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EFFECTS OF AN ENZYME-ENHANCED RINSE OF CREAM STORAGE TANK ON DAIRY WASTEWATER

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EFFECTS OF AN ENZYME-ENHANCED RINSE OF CREAM STORAGE TANK ON DAIRY WASTEWATER

By

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ABSTRACT

EFFECTS OF AN ENZYME-ENHANCED RINSE OF CREAM STORAGE TANK ON DAIRY WASTEWATER

By

Sirinart Thanesvorakul

An enzyme-based cleaner, Xzyme™, was applied to a model cream tank rinse to assess the effects on the components, strength and treatability of the model wastewater stream. Analysis of biological oxygen demand (BOD) was carried out using model cream tank rinse water treated with and without 10% enzyme cleaner. At dilution rates of 1:60,000, 1:80,000, and 1:100,000, the reduction in BOD for the treated cream compared to the untreated sample was not found to be significantly different ($p<0.05$). The ability to degrade milk proteins and fats by Xzyme™ was observed using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and thin layer chromatography (TLC), respectively. The SDS-PAGE gels showed extensive degradation of milk proteins in the sample lanes treated with Xzyme™. The higher molecular weight bands representing native milk proteins disappeared accompanied by the appearance of numerous bands representing milk protein fragments. Similar band patterns for the Xzyme™-treated and untreated samples on TLC plates indicated no lipolytic enzyme activity from the enzyme-based cleaner.

I dedicate this work to my parents and my sister and brother
for their love and support.

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ABBREVIATIONS

AA:	Amino Acid
AF:	Anaerobic Filter
AFBR:	Anaerobic Fluidized Bed Reactor
ANOVA:	Analysis of Variance
BOAAS:	Buffered Organic Acid Anionic Surfactant
BOD:	Biological Oxygen Demand
BSA:	Bovine Serum Albumin
CIP:	Clean-In-Place
COD:	Chemical Oxygen Demand
DAF:	Dissolved Air Floatation
DO:	Dissolved Oxygen
EPS:	Exopolysaccharide
GGA:	Glucose-Glutamic Acid
LCFA:	Long-Chain Fatty Acid
NFDM:	Non-Fat Dry Milk
NPN:	Non-Protein Nitrogen
POTW:	Publicly Own Treatment Works
SBR:	Sequencing Batch Reactor
SCFA:	Short-Chain Fatty Acid
SDS:	Sodium Dodecyl Sulfate
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TLC:	Thin Layer Chromatography
TS:	Total Solids
TSS:	Total Suspended Solids
UF:	Ultrafiltration
USB:	Upflow Sludge Blanket
USEPA:	United States Environmental Protection Agency

INTRODUCTION

Dairy wastewater is generated from many locations in the processing plant, including milk receiving, storage, processing, cleaning and maintenance. Dairy wastewater is defined as the wastewater typically containing high concentrations of organic materials originating from milk (i.e. lactose, fats and proteins) (Perle *et al.*, 1995 and Baick *et al.*, 1992). The strength of the wastewater is characterized by biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Some dairy processors send wastewater directly to a local wastewater treatment plant or may be required to install a pretreatment process. Local wastewater treatment capacity may require other processors to install a full scale treatment system to reduce the wastewater strength before releasing the treated effluent to natural waters.

Cleaning chemicals, such as chlorinated alkaline cleaner, used in the dairy processing plants are corrosive and toxic. Enzyme-based cleaning systems have been developed and tested in several parts of the dairy processing industry. The enzyme-based cleaners have been tested on filtration systems (Arguello *et al.*, 2002; Munoz-Aguado *et al.*, 1996; Smith and Bradley, 1987), equipment surfaces (Graßhoff, 2002; Potthoff *et al.*, 1997), and in wastewater treatment systems (Loukidou and Zouboulis, 2001; West, 1988).

To their advantage, the enzyme cleaners are safer to use since they are not toxic, corrosive or irritating. The enzymes also work near neutral pH and at biological temperatures (25°C-42°C) as opposed to pH levels of12-13 and

temperatures of 65°C-80°C in a typical alkaline-based cleaning system. With this lower working temperature range, the life of the equipment and gaskets can also be prolonged. The components of the enzymatic cleaning systems are biodegradable and add little to the BOD/COD content of wastewater (Stilwell *et al.*, 2000 and Potthoff *et al.*, 1997).

The purpose of this research was to evaluate the use of an enzyme-based cleaner, Xzyme™ (Renew Systems Inc., Bay City, MI), on model cream tank rinse and the effects on the dairy wastewater strength. Preliminary testing using enzyme-based cleaner as an adjunct to rinse water was carried out on the first burst rinse of cream storage tank at an industrial site producing ice cream. With the hope to use the enzyme-based cleaner as an adjunct to the rinse water and look for the cleaner's effectiveness, the objectives of this work were: (1) to investigate if Xzyme™ would enhance the treatability of the waste as measured by biological oxygen demand (BOD), and (2) to investigate the effects of Xzyme™ on dairy components, mainly milk proteins and fats. If the research indicated that the enzyme-based cleaner was useful, the dairy plants would benefit in many ways, including reducing wastewater strength and lowering the surcharges imposed by wastewater authority.

LITERATURE REVIEW

DAIRY SOIL CHARACTERISTICS

The majority of the soil deposited in dairy plant process equipment consists of milk constituents. Organic milk residues are comprised mostly of lactose, protein, and milkfat. Inorganic residues include the minerals contained in milk, such as calcium and magnesium. Lactose is easily removed with rinse water due to its solubility. Most of the milkfat, with a melting point of approximately 32°C, is also easily removed with a warm water rinse. On the other hand, proteins may be difficult to remove, especially, if they become cooked-on or denatured during heat treatments, such as pasteurization or sterilization. Denatured proteins adhere to surfaces and can combine with minerals and constituents of cleaning water and form a residue known as milkstone (Mauck *et al.*, 1993). Milkstone appears as white or grayish film and is usually a porous deposit which will harbor microbial contaminants. Acid cleaner will remove the milkstone by dissolving alkaline minerals (e.g. calcium, potassium, or sodium) and remove the film. To prevent or reduce milkstone deposits, product heating surfaces should be cooled before and immediately after emptying of heated processing vats. Foams and other products should be rinsed with warm (not hot) water after production shift before they dry (Marriott, 1999). Drying enhances film formation and attachment of biofilm.

TYPICAL CLEAN-IN-PLACE (CIP) CLEANING PROCEDURES FOR STORAGE TANK IN A MILK PROCESSING PLANT

Typical guidelines for the practice of cleaning and sanitizing in fluid milk processing plants can be obtained from The Dairy Practices Council (Mauck *et al.*, 1993). After use, the storage tank is immediately pre-rinsed with warm, clean water (approximately 110°F or 43.3°C) to remove as much of the free soil as possible. This pre-rinse prevents residues from drying on and adhering to equipment, and therefore minimizing film and/or milkstone formation. The next step is to clean with chlorinated alkaline cleaner (1500-1800 ppm alkaline, 30-50 ppm chlorine, at 145°F (62.8°C) for 15 min) to saponify any residual fat and solubilize proteins. Then the equipment is rinsed with cold water followed by an acidified rinse (pH 4.0-5.0) for mineral removal. The acid rinse leaves the surfaces at a slightly acidic pH which improves bactericidal action of many chemical sanitizers. The equipment will be sanitized just prior to use with chemicals (e.g. chlorines, acids, and quaternary ammonium compounds) or heat (approximately 170°F (76.7°C) water) (Mauck *et al.* 1993).

DEFINITION AND CHARACTERISTICS OF DAIRY WASTEWATER

Dairy wastewater is defined as wastewater typically containing high concentrations of organic materials originating from milk (i.e. lactose, fats and proteins) (Perle *et al.*, 1995 and Baick *et al.*, 1992). The wastewater is

characterized as having a high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total solids (TS); and is sometimes high in nitrogen (N) and phosphorus (P) content (Donkin, 1997). The pH is variable (pH 4.4-9.4) and temperature is elevated. Typical characteristics of dairy waste generated from different products are listed in Table 1 (Hale *et al.*, 2003).

Table 1: Typical characteristics of various dairy products.

Product	Total solids (g/L)	Fat (g/L)	Protein (g/L)	Lactose (g/L)	Total N (g/L)	P (mg/L)	BOD_5 (mg/L)	COD (mg/L)
Whole milk	125	35	36	47	6	950	114000	183000
Skimmed milk	92	0.5	36	47	6	980	90000	147000
Cream 30%	379	315	28	33	4	672	400000	750000
Sweet whey	62	0.5	7.5	47	1.2	490	42000	65000
Casein whey permeate	48	-	0.7	-	0.4	-	26400	44000
Skimmed milk powder (/kg)	957	10	350	519	60	9950	700000	950000

Source: Hale *et al.* (2003).

Biological oxygen demand (BOD) represents the readily decomposable organic content of wastewater, and is determined by measuring the amount of oxygen utilized during the decomposition of organic material by microbial population, over a specified time period, usually 5 days. The BOD measured for 5 days are referred to as BOD_5. Chemical oxygen demand (COD) is defined as the amount of a specified oxidant, dichromate ion ($Cr_2O_7^{2-}$) that reacts with the

sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. Both organic and inorganic materials are subject to oxidation by this methodology (APHA, 1998).

SOURCES OF DAIRY WASTEWATER

Sources of dairy waste range from milk receiving, storage, to product processing and even product defects/returned product. Trucks are usually the transport of choice to transport raw milk from the farm to the processing facilities. Wastewater generated from milk receiving typically results from incomplete drainage and washing of the truck tanker (Table 2) and receiving equipment. After the raw milk has been received, it is cooled in a plate heat exchanger using chilled water and then stored in insulated tanks. At this point, waste is also generated from the cleaning and sanitizing of storage vessels (Table 3) and transfer lines. During the processing of milk, waste can be produced from spillage, heat deposition (i.e. milkstone), sludge from the milk separator, and milk and water purge at the start and end of production. Loss of products that do not meet the required specification is also another factor.

Table 2: Example of the effluent from clean-in-place (CIP) of a 12,000 liter tanker.

Description	Amount of water	pH	COD
Pre-rinse water	1650 L	8.6	330 mg/L

Source: Hale *et al.* (2003).

Table 3: Example of the effluent from CIP of a 100,000 liter raw milk storage tank.

Description	Amount of water (L)	pH	Fat (%)	COD (mg/L)
Pre-rinse water	650	8.6	0	530
Final rinse water	1200	7.6	0	320

Source: Hale *et al.* (2003).

Milk consisting of fat, protein, lactose and other minor components contribute organic load to the wastewater. However, routine operating, cleaning and maintenance functions may also add to the contamination of water providing inorganic material. Examples of these contaminants are: (1) sodium hydroxide, sodium hypochlorite, quaternary amine cleaners, and surfactants from cleaning and sanitizing, (2) lubricants, (3) refrigerants, and (4) propylene glycol from the coolant system (Bowie, 1988).

WASTEWATER TREATMENT METHODS

Currently, several available treatments have been documented in the literature as ways to treat dairy wastewater. Generally, the treatment of dairy wastewater is divided into three parts: physical, biological and advanced treatments. They are often referred to as primary, secondary and tertiary treatment (Amos, 1997). However, a pretreatment step is often required.

Pretreatment process

The purpose of the pretreatment is to remove suspended solids and/or condition the wastewater for further treatment. Examples of pretreatment processes are (1) screening, (2) gravity settling, (3) and neutralization.

Screening is usually achieved early in the treatment process. Screens are installed at the entrance of the wastewater treatment plant to remove solids of various types and sizes. The screening helps protect the equipment from large suspended solids, and reduce the solid loading and risk of plugging in the downstream treatment.

Gravity settling separates grit from organic matter. Grit is the heavy mineral material in raw sewage, and may contain sand, gravel, small fragments of metal, and other small inorganic solids. The settling process is accomplished by exploiting the specific gravity of the materials in the wastewater. The grit in wastewater has a specific gravity in the range of 1.5-2.7 while the specific gravity is 1.02 for the organic matter. Differential sedimentation thus occurs, separating grit from organic matter.

Neutralization is usually carried out to enhance the quality of dairy wastewater prior to discharge to the municipal sewer system or chemical or biological treatment. Neutralization mainly attempts to level out the fluctuations in pH, flow, and pollutant. The biological system in secondary treatment needs to be maintained within the pH range of 6.5-8.5. To achieve neutralization, equalization tanks are employed. The tanks are equipped with waste water

samplers, pH controllers and corrective dosing units to ensure proper measurement of wastewater variables and subsequent adjustment of pH (Liu & Liptak, 2000; Ramalho, 1983; and Walker, 2001).

Primary Treatment

The physical/chemical treatment is completed in a three-step process consisting of coagulation, flocculation and liquid-solid separation. Coagulation is achieved via the precipitation of the waste components by adding chemicals such as iron or aluminum salts. These chemicals react with suspended solids in the wastewater to form very small particles, called floc. The next step involves flocculation, which is accomplished by gentle mixing of wastewater to aid in floc growth. These denser floc particles will then pass through the liquid-solid separation process. The liquid-solid separation is usually accomplished by means of gravity settling or a process of aeration such as air floatation or dissolved air floatation (DAF) (Figure 1). The lighter particles will rise to the top in the air systems due to their attraction to small bubbles created by the pressurized air applied. These air bubbles are 40-70 microns in size. Thus, as the lighter particles are lifted with the air to the top, they are scraped off with a sludge scraper. The denser particles settle at the bottom of the tank and are removed by a sludge transfer pump (Walker, 2001).

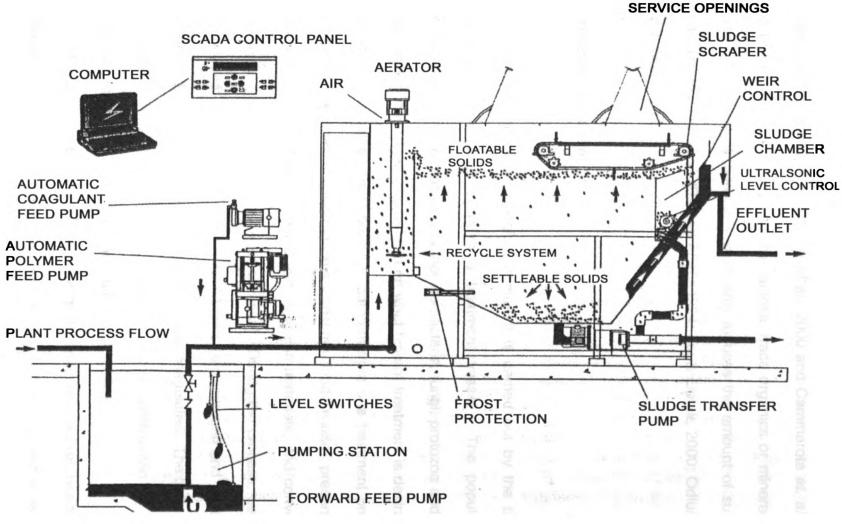

Figure 1: Schematic Diagram of Dissolved Air Floatation Unit.

The physical/chemical treatment, however, is an expensive method because of the chemical cost (Vidal *et al.*, 2000 and Cammarota *et. al.*, 2001). The process will not remove soluble lactose, trace organics or minerals in the waste. The physical/chemical system only reduces the amount of suspended solids and BOD (5-10%) (Barnett *et al.*, 1994; Liu & Liptak, 2000; Odlum, 1991; and Walker, 2001).

Secondary Treatment

The secondary or biological treatment is carried out by the biological activity of microorganisms within a treatment vessel. The population of microorganisms in the reactor may include bacteria, fungi, protozoa and rotifers, algae, and invertebrates. Depending on what type of treatment is desirable, the population of microbes could be made different from one treatment process to another. Essentially, microorganisms consume food (waste) present in the wastewater in the forms of organic and inorganic materials, and convert them into various gases and cell tissue (or biomass). During the process, wastes are decomposed; carbonaceous BOD and nutrients (e.g. nitrogen and phosphorus) are removed, and nonsettlable colloidal solids are coagulated. The dairy industry may employ aerobic or anaerobic treatment, or a combination of both for biological treatment (Amos, 1997 and Liu & Liptak, 2000).

Microbial population in the biological treatment vessel needs to be acclimated to the waste being treated. Acclimation is a period in which the

microorganisms adapt to a new environment and biodegradation of a molecule is not detected (Buitron and Capdeville, 1995). Once acclimated, the biomass work at the optimum performance (Lau *et al.*, 1996, Omil *et al.*, 1995, Thiem and Alkhatib, 1988), i.e. degrading the wastewater faster, reaching a steady state faster, and being more tolerant to sudden changes in the load (Panswad and Anan, 1999). Without acclimation, the biomass activity could be inhibited by its own organic substrates and steady-state condition would not be attained (Di Palma *et al.*, 2002 and Beccari *et al.*, 1983).

Aerobic treatment comes in the forms of such technologies as trickling filters, activated sludge, and sequencing batch reactors. An aerobic system requires oxygen for microorganisms as they break down the organic matter in the wastewater. The end products produced from aerobic system are carbon dioxide, water and bacterial biomass. The aerobic treatment is more suitable for the low to moderate strength wastewaters due to increased costs of equipment and power for aeration. Examples of low to moderate strength wastewaters are domestic waste, cheese waste, and milk powder/butter with BOD_5 of 400, 8000, 1500 mg/L, respectively (Barnett *et al.*, 1994 and Donkin, 1997)

Activated sludge process is probably the most common aerobic treatment (Figure 2). The goal for the process is to remove organic matter from wastewater by employing a mass of microorganisms (activated sludge). The activated sludge is a flocculated mass of microbes comprised mainly of bacteria and protozoa (Liu and Liptak, 2000). The activated sludge is kept in suspension in an aeration tank and allowed to grow as the biomass consumes the organic material

in the wastewater. The sludge is then settled out in a clarifier. A portion of activated sludge is recycled back to the influent end of the reactor to maintain the required activated-sludge concentration while the excess sludge is removed. The end products from this process treatment are carbon dioxide, water, excess sludge, and the clarified effluent for discharge or further treatment (Henze *et al.*, 2002, and Stephenson and Blackburn, Jr., 1998). BOD reduction in dairy wastewater can vary from 70% to 99% (Odlum, 1991 and Fang, 1990) while COD reduction can be as high as 90% to 98% (Carta *et al.*, 1999; and Donkin and Russell, 1997).

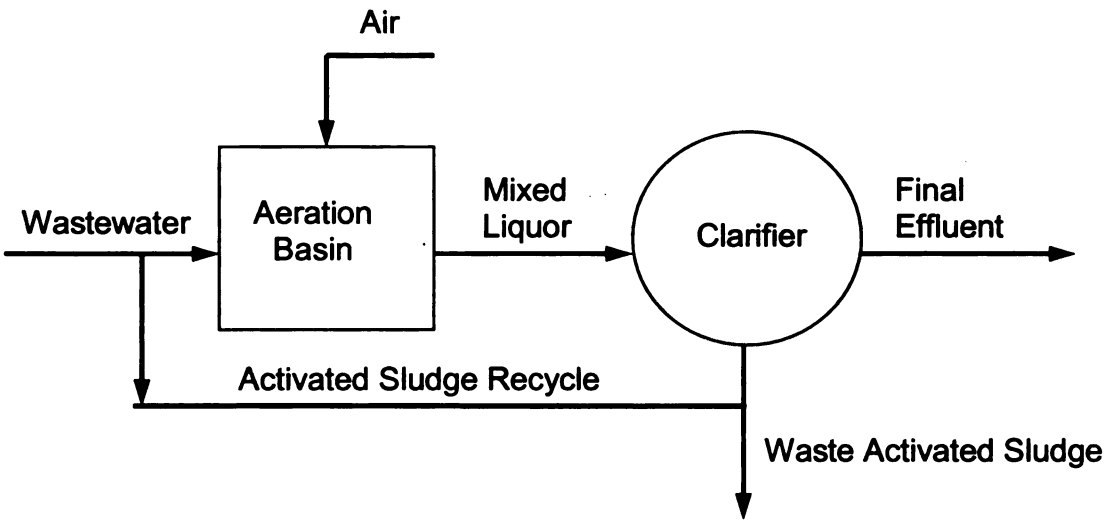

Figure 2: Activated Sludge Process.

The disadvantages to the aerobic treatment process are that often bulking and excessive biomass growth arise (Barnett *et al.*, 1994; Donkin and Russell, 1997; and Martin and Zall, 1988). In the activated sludge system treating dairy wastewater, bulking may be defined as the condition in which the flocs are not of

a suitable structure and size distribution to allow for effective settling and compaction in a clarifier (Donkin, 1997). This may be due to the outgrowth of filamentous bacteria. The problem can be improved by treating the waste activated sludge returning from the clarifier with chlorine to reduce filamentous bacterial populations. However, a fresh culture of bacteria must be added daily to maintain an effective biomass (Stengel, 1988).

Sequencing batch reactor (SBR) is a semi-continuous activated sludge process comprised of five steps: fill, react, settle, draw and idle (Figure 3). A sequence of the SBR process used with dairy wastewater is: (1) wastewater discharged into SBR tank, (2) treatment occurs in the SBR tank, (3) biomass solids are allowed to settle, (4) treated effluent is discharged via a decanter, and (5) sludge is further treated in sludge digester/thickener. Up to 98% and 93% reduction in BOD and COD, respectively, can be achieved (Norcross, 1998 and Samkutty *et al.* 1996). In 1987, The Kroger Co. examined the choices to treat waste from their fluid milk plant and found the SBR process to be the most cost effective. Other advantages to the SBR process include excellent settling characteristics of the biomass, elimination of the clarifier and sludge return pump stations, and highly resistant to organic shock loading (Schulte, 1988). Organic shock loading is a sudden change in organic loading rate, resulting from instances such as heavy rainfall or changes in operating conditions in the processing plants.

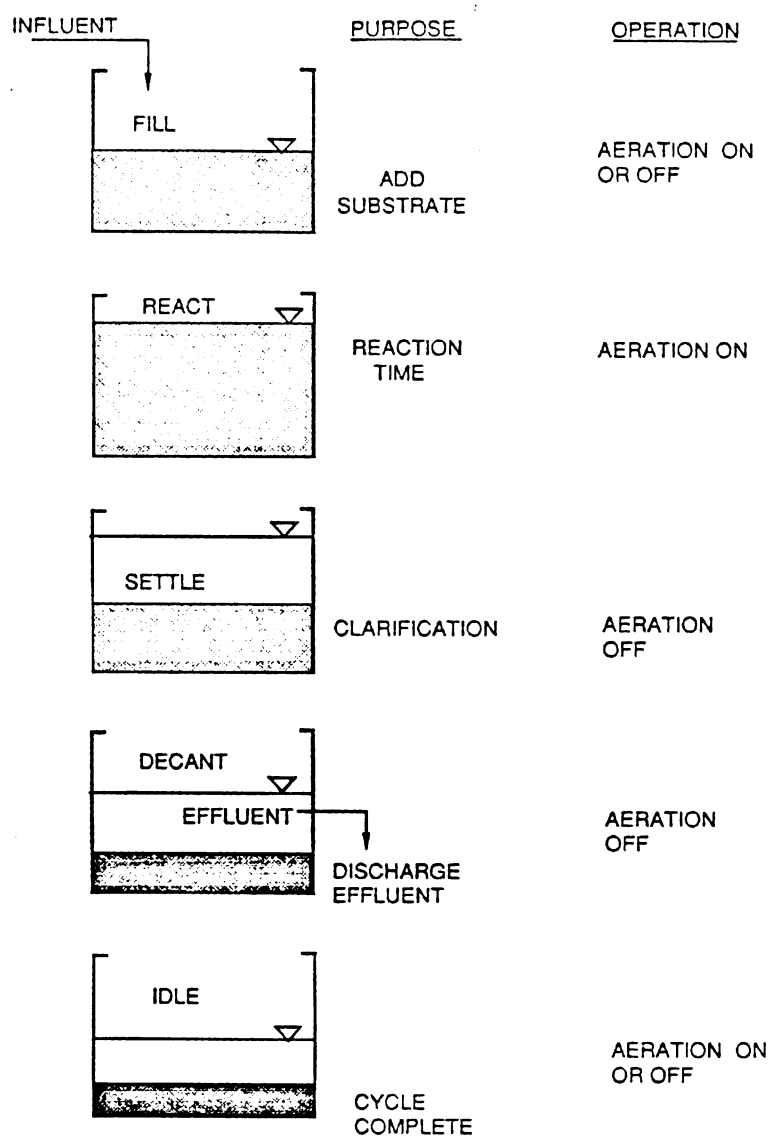

Figure 3: Sequencing Batch Reactor Operation Sequence.

Trickling filters or biofiltration is another form of an aerobic process in which the bacteria population is attached to a solid media in a tank (Figure 4). Microorganisms then act on the dairy wastewater that trickles through. Bacteria then colonize and form more biomass as more foods are utilized. Eventually the

biomass sloughs off naturally as the bacteria in the interior die due to lack of oxygen. The biomass is removed from the treated wastewater in a clarifier. Depending on the loading rates, BOD removal in dairy wastewater varies from 80%-95%. Trickling filters are more energy efficient and recover from shock loadings faster than activated sludge. However, the filters have lower purification efficiency, higher construction cost per unit volume treated, and no control of oxygen supply (Odlum, 1991 and Amos, 1997).

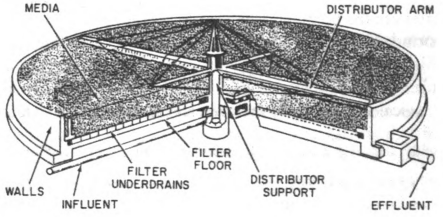

Figure 4: Cross Section of Trickling Filter Media.

Anaerobic treatments have gained favor over the years due to the ability to treat high organic concentration wastewaters. The process removes BOD and COD without the need of aeration, thus saving space and energy input. The anaerobic process is not limited by rate of oxygen diffusion between gas and liquid phases. The amount of excess sludge produced is considerably lower than that of aerobic processes (Perle *et al.*, 1995). The less sludge volume generated

is due to the fact that less than 5 percent of the biodegradable organic matter is converted to cell material and about 90% of the biodegradable fraction can be converted to a usable end-product in the form of methane gas (Obayashi and Gorgan, 1985).

The anaerobic treatment uses a series of microbial catabolisms in the absence of oxygen to produce biogas (carbon dioxide, methane, nitrogen and hydrogen), organic acids, sludge, and water (Hwang and Hansen, 1998). However, hydrogen sulfide and ammonia which give off a foul odor may also be produced (Amos, 1997). The degradation process can be roughly divided into three steps (Figure 5). The hydrolysis step is by extracellular enzymes of a group of heterogenous and anaerobic bacteria while the latter two steps are brought about by acetogenic bacteria and methanogens, respectively. Examples of the anaerobic process are anaerobic contact process, the upflow sludge blanket (USB) reactor, and the anaerobic filter (Liu and Liptak, 2000).

The principle of the anaerobic contact process is similar to that of the activated sludge treatment (Figure 6). Wastewater is mixed with anaerobic sludge culture in the reactor. The sludge is then removed from the treated effluent in a clarifier and a portion is recycled back to the anaerobic reactor. Ripley and Totzke (1988) evaluated the anaerobic contact process at Gold Bond Ice Cream (Green Bay, WI) where milk, cream, and ice cream were processed. The BOD level was high (7,000 to 9,000 mg/L). By the end of the study, BOD removal of 99% (from 9,870 mg/L to an average of 94.5 mg/L) was achieved while COD level was reduced from 16,630 mg/L to an average of 656 mg/L.

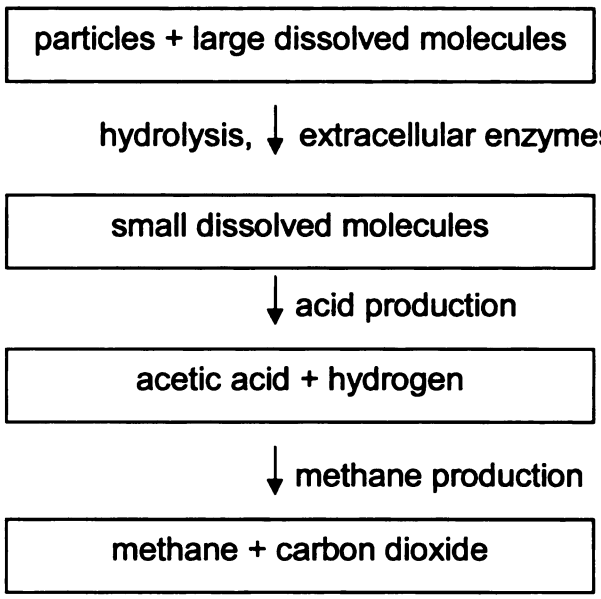

Figure 5: Anaerobic Degradation Process.

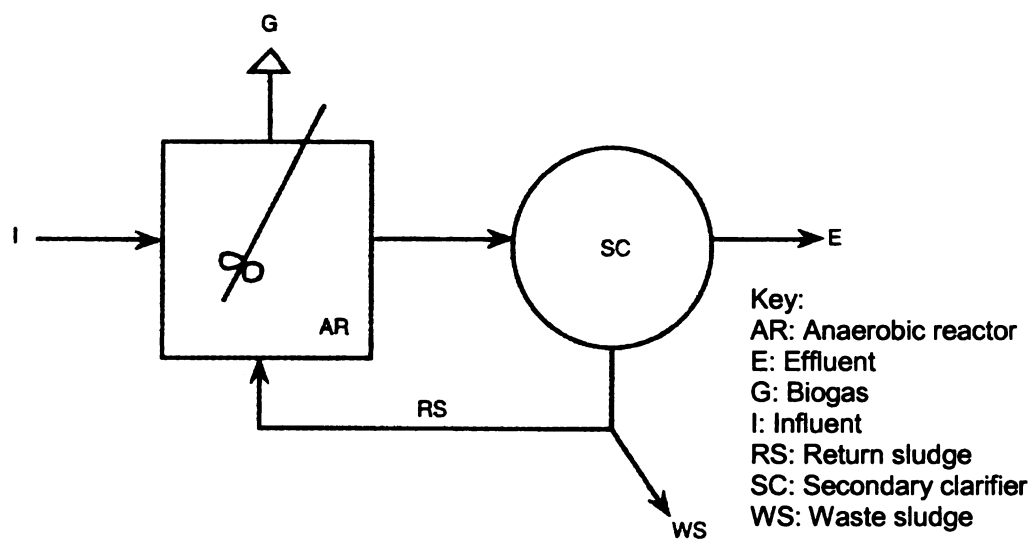

Figure 6: Anaerobic Contact Process.

For the anaerobic filter (AF) reactor, microorganisms grow on supporting media (Figure 7). The wastewater can be fed from the top (downflow filter) or the bottom (upflow filter). The active biomass is retained for an extended time period compared to other methods. Only when the filter is saturated is the biomass removed (Liu and Liptak, 2000).

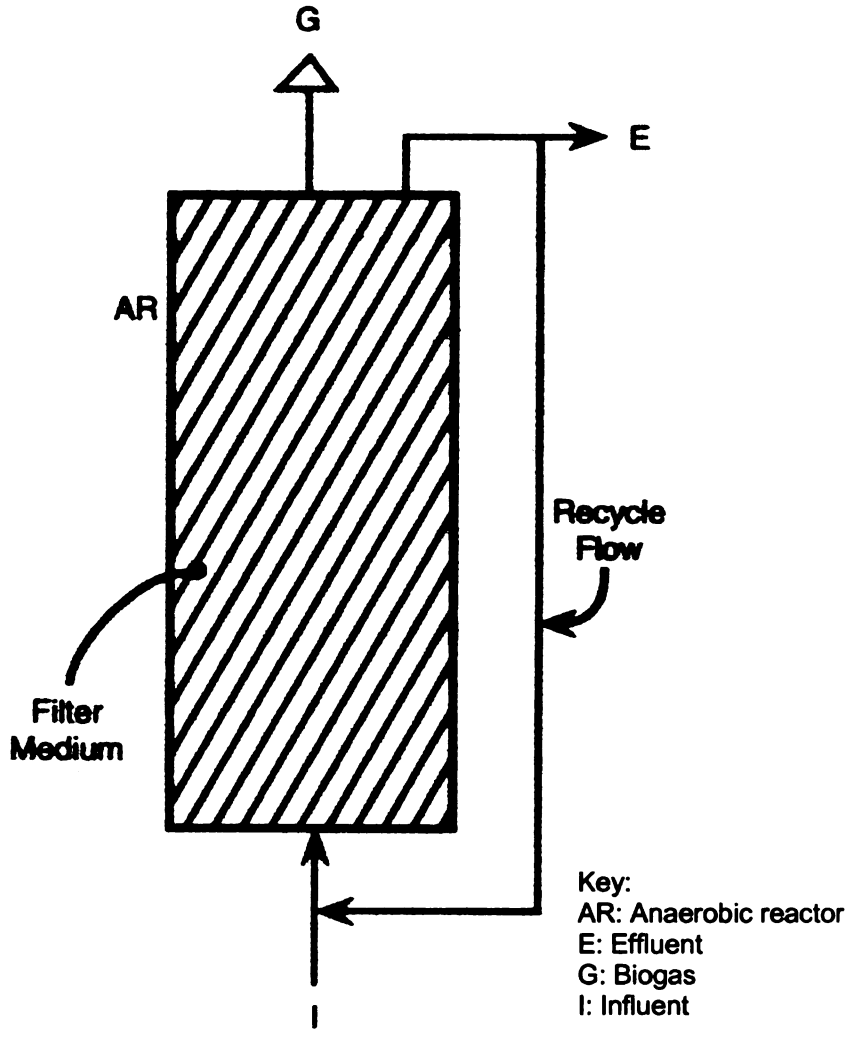

Figure 7: Anaerobic Filter (AF).

The upflow sludge blanket (USB) reactor system is a suspended-growth reactor as well as a fixed-biomass reactor. There are 3 distinct zones (Figure 8): 1) sludge zone, 2) sludge blanket or clarification zone, and 3) gas-liquid separation zone. The wastewater is pumped in from the bottom and passes through the sludge zone where about 80-90% of the waste treatment occurs. The waste stream then moves on to the sludge blanket zone, where the biomass exists in the form of lighter and less dense grains. The gas bubbles produced by the biomass aid in mixing and help carry the solid particles up to a gas-liquid separator above the sludge blanket. The gas-liquid separator separates biogas, sludge and effluent (Liu and Liptak, 2000, Obayashi and Gorgan, 1985, and Stronach *et al.*, 1986).

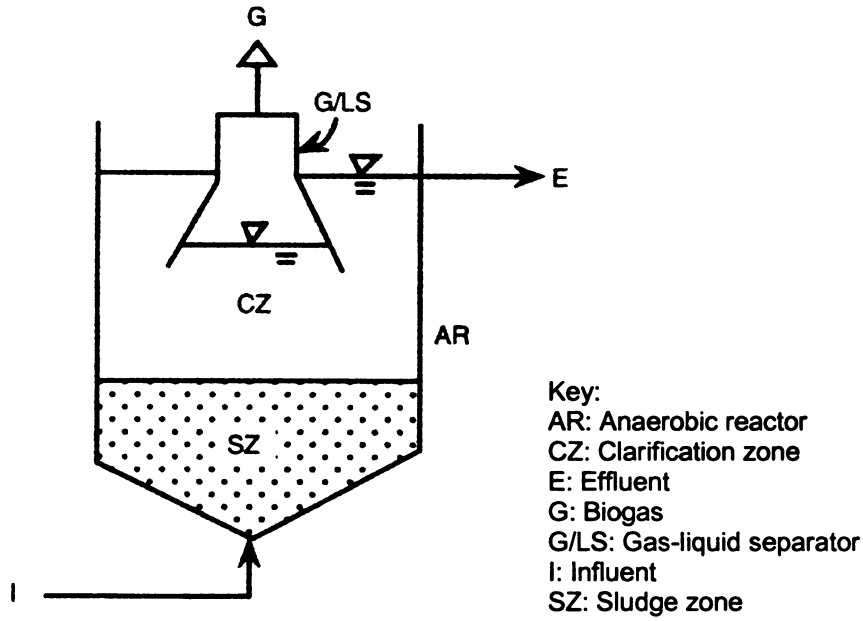

Figure 8: Upflow Sludge Blanket (USB) Reactor.

The disadvantages of the anaerobic systems are that complications often arise from sludge floatation (Vidal *et al.*, 2000) and the microbial population. Anaerobic populations are very sensitive to certain compounds and chemicals and more sensitive to pH and temperature than aerobic organisms. Methanogenic microorganisms, which tend to stick onto fat particles, create a problem with sludge floatation, especially during the peak loadings (Grootaerd *et al.*, 1999). Acclimation could be another factor in the operation of anaerobic treatment system as seen from the work of Perle and coworkers (1995). Perle *et al.* investigated the anaerobic degradation of dairy wastewater and found that reactors that were not acclimated with caseins exhibited a slow rate of digestion, resulting in inefficient process treatment. Additionally, milk fat and its by-products from degradation, mainly long chain fatty acids, were found to reduce the activity of methanogens as seen from lower ATP level. The authors suggested that the milk fat concentration had to be below 100 mg/l for the anaerobic digestion process to be successful. Tay and Zhang (1992) conducted a study to investigate the effects of different shock loads (e.g. organic, hydraulic, or toxic) on different anaerobic treatment processes (USB, AF and AFBR (anaerobic fluidized bed reactor)) and found that anaerobic filter performed poorly with sudden high organic loading rate while USB did not recover fully from toxic shock even after 12 hr.

Tertiary Treatment

The goal of tertiary treatment (also referred to as "advanced wastewater treatment") is primarily to further treat or polish the effluents to meet a more stringent limit such as 20 mg/L BOD for discharge to a natural stream. The process will remove ammonia, nitrates, phosphates, and/or other soluble materials from the effluents that have undergone chemical and/or biological treatment. Filtration, ion exchange, and ammonia stripping are some of the practices employed at this final stage (Amos, 1997).

Filtration can remove up to 99% of the suspended solids which have not been removed in primary or secondary treatments. Sand, anthracite, and diatomaceous earth are the most commonly employed filter media. Ion exchange is a process where ions, which are held to functional groups on the surface of a solid by electrostatic forces, are exchanged for ions of a different species in solution. Therefore, ion exchange is a very useful tool to completely remove metal ions from the effluent. Ammonia stripping can be achieved via a nitrification-denitrification process. The nitrification-denitrification process is a modification of the conventional activated sludge system. Longer detention time in the reactor allows the complete conversion of ammonium nitrogen to nitrates in the presence of nitrifying bacteria (Nitrosomonas and Nitrobacter). Denitrification, which is an anaerobic process, converts nitrites and nitrates to nitrogen gas and nitrogen oxide using denitrifying bacteria (facultative heterotrophic microorganisms) (Ramalho, 1983).

USE OF ENZYMES IN DAIRY PROCESSING PLANTS

Enzymes are biological catalysts and are defined as proteins with catalytic properties. They increase the rate of reaction by factors of 10^6 to 10^{12} compared to the uncatalyzed reactions, and only a small amount is required. Enzymes also possess specific ability to convert a particular substrate to a required product, without unwanted side reactions, in mild conditions (temperatures below 100°C, atmospheric pressure, and nearly neutral pH's). They are of natural origin and non-toxic (Henderson, 2000; and Voet and Voet, 1995).

While the traditional cleaning system in dairy processing plants often involves the use of caustic and alkali cleaners, a new generation of cleaners sometimes incorporates enzymes into the cleaning compounds. Over the years, dairy processing plants have found use for the enzyme-based technology in certain areas of the plants.

One of the enzyme-based cleaners that was released in recent years is P3-paradigm from Henkel-Ecolab. This particular enzyme-based product is a two-component cleaning system that is composed of enzymes (proteases), surfactants, a buffer and complexing agent. The proteases remove protein while surfactant removes the milk fat. The complexing agent is formulated to prevent the deposit of mineral on the equipment surfaces. However, P3-paradigm is for use on cold process surfaces only, such as raw milk tankers and milk storage tanks (Potthoff *et al.*, 1997).

Membranes used in filtration systems and stainless steel equipment are some of the areas in the dairy processing plants that have also been tested with the use of enzymatic cleaner. In the filtration systems, the problem often arises because of the fouling of the membrane due to protein deposition and mineral precipitation. Initially, Smith and Bradley, Jr. (1987) examined the activity of four enzyme-based cleaners for ultrafiltration (UF) systems against proteins in skim milk and whey. Two of the four formulations failed to hydrolyze caseins or whey proteins while the other two were effective, producing an increase in non-protein nitrogen (NPN) content with a decrease in protein. Ganesh Kumar and Tiwari (1999) further explored the use of alkaline protease as a UF membrane cleaner additive to the alkaline cleaner. The authors found that with the addition of alkaline protease at 5 g/L, the water flux was restored to 100% within a period of only 30 min. The short period for the restoration of membrane water flux indicated that the enzyme effectively hydrolyzed milk proteins so that the smaller peptides were easily washed off.

Argüello *et al.* (2002) observed >90% cleaning efficiencies on ultrafiltration (UF) membrane with the commercial enzyme formulation Alcalase (Novo Nordisk A/S) in short operating times of 20 min although some residues still remained. Additionally, an increase in cleaning efficiency of a polysulfone UF membrane, which was fouled by bovine serum albumin and reconstituted whey protein concentrate, was achieved with the combined use of enzyme and detergent as observed by Muñoz-Aguado *et al.* (1996).

Biofilm formation on equipment is also a major issue in the sanitation of the dairy processing plant. In a dairy processing plant, biofilm is usually formed on the surface where organic or inorganic material is deposited due to improper cleaning and sanitizing. This layer is often called conditioning layer. Once the bacteria are introduced, they attach themselves to the deposit via fimbriae, pili, flagella or exopolysaccharides (EPS). EPS is produced and secreted by the bacteria once attached to solid surfaces. Multilayers of bacterial cells entrapped within the EPS-containing matrices develop within the biofilm. Further increase in the size of biofilm takes place by the deposition or attachment of other organic and inorganic solutes and particulate matter to the biofilm from the surrounding liquid phase. If biofilm is released into the product, the product will be contaminated and its shelf-life decreased because biofilm can contain foodborne pathogens and spoilage organisms (Ganesh Kumar and Anand, 1998).

Several authors have attempted to incorporate the use of enzymes in a cleaning protocol to remove biofilms. Flint *et al.* (1999) experimented with a variety of ways to remove and inactivate the thermo-resistant streptococci that colonize the stainless steel surfaces. The application of proteolytic enzyme trypsin (1%) together with a commercial enzyme cleaner (Paradigm, Ecolab, Hamilton, New Zealand) as found to be more effective than acid or alkali cleaning with a consistent reduction of the bacterial numbers by more than $\log_{10}$ 2 cells cm^{-2}. Another study by Graßhoff (2002) also showed that commercial enzyme preparations (Savinase®, Properase 1600 L® and Esperase®) could effectively clean milk pasteurizers but with the need of an acid pre-treatment step (15 min,

0.5% nitric acid, 60°C). The acid pre-treatment removed the mineral components leaving the organic components to be degraded by the enzymes.

To their advantage, the enzyme cleaners are safer to use since they are not toxic, corrosive or irritating. The enzymes also work near neutral pH and the cleaning system is more energy efficient because enzymes work at biological temperatures (25°C-42°C) as opposed to 65°C-80°C in a typical cleaning system. With this lower working temperature range, the life of the equipment and gaskets can also be prolonged. The components of the enzymatic cleaning system are biodegradable and do not add much to the BOD/COD content of wastewater (Stilwell *et al.*, 2000 and Potthoff *et al.*, 1997).

Graz and McComb (1999) reviewed the potential use of enzymes in a CIP system in South Africa. As of the publication date, only two products for enzymatic CIP were marketed in South Africa. The cleaning process involved an initial rinse, a wash with an alkaline builder to increase the pH of the system and saponify lipids, and finally a wash with the enzyme mixture to remove protein residues. However, both products have not been truly successful.

In the United States, several companies manufactured enzyme-based products, including Ecolab's Solodigm™ (St. Paul, MN), Koch Membrane Systems' KochKLEEN® (Wilmington, MA), and Alconox's Terg-A-Zyme® (White Plains, NY). All the cleaners mentioned above have one characteristic in common – they contain protease, thus enabling them to break down proteinaceous soils.

APPLICATION OF ENZYMES IN WASTEWATER TREATMENT

Over the years, there have been studies where enzymes are used in conjunction with detergents/cleaners to enhance the wastewater treatment or to pre-treat the wastewater. Liu *et al.* (1998) observed the effects of detergents and surfactants in the wastewater on the biodegradation of butterfat. Without the presence of detergents, removal of COD and BOD was slow and incomplete. Jung *et al.* (2002) observed the use of an enzymatic pre-hydrolysis step with fermented babassu cake containing *Penicillium restrictum* lipases. The babassu cake was a solid waste obtained from the Brazilian babassu oil industry. The cake served as substrate for *Penicillium restrictum* in the solid-state fermentation to produce lipases (Cammarota *et al.*, 2001). With dairy wastewater pre-hydrolyzed, the COD removal efficiency in batch activated sludge systems was maintained at 93%, 92% and 82% when oil and grease concentration in the reactor was increased from 400, 600, to 800 mg/L, respectively. Without the wastewater pre-hydrolysed, the COD removal efficiency fell (86%, 75% and 0% for 400, 600, and 800 mg/L oil and grease loading, respectively).

Enzymes are also used as a supplement to aid in the biodegradability of dairy and cheese whey wastewaters as observed by Loukidou and Zouboulis (2001). Laboratory-scale sequencing batch reactor was the test method used to treat the wastewater. The bacteria/enzyme blend was mixed with dairy wastewater or cheese whey and treated for a period of 5 or 7 days. Unpleasant

smell disappeared, and BOD reduction of 95% was achieved while COD reduction was satisfactory (from around 4400 mg/L to approximately 1000 mg/L).

Windsor Creameries (Llanelli, Wales) found the application of an enzyme/microbial treatment process useful for its ice cream processing plant (West, 1988). Typically, the wastewater from Windsor Creameries generated over 50% fat solids content with BOD as high as 20,000 mg/L. With the use of Combizyme produced by Biocatalysts, Windsor Creameries was able to reduce the BOD load to 200 mg/L after a few weeks of aerobic treatment operation. After a year, the BOD was further reduced to 21 mg/L. This enzyme blend, Combizyme, is a combination of viable non-pathogenic bacteria, hydrolytic enzymes and inorganic nutrients. The enzyme formulation included lipase which degraded fat. The fat degradation products were then utilized more easily by bacteria in the aerobic treatment tank, making the system more efficient in treating high fat wastewater.

Kikkoman Foods Inc. (Walworth, WI) represented another industry that decided to investigate the use of a commercial enzyme-surfactant composition in their wastewater pretreatment facility (Podella *et al.*, 2000). With the growing production levels, Kikkoman pretreatment facility was overloaded and the Fontana/Walworth publicly owned treatment works (POTW), who received and treated the discharge from the Kikkoman's plant, could not handle the wastewater load. Faced with the decision to upgrade its own pretreatment facility (at a cost of $2.5 million) and the POTW ($1.5 million), Kikkoman decided to test the enzyme-surfactant compound in its pretreatment system onsite. After 6

months of use, BOD and total suspended solids (TSS) were reduced by 60% and 44%, respectively, and odor was eliminated. A bench study was conducted for COD to better understand why BOD was reduced as well as TSS. Typically, increased microbial activity needed to digest BOD to this 60% reduction would result in a significant increase in TSS in the form of biomass. From the COD experiment, it was found that a decrease of 1550 mg/L COD was achieved in just 8 hours compared to 24 hours for the control. The ratio of biomass (as TSS) created to nutrient consumed was calculated for both control and treated samples after 8 hours. The results showed that the control treatment required 2.2 times more biomass than treated sample to oxidize a given amount of biologically available COD. Kikkoman Foods Inc. was able to avoid large capital investment to upgrade either its pretreatment facility or the POTW, and also saved nearly $500,000 in surcharges over the first 4.5 years.

POTENTIAL USE OF ENZYME AS A PRE-RINSE IN A DAIRY PLANT

Wastewater surcharges have become a significant issue, raising processing costs. Discharges of processing wastewater into municipal sewer or onto land have become increasingly difficult because of the high strength wastewater generated and more stringent enforcement of the environmental laws. Questions now arise as to how and what the dairy industry could do to improve on the quality of the wastewater before the release either to the municipal treatment system or to the natural streams. Many dairy operations have decided

to install their own wastewater treatment plants while smaller producers still have to discharge and pay for the surcharges. An investigation into the use of an enzyme-based cleaner, Xzyme™, on a storage tank could provide an insight on the effects of the cleaner. The enzyme-based cleaner could potentially clean as well as carries the effect downstream as the waste travels to the wastewater treatment facility. The effects could be reducing the wastewater strength, eliminating odor, or simply breaking down complex organic molecules into simpler forms.

EXPERIMENTAL PROCEDURES

BIOLOGICAL OXYGEN DEMAND DETERMINATION

The biological oxygen demand (BOD) was determined according to the *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998).

1) Dilution Water

Deionized water was aerated overnight. For the desired volume of water, 1 ml each of phosphate buffer (0.06 M KH_2PO_4, 0.12 M K_2HPO_4, 0.12 M $Na_2HPO_4 \cdot 7H_2O$, 0.03 M NH_4Cl), 0.09M magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.25 M calcium chloride ($CaCl_2$), and 0.25 M ferric chloride ($FeCl_3 \cdot 6H_2O$) solutions was added per 1 L of water.

2) Seed Control

Seed control BOD bottles contain only seeded dilution water. The seed is used when there are not enough bacteria in the water to act on the waste. BODseed Polyseed Inoculum was purchased from Hach Company (Loveland, CO) and served as seeding material. The Polyseed is a proprietary consortium of at least 12 species of cocci and rod-shaped bacteria commonly found in wastewater (Confer and Logan, 1997). The preparation was carried out according to the manufacturer's instructions. One capsule of the Polyseed was aerated with 500 ml prepared dilution water for 1 hr, and allowed to settle for 15

min. The seed solution was drawn from the middle portion of the bottle. Preliminary testing suggested that the optimum volumes of seed water for the seed control bottles were 10, 15 and 25 ml per 300-ml BOD bottle. Therefore, 3 BOD bottles were prepared with 10, 15 and 25 ml of seed solution. For the sample bottles, 2 ml of the seed preparation was used per 300 ml of test solution. Because the water was seeded, a seed correction factor was determined to correct the oxygen uptake for the BOD calculation. The seed correction factor was calculated as follows:

$$\text{Seed correction factor} = \frac{V_1*(DO_1-DO_2)}{V_2}$$

where:

DO_1 = dissolved oxygen of seed control before incubation, mg/L
DO_2 = dissolved oxygen of seed control after incubation, mg/L
V_1 = volume of seed in BOD sample, mL
V_2 = volume of seed in seed control, mL

The average seed correction factor was determined from the 3 seed control bottles and used in the calculation of BOD.

3) Glucose-glutamic Acid Check

Glucose-glutamic acid (GGA) check is intended to be a reference point for evaluation of dilution water quality, seed effectiveness, and analytical technique. A 2% (v/v) dilution of a 50:50 mixture of 150 mg glucose/L and 150 mg glutamic acid/L standard solution (LabChem Inc., Pittsburgh, PA) was prepared in 300-ml BOD bottles for their BOD determination.

4) Sample Preparation

Table 4: Preparation of samples for BOD determination.

	Dilution	Seed (ml)	Sample (ml)	Xzyme (ml)	GGA (ml)	Total Volume (ml)
Diltuion water	-	-	-	-	-	300
Seed control	1:30	16.7	-	-	-	500
	1:20	25.0	-		-	500
	1:15	33.3	-	-	-	500
GGA[a]	1:50	3.3	-	-	10.0	500
Xzyme™ only	1:5000	3.3	-	100.0[c]	-	500
Untreated Cream[b]	1:100,000	3.3	5.0[d]	-	-	500
Untreated Cream[b]	1:80,000	3.3	6.3[d]	-	-	500
Untreated Cream[b]	1:60,000	3.3	8.3[d]	-	-	500
Treated Cream[b]	1:100,000	3.3	5.0[d, e]	-	-	500
Treated Cream[b]	1:80,000	3.3	6.3[d, e]	-	-	500
Treated Cream[b]	1:60,000	3.3	8.3[d, e]	-	-	500

[a] 2 replicates
[b] 3 replicates
[c] from 1:1000 initial dilution of Xzyme™
[d] from 1:1000 initial dilution of cream
[e] treated with 0.06% Xzyme™

Treated and untreated cream samples were prepared in the laboratory. Heavy whipping cream was purchased from a local grocery. A 1:6 dilution of cream was treated with 10% Xzyme™ (Renew Systems, Bay City, MI) (16.4 ml cream, 10 ml Xzyme™ and 73.6 ml deionized water for a total of 100 ml). The 1:6 treated cream preparation was stirred overnight in a cold room, and was serially diluted to 1:100 and 1:1000. The 1:1000 treated cream thus contained 0.06% Xzyme™, and was used as samples in subsequent BOD preparation (Table 4). Untreated cream was prepared by mixing 16.4 ml cream with 83.6 ml

deionized water (1:6 dilution) and serially diluting to 1:100 and 1:1000. The 1:1000 untreated cream was used as samples for the preparation of the BOD bottles. Xzyme™-only samples were made by serially diluting 1:10 (10 ml Xzyme™ and 90 ml deionized water) concentration to 1:100 and 1:1000, respectively.

Samples were prepared in 500-ml volumetric flasks (Table 4) and slowly added to the 300-ml BOD bottles until completely filled. Nitrification inhibitor was added to every bottle, except the dilution water bottle. Sample BOD bottles were incubated at 20°C along with seed controls, dilution water, and GGA bottles.

5) Dissolved Oxygen (DO) Determination

The dissolved oxygen was measured every 24 hr for 5 days. The measurement was taken using an oxygen probe (Model 97-08-99, Orion Research, Inc., Beverly, MA) connected to a Corning pH/ion meter 150 (Corning Science Products, Corning Limited, Essex, England).

6) Biological Oxygen Demand Determination

$$BOD = \frac{(DO_1 - DO_2) - \text{seed correction factor}}{P}$$

where:

DO_1 = dissolved oxygen of sample before incubation, mg/L
DO_2 = dissolved oxygen of sample after incubation, mg/L
P = decimal volumetric fraction of sample used

DETERMINATION OF PROTEOLYTIC ACTIVITY OF XZYME.

1) Preparation of samples

Non-fat dry milk (NFDM) powder contains 35% protein. A solution of NFDM powder was prepared by adding 1.43 g NFDM powder and 1 ml of Xzyme™ and making to 100 ml deionized water. The prepared protein solution thus contained 5 mg/ml protein concentration. The Xzyme™-treated NFDM powder sample was then stirred for 1/2 hr and left overnight at room temperature. On the day of the experiment, 100 µl of sample buffer (50 mM HEPES, 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol and 0.2% (w/v) bromophenol blue) were mixed with 400 µl of treated sample to give the final concentration of protein of 4 mg/ml. As a control, NFDM powder (0.0114 g) was reconstituted with a mixture of sample buffer (200 µl) and deionized water (800 µl). The final protein concentration in the control was also 4 mg/ml. All prepared samples were heated in boiling water for 5 min.

2) Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the proteolytic enzyme activity in Xzyme™ cleaner on milk proteins. Electrophoresis was run in a Mini-PROTEIN® II Electrophoresis Cell (Bio-Rad, Hercules, CA) filled with running buffer (0.05 M Tris base, 0.384 M glycine, and 1% (w/v) SDS). The acrylamide concentrations of stacking and resolving gels were 4% and 14%, respectively. The ratio of acrylamide-to-bis-

acrylamide for stacking and resolving gel were 32.7:1 and 32:1, respectively (Tables 5 and 6).

Table 5: Formulation of the SDS-PAGE stacking gel.

	Volume (ml)
50% (w/v) Acrylamide/Bis-acrylamide	4.2
Laemmli A (1.5 M Tris base)	3.7
Glycerol	1.8
Deionized Water	5.3
10% Ammonium persulfate	50*
Temed	6*

*microliters (μl)

Table 6: Formulation of the SDS-PAGE resolving gel.

	Volume (ml)
49.5% (w/v) Acrylamide/Bis-acrylamide	0.8
Laemmli B (0.5 M Tris base)	2.5
Deionized Water	6.1
10% Ammonium persulfate	100*
Temed	20*

*microliters (μl)

About 5 μl of each protein sample were loaded to a sample well, together with low-range and broad-range pre-stained SDS-PAGE standards (Bio-Rad, Hercules, CA). The gel was run at 90 mA current and 300 v constant voltage for 3 hr. The gel was then stained for 1 hr with Coomassie Brilliant Blue R-250 solution (0.25% (w/v) in 9:45:45 v/v/v of acetic acid:methanol:water) and de-stained for 1 hr in acetic acid-methanol-water (1:1:8 v/v/v) solution. Digital photographs of the gels were taken after the gels were dried in cellophane gel drying film (Idea Scientific Company, Minneapolis, MN).

DETERMINATION OF LIPOLYTIC ACTIVITY OF XZYME

1) Sample Preparation

Thin layer chromatography (TLC) was used to assess the lipolytic activity of Xzyme™. All samples were prepared using deionized water and heavy whipping cream purchased from a local grocery. Samples of both untreated and Xzyme™-treated cream were maintained at 24°, 37°, and 50°C. Untreated cream was diluted to 1:6 (16.4 ml cream with 83.6 ml deionized water) and maintained at the temperature points for 3 hr. For treated cream samples, 1:6 cream dilution was treated with 10% Xzyme™ (16.4 ml cream, 82.6 deionized water, and 10 ml Xzyme™), stirred and held at 24°, 37°, and 50°C for 3 hr. As a positive control, 1:6 cream dilution was treated with 0.5 g lipase (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 3 hr.

2) Sample Extraction

The samples were evaluated for the lipid classes (mono, di-, and triglycerides) by first extracting the lipid using the methods described by Bligh and Dyer (1969). In a separatory funnel, 15 ml of sample was mixed with 17 ml chloroform and 34 ml methanol for 2 min. Then, chloroform (17 ml) was added and mixed for 30 s, followed by the addition of deionized water (17 ml) and mixing of 30 s. The lipid mixture was then filtered through No. 1 Whatman Filter Paper (Whatman International Ltd., Kent, United Kingdom) into another reparatory funnel. The layers were allowed to separate. The bottom layer was

the lipid-chloroform layer while the top layer was the methanol-water layer. The chloroform layer was then transferred to a tared and pre-weighed beaker. The chloroform layer was evaporated in a 60°C water bath in the fume hood, cooled, and dried in a desicator.

3) Sample Development

The procedures for TLC analysis followed that outlined by Kates (1972). The lipid extract (0.1 g) was dissolved in chloroform (2.5 ml) to make a 4% (w/v) lipid solution. The lipid solution was then developed using thin layer chromatography (TLC). For each spot on the TLC plate (Silica Gel plate, Wheaton Inc., Clifton, NJ), 2 µl (containing 80 µg lipids) of each preparation were loaded along with the standard mixture of mono-, di-, and triglycerides (Sigma-Aldrich, St. Louis, MO). The spots were 1.5 cm apart and 1.5 cm from the bottom of the plate. A solvent system of petroleum ether, diethyl ether and acetic acid in a 70:30:1 (v/v/v) ratio was prepared and allowed to saturate the chromatographic chamber before a TLC plate was put in. The plate was allowed to develop until 2 cm was left on the top of the plate. The TLC plate was then dried in 110°C oven for 5 min.

4) Sample Detection

The lipid fractions were stained and visualized in a closed container saturated iodine vapor in a closed container for 5 min. Digital photographs of the TLC plates were taken immediately after the visualization.

STATISTICAL ANALYSIS

To investigate the effects of Xzyme on BOD, the statistical analysis software (SAS) was used (SAS Institute Inc., Cary, NC). Mixed model ANOVA with repeated measurements was used to analyze the differences between treatment, experiment, day, and their two way and three way interactions for each cream dilution level. Modeling the covariance structure of the same experiment unit in sequence of days was considered in repeated statement of mixed model procedure. The Tukey method was employed to adjust the multiple test *p*-values for the differences of all pairwise comparisons. The *p*-value less than or equal to 0.05 after Tukey adjustment is considered significantly different.

RESULTS AND DISCUSSION

BIOLOGICAL OXYGEN DEMAND

Biological oxygen demand (BOD) is one of the most common standard tests employed in assessing the water quality and pollutant level in water. Its widest applications are in measuring the strength of water pollution and waste loading to treatment plants as well as determining the relative oxygen requirement of treated effluents or polluted waters (Kumar *et al.*, 1999). Biological oxygen demand is determined by measuring the amount of oxygen utilized during the decomposition of organic material by a microbial population over a specified time period, usually 5 days. The microorganisms utilize the waste for the synthesis of complex molecules such as proteins and polysaccharides which are needed to build new cells. In the process, enzymes are synthesized as well to break down the waste to monomers because microorganisms cannot take up intact proteins, fats, or carbohydrates.

According to the *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998), dissolved oxygen (DO) measurements will have to meet the following criteria in order to be calculated into BOD: 1) the oxygen uptake must be at least 2 mg/L, and 2) the residual oxygen must be at least 1 mg/L. Any oxygen measurements that did not meet these conditions were not included in the calculations of BOD.

Preliminary studies began with a series of experiments to determine the concentration range of the samples. For Xzyme^{TM}-treated cream, the enzyme-based cleaner was added at 1% concentration to the 300 ml capacity of BOD bottle (i.e. 3 ml of cleaner per 300-ml BOD bottle). Figures 9 and 10 show the dissolved oxygen (DO) profiles for the untreated and treated cream, respectively, at various dilutions. From the high to low sample concentrations, the measured DO for the untreated cream still did not meet the requirements for the calculations of BOD. None of the dilutions had 1 mg/L residual DO left in the bottle at the end of 5 days. As observed later, the DO measurements could be translated into BOD only at 1:60,000 dilution and higher. For the treated cream, there was an increase in DO consumption as the dilution was increased. At 1:3000 and 1:5000, almost no oxygen uptake was observed (Figure 10) at the end of 5 days. At 1:9000, a less than 2 mg/L oxygen uptake was observed while at 1:15,000 and 1:50,000, DO dropped to below 1 mg/L at Day 5. Therefore, none of the DO numbers met the criteria for calculating BOD.

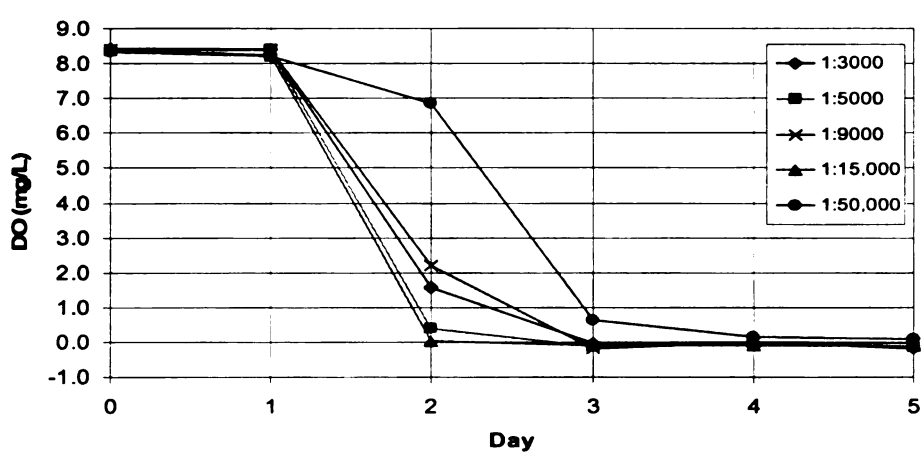

Figure 9: Dissolved oxygen profile of untreated cream over a period of 5 days at various concentrations.

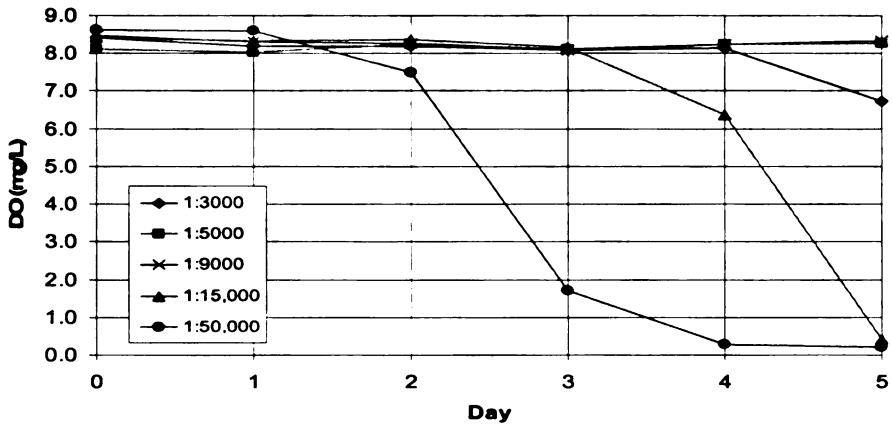

Figure 10: Dissolved oxygen profile of 1% Xzyme™-treated cream over a period of 5 days at various concentrations.

While all samples were treated with 1% enzyme-based cleaner, the samples contained different amounts of organic matter due to the dilutions made. Therefore, there was an inhibition of bacterial activity in the wastewater at higher cream concentrations. Perhaps, the inhibition resulted from an interaction between the cleaner, cream components, and the bacteria. Surfactant which is a component of a typical cleaner has been known to exhibit an antimicrobial property. The mechanism of microbial inactivation involves the adsorption and diffusion of surfactant through the cell wall, altering the cytoplasmic membrane's semi-permeable properties. The hydrophobic moiety of surfactants is inserted into the apolar fatty acid domain of the membrane phospholipids, causing increased permeability and leakage, while the hydrophilic chain can bind to the polar head group of phospholipids. The disruption of the membrane leads to leakage of potassium ions and other constituents necessary to maintain cell functions, thus causing cell death. (Cserhati, 1995).

Perhaps, a synergistic effect of surfactant and cream protein components exerts a detrimental effect on bacteria as observed by Restaino and coworkers (1994). The authors conducted research on the effect of a buffered organic acid anionic surfactant (BOAAS) against various bacterial strains on the countertop surface in the presence of 0.5% bovine serum albumin (BSA). A buffered organic acid anionic surfactant (BOAAS) concentration range of 0.6-1.75% was studied while BSA concentration remained 0.5% for all samples. The authors observed a 100-fold reduction of *Staphylococcus aureus* on the protein-coated countertop surface when 1.75% BOAAS was applied while at lower concentrations of BOAAS, the effect of BOAAS on *S. aureus* reduction decreased. With an increase in the ratio of BOAAS-to-BSA, a greater reduction of *S. aureus* was achieved. Therefore, in this study, with increased surfactant-to-protein ratio in 1:5,000 dilution sample compared to 1:50,000, perhaps bacterial growth was inhibited and thus no oxygen uptake was observed (Figure 10). However, possible mechanisms as to why bacteria are inactivated need to be further studied.

When the present sample preparation and dilutions failed to obtain the DO numbers that met the BOD criteria, an experiment with a change in sample preparation for the Xzyme™-treated cream was attempted. Instead of adding 1% cleaner concentrate to 300-ml sample preparation, a mixture of the cleaner and cream was prepared (1:6 cream dilution treated with 10% Xzyme™ and diluted to 1:1000 thus having 0.06% cleaner) and kept as stock solution. Dilutions for the BOD bottles were then made from this solution. Again, various dilutions were

attempted in order to obtain the DO numbers that fall within the criteria to calculate BOD. Figure 11 revealed the results obtained from the trial experiment. As the dilution was increased from 1:4000 to 1:40,000, the oxygen uptake rate seemed to slow down. However, at the end of 5 days, the DO decreased to below 1 mg/L for all dilutions.

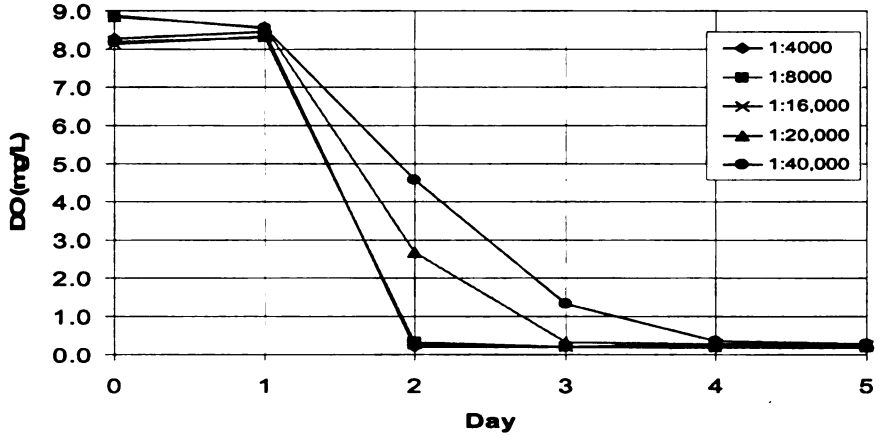

Figure 11: Dissolved oxygen profile of Xzyme™-treated cream prepared with 1:1000 cream containing 0.06% Xzyme™.

From previous studies shown, therefore, more attempts were made at higher dilutions and found that at 1:100,000, 1:80,000, and 1:60,000, the DO measurements all fell within the required criteria; that is: 1) oxygen uptake of at least 2 mg/L and 2) residual oxygen of at least 1 mg/L. Therefore, these DO numbers could be used in the equations to calculate BOD. Table 7 shows the average BOD values for 1:100,000, 1:80,000, and 1:60,000 dilutions. Between the Xzyme™-treated and treated samples, there were no significant differences found in terms of BOD for any dilution tested. The Xzyme™ -treated sample did

not improve the treatability of the waste using this protocol. Dilutions were averaged and significant difference between the untreated and Xzyme™ -treated samples was found at day 2, but not days 3-5.

Table 7: Biological oxygen demand (BOD) of cream samples over a period of 5 days (p<0.05).

	Dilution	BOD (mg/L) Day					
		0	1	2	3	4	5
Untreated cream	1:100,000	n/a*	n/a	346(±35.8)	490(±33.8)	556(±33.8)	584(±33.8)
Treated cream	1:100,000	n/a	n/a	283(±37.4)	438(±33.8)	529(±33.8)	578(±33.8)
Untreated cream	1:80,000	n/a	n/a	299(±36.7)	459(±35.1)	496(±37.3)	505(±39.8)
Treated cream	1:80,000	n/a	n/a	259(±41.2)	396(±35.1)	501(±36.7)	526(±41.0)
Untreated cream	1:60,000	n/a	n/a	298(±33.6)	363(±35.8)	421(±39.6)	468(±59.6)
Treated cream	1:60,000	n/a	n/a	225(±32.9)	403(±34.1)	468(±48.1)	n/a

*not applicable
**standard errors are given in parenthesis

From the data present in Table 7, biological oxygen demand was observable starting at Day 2 because the DO uptake on Day 1 was not 1 mg/L or more (Appendix A, Table A.1, Figure A.1). One of the reasons that could explain this observation could be that the seed material was not acclimated to the prepared dairy waste. The seeding material was obtained from commercially prepared inoculum from Hach Company ("Polyseed Inoculum," Loveland, CO), which is United States Environmental Protection Agency (USEPA)-approved. Because the seed was not acclimated, a lag period in which the organisms adjust to a new environment could occur (Ramalho, 1983). Although the lack of acclimation seems a possible reason for the slow early growth (or lag period),

several studies of the prepared seed supported the use of unacclimated prepared inocula. Comparable, reliable, and reproducible results were obtained from both unacclimated inocula and acclimated inocula and acclimated inocula from activated sludge treating domestic waste (Fujita, 1995; Kumar *et al.*, 1999; Manoharan *et al.*,2000; McDowell and Zitrides, 1979; Paixao *et al.*, 2000; and Paixao *et al.*, 2003)

Figures 12(a)-(c) reveal how the BOD increased over a period of 5 days. In all three curves, BOD was not reported for days 0 and 1. One notable observation from the figures was that as the dilution was lowered, i.e. more cream in the sample, the BOD decreased. This was not expected since increased concentration of organic material in the wastewater should exert more oxygen demand, thus increasing the BOD.

As seen, at 1:100,000 dilution, the Xzyme^{TM}-treated sample BOD started out at day 2 at 283 mg/L while for 1:80,000 and 1:60,000 dilution, the BOD were 259 and 225 mg/L, respectively. Similarly, untreated sample BOD for 1:100,000 dilution was 346 mg/L at day 2 while it was 299 and 298 for 1:80,000 and 1:60,000 dilution, respectively. Over a period of five days, the untreated sample curve slowed down and flattened while the treated curve accelerated. Eventually, the two lines intersected at day 5 for 1:100,000 dilution, at day 4 for 1:80,000 and between day 2 and day 3 for 1:60,000. In addition, the untreated samples also had higher BOD_5 value at 1:100,000 (584 mg/L) dilution than 1:80,000 (505 mg/L) and 1:60,000 (468 mg/L) dilutions.

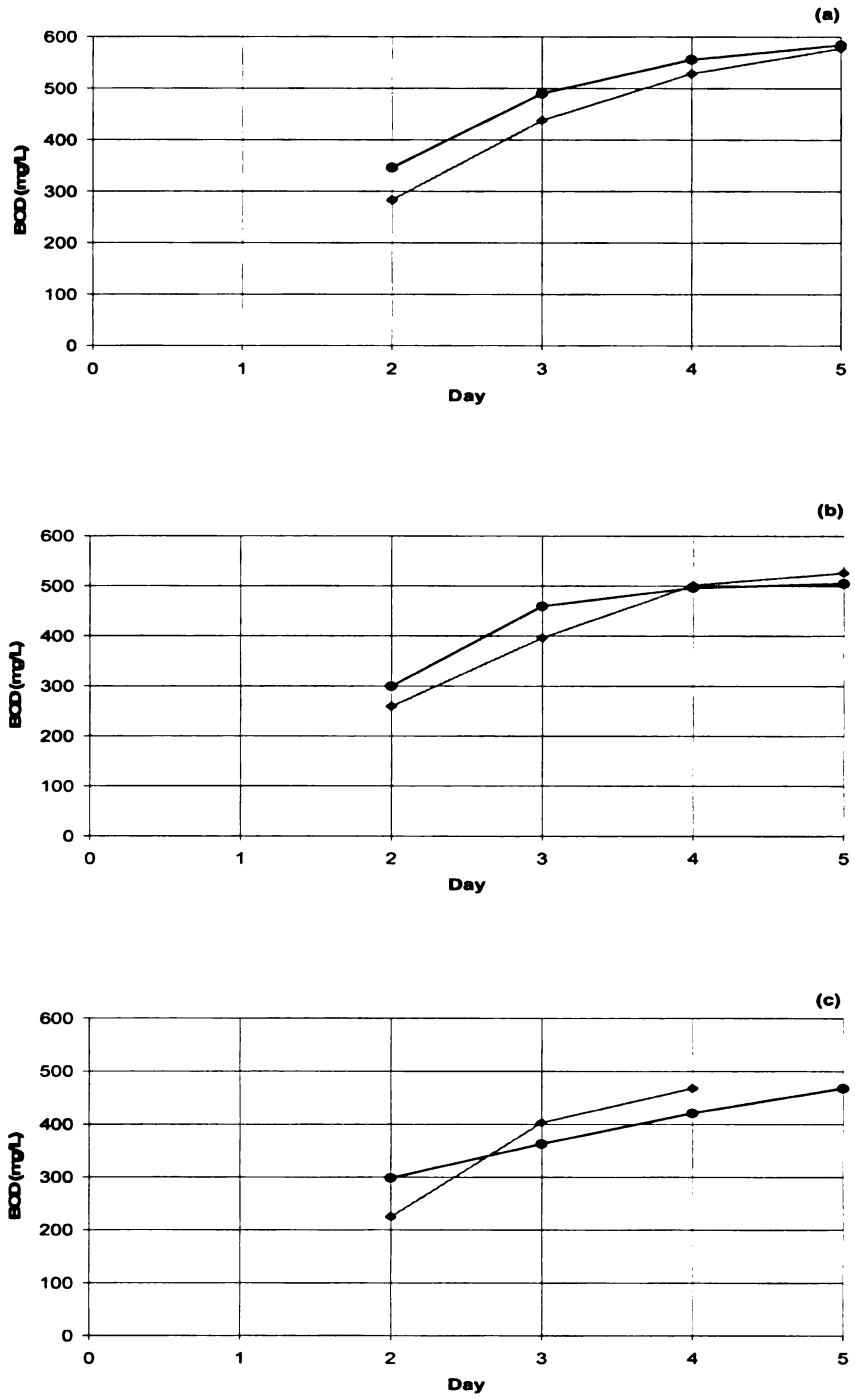

Figure 12: BOD values of Xzyme™-treated and untreated cream over a period of five days. (a) 1:100,000 dilution, (b) 1:80,000 dilution and (c) 1:60,000 dilution. (•) untreated cream, (♦)Xzyme™-treated cream.

A possible explanation for the increase in the acceleration for the treated cream curve could be that there was inhibition that occurred. The inhibition slows the rate of oxidation. An inhibition/toxicity test would be required to identify which compound was inhibitory. However, the only difference between the untreated and treated cream was the enzyme-based cleaner mixed in with the treated cream. Any component of the cleaners could be responsible for the faster oxygen consumption in the treated cream curve. The most likely component could be the proteolytic enzymes present in the cleaner. From the results obtained in the study of the proteolytic activity of the enzyme-based cleaner (see "Determination of proteolytic activity of Xzyme^{TM}"), there was a breakdown of milk proteins by proteolytic enzymes present in the cleaner. The accumulation of the intermediate fragments resulting from the hydrolysis could slow down the degradation. Therefore, with the breakdown of proteins in treated cream, the bacteria could assimilate the organic material faster, thus resulting in increased rate of oxygen consumption.

Dairy cream contains 35-40% fat. Lipids are known to have low biodegradability. When fat is degraded, the products resulting from the hydrolysis are glycerol, long-chain fatty acids (LCFA), and short-chained fatty acids (SCFA). In several studies, lipids were shown to exhibit slow rate of hydrolysis and certain levels of lipids also could be inhibitory. Perle *et al.* (1995) studied the degradation of dairy wastewater and measured ATP content to assess the physiological activity of bacteria. With added milk fat in the range of 0-1500 mg/L, the ATP content fell from 17.3 mol/L x 10^{-8} to 5.68 mol/L x 10^{-8},

suggesting a reduction in total physiological activity of bacteria in the treatment system. In addition, long-chain fatty acids are reported to be inhibitors of various microorganisms. The inhibitory effect increases with the number of double bonds and cis-isomers which are abundant in natural lipids. The overall conversion rate of lipids is limited by the conversion of LCFA (Rinzema *et al.*, 1993). In addition to LCFA, short-chain fatty acids (SCFA) are also present in milk. Hirshfield *et al.* (2003) conducted a review of weak organic acids (i.e. acetic, propionic and butyric acids) and their effects on bacteria. Weak acids have been known to act as preservatives in foods. Possible mechanisms of how the weak acids prevent the growth of bacteria were detailed. Weak acids can be charged or uncharged. The uncharged form of weak acid is lipid permeable and can freely diffuse into the cytoplasm of microbial cells via membrane proteins. Weak acid's undissociated form might interfere with the function of the membrane proteins. Furthermore, weak acid dissociates to give a proton (H^+) and an anion (A^-). Anions inhibit the function of cells by increasing the osmolarity of the cytoplasm. High levels of weak acid anions could also influence the activity of enzyme reactions within the cytoplasm, thus hindering the metabolism of the cell. Therefore, in the present study there could be a possibility that the accumulation of LCFA and SCFA in the model waste might inhibit or slow down the activity of microorganisms present in the seed, thus slowing down the rate of reaction. The slow rate of reaction could explain why BOD was lower at 1:60,000 than at 1:100,000 and 1:80,000 dilution.

Another perspective as to why BOD decreased with increased concentration of cream might involve proteins and their intermediates from protein hydrolysis. Ubukata (1998) studied the effect of the accumulation of protein intermediate products in a batch activated sludge reactor compared to that of amino acid (AA) mixture. The protein intermediate tested in this study was peptone which is a protease degradation product of milk casein and comprises about 24% free AA and about 76% peptides. In this case, the peptone's molecular weights used were less than or about 1000 daltons. The author showed the removal rate of peptone by activated sludge decreased with time and increasing initial peptone concentration. On the other hand, the removal rate of AA mixture by activated sludge was not affected by initial concentration of AA; that is, the removal rate stayed the same no matter how much the initial AA concentration was. In the present study, there was a possible accumulation of intermediate products of protease hydrolysis on milk proteins in this study. The accumulation of intermediate protein fragments could be more with less diluted cream. This increase in initial concentration of intermediate protein fragments might explain why BOD was decreased with increased concentration of cream. Furthermore, Confer and Logan (1997) also studied the degradation of bovine serum albumin (BSA) using commercial BOD-test inoculum and observed the intermediate-size fragment (2,000-10,000 Da) accumulation in the reactor. The accumulation peaked at the mid-exponential growth of the culture in the reactor and disappeared later. In the present study, some of the protein fragments fall into the intermediate-size category (Figure 13).

Therefore, the accumulation of intermediate size fragment could slow down the bacterial activity, thus result in a decrease in BOD at the end of day 5 for the decreased dilution.

DETERMINATION OF PROTEOLYTIC ACTIVITY OF XZYME™.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the proteolytic activity of the Xzyme™ cleaner. With the same starting protein concentration for each sample run on SDS-PAGE, the bands were compared to each other and to the molecular weight standards.

Figure 13 revealed that milk proteins from NFDM powder were degraded by the Xzyme™ cleaner. There are four distinct bands (*a*, *b*, *c* and *d*) in Lanes 2, 6 and 7 that represent parts of milk protein fractions. Band *a* represented a concentrated amount of α- and β-caseins, which range from 23,000-25,000 daltons (Da) in molecular weight (Table 9, Fox and McSweeney, 1998 and Figure 14, Mistry and Hassan, 1991). Bands *b*, *c*, and *d* were κ-casein, β-lactoglobulin and α-lactalbumin, respectively, and band *e* was one of the fractions of whey protein, serum albumin. Lanes 4 and 5 were loaded with Xzyme™-treated samples, and visual observation indicated extensive degradation of α- and β-casein fractions as well as some κ-casein. β-lactoglobulin and α-lactalbumin were also broken down into smaller peptides. Bands in the region of *f* could represent these smaller peptides with molecular weight less than 7,000 Da.

The degradation of milk protein could potentially have beneficial effects on the wastewater treatment since cleavage of peptide bonds by protease is the main enzymatic activity of treatment processes such as activated sludge system (Kim *et al.*, 2002). Because proteins present are polypeptides, the indigenous bacteria in wastewater treatment plants must hydrolyze the polypeptides into

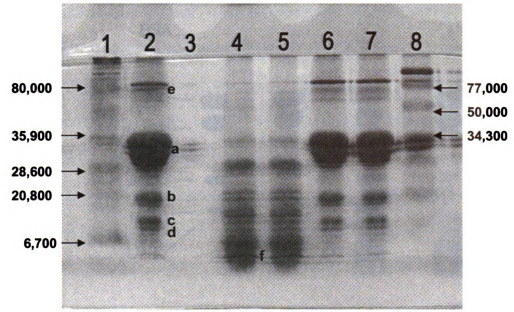

Figure 13: SDS-PAGE gel of Xzyme™-treated and untreated NFDM powder. Lane 1: broad range standards; lane 2: untreated sample; lane 3: Xzyme™ only; lanes 4 and 5: Xzyme™-treated samples; lanes 6 and 7: surfactant-treated samples; lane 8: low range standards. Bane *a*: α- and β-caseins; band *b*: κ-casein, band *c*: β-lactoglobulin; band *d*: α-lactalbumin; band *e*: serum albumin; band *f*: smaller degradation products. Numbers are molecular weights in Da.

Table 8: Molecular weights of milk proteins.

	Molecular Weight
Caseins	
α_{s1}-B 8P	23,614
α_{s2}-A 11P	25,230
β-A^2 5P	23,983
κ-B 1P	19,023
Whey Proteins	
α-la-B	14,176
β-lg-B	18,363
Serum albumin	66,276

Source: Fox and McSweeney (1998).

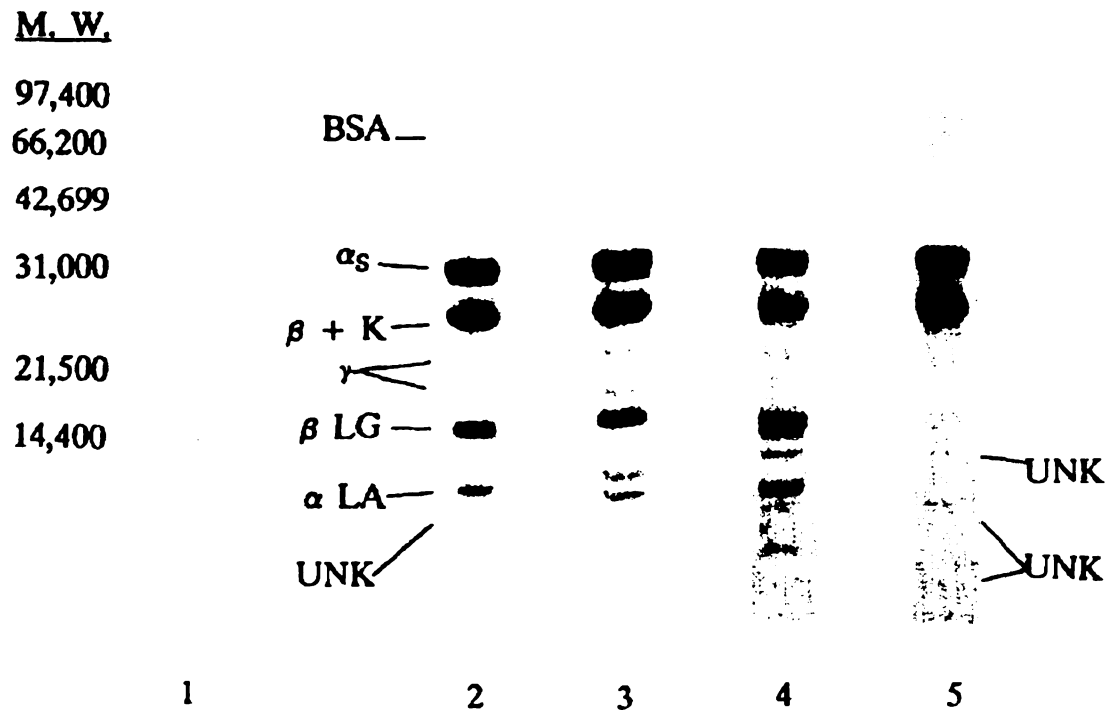

Figure 14: SDS-PAGE gel of skim milk and delactosed high milk powder. Lane 1: molecular weight (MW) standards: phosphorylase b, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, egg white lysozyme; lane 2: skim milk; lane 3: delactosed high milk protein powder; lane 4: protein isolate; and lane 5: sodium caseinate. BSA = bovine serum albumin, α_s = α_s-casein, β+κ = β- plus κ-caseins, γ = γ-casein, β-LG = β-lactoglobulin, α-LA = α-lactalbumin, and UNK = unknown (Mistry and Hassan, 1991).

monomers (i.e. amino acids) before the compound can be transported across the bacterial membrane. This step requires cell-wall associated enzymes and/or extracellular enzymes (Coulibaly *et al.*, 2002). With the protein hydrolyzed to amino acids, the time required for an activated sludge to achieve 90% removal rate of proteinaceous material was cut in half compared to the time for removal of peptone (a protease degradation product containing 24% free amino acids and about 76% peptides with molecular weights less than or equal to 1000 Da) (Ubukata, 1998). Confer and Logan (1997) studied the release of protein

hydrolytic fragments of bovine serum albumin in continuous and batch suspended cultures and in fixed-filmed reactor systems. The authors found that hydrolyzed BSA of intermediate sizes (2,000-10,000 Da) could accumulate in the treatment system. Twenty-five hours were needed to completely degrade this intermediate-molecular-weight protein. Therefore, if the proteins in the wastewater were degraded to smaller peptides or even amino acids before the release of wastewater to the biological treatment system, the effect could prove to be beneficial because of improved digestibility and decreased treatment time.

DETERMINATION OF LIPOLYTIC ACTIVITY OF XZYME

Lipids are organic compounds insoluble in water, but soluble in organic solvents. Neutral lipids are formed by esterification of one or more fatty acid residues with a glycerol molecule (Nichols and Sanderson, 2003). Thin layer chromatography (TLC) was applied to determine the activity of lipolytic enzymes in Xzyme cleaner on treated and untreated cream. Lipase catalyzes the hydrolysis of triglycerides to free fatty acids and glycerol.

Figures 15-17 showed the bands resolved on a TLC plate. Lipase-treated samples served as positive controls and were the only samples that showed bands resulting from lipolytic activity. Visual observation indicated the triglyceride fraction in the lipase-treated cream became less intense in color development than in the untreated cream.

Lipid hydrolysis was carried out at three different temperatures, 23, 37, and 50°C (Figures 15-17). The first experiment involved doing the hydrolysis at 23°C or room temperature (Figure 15). No degradation was observed. Another experiment was run at 37°C, the same temperature at which the lipase-treated cream was hydrolyzed and no hydrolysis was apparent on the TLC plate (Figure 16). Lastly, increasing temperature to 50°C also did not benefit the cleaner in terms of lipase activity (Figure 17). Therefore, the information obtained from the lipid analysis at different temperatures confirmed that the enzyme-based cleaner did not contain a lipase capable of hydrolyzing neutral milk lipids.

Although there was no lipolytic activity from the enzyme-based cleaner, the degradation of lipids prior to the biological treatment could prove to be useful. Wastewater containing lipids can pose problems with sludge floatation and odors. Fats also cause surface scum formation in settling tanks, digesters and pipe interior surfaces. In severe cases, the lipids might eventually clog and require shutdown of the treatment plants (Liu *et al.*, 2003). Masse *et al.* (2001) investigated the effects of pretreatment of fats from slaughterhouse wastewater with lipases from different sources (plant, bacterial and animal (pancreatic) origin). The authors found that pancreatic lipase worked better than bacterial lipase to reduce the average fat particle size after 4 hr of pretreatment while plant lipase did not reduce fat particle size. Treatment with pancreatic lipase increased the free long-chain fatty acid concentration, indicating some solubilization of the pork fat particles, which is desirable for waste treatment. Cammarota *et al.* (2001) and Jung *et al.* (2002) also studied the pre-hydrolysis of dairy wastewater with lipase and found that even at 700-800 mg/L of oil and grease, the COD removal efficiency could be maintained and was as high as 90%. Jung *et al.* (2002) noted also that with prehydrolyzed wastewater containing 400 mg oil and grease/L, the biomass growth in the reactor was 1.6 times more than the control. Therefore, enzymatic pre-hydrolysis enhanced the activity and growth of the biomass in the treatment reactor.

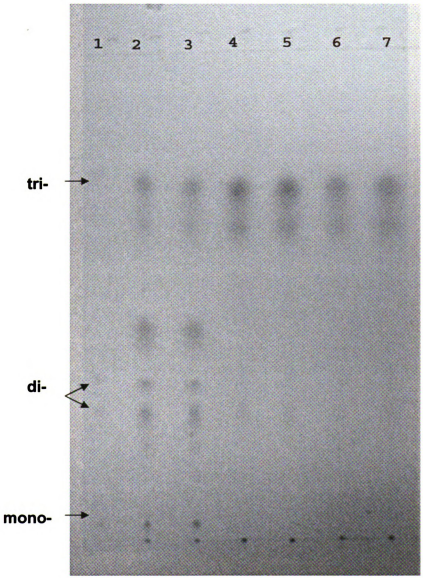

Figure 15: Separation of Xzyme™-treated and untreated dairy cream together with neutral lipid standards on TLC. The sample digestion was carried out at 23°C. Lane 1: tri-, di-, and monoglyceride standards; lanes 2 and 3: lipase-treated cream; lanes 4 and 5: untreated cream; lanes 6 and 7: Xzyme™-treated cream.

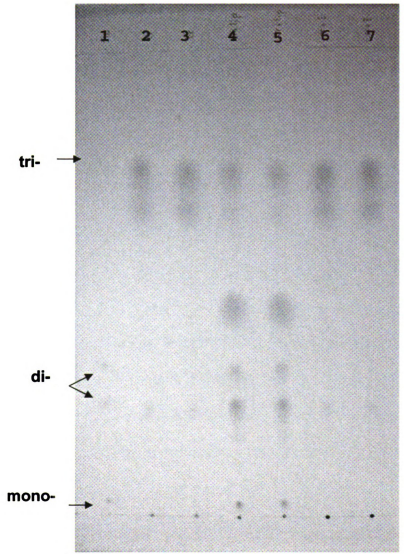

Figure 16: Separation of Xzyme™-treated and untreated dairy cream together with neutral lipid standards on TLC. The sample digestion was carried out at 37°C. Lane 1: tri-, di-, and monoglyceride standards; lanes 2 and 3: untreated cream; lanes 4 and 5: lipase-treated cream; lanes 6 and 7: Xzyme™-treated cream.

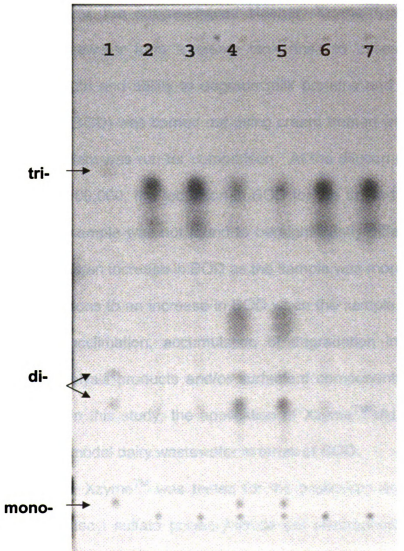

Figure 17: Separation of Xzyme™-treated and untreated dairy cream together with neutral lipid standards on TLC. The sample digestion was carried out at 50°C. Lane 1: tri-, di-, and monoglyceride standards; lanes 2 and 3: untreated cream; lanes 4 and 5: lipase-treated cream; lanes 6 and 7: Xzyme™-treated cream.

CONCLUSIONS AND RECOMMENDATIONS

In this study, the enzyme-based cleaner, Xzyme^{TM}, was applied to a model dairy wastewater from a cream tank rinse to assess effects on the wastewater strength and ability to degrade milk proteins and lipids. Biological oxygen demand (BOD) was carried out using cream treated with 10% Xzyme^{TM}. The untreated cream was run for comparison. At the dilution rates of 1:60,000, 1:80,000, and 1:100,000, the reduction in BOD for the treated cream compared to the untreated sample was not found to be significantly different (p<0.05). In addition, there was an increase in BOD as the sample was more diluted. Several possible explanations to an increase in BOD when the sample was more diluted could be seed acclimation, accumulation of degradation intermediates, and inhibition of hydrolysis products and/or surfactant components. Based on the results obtained in this study, the application of Xzyme^{TM} did not enhance the treatability of the model dairy wastewater in terms of BOD.

In addition, Xzyme^{TM} was tested for the proteolytic and lipolytic activity using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and thin layer chromatography (TLC), respectively. Non-fat dry milk (NFDM) powder was subjected to hydrolysis by the enzyme-based cleaner and the SDS-PAGE gels were visually inspected. The degradation was observed in the sample lanes treated with Xzyme^{TM}. The higher molecular weight bands disappeared and smaller fragments were accumulated as compared to the

untreated sample. Therefore, Xzyme™ contains proteolytic enzymes capable of breaking down milk proteins into smaller molecules.

Cream was also treated with Xzyme™ and the degradation of milk lipids was analyzed. Lipids were then extracted and run on the TLC plates. Xzyme™ does not contain lipolytic enzymes capable of hydrolyzing neutral milk lipids because there was no lipid degradation as observed from the similar band pattern on the TLC plate for the enzyme-treated and untreated samples.

Future studies related to this research are recommended as follows:

1. Investigate the effects on biological oxygen demand (BOD) of acclimating the seed culture to the Xzyme™ treatment. Side-by-side comparison would clarify if the unacclimated seed were responsible for the lag period during the first 2 days of BOD.
2. Due to the proprietary mix of Xzyme™, the manufacturer could carry out an inhibition/toxicity test on the seed culture to determine if any of the cleaner components or cream components affects the bacterial activity. The inhibition/toxicity test could be done on individual compounds, or mixtures such as surfactant and protein or surfactant and fatty acids.
3. From the information provided by the manufacturer, the enzyme-based cleaner tested is expected to have superior stability upon long period of storage. Therefore, another study could be proposed to determine

the shelf-life (i.e. - the retention of activity of the enzyme system) compared to products of the same kind from other manufacturers.

4. A long-term study using a laboratory-scale bioreactor could be conducted to observe the long-term effects of Xzyme™. Samples would be withdrawn and tested everyday for a 5-day biological oxygen demand (BOD_5) and other parameters commonly tested on wastewater (e.g. chemical oxygen demand (COD), total suspended solids (TSS), nitrogen, and phosphorus). The proposed project would mimic the conditions of the real wastewater treatment process. If the result was a reduction in BOD over a long reactor run, Xzyme™ could then be proven to work.

APPENDICES

APPENDIX A

Table A.1: Dissolved oxygen (DO) measurements of cream samples over a period of 5 days (p<0.05).

	Dilution	DO (mg/L)					
		Day					
		0	1	2	3	4	5
Untreated cream	1:100,000	8.548(±0.283)	8.579(±0.280)	5.283(±0.280)	3.275(±0.180)	2.598(±0.280)	2.288(±0.283)
Treated cream	1:100,000	8.540(±0.284)	8.403(±0.282)	6.036(±0.282)	3.612(±0.282)	2.642(±0.282)	2.176(±0.288)
Untreated cream	1:80,000	8.568(±0.391)	8.581(±0.385)	4.986(±0.384)[1]	2.558(±0.384)	1.603(±0.385)	1.175(±0.391)
Treated cream	1:80,000	8.452(±0.393)	8.297(±0.388)	6.135(±0.388)[1]	2.969(±0.388)	1.626(±0.388)	0.952(±0.393)
Untreated cream	1:60,000	8.659(±0.365)	8.576(±0.356)	4.223(±0.355)	1.535(±0.355)	0.720(±0.356)	0.458(±0.365)
Treated cream	1:60,000	8.550(±0.368)	8.516(±0.360)	4.928(±0.360)	1.908(±0.360)	0.529(±0.360)	0.360(±0.368)

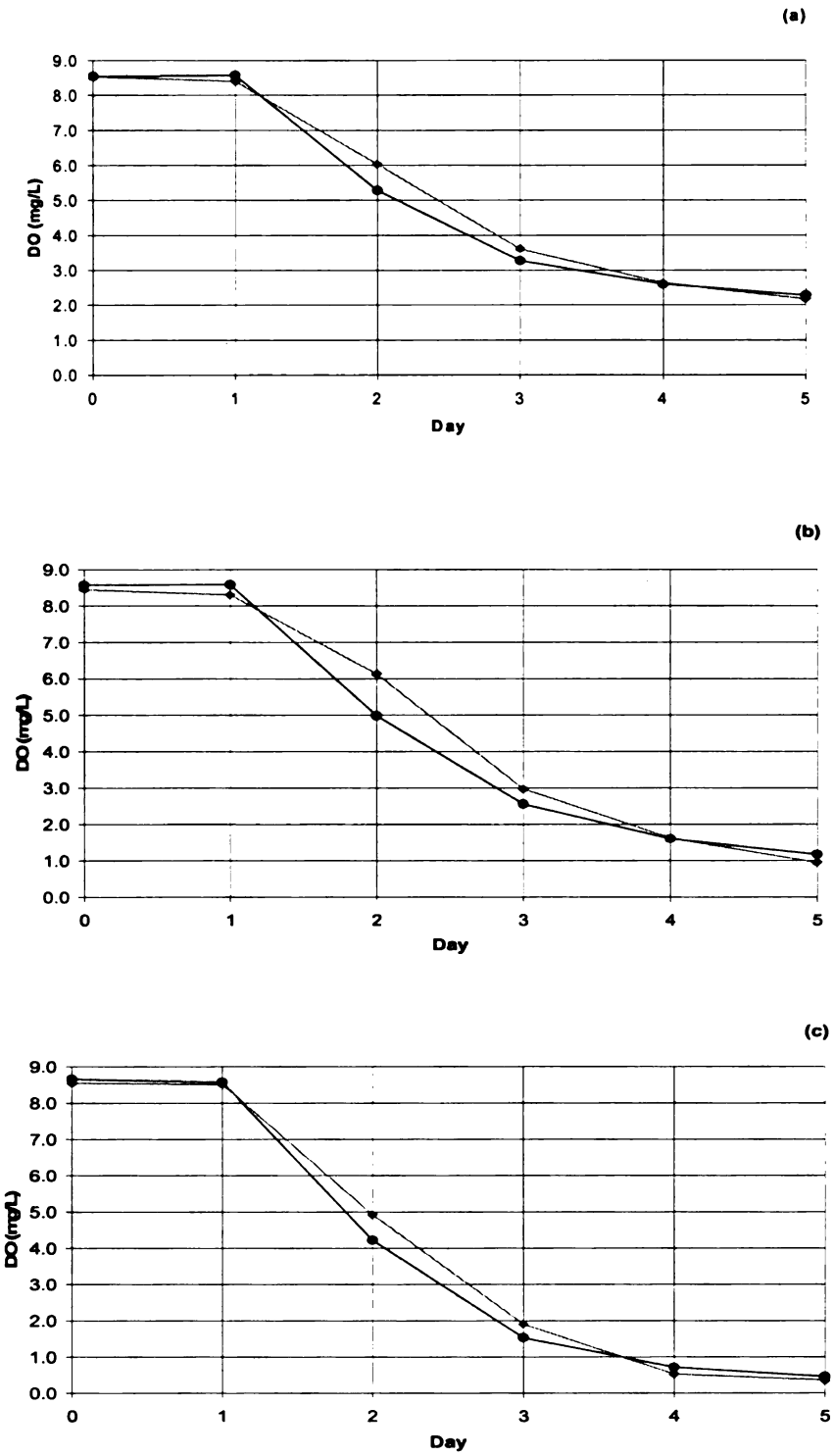

Figure A.1: Dissolved oxygen profile of Xzyme™-treated and untreated cream over a period of 5 days. (a) 1:100,000 dilution, (b) 1:80,000 dilution and (c) 1:60,000 dilution. (•) untreated cream, (♦)Xzyme™-treated cream.

APPENDIX B

Table B.1: Dissolved oxygen (DO) of dilution water, seed controls, glucose-glutamic acid (GGA).

	Dilution	DO (mg/L) Day					
		0	1	2	3	4	5
Dilution water	-	8.722(±0.112)	8.710(±0.112)	8.550(±0.112)	8.419(±0.112)	8.296(±0.112)	8.262(±0.112)
Seed control	1:30	8.603(±0.087)	8.166(±0.087)	6.938(±0.087)	6.462(±0.087)	6.197(±0.087)	6.135(±0.087)
Seed control	1:20	8.614±0.087)	7.890(±0.087)	6.183(±0.087)	5.488(±0.087)	5.132(±0.087)	5.011(±0.087)
Seed control	1:15	8.636(±0.087)	7.646(±0.087)	5.365(±0.087)	4.412(±0.087)	4.002(±0.087)	3.839(±0.087)
Xzyme™	1:5000	8.590(±0.202)	8.546(±0.202)	7.684(±0.202)	5.245(±0.202)	4.026(±0.202)	3.628(±0.202)
Glucose-glutamic acid	1:50	8.578(±0.063)	8.471(±0.063)	5.526(±0.063)	5.174(±0.063)	4.987(±0.063)	4.812(±0.063)

*standard errors are given in parenthesis.

Table B.2: Biological oxygen demand (BOD) of dilution water, seed controls, glucose-glutamic acid (GGA).

	Dilution	BOD (mg/L)
Dilution water	-	0.4(±0.064)
Seed control	1:30	73.8(±0.374)
Seed control	1:20	72.0(±1.673)
Seed control	1:15	72.0(±0.775)
Xzyme™	1:5000	22.7(±0.333)
Glucose-glutamic acid (GGA)	1:50	158.6(±1.939)

*standard errors are given in parenthesis.

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