig. 26). In case of Sal DOC and Mahua extract, the lipase production was found to be much better compared to the media supplemented with or without tributyrin. There was minimal production of lipase by S1, in media without any additional substrates i.e. nutrient broth only. This shows that lipase can be produced more and early also, with supplementation of additional natural substrates. Control tests were also done, in which enzymes was first boiled,

at 100 ºC, for 10 minutes and then usual assay was performed using p-nitrophenyl palmitate as substrate; to confirm the enzyme activity. We have found zero activity in the control sets (Boiled enzyme preparation, data not shown). Since maximum lipase production was observed in case of Sal DOC after 24 hr of fermentation, it was used as substrate for further optimization studies.

4.3.3.4. Effect of pH on lipase production by strain S1 in nutrient broth containing 3% Sal DOC extract.

To check the influence of pH on lipase production in the presence of Sal DOC, lipase production was carried out at varying pH as earlier experiment was performed without pH adjustment. The lipase production was found be highly affected by pH variation as shown in Fig. 27. Maximum lipase production (195 U/ml/min) was observed at pH 8.0. An increase and decrease in pH significantly affected enzyme production. Lipase activities were found to be 63, 111, 39 U/ml/min at pH 7.0, 9.0 and 10.0 respectively (Fig. 27). There was no growth at pH 11.0 and 12.0, thus no enzyme activity.

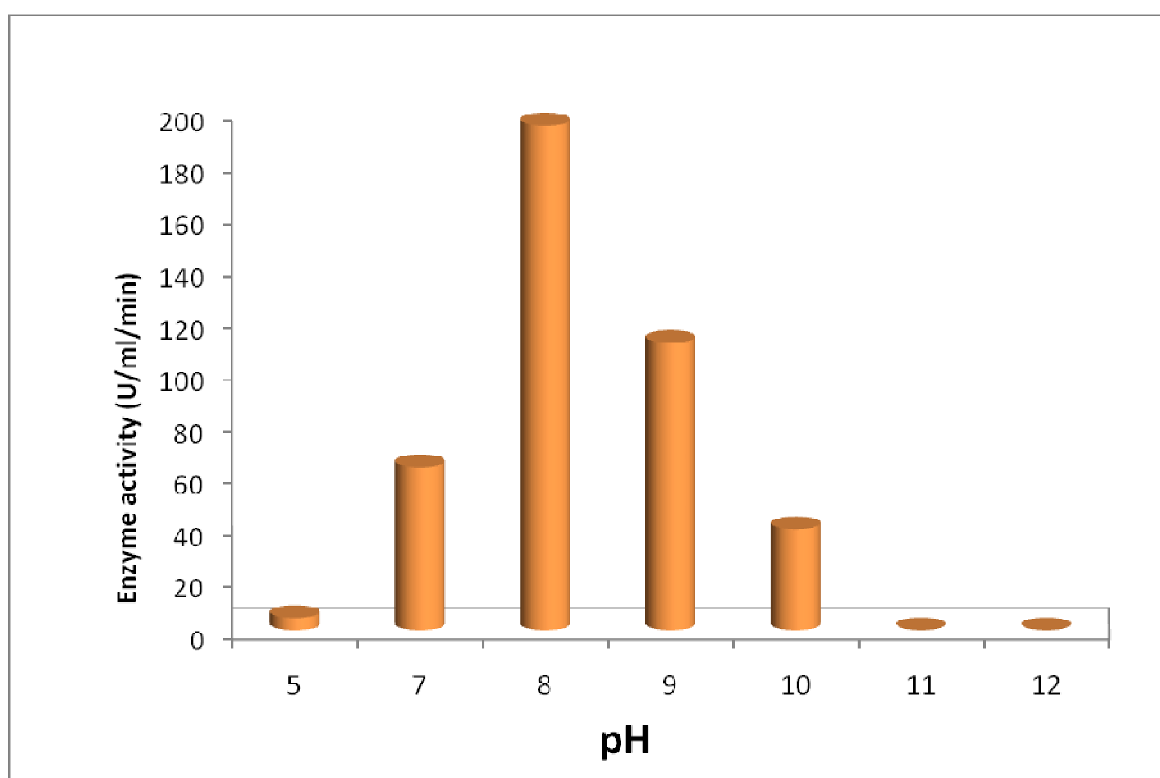


Fig. 27: Effect of different pH on lipase production by strain S1 in nutrient broth containing 3% Sal DOC extract.

4.3.4. Changes in pH of fermentation media by strain S1

The pH of fermentation media may change during fermentation due to production or utilization of acidic or alkaline compounds. Some samples from fermented media were aseptically withdrawn after 24 hr of growth and pH checked. As presented in Table 15, the changes in initial pH of the media during growth and lipase production were observed in case of S1 strain also. The strain was shown to change the pH of both low (acidic) and high value (alkaline) to nearby alkaline range (8.0 – 9.5) except pH 5.0 sample as observed after 24 hr of growth at 30 ºC. Because, the enzyme production was also found to be maximum at pH 8.0 - 9.0, this supported the findings. This property was shown to be very interesting, and have useful applications in the treatment of wastewater. This will reduce the cost of treatment, by minimizing the steps required for usual treatment, especially in case of dairy wastewater treatment, where the pH is first neutralized with some chemicals and then additional treatments/steps are done.

NO.	Culture conditions	Initial pH of the culture media	Final pH of the culture media after 24 hr of growth (S1)
1-	Nutrient broth + Sal DOC + S1	5	6.65
2-	Nutrient broth + Sal DOC + S1	7	8.00
3-	Nutrient broth + Sal DOC + S1	8	8.28
4-	Nutrient broth + Sal DOC + S1	9	8.45
5-	Nutrient broth + Sal DOC + S1	10	8.51
6-	Nutrient broth + Sal DOC + S1	11	8.60
7-	Nutrient broth + Sal DOC + S1	12	9.50

Table 15: Changes in the initial pH of the media, after 24 hr of growth.

The optimized culture conditions for lipase production from strain S1 were established as following: Nutrient broth containing 3% Sal DOC, pH 8.0. Under optimized conditions, 195 U/ml/min of lipase activity was obtained in 24 hr of fermentation.

Since this lipase preparation was required for dairy wastewater treatment, it was worthwhile to look for its enzymatic characteristics.

4.3.5. Enzyme characteristics

4.3.5.1. pH optima

To determine the pH optima of the enzyme, lipase assay was done at different pH values. The source of the enzyme was from media of pH 8 and 9, supplemented with 3% Sal DOC. The enzyme has the optimum range from pH 8.0 to 10.0, with maximum activity at pH 10 in both the cases.

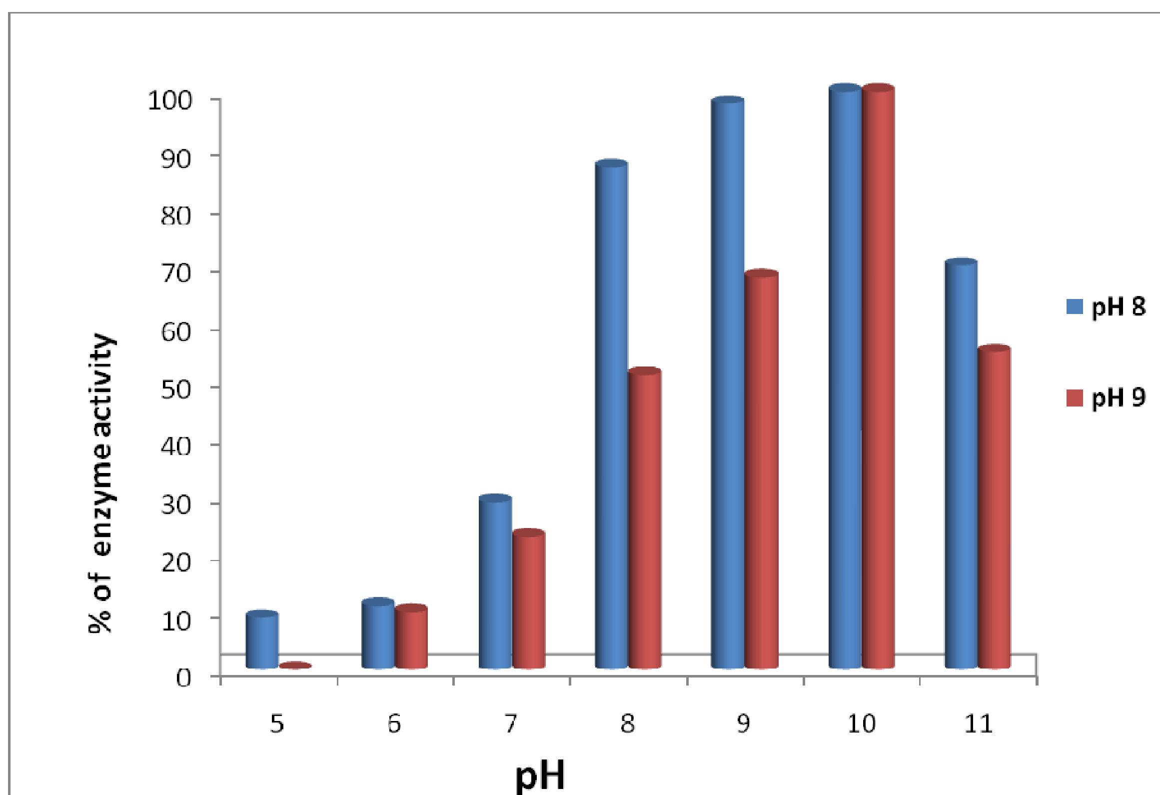


Fig. 28: pH optimum of lipase produced in nutrient broth containing 3% Sal DOC.

4.3.5.2. Temperature optima

To study the optimum temperature of lipase activity, assay was performed at different temperatures. The good enzyme activity was observed in the temperature range from 45 °C to 55 °C, optimum being 55 °C.

The same temperature and pH optima were also reported in case of Different species and strain of *Pseudomonas* (Brune et al., 1992; Rashid et al., 2001; Gilbert et al., 1991).

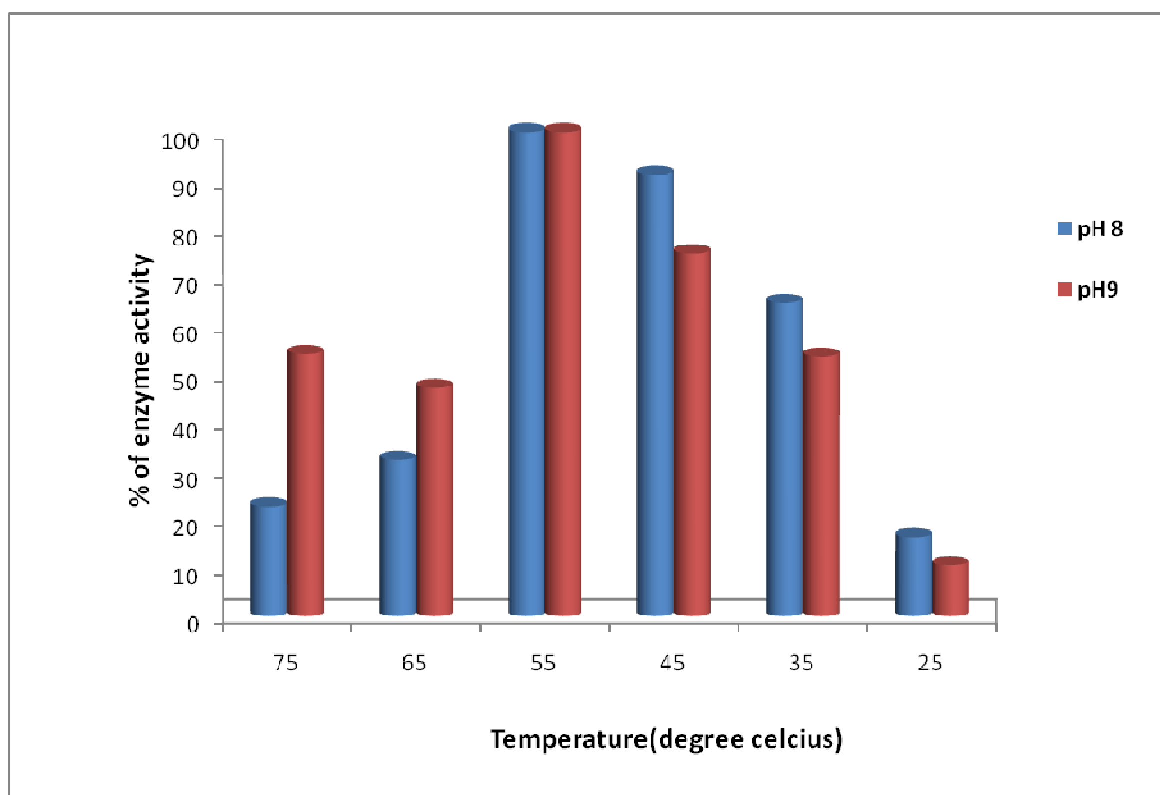


Fig. 29: Temperature optimization of lipase activity at pH 8 and pH 9.

4.3.5.3. Stability of enzyme at 4°C

For any enzymatic application, it is important to check the stability of the enzyme. In the present study, the crude enzymatic preparations (pH 8.0 and 9.0) were stored at 4 °C for one week to check the stability of the lipase. The samples were withdrawn at various time intervals and assayed for remaining lipase activity. The results in Fig.18, shows that the enzyme is moderately stable at 4 °C having 65% of initial activity after 144 hr of storage.

When the enzyme was stored at -20 ºC, it was found to remain stable for long duration (18 days), with 64% of initial activity.

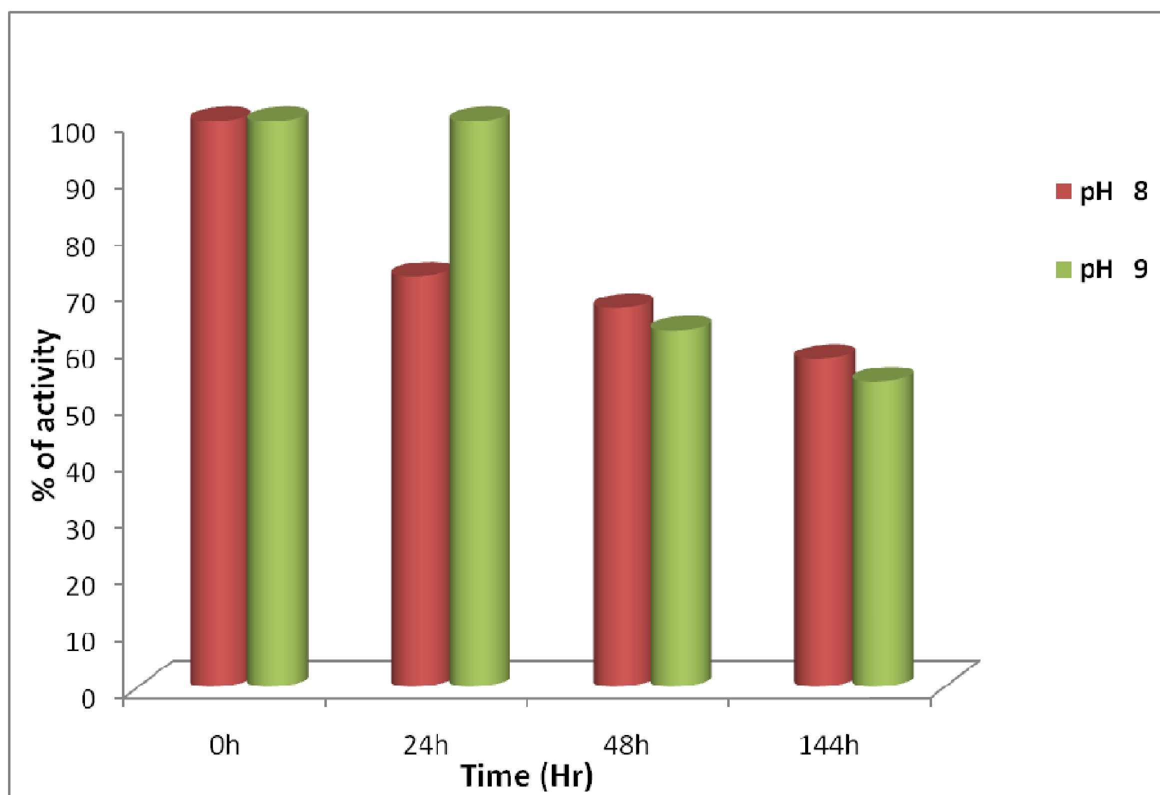


Fig. 30: Stability of S1 lipase at different intervals during storage at 4 ºC

4.3.5.4. <u>Thermal stability.</u>

Since the enzyme was found to be more active at 55 ºC, the study was undertaken to check its stability at 55 ºC. The crude enzyme preparation was kept at this temperature and aliquots of sample were withdrawn at various times (0, 5, 10, 20, 40, 60, 80, 100 minutes). Remaining lipase activities were measured under standard assay conditions. Fig. 31, shows the stability of lipase at 55 ºC. It lost about 25 percent of initial activity in 5 minutes. After 40 minutes, the enzyme activity remained stable with 30% of initial activity. Similarly, *Alcaligenes* sp. lipase with pH optima of 9.0, temperature optima of 55 °C, also showed 60% of residual activity at 60 °C after 10 minutes (Brune et al., 1992). Liu et al., (2009) have also reported similar observations in case of *Burkholderia* lipase.

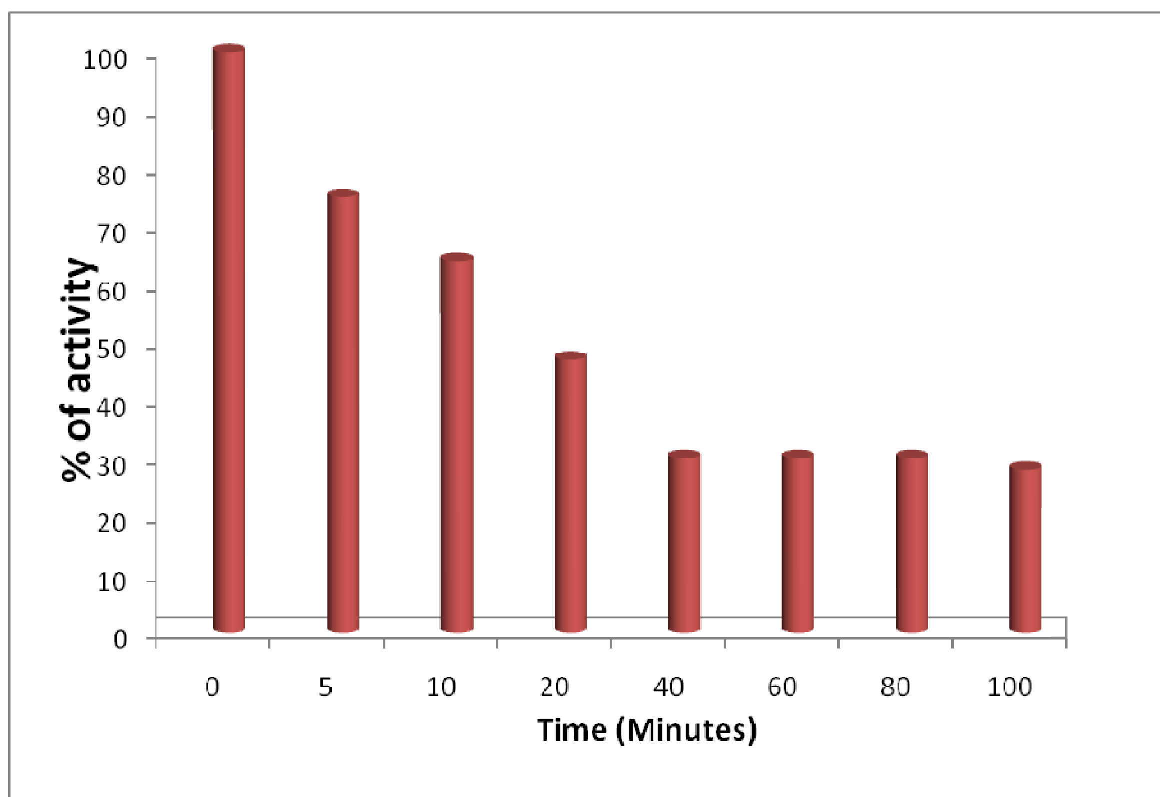


Fig. 31: Thermal stability of S1 lipase at 55°C

4.4. Treatment of raw dairy waste water

Biological degradation is one of the most promising options for the removal of organic material from dairy wastewaters. From the four strains obtained after primary screening, a potential strain was selected based on the efficiency to produce maximum lipase activity. The bacterial strain designated as S1 was thus selected and used for dairy wastewater treatment and lipase production. In an attempt to overcome some of the problems associated with traditional chemical and biological waste treatment systems, recent research has focused on the environmental applications of enzymes that have been isolated from their parent organisms. In view of this, studies have also been conducted on dairy wastewater treatment by using the lipase produced by strain S1.

4.4.1. Microbial treatment of dairy wastewater

Lipase producing microbes are reported to be used for biological treatment of dairy waste water. For example, both *P.aeruginosa* LP602 cells and its lipase were shown to be usable

for lipid rich wastewater treatment (Mongkolthanaruk et al., 2002). Lipases are utilized in aerobic wastewater treatment processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport.

The isolated and purified strain S1 was used for the treatment of dairy wastewater sample collected from dairy wastewater treatment plant of Delhi Milk Scheme. The results presented in Fig. 32, 33, 35 and 36, clearly shows that there is remarkable decrease in the COD, fats & oils and TSS contents after treatment with strain S1. The strain could reduce the COD and TSS of waste water by 93% and 47% respectively after 96 hr of treatment (Fig. 33, 35). The oil and grease content was also shown found to be reduced up to 75% of initial value. The similar results were also reported by Bhumibhaman et al., (2002) by using mixture of three microbes containing *Bacillus sp, Acinetobacter sp* and *Pseudomonas sp.* COD reduction was highest than other two parameters because microbes (added as well as already present in the wastewater) use organic compounds for their growth. The same process also happened in case of control, where microbe already present in the waste water, might have used the organic matter for their growth. Since control was also kept under similar conditions like test sample i.e. with shaking of 200 rpm. This speed might had provided, enough oxygen for growth of microbes (already present in the wastewater), resulting in COD reduction. One control flask was also kept under similar experimental conditions but without shaking, the samples were withdrawn at 0 and 96 hr, and surprisingly, there was negligible reduction of COD (less than 5%). This might be due to the fact that without shaking, microbes could not get enough oxygen to grow resulting in lesser reduction of organic matter (COD).

4.4.2. Enzymatic treatment of dairy waste water

Enzymes and pure cultures have been used to increase hydrolysis during or prior to biological treatment process (Lanciotti et al., 2005; Cammarota et al., 2006). Such pretreatment methods generally consist of the cultivation of the lipase producing microbial strains in the effluents or direct addition of crude or pure lipase preparations in the sample. The same approach of addition of direct crude lipase preparation was attempted in the present study also. High lipase activity is a critical prerequisite, if the enzymes are to be applied to industrial effluent treatment (Cammorata et al., 2006). Treatment of effluent by using enzymes was also reported by Cammorata et al., (2001), Leal et al., (2002) and Jung et al.,

(2002). As shown in Fig. 31-36, there was significant reduction in COD, TSS and oil & grease compared to control. Since in case of enzymatic treatment, there was 86% reduction of COD in 48 hr (Fig. 32), and thereafter it remained almost constant in 96 hr (Fig. 33), an experiment was set up to check earlier COD removal as enzymes are known to be fast in their reactions. Samples were withdrawn every four hr interval during the treatment. It was found that there were 40 and 60% reduction of COD in first 4 and 8 hr respectively as shown in Fig. 34. In 12 hr of treatment this reduction reached to 86 % which remained constant in subsequent time interval of 24 hr, 48 hr and 96 hr. This experiment confirmed that enzymatic treatment was very fast as compared to the microbial treatment. There was 45 and 75% reduction of TSS and O&G after 96 hr of enzymatic treatment as shown in Fig. 35 and 37. Cail et al., (1986) have reported the use of mixture containing protease, amylase, cellulase, lipase and *Bacillus subtilis* used to treat wastewater with high lipid content. This mixture increased the COD reduction from 59% in the control to 78%, increased grease removal from 47% to over 70%, and improved solids reduction from 34% to over 70%.

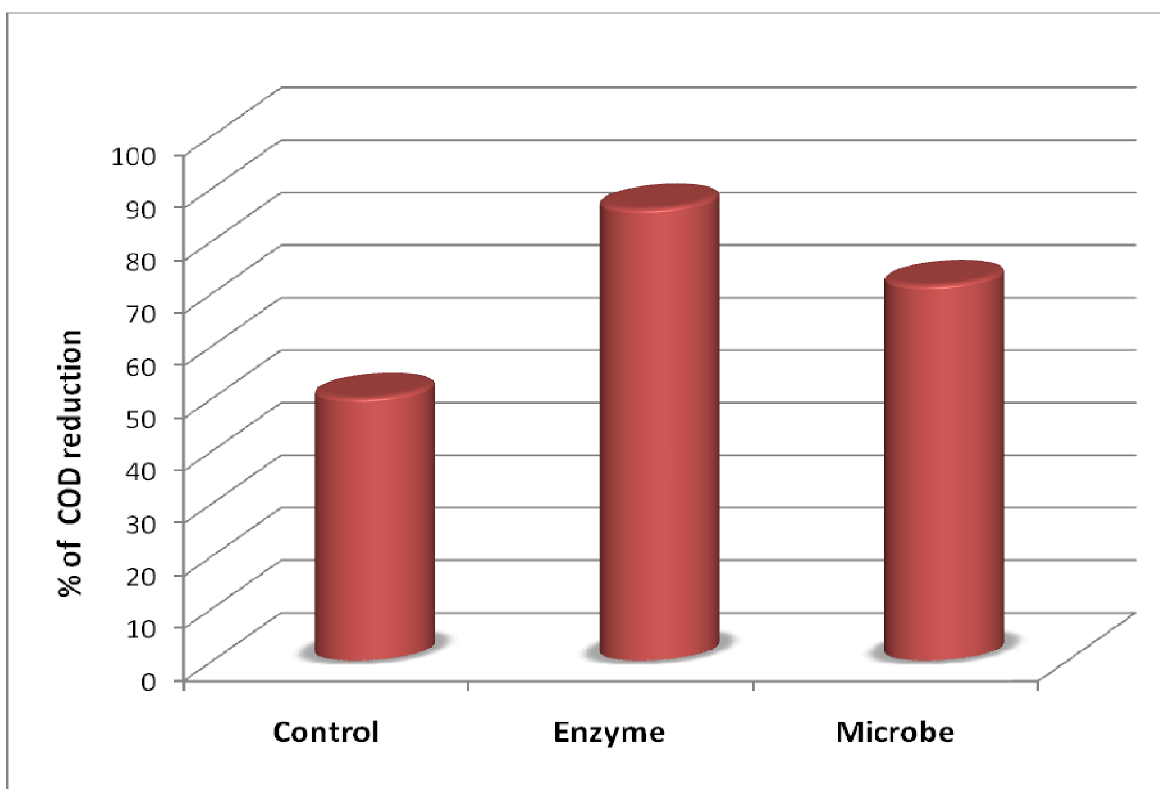


Fig. 32: COD reduction after 48 hr of wastewater treatment.

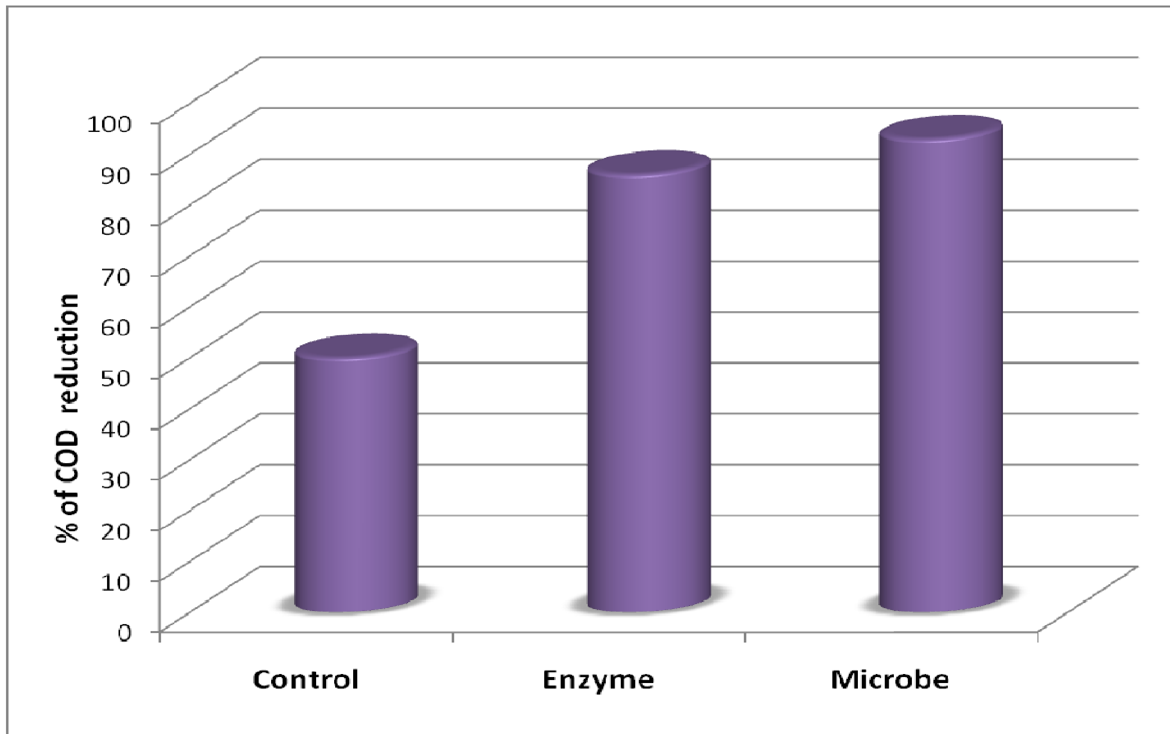


Fig. 33: COD reduction after 96 hr of wastewater treatment.

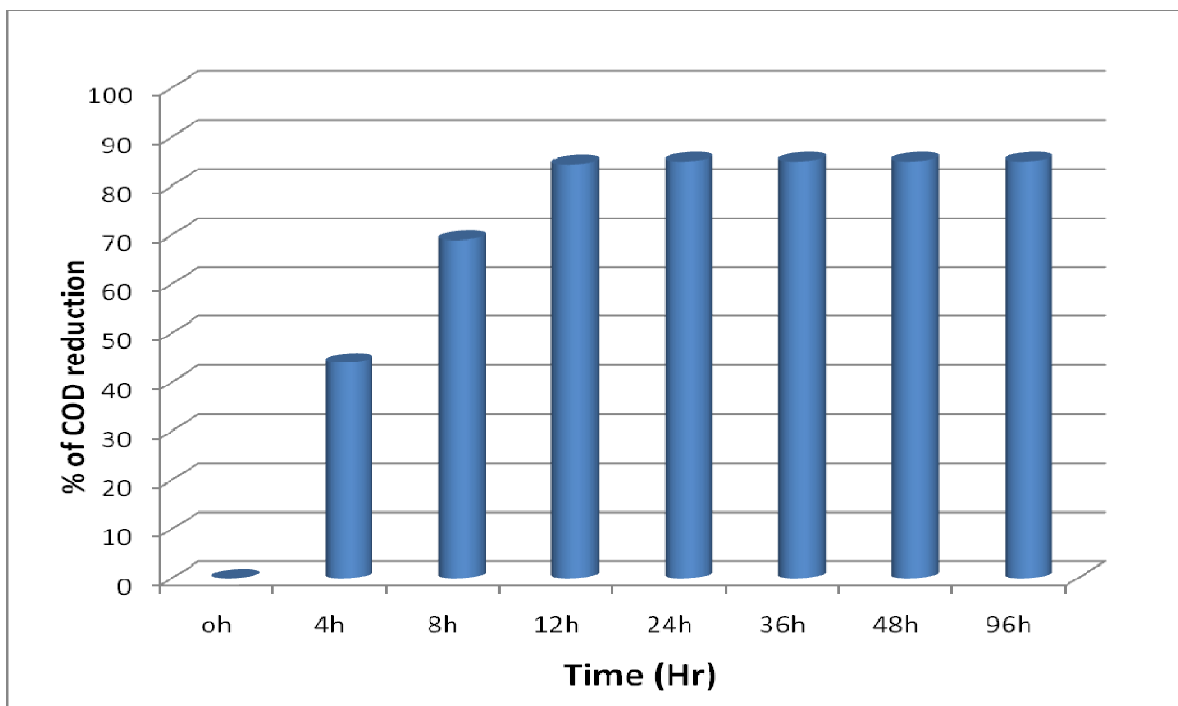


Fig. 34: Reduction of COD of wastewater by using enzymatic treatment.

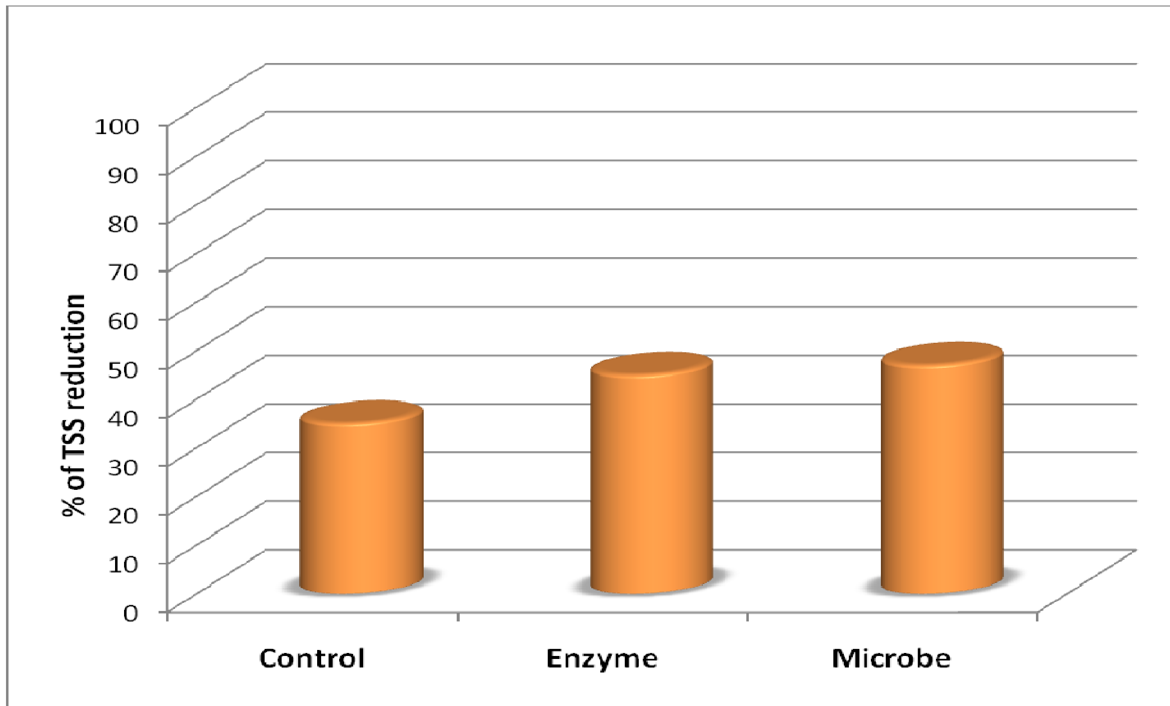


Fig. 35: TSS reduction after 96 hr of wastewater treatment.

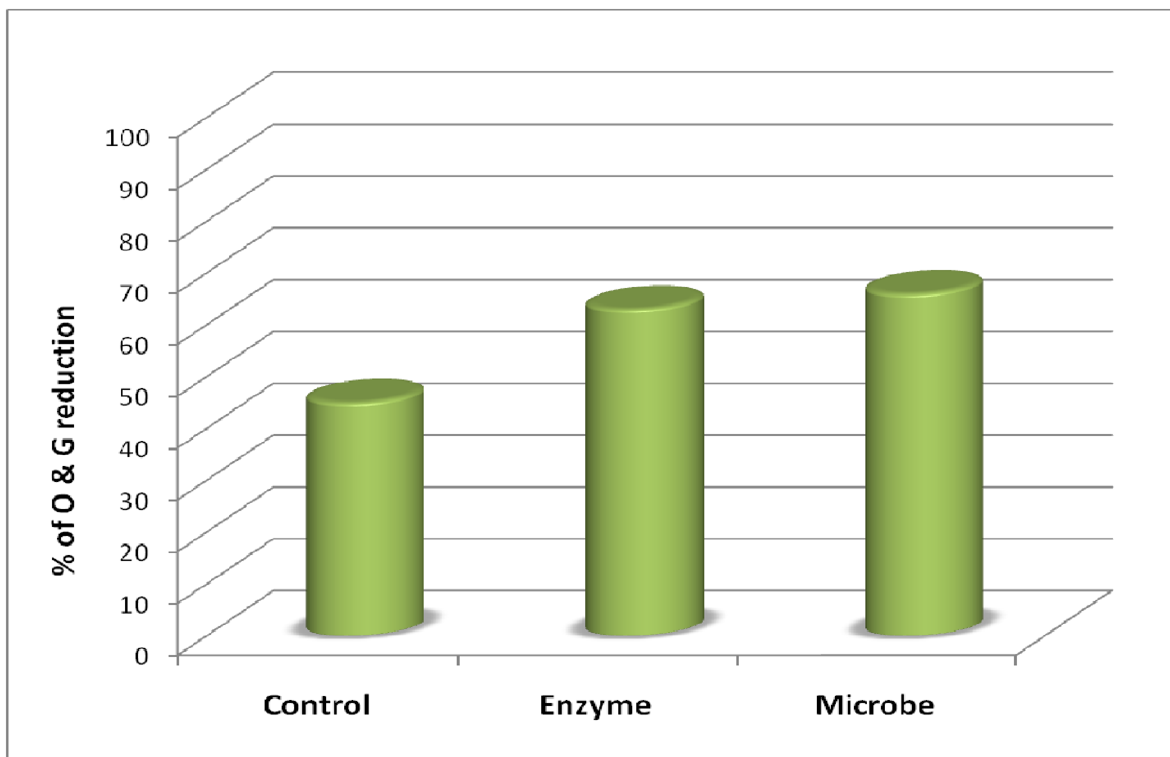


Fig 36: Oil and grease reduction after 24 hr of treatment.

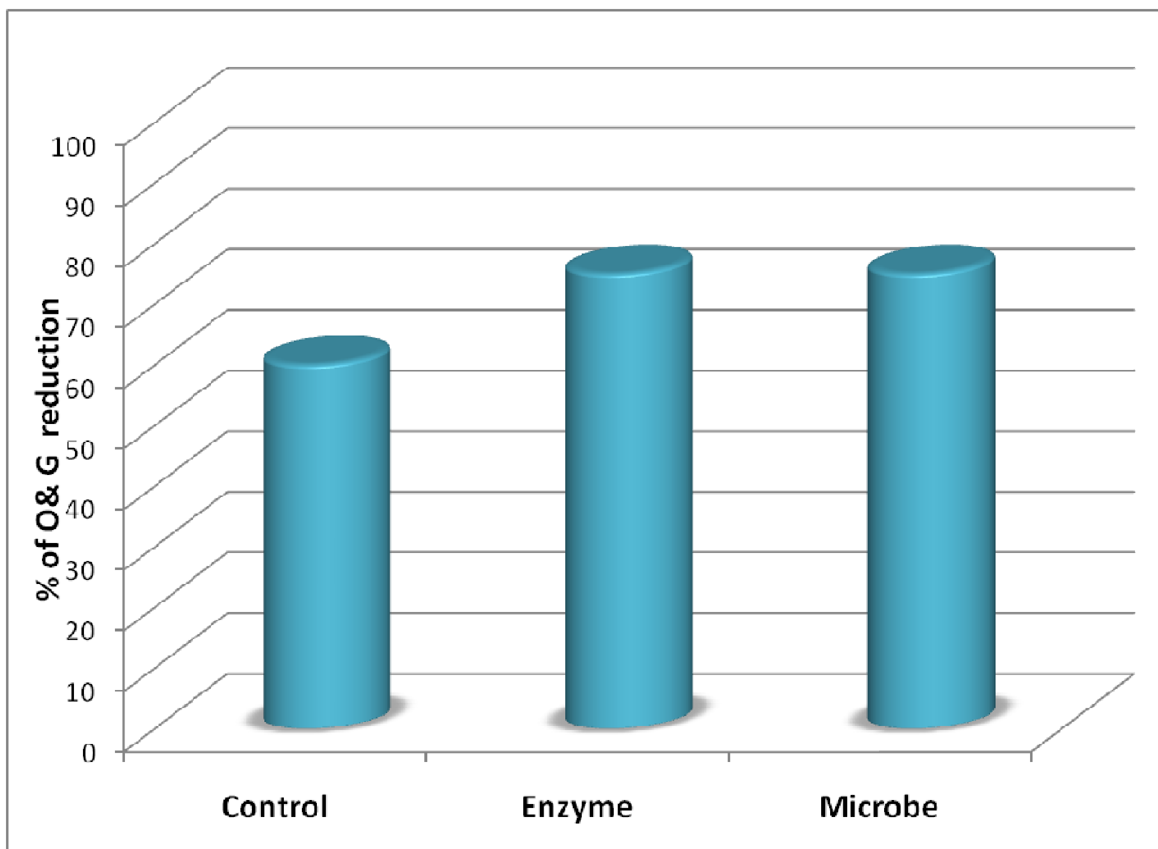


Fig. 37: Oil and grease reduction after 96 hr of treatment.

CONCLUSION

REFERENCES

AND

APPENDIX

Conclusion

In the present work, analysis of physicochemical and biological characteristics of the dairy wastewater was attempted. Isolation of lipase producing microbes from the samples collected from the dairy wastewater was also carried out in order to find out a suitable treatment method to reduce the organic load. Following are the overall outcomes of the thesis:

1. The collected dairy wastewater sample found to contain 1200 mg/L of COD, 850 mg/L of BOD, 425 and 219 mg/L of TSS and oil and grease respectively along with 20.2 mg/L of total nitrogen, total phosphorus content of 2.4 mg/L and 7500 MPN/100 ml waste water of total coliforms.
2. A total of eight microbial strains could successfully be isolated and purified.
3. Among the purified strains, four strains were found to be good lipase producers.
4. Strain S1, showing maximum lipase production was selected as potential strain for lipase production as well for dairy wastewater treatment.
5. For increased lipase production form strain S1, further optimization in terms of culture conditions was also attempted. Under optimized conditions, 195 U/ml/min of lipase activity was obtained in 24 hr of fermentation.
6. The enzyme was found to have a pH and temperature optimum of 10.0 and 55 °C respectively. The enzyme was found to be quite stable if stored at low temperature. But it retained only 30% of its initial activity after heating for 40 minutes at 55 °C.
7. Both the microbe (strain S1) and its enzyme were found to be very effective when tested for oil rich dairy wastewater treatment. Studies showed that the microbe could reduce COD, TSS, and Oil and grease content by 93%, 47%, and 75% respectively while lipase enzyme was found to reduce 86%, 45% and 75% of COD, TSS and O&G content respectively.
8. Further optimization in the form of temperature, nitrogen, carbon sources, addition of surfactants shaking speeds etc. can be looked on, for efficient and fastest removal of oil and grease and organic content from the waste water. After that study can also be extended to large scale treatment of dairy wastewater.

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Appendix

I. Biochemical oxygen demand (BOD):

The BOD is used as an approximate measure of the amount of biochemically degradable organic matter present in a sample. The 5- day incubation period has been accepted as the standard for this test (although other incubation periods are occasionally used). The BOD determined by the dilution method presented here has come to be used as an approximate measure of the amount of bio-chemically degradable organic matter in a sample. The dilution method of determining BOD is the one most generally used. The dissolved oxygen content of the liquid is determined before and after incubation for 5 days at 20 °C. The difference gives the BOD of the sample after allowance has been made for the dilution, if any, of the sample.

Sample handling:

The test should be carried out as soon as possible after samples have been taken. If samples are kept at room temperature for several hours, the BOD may change significantly, depending on the character of the samples. In some instances it may decrease and in others it may increase. The decrease at room temperature has occasionally been found to be as much as 40% during the first 8 hr of storage. If samples cannot be dealt with at once they should, whenever practicable, be stored at about 5 °C. In the case of individual samples collected over a long period, it is desirable to keep all the samples at about 5 °C until the composite sample can be made up for the BOD determination. Samples must be free from all added preservatives and stored in glass bottles.

Apparatus:

- Incubation bottles. It is recommended that narrow-mouthed, glass stopper bottles of a nominal capacity of 300 ml are used, and it is essential that the bottles are clean. New bottles should be cleaned with either 5 mol/L hydrochloric or sulphuric acid and thoroughly rinsed. In normal use, bottles are kept clean by the acidic iodine solution of the Winkler procedure and require no treatment apart from thorough rinsing with tap water and distilled water. Special cleaning may be necessary in some cases, but

the use of chromic acid is not recommended because traces of chromium may remain in the bottle. Some analysts prefer to use bottles of about 125 ml capacity, thus reducing the incubator space required. There is evidence, however, that with samples of some types the size of bottles (i.e. the ratio of the glass surface to the volume of liquid) may influence the result. The analyst wishing to use small bottles must, therefore, be satisfied that such a procedure gives results similar to those obtained by use of bottles of standard size. As a precaution against drawing air into the dilution bottle during incubation, a water seal is recommended. Satisfactory water seals are obtained by inverting the bottles in a water-bath or adding water to the flared mouth of special BOD bottles.

- Incubator or water-bath. The temperature of incubation should be 20 ± 0.5 °C. A water bath or constant temperature room is usually employed. Incubation must be carried out in the dark. Some samples may contain algae which, if incubated in the light, would give off oxygen by photosynthetic action, and thus interfere with the BOD determination.

Reagents:

- ✓ Dilution water.
- ✓ Ferric chloride solution: dissolve 0.125 g ferric chloride, $FeCl_3.6H_2O$, in 1 litre water.
- ✓ Calcium chloride solution: dissolve 27.5 g anhydrous calcium chloride, $CaCl_2$, (or equivalent if hydrated calcium chloride is used), in 1 litre water.
- ✓ Magnesium sulphate solution: dissolve 25 g magnesium sulphate, $MgSO_4.7H_2O$, in 1 litre water.
- ✓ Phosphate buffer stock solution: dissolve 42.5 g potassium dihydrogen phosphate, KH_2PO_4, in 700 ml water and add 8.8 g sodium hydroxide. This should give a solution of pH 7.2 which should be checked. Add 2 g ammonium sulphate, and dilute to 1 litre.

Add 1 ml of each reagent to each litre of freshly distilled (or deionised) water. Bring the water to incubation temperature 20 ± 1 °C and saturate with oxygen by bubbling air through it or by shaking the partially filled bottle, and use as soon as possible. Discard any dilution water remaining unused and clean the bottle, preferably with a sterilizing agent. Thoroughly wash and rinse free from residual traces of the agent, and store out of direct sunlight. Stocks

of dilution water should never be "topped up" with fresh solution. Satisfactory dilution water, when incubated with or without a seed under standard conditions should not absorb more than 0.2 mg/L of oxygen, and in any case must not absorb more than 0.5 mg/L. A high oxygen uptake may sometimes be associated with the presence of water soluble organic vapors in the laboratory atmosphere. Water for dilution should therefore be distilled (or deionised) and used in a room from which volatile organic compounds are excluded. Air used for aeration must be as clean as possible.

Procedure:

1. Pretreatment of dilution water by seeding is sometimes necessary

2. Samples that have been stored in a refrigerator should be allowed to reach room temperature before dilutions are made. All samples must be well mixed just before dilution.

3. In preparing dilutions for the BOD test, the following principles must be strictly adhered to:

a- The sample and dilution water must be mixed thoroughly, but violent agitation leading to the formation of minute air bubbles must be avoided.

b- The diluted mixture is transferred to two incubation bottles (more if replicate results are required) by siphoning or by careful pouring. If a siphon is used, at least 50 ml of mixture must be discarded before the first bottle is filled. Bottles must be filled completely, allowed to stand for a few minutes and then tapped gently to remove bubbles. The stoppers are then inserted firmly without trapping air bubbles in the bottle.

c- Bottles of the dilution water used in the test must be prepared at the same time as the sample dilutions to permit a determination of the blank.

4. Determine the initial concentration of dissolved oxygen in one bottle of the mixture of sample and dilution water, and in one of the bottles containing only dilution water. Place the other bottles in the incubator (those containing the sample, or the mixture of sample and dilution water, and that containing the plain dilution water to act as a blank, unseeded or seeded in accord with previous steps).

5. Incubate the blank dilution water and the diluted samples for 5 days in the dark at 20 °C. The BOD bottles should be water-sealed by inversion in a tray of water in the incubator or by use of a special water seal bottle. Although it is known that the BOD of some samples is

increased if the liquid is agitated during the incubation, it is not at present suggested that agitation should be provided.

6. After 5 days determine the dissolved oxygen in the diluted samples and the blank using the azide modification of the iodometric method or an electrometric method, those dilutions showing residual dissolved oxygen of at least 30 % of the initial value and a depletion of at least 2 mg/L should be considered the most reliable.

Calculation:

BOD = {(D1 – D2) – (B1 – B2)}/ P

Where:

D1 = DO of diluted sample immediately after preparation (mg/L)

D2 = DO of diluted sample after 5 days' incubation

B1 = DO of dilution of Blank before incubation;

B2 = DO of dilution of Blank after incubation;

P = decimal fraction of sample used.

II. Chemical oxygen demand (COD):

The chemical oxygen demand (COD) is the amount of oxygen consumed by organic matter from boiling acid potassium dichromate solution. It provides a measure of the oxygen equivalent of that portion of the organic matter in a water sample that is susceptible to oxidation under the conditions of the test.

Principle:

The sample is boiled under reflux with potassium dichromate and silver sulphate catalyst in strong sulphuric acid. Part of the dichromate is reduced by organic matter and the remainder is titrated with ferrous ammonium sulphate.

Apparatus:

- ✓ A reflux apparatus consisting of a 250-ml Erlenmeyer flask (500 ml if large samples are used) with ground-glass neck, and a 300-mm double surface condenser (Liebig, Friedrichs, West or equivalent) with a ground-glass joint. Since absolute cleanliness is essential, flasks and condensers should be protected from dust by inverted cups when not in use. The glassware must be used exclusively for COD determinations.

- ✓ A heating mantle or hotplate.
- ✓ A hotplate producing at least 1.5 W cm-2 of heating surface to ensure adequate boiling of the liquid in the flask. Heating mantles are preferred because they prevent the problem of overheating.

Reagents:

- ✓ Sulphuric acid (d =1.84).
- ✓ Standard potassium dichromate solution, 0.0417 mol/L. Dissolve 12.259 g of $K_2Cr_2O_7$ primary standard grade, dried at 103 °C for 2 hr, in distilled water and dilute to 1.000 litre.
- ✓ Dilute standard potassium dichromate solution, 0.00417 mol/L. Dilute 100 ml of the standard potassium dichromate solution to 1.000 litre.
- ✓ *Standard ferrous ammonium sulphate solution, 0.250 mol/L.* Dissolve 98 g of Fe $(NH_4)_2(SO_4)_2.6H_2O$ analytical grade crystals indistilled water. Add 20 ml of H_2SO_4 (d=1.84), cool and dilute to 1.000 litre.
- ✓ Dilute standard ferrous ammonium sulphate solution, 0.025 mol/L. Dilute 100 ml of the standard ferrous ammonium sulphate solution to 1.000 litre. Standardise daily against the dilute standard potassium dichromate, 0.00417 mol/L.
- ✓ Silver sulphate, reagent powder. This reagent may be used either directly in powder form or in saturated solution, or it may be added to the sulphuric acid (about 5 g of Ag_2SO_4 to 1 litre of H_2SO_4; 1–2 days are required for dissolution).
- ✓ Mercuric sulphate, analytical grade crystals.
- ✓ Ferroin indicator solution. Dissolve 0.695 g of ferrous sulphate, $FeSO_4.7H_2O$, in water. Add 1.485 g of 1, 10- phenanthroline monohydrate, shaking until dissolved. Dilute to 100 ml. This solution is also commercially available.
- ✓ Sulphamic acid, analytical grade (required only if the interference of nitrites is to be eliminated).
- ✓ Anti-bumping granules that have been previously heated to 600 °C for 1 hour.

Procedure:

1. Place in an Erlenmeyer flask 20.0 ml of the sample or an aliquot diluted to 20.0 ml with distilled water.
2. Add 10.0 ml of standard potassium dichromate solution, 0.0417mol/L, and a few anti-bumping granules. Mix well.
3. Add slowly, with caution, 30 ml of concentrated H_2SO_4 containing silver sulphate, mixing thoroughly by swirling while adding the acid. If H_2SO_4 containing silver sulphate is not used, add 0.15 g of dry silver sulphate and then, slowly, 30 ml of concentrated H_2SO_4.

Note: If the liquid has not been well mixed local heating may occur on the bottom of the flask and the mixture may be blown out of the flask.

4. Attach the condenser to the flask and reflux the mixture for 2 hr. Allow to cool and then wash the condenser with distilled water.

5. Dilute the mixture to about 150 ml with distilled water, cool to room temperature, and titrate the excess dichromate with standard ammonium ferrous sulphate using 2–3 drops of ferroin indicator. Although the quantity of ferroin is not critical, do not vary it among different samples even when analysed at different times. The end-point is when the colour changes sharply from blue-green to reddish-brown, even though the blue-green may reappear within several minutes.

6. Reflux in the same manner a blank consisting of 20 ml of distilled water together with the reagents and titrate as in step 5, above.

Calculation:

COD = {(a – b) × c × 8000} / V mg/L

Where:

a = ferrous ammonium sulphate (ml) used for blank

b = ferrous ammonium sulphate (ml) used for sample

c = molarity (mol/L) of ferrous ammonium sulphate

v = volume of sample (ml).

III. Total suspended solids (TSS):

The term "total suspended solids" (TSS) applies to the dry weight of the material that is removed from a measured volume of water sample by filtration through a standard filter. The test is basically empirical and is not subject to the usual criteria of accuracy.

Apparatus:

- Filter holder. All of the following types of filter holder are suitable:
- Glass-fiber filter discs, Whitman GF/C or equivalent, of a size compatible with the filter holder.
- Suction flask, 500-ml capacity.
- Drying oven, 103–105 °C.
- Desiccators.
- Analytical balance, capacity 200 g (or more), accuracy 0.1 mg.
- Vacuum pump or aspirator.

Procedure:

Preparation of glass-fiber filter discs

1. Place a filter disc on the filter holder. Assemble filter holder in suction flask apparatus, connect to vacuum source and apply vacuum.
2. Wash the filter disc with three successive 20-ml portions of distilled water. Continue to apply vacuum for 2–3 minutes after the water has passed through the filter. Discard the filtrate.
3. Remove the filter paper from the membrane filter funnel or the Buchner funnel and place it on a supporting surface in a drying oven. If the Gooch crucible/ filter combination is being used, place it in the drying oven. The oven should be maintained at 103–105 °C and drying should be continued for at least 1 hr.
4. Cool the filter or Gooch combination in a desiccator and weigh it on an analytical balance.
5. Repeat the cycle of drying, desiccating and weighing until the weight loss between two successive series of operations is less than 0.5 mg.
6. Store filter(s) or Gooch crucible(s) in the desiccator until required.

Sample analysis

1. Remove the filter disc or Gooch crucible from the desiccators, weigh it and record its weight.
2. Place the filter in the filter holder and assemble the filter holder in the suction flask apparatus. Connect to the vacuum source and apply vacuum.
3. Wet the filter with a few drops of distilled water to seat the filter.
4. Shake the sample vigorously and measure out 100 ml in a 100-ml graduated cylinder or volumetric flask. Pour this portion of the sample into the filter funnel, being careful not to disturb the seating of the filter disc.
5. Rinse out the measuring flask or cylinder with a small quantity of distilled water. If the sample is very low in suspended material, a larger volume of sample may be used.
6. When filtration is complete, carefully remove the filter disc from the filter holder with tweezers (or remove the Gooch crucible from its supporting socket with a pair of tongs) and places it in the drying oven. Dry for at least 1 hr at 103– 105 °C. Cool in a desiccator and weigh.
7. Repeat the drying, desiccating and weighing cycle until the weight loss between two successive weightings is less than 0.5 mg.
8. Record the final weight obtained.

Calculation

Total suspended solids (mg/L) = $\{(A - B) / C\} \times 106$

Where

A = weight of filter + solids (g)

B = weight of filter (g)

C = volume of sample filtered (ml)

IV. Oil and Grease (O&G):

The determination of oil and grease includes all the substances that are extractable by the specified solvent. General, the substances extractable are oil, fat and waxes .It is to be noted that the results obtained indicates only the non-volatile fraction of these materials. Determination of the volatile fraction is beyond the scope of this book.

Principle:

Oil, grease and other extractable matters are dissolved in suitable solvent and separated from the aqueous phase. The solvent layer is then evaporated and the residue is weighed as oil and grease.

Reagents:

1. Sulphuric acid 1+1: Add carefully 250 ml. conc.H_2SO_4 to 250 ml. distilled water and cool.
2. Petroleum ether: Boiling point 35 ^oC to 60 ^oC.

Procedure:

1. Place 1000 ml or appropriate volume of the sample in a suitable separating funnel.
2. Add 5 ml sulphuric acid per litre of sample.
3. Rinse the sample bottle with 15 ml. petroleum ether and add the rinsings to the separating funnel. Add further 25 ml ether to the funnel and shake vigorously for 2 minutes.
4. Draw the aqueous phase in to a clear container and transfer the ether layer to a clean, tared distilling flask.
5. Continue the extraction twice and add the ether extracts to the distilling flask.
6. Distill off the ether in the distilling flask .Dry in water bath.
7. Cool in a dessicator and weigh.

Calculation:

$$\text{mg/L oil and grease} = \frac{\text{mg.residue in the distilling flask}}{\text{ml.sample taken for determination}} \times 1000$$

V. Total Nitrogen water and waste water (Total Kjeldahi Nitrogen):

Principle:

The nitrogen of the organic matter is converted into ammonium sulphate when treated with sulphuric acid in the presence of copper sulphate catalyst. An excess of alkali is then added (to liberate the ammonia from ammonium sulphate) and distilled and the distillate is either

treated with Nessier reagent or titrated with standard sulphuric acid after absorbed in boric acid solution.

Equipment:

- Pipettes of 1 ml, 5 ml, 10 ml.
- Kjeldahl flask, 500 ml.
- Mantle Heater.
- Distillation flask with condenser, 500ml.
- Conical flask, 250 ml,

Reagents:

- Sulphuric acid concentrated (L. R. grade).
- Copper sulphate solution (10%).
- Potassium sulphate crystal (L .R. grade).
- Phenolphthalein indicator solution.
- Sodium hydroxide solution (50%).
- Boric acid solution (2%).
- Methyl red indicator solution.
- Standard sulphuric acid solution 0.02 N.
- Sulphuric acid – N.

Reagent preparation:

1. Copper sulphate solution 10 %: Dissolve 10 g copper sulphate of (L.R grade) ($CuSO_4.5H_2O$) in 100 ml. distilled water.
2. Phenolphthalein indicator solution: Dissolve 500 mg Phenolphthalein (L.R grade) in 50 ml ethyl or isopropyl alcohol (G.R. grade)and add 50 ml distilled water .Add 0.02 N sodium hydroxide solution drop wise until a faint pink colour appears.
3. Sodium hydroxide solution 50 %: Dissolve 100g of (L.R. grade) sodium hydroxide n 200 ml distilled water. Place it in a rubber stopper bottle.
4. Boric acid solution 50 %: Dissolve 10 g boric acid [H_3BO_3 (L.R. grade)] in ammonia free distilled water and dilute to 500 ml.

5. Methyl red indicator solution: Dissolve 50 mg of analytical laboratory reagent grade methyl red in 100 ml ethyl alcohol (G.R. grade).
6. Standard sulphuric acid solution (0.02 N): Dilute appropriate volume of 1N, H_2SO_4 (L.R. grade) (about 20 ml) to 1000 ml in a volumetric flask with carbon di-oxide free distilled water to give a 0.02 N solution.
7. Sulphuric acid N: place 28 ml concentrated sulphuric acid (L.R. grade) in a1000 ml volumetric flask and make up to the mark with carbon di-oxide free distilled water.

Procedure:

a) Digestion:

Place 100 ml of the sample in a Kjeldahl flask. Dilute to about 200 ml with ammonia free distilled water. Add 10 ml concentrated sulphuric acid and add 1 ml copper sulphate solution.

If the organic matter is hard to destroy add 20 ml conc.H_2SO_4 and 5g Potassium sulphate. Add a few glass beads and boil under hood, until the solution becomes clear. Allow to cool.

b) Distillation:

i. Transfer the contents of the flask carefully into a distillation flask and dilute it about 300 ml.
ii. Add 4-5 drops phenolphthalein indicator and titration with 50 % NaOH (colour is now violated).
iii. Start the distillation after immersing the tip of the condenser in 50 ml boric acid solution in a conical flask.
iv. Collect about 200 ml of the distillate.

Titration:

Add 0.5 ml methyl red indicator solution to the distillate. (The colour is now yellow). Titrate against 0.02 N-sulphuric acids. The end point is the appearance of red colour. Conduct a blank also.

Calculation:

(Total Kjeldahl nitrogen as N (mg/l)

$$= \frac{1000 \times 0.28 \times (0.02 \text{ ml. N H2SO4 for sample} - 0.02 \text{ ml. N H2SO4 for blank})}{\text{ml. sample taken for titration}}$$

VI. Phosphate in water and waste water (Stannous Chloride Method)

Principle: Ammonium molybdate reacts with phosphate to form molybdophosphoric acid which is reduced to a blue coloured complex in a molybdenum blue by the addition of stannous chloride.

Interference: Arsenic, Flouride, Sulphide, Thiosulhate and Molybdate causes negative interference. Sulphides may be removed by oxidation with bromine water.

Equipment

1) Nessler tube-50 ml.
2) Pipet of 5 ml and 10 ml.
3) Spectrophotometer to be used at 690 nm.

**Reagent **

1) Ammonium molybdate (L.R. grade).
2) Stannous chloride solution.
3) Glycerol L.R. grade.
4) Anhydrous potassium dihydrogen phosphate.

Reagent preparation

1. Ammonium molybdate solution: Dissolve 25 g of an (L.R. grade) ammonium molybdate in 200 ml distilled water.
2. Add 250 ml concentrated sulphuric acid (L.R. grade) to 400 ml distilled water carefully and cool it. Now ammonium molybdate solution is added to the diluted acid volume is made with distilled water to 1000 ml.

3. Stannous chloride solution: Dissolve 2.5 g fresh stannous chloride an (L.R. gread) in 100 ml glycerol and heat gently on a water bath.

4. Phosphate stock solution: Dissolve 439 mg anhydrous analytical laboratory Grade potassium dihydrogen phosphate in 1000 ml distilled water. Add two drops of toluene as a preservative.

   1.0 ml =100 µg p = 306 µg PO_4

5. Phosphate standard solution: Pipete 10 ml stock solution into 1000 ml volumetric flask and make up to the mark with distilled water.

   ml = 1 µg P = 3.06 µg PO_4

Procedure

1. Ensure that the spectrophotometer is within calibration data.

2. Into a series of 50 ml Nessler tubes, taking 2 ml, 4 ml, 6 ml, 8 ml, 10 ml of phosphate working solution and make up to 50 ml including a nessler tube containing 50 ml distilled water as a blank.

3. To the blank, standard and sample add 2 ml ammonium molybdate solution, and 5 drop stannous chloride solution mixing after each addition.

4. After 10 minutes, measure the color using a Spectrophotometer at 960 nm.

5. Preparing a calibration curve and find out the number of microgram of 'P' equivalent to the observe optical density of the sample.

Calculation

P mg/l =Absorbance of sample × Mean factor.

Express the result as mg phosphate as, p / lit of sample.

If it has to be expressed n terms of PO_4 multiplying by 3.06

i.e. PO_4 mg /L = P × 3.06

VII. Most Probable Number (MPN)

One fast quantitative method for routinely examining water for fecal organisms is the most probable number (MPN) technique. Adapted as a standard method for the examination of drinking water and wastewater by American public Health Association, it is basically a statistical approach to the quantitative estimation of bacterial numbers. Samples are serially diluted to the point at with there are a few or no viable microorganisms. The detection of this end point is based on multiple serial dilutions that are inoculated in to a suitable growth medium. Statistical table are available to estimate the size of bacterial populations based on the number of replicate tube (3, 5, or 10) of each dilution. The MPN method has the advantage of being a bit faster for the microbiologist to perform and evaluate. This exercise can be done in concert with Exercises 17, 19, 57, and 59.

Procedure

1. Add 1 ml of the water sample containing *E.coli* to a 99 ml water dilution bottle. This is your dilution 1 bottle (1×10^{-2}).
2. Shake the bottle vigorously, and aseptically transfer 1 ml of dilution to a second 99 ml dilution bottle (1×10^{-4}). Sake the bottle vigorously.
3. For each of the three bottles (original sample, dilution 1, and 2), inoculate tube as follows:
    a) Add 10 ml from each bottle to each of three tubes of double-strength phenol red lactose broth with Durham tubes.
    b) Add 1 ml from each bottle to each of three tubes of single-strength phenol red lactose broth with Durham tubes.
    c) Add 0.1 ml from each bottle to each of three tubes of single-strength phenol red lactose broth with Durham tubes.
4. Incubate the tubes at 37°C for 24 hr.
5. After incubation, examine the tubes for acid and gas production. Record your results and then select the dilution in which the tubes are neither al positive nor all negative.

Using the series with mixed results, compare with the MPN table (Table I) and record the most probable number of coliforms in your sample. Multiply by the dilution factor if necessary

Number of Tubes Giving Positive Reaction			MPN Index Per 100 ml	95 % Confidence Limits	
3 of 10 ml each	3 of 1 ml each	3 of 0.1 ml each		Lower	Upper
0	0	1	3	0.5	9
0	1	0	3	0.5	13
1	0	0	4	0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	260	71	2400
3	3	2	1100	150	4800

Table I: MPN determination from multiple tube tests.

Source: Standard methods for the examination of water and wastewater.

(New York: APHA, 1995).