RAPID DEGRADATION OF FOG DISCHARGED FROM FOOD INDUSTRY WASTEWATER BY LIPOLYTIC FUNGI AS A BIOAUGMENTATION APPLICATION

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Degree of Doctor of Philosophy

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DECLARATION

I declare that this is my own work and this thesis does not incorporate without acknowledgement any material previously submitted for a Degree or Diploma in any other University or institute of higher learning and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where the acknowledgement is made in the text.

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Dr. (Mrs.) Chandrika M. Nanayakkara Date: ……..

ABSTRACT

Rapid Degradation of FOG Discharged from Food Industry Wastewater by Lipolytic Fungi as a Bioaugmentation Application

Fats, oils, and grease (FOG) congregate in grease trap devices as a result of culinary activities in the foodservice industry. FOG is considered to be slowly biodegradable particulate (sbpCOD) organic matter and may require enzymatic or hydrolytic conversion to form readily biodegradable soluble organic matter (rbsCOD). The existing treatment methods are claimed on water-based hydrolysis of FOG to form long chain fatty acids. The long chain fatty acids discharged into wastewater treatment systems create functional difficulties, especially the inhibitory effect caused by the accumulation of such fatty acids. In order to overcome the issues associated with water-based treatment systems, FOG was extracted from the waste and solid-state degradation was performed by lipolytic fungi in a tray-type reactor as a novel approach of bioaugmentation. In the reactor, each 10 mg/g dry weight of FOG (substrate) was mixed with 1% w/v of coir fiber for proper aeration. Then the reactor was inoculated with 1 mL of spore suspension (1×10^7 spores/mL) of lipolytic fungi. The isolated lipolytic fungi were *Aspergillus niger, Geotrichum candidum, Aspergillus fumigates, Fusarium proliferatum* and *Penicillium citrinum.* The optimum conditions to degrade grease trap waste by solid-state degradation: initial moisture content of FOG should be 25 – 35% of weight; temperature 30°C; pH should be between 6–7; the reactor moisture condition for continuation of degradation process should be maintained around 65%. The higher degradation efficiencies (>80%) were recorded by these fungi isolates. As a practical application of the developed methodology, solid-state degradation was performed with raw grease trap waste (without extraction of FOG) in room temperature without adjusting the pH. The recorded pH for grease trap waste varied between 4.5–6.5 and most abundant fatty acids present in grease trap waste were palmitic acid 49.5% (w/w) and oleic acid 33% (w/w). Within 72 h of post-incubation, degradation efficiency of about 50% was recorded by fungal isolates. The degraded residue can be used as an inoculum for the degradation of the second set of grease trap waste. Therefore, once the degradation cycle is started, continuous inoculation for the rest of the degradation process would not be needed. The feasibility of using the developed protocol for FOG degradation was tested with a laboratory-scale tray type reactor, and it was operated successfully.

Keywords: Fatty acid methyl esters, grease trap waste, lipase, long-chain fatty acid, solid state degradation

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CHAPTER 1

1 INTRODUCTION

1.1 Background of the study

Fats, oils and grease (FOG) congregate in grease trap devices as a result of culinary activities in the food service industry. In Sri Lanka, the most common practices in food service industry for this aggregated grease trap waste are: (i) pumping up to a gully bowser (ii) drawing up manually by labours and handing over to the local authority in the area to dispose of with municipal solid waste. In addition, some patented chemical/biological formulations are used to dissolve/emulsify the floating scum and hardened grease without actually treating the pollutant. Eventually, the ultimate disposal point of the untreated waste is the environment (soil and water) and pollution caused by the grease trap waste is continued. This issue was confirmed by small and medium-sized hotels which have been registered for the SWITCH-Asia 'Greening Sri Lanka Hotels' project (2009–2013). Most of the hotels that operate at a relatively large-scale have their own wastewater treatment plants. Such hotels also raised the issue of inability to treat fat oil and grease (FOG) in conventional biological treatment systems (Ratnayake and Mittapala, 2011).

Some technologies are available for FOG treatment/management such as biodiesel and biogas production, which have received increased attention to be used as an energy source. Nevertheless, use of such higher biotechnological applications requires substantial capital outlay and demand for higher technical input with closer supervision. Consequently, it may discourage the industry personnel from the implementation of such advanced biotechnological methods, if it is not a part of their core business.

In Sri Lankan context, technical limitations and higher capital investment discourage the industry personnel from implementing advanced technological methods. Therefore, there is a growing demand for cost-effective FOG treatment/management methods to minimize the adverse impact on water and soil. The present study was aimed at finding a sustainable solution to the persistent problem of FOG in wastewater. The objective of the present research is to contribute to the development of a feasible technology to treat/degrade FOG. To this end, from different sources such as grease trap waste, lipolytic fungi were isolated.

Based on their lipolytic activity, best five fungi species were selected and inocula were prepared. The lipid degradation efficiencies were determined at different pH and moisture levels using FOG as the main carbon source. The feasibility of using the developed biotechnological application for degradation of FOG was also tested using raw grease trap waste.

1.2 The justification for the study

The present study identifies the existing research gaps in biological treatment systems in the food service industry wastewater. In conventional wastewater treatment systems (aerobic or anaerobic), FOG is separated by installing a grease trap/grease interceptors or dissolved air flotation (DAF) unit. In food service industries, where they treat the effluent by anaerobic treatment, there is a tendency to hydrolyze FOG into long-chain fatty acids (LCFA) due to an added advantage of increasing the methane yield. However, LCFA inhibits methanogenesis, which in turn discourage the use of FOG as an energy source for methane production. The aerobic treatment of FOG is not appealing due to the high cost to maintain aerobic conditions during the treatment process. Moreover, grease trap waste is a mixture of lipids, fatty acids, food particles, detergents and therefore characteristics may change from one location to another. Consequently, in most food service industries, though they have a wastewater treatment plant, grease trap waste is disposed of with municipal solid waste (without proper treatment) which may cause negative impacts on the environment. Therefore, it is necessary to find an effective method to treat FOG collected in the grease trap before disposal.

1.3 Research gap of using biological treatment methods

In biological treatment methods, most of the researchers have shown promising results in the production of enzymes with higher lipase activity (Santis-Navarro *et al*., 2011; Kumar and Kanwar, 2012; Parihar, 2012). These enzymes have been used to catalyze the initial hydrolysis step of FOG to form long-chain fatty acids (LCFA) (Valladao *et al*., 2007; Alexandre *et al*., 2011). These LCFA need further conversion via β oxidation to form acetate and then convert into methane and carbon dioxide by methanogens. The Figure 1.1, presented in this section, has been developed using reported literature on anaerobic degradation path of FOG (Li *et al*., 2005; Weng and Jeris, 1976; Palatsi *et al*., 2012).

The addition of enzyme accelerates the hydrolysis of FOG to form long chain fatty acids, it may have a propensity to accumulate the LCFA in the system. The β oxidation path has been

identified as limiting step of the anaerobic degradation of FOG (Pereira *et al*., 2004; Jeganathan *et al*., 2006). Consequently, accumulation of LCFA and toxicity effects has become unavoidable impact in anaerobic degradation path of FOG.

However, the unfavourable effects on methanogenic bacteria due to the accumulation of LCFA is also documented in the literature, notably the high mortality due to toxicity (Hwu and Lettinga, 1997; Alves *et al*., 2001; Shin *et al*., 2003; Kim *et al*., 2004; Long *et al*., 2012). The possible recovery processes to overcome this toxicity, such as precipitation with soluble calcium (Hanaki *et al*., 1981; Koster, 1987), use of bentonite (Mouneimne *et al*., 2004), adsorption with iron-containing clay (Ivanov *et al*., 2002) or zeolite (Nordell *et al*., 2013), use of high dilution ratio with water: active inocula (Palatsi *et al*., 2009; Wu *et al*., 2015), have been studied in detail. Most of these recovery measures have been developed to reduce the toxicity of LCFA were claimed by reducing the bioavailability of LCFA.

However, to date, the exact level and nature of the inhibitory effect of LCFAs on methanogenic bacteria is not well understood (Valladão *et al*., 2011; Long *et al*., 2012; Wu *et al*., 2015).

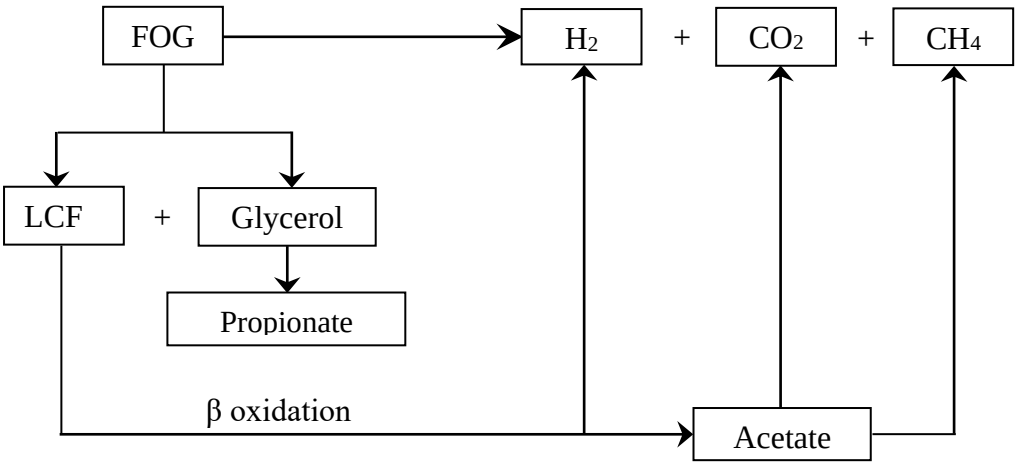


Figure 1.1: FOG degradation cycle under anaerobic condition

1.4 Objectives

The development of rapid biodegradation method without stressing the existing wastewater treatment system would encourage the industry personnel for pollution control aspects. The main objective of the present research is to contribute to the development of a feasible

technology to treat/degrade FOG. To strengthen the overall objective, three specific objectives have been defined:

1. Determination of the composition of wastewater
2. Investigation of biodegradation of high fat, oil and grease by lipolytic fungi
3. Development of optimum condition for fat, oil and grease degradation under ambient conditions.

1.5 The content of the thesis

This thesis provides an insight of existing problems related to FOG treatment/disposal and discusses the limitations of existing biological treatment methods to treat FOG. Also, advantages and feasibility of a developed protocol for the degradation of FOG have been discussed, and conclusions were made on results obtained.

Chapter 2: A Literature review on wastewater characteristics, FOG treatment methods, lipolytic fungi and bioremediation methods were compiled.

Chapter 3: Materials and methods used for the development of experimental set-up for effective biodegradation, environmental factors affecting optimum degradation are described in this chapter.

Chapter 4: Results and Discussion: wastewater characteristics of food service industry wastewater, the fattyacid profile of FOG were analyzed, and FOG degradation efficiency under optimum conditions was determined. The degradation of actual grease trap waste in a tray-type reactor was presented.

Chapter 5: Main conclusions of the study and recommendations for further research and development are included in the last chapter.

CHAPTER 2

2 LITERATURE REVIEW

Initially, the literature review has been carried out for the identification of research need/gaps, formulation of research questions and objectives. In this context, the past research data and information related to the source of oils/fats rich wastewater, their characteristics and treatment methods have been reviewd. It was used to compare the field data (preliminary field visits) with similar industries that have higher oil/fat content in wastewater. In addition, the data/information from the literature review was useful in identification of advantages and disadvantages of each method that hasbeen proposed for FOG removal/treatment (physical, chemical and biological).

The literature data/information have been gathered and reviewed for identifying research problems to development of research methodology; types of lipolytic microorganisms, environmental factors affecting their growth, metabolism and FOG removal/degradation,etc. In addition, personal communication methods have been used in the development of methods/techniques for analytical quantification.

Finally, literature survey was carried out to gather data and information from similar disciplines of research, used them to compare the results/observations from present study, and included under results and discussion.

2.1 Fats, oils and grease (FOG) rich wastewater generation and characteristics

Foodservice industries are considered the major consumers of vegetable oils and animal fats. Oils and fats are mainly used to enhance the taste, flavour,and texture of the food product (Omar *et al*., 2013). Nevertheless, with the change in lifestyle of the urban population, where home cooking has been replaced by takeaway food and restaurant meals, a large number of small hotels and restaurants have been opened up in many cities. As a result, the amount of fat and oil to be discharged with kitchen wastewater has also increased.The spent FOG comes out from the commercial kitchen in two basic forms viz., yellow grease and brown grease (Gaur *et al*., 2010). In general, yellow grease can be defined as the inedible, unadulterated FOG that remains in the containers after kitchen operations such as bulk deep-frying (Makkar

and Cammerota, 2002). This yellow grease usually does not contribute to elevated FOG levels in wastewater. The brown grease is mainly generated because of cleaning of dishes, pots, pans and utensils. The brown grease can be found as recovered materials from grease traps, and the majority of brown grease had been adulterated and in contact with food particles, detergents/cleaning solutions,etc. (Kiepper, 2001). The characteristics of wastewater discharge from food service industries especially from fast food restaurants, fish processing, poultry/slaughterhouse and dairy industries have shown in the (Table 2.1). Although, FOG discharged levels from restaurant wastewater varies from one location to another, BOD and COD levels of wastewater discharged from restaurants exceed 2000 mg/L (except few locations). As noted by Chun and Hsu (1999), considerable amount of FOG is discharged by pot and pan washing. The wastewater discharged from dairy industry contributes to high BOD and COD levels; however, FOG levels in wastewater are relatively low (Table 2.1).

2.2 Treatment methods of fats, oils and grease (FOG) rich wastewater

The oils/fats remain in the commercial kitchen, i.e., yellow grease may have beneficial uses such as the production of pet foods, surfactant biodiesel etc. However, the beneficial use of brown grease is hardly reported in the literature. Therefore, most of the treatment methods have been proposed to treat brown grease. Figure 2.1 provided a summary of FOG generation and treatment/management methods/options available in literature at a glance.

2.2.1 Physical treatment

In most of the food service industries, main technique for separating FOG from the wastewater stream is accomplished through gravity separation by the installation of grease traps/interceptors (physical treatment). Grease traps/receptors can be found in a treatment facility as a rectangular tank where the wastewater passes at a pre-determined rate during a pre-defined time period, fat/oil particles are risen to the water surface (gravity separation of floatable fat/oil). Due to elevated FOG levels in wastewater, numbers of baffles in the grease trap device needs to increase to retain the FOG. However, this method recurrently becomes aesthetically unappealing and causes nuisance in the vicinity of the treatment facility.

Dissolved air flotation system (DAF) is used to separate oil droplets dispersed in water. In this method, air is dissolved into wastewater under pressure. When the pressure is released, dissolved air releases itself into the water column in the form of micro-bubbles. These micro-

bubbles aggregate at the water-oil/fat interphase and separate the two phases from each other facilitating separation (Cammarota and Freire, 2006). Thus physical separation methods help to separate oil and grease from the main wastewater stream. However, the separated oil and grease has to be treated further or disposed of properly.

2.2.2 Chemical treatment

In chemical hydrolysis of oil and grease, i.e., treating wastewater with alkali has two objectives:

(i) neutralize the acidity of wastewaters

(ii) maintains an alkaline pH which facilitates the degradation of fat in the subsequent feeding tank.

The addition of alkali reduces the amount of fat in the effluent. The effects of various chemical hydrolysis [HCl, NaOH and $Ca(OH)_2$] and biological hydrolysis have been examined by Masse´ *et al*., (2001) who reported that both acid and alkaline pretreatment had reduced lipid in the sludge phase by 28%.Lin *et al*., (2009) reported that chemical hydrolysis with NaOH increases the ratio of soluble COD to total COD and reduces the volatile solid content during anaerobic digestion. However, these chemical methods remove the floating scum and hardened grease adhering to treatment plant equipment but transfer the pollutant from solid to the liquid phase, without actually treating the FOG.

2.2.3 Biological treatment

In biological treatment methods, biodegradation of fat and oil using lipolytic microbes in wastewater and/or enzymatic applications such as lipase has been a concern of many scientists in past few decades. Lipases are an array of enzymes which catalyses the hydrolysis of long-chain triglycerides, i.e., the parent compound of oils and fats. Hence, microbial lipases are in the focal point of oils/fats biodegradation research and development of associated enzyme technologies.

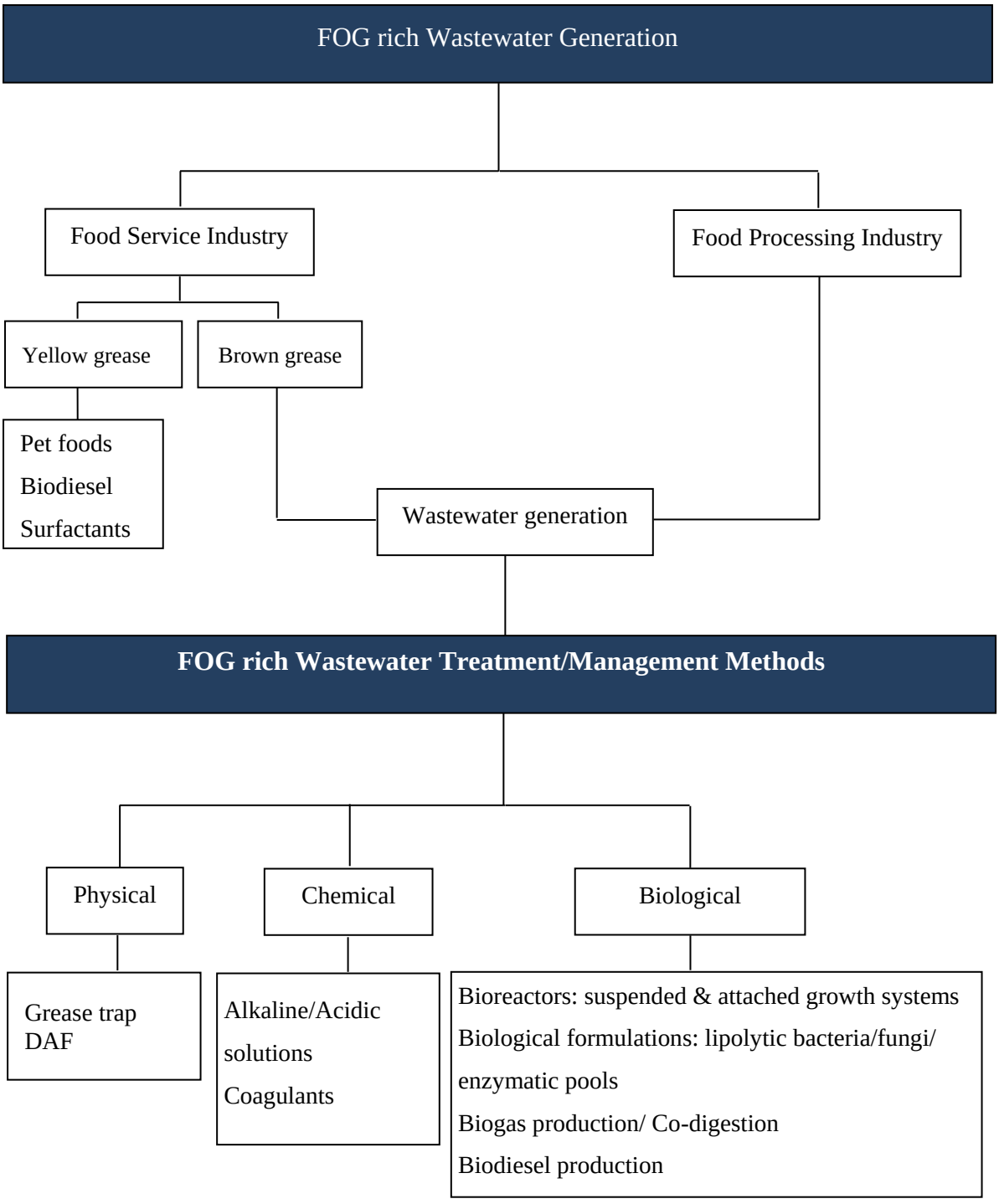


Figure 2.1: Oils/fats rich wastewater generation and treatment/management methods

Table 2.1: The characteristics of FOG rich wastewater from food service and food processing industries

Country	Sample type C-composite G-grab	pH	BOD_5 (mg/L)	COD_{tot} (mg/L)	TSS (mg/L)	VSS (mg/L)	TN (mg/L)	TKN (mg/L)	TP (mg/L)	FOG (mg/L)	Reference
Restaurants wastewater											
USA	C-24 hrs, 10 samples	4.7–10	4,000 (2,000–6,500)	7,900 (3,900–17, 000	3,900 (1,700–8,300)	3,800 (1,700–8,300		51 (14–82)	29 (20–4)	5,100 (2,200–9,900)	Schumidt *et al*.(1974)
	C-24 hrs, 7 samples	5.7–9.8	2,000 (1,200–4,700)	3,800 (1,700–8,200)	1,400 (46 – 3,000)	1,400 (440–2,900)		85 (55–100)	28 (22–41)	1,100 (230–2,900)	
Hong Kong	Pot & pan washing 4 - G samples			873–67,853						117–19,850	Chun and Hsu,(1999)
	Dish Washing 4 - G samples			4,695–25,304						365–6,827	
	Meat soaking and defrosting 5 - G samples			71–2,875						11–161	
	Floor washing 2 - G samples			13,320–24,142						2,478–8,641	
Canada		6.4 –7.0		87,480 (74,000–100,960)	82,250 (68500–96,000)	51,250 (41,600–60,900)			286 (240–355)	83,000 (52,000–114,000)	Jeganathan *et al*. (2006)
Taiwan	3 - G samples (Municipal Kitchen wastewater			80, 582				985	432	9,865	Kuo & Chan (2007)
Japan	3 G samples	5.6 ± 0.1	2,500 ± 12		110 ± 6					22,000 ± 17	Rashid & Imanaka (2008)

Country	Sample type C-composite G-grab	pH	BOD_5 (mg/L)	COD_{tot} (mg/L)	TSS (mg/L)	VSS (mg/L)	TN (mg/L)	TKN (mg/L)	TP (mg/L)	FOG (mg/L)	Reference
China	14 – G samples during busy hrs (12–15 hrs)			2,173 ± 176						529 ± 185	Chan (2010)
China	3 – G samples	6.1–8.0	600 – 2,500	750–5,800	250–650			4.8–10.4		500–4,700	Kang *et al*. (2011)
Fish processing wastewater											
Denmark	3 – G samples	9.02 ± 0.21		4200 ± 300		400		0.0		1000 ± 100	Maya-Altamira *et al*. (2008)
Brazil	7 – G Samples	6.3 ± 0.7		6612 ±4336						1,470 ± 1,188	Alexander *et al*. (2011)
Slaughter house wastewater											
Brazil	3 – G samples	6.3–6.6	1,300–2,300	2,000 – 6,200	850–6, 300	66–5,250		50–210	15–40	40–600	Caixeta *et al*. (2002)
Brazil	8 – G samples	6.5–7.0	1,780 ± 543	3102 ± 688	2,457 ± 332	1,782 ± 301		186 ± 27	76 ± 36	375 ± 151	Del Nary *et al*. (2007)
Denmark	3 – G samples	7.31 ± 0.22		10,400 ± 100		4,200 ± 300		400		1,300 ± 100	Maya-Altamira *et al*. (2008)
Brazil	3 – G samples	5.4 ± 0.3	638 ± 17	1,010 ± 23				216 ± 12	14 ± 7	10	Valladao *et al*. (2011)
USA	3 – G samples			10,000	3,347	2,667				14,297	Dassev & Theegala (2012)
Canada	3 – G samples	4.90 –	610 –	1,250 –15,	300 –		50 –841				Bustillo-

Country	Sample type C-composite G-grab	pH	BOD_5 (mg/L)	COD_{tot} (mg/L)	TSS (mg/L)	VSS (mg/L)	TN (mg/L)	TKN (mg/L)	TP (mg/L)	FOG (mg/L)	Reference
		8.10	4,635	900	2,800						Lecompte *et al.* (2014)
Dairy wastewater											
USA	C – 24 hrs, 15 samples	8.4 ± 1.8	1,856 ± 1,335	2,855 ± 1,646	976 ± 833	703 ± 479		91.4 ± 39.3	71 ± 40	As VFA 147 ± 115	Danalewich *et al.* (1998)
India	3 – G samples	5.5–7.5	350–600	1,500 – 3,000	250–600						Sakar *et al.* (2005)
Poland	Apparatus room 3 – G samples	10.37	3,470	14,639.5	3,821.2					3,105.2	Janczukowicz *et al.* (2008)
	Butter Section 3 – G samples	12.08	2,423.3	8,925.9	5,066.5					2,882.	
	Milk reception point 3 – G samples	7.18	797.6	2,542.9	653.6					1,056.8	
Italy	3 – G samples	5.3–7.0	380–702	662–1,293	275–450			5.9–36.7	0.79–6.84		Tocchi *et al.* (2012)

i. Bioreactors: suspended and attached growth system

A single or mixed culture of lipolytic bacteria is cultivated in bioreactors in the form of attached or suspended growth system. Depending on type and nature of wastewater, either aerobic or anaerobic bioreactoris used, and reactor condition is enhanced to facilitate the better performance of microbes. For instance, in 1998, Wakelin and Forster treated two fast food restaurant wastewater of having an organic loading rate of 5 kg $m^{-3}d^{-1}$ by using a consortium of gram-negative bacteria which had been isolated from grease trap residues from fast food restaurants and activated sludge. In this study, they used a novel bioreactor, the weir tank reactor, and two different mixed microbial cultures in the form of suspended growth. The reactor achieved a high removal rate (84 -96%) of fat, oil and grease, irrespective of the microbial inoculums. This high performance was attributed to aeration and periodic removal of a portion of microbial solids from the weir tank. The remarkable fact of weir reactor operation was that each trial was initially operated as a batch process for 3-4 days during the start-up period, to encourage acclimatization of each microbial inoculum to the fast food restaurant wastewater.

The study done by Rashid and Imanaka (2008) did not apply micororganisms directly to the wastewater. Bacteria were grown independently in porous rocks at 30 °C for 10 h by taking advantage of eliminating the acclimatization step. The porous rock was used as a bed for five isolated bacteria, in which the rock was dipped in the grease trap and continuously supplied with air. The microorganisms immobilized in the pores, subsequently degrade the food waste and grease. The results indicated that these isolates collectively were able to decrease the suspended solids from 102 to 40 mg/L and grease from 21,500 mg/L to 4 mg/L.

The study carried out by Keenan and Sabelnikov (2000) for bakery wastewater, experienced the advantage of using attached growth system. In their first trial, a continuous culture of bacteria (chemostat) was added to the treatment tank and oil and grease content in the outflow were still greater than the allowable level. Therefore, at the second trial, to increase cell concentration in the treatment tank, a solid stratum to support the bacterial growth, a biological filter, was incorporated. As a result,dramatic reduction of oil and grease in the bakery waste, from approximately 1.5 g/L to less than 0.03 g/L, was achieved.

Although in reported literature it was able to achieve high removal efficiency, most of these investigations have been carried out under controlled laboratory conditions. Therefore, it is timely to design and conduct pilot-scale investigations to make industrial applications realistic and effective.

ii. Biological Formulations

There are research reports and patents describing the use of microorganisms and/or enzyme pools developed in the laboratory for the biological treatment of effluents with high fat and oil concentrations (Table 2.2). Consequently, patented catalytic formulations, which can break down oils/fats rapidly into more usable form by microbes, have been introduced into wastewater treatment industry. These commercial products have been tested by immersing in the upper layer of greasetraps and reported hydrolysis rates of greater than 90%. Specifically, a bacterial density of 10^7 to 10^9 cells/mL employed for 12–72 h under and oxygen concentration of 4 – 8 mg/L was found to be the best combination of factors (Cammarota and Freire, 2006).

Table 2.2: Use of biological formulations (microorganisms and/orenzymatic pools) for the treatment of wastewater with high FOG contents

Enzyme/Product Name	Microorganism	Reference
Hydrolases, lipase, protease and amylase	*Penicillium restrictum*	Cammarota *et al.* (2003)
Amerzyme-A-100	*Bacillus subtilis*, a protease and amylase producing bacterium, and *Aspergillus niger*, lipase, cellulase and pectinase producing bacterium.	Mendez *et al.* (2005)
Enzyme complex	*Aerobacter aerogenes, Bacillus subtilis, Cellulomonas biazotea, Nitrosomonas sp., Nitrobacter winogradskyi, Pseudomonas stutzeri*	Cammarota *et al.* (2001)
Lipolytic enzymes	Microbial consortium derived from a mixture of wastewater sludges	Santis-Navarro *et al.* (2011)
Lipase	*Pseudomonas aeruginosa* LB-2	Parihar, 2012
Lipase	*Penicillium chrysogenum* SNP5	Kumar *et al.* (2012)

On the other hand, in an economical point of view, use of such products in intensive industrial applications rather depends on the reduction of production costs (Sharma, 2012). Therefore, research for selection of highly productive strains, and development and optimization of the product to be traded are needed.

iii. Solid State Fermentation

Use of microbial enzyme preparations obtained by solid-state fermentation (SSF) to treat food industry wastewater has received great attention. SSF culture medium can be prepared

using cheap organic waste material: agro-industrial residues such as rice or wheat bran, cereal husk and other vegetal-origin complex materials. As noted in literature, use of fungi species has an added advantage, as environmental conditions of solid-state fermentation are similar to those of their natural habitat. Therefore, it enables them to excrete large amounts of enzymes. Moreover, as pointed out by Cammerota and Frier (2006), solid-state fermentation systems have several advantages; the space requirement is relatively small in comparison with the product yield, high volumetric productivity, and can be applied directly. This approach has been tested by using fungi (*Penicillium restrictum*) as a single culture in dairyand poultry/ slaughterhouses industries which have a relatively high fat and oil content in wastewater (Table 2.3).

Table 2.3: Use of solid-state fermentation for the production of hydrolases using *P. restrictum*

FOG concentration	Reactor type	Reference
Dairy effluent Oil and Grease: 250 mg/L.	Anaerobic reactor	Leal *et al*. (2002)
Dairy effluent Oil and Grease: 400, 600, 800 mg/L	Batch activated sludge systems	Jung *et al*.(2002)
Dairy effluent Oil and Grease: 400–800 mg/L	Continuous activated sludge reactor	Rosa (2009)
Poultry slaughterhouse Oil and grease: 150–1200 mg/L	Anaerobic reactor	Valladão *et al*. (2007)
Dairy effluent 1200 mg of oil and grease/L	Anaerobic reactor	Rosa *et al*. (2009)
Poultry slaughterhouse 800 mg oil and grease (O&G)/L	Anaerobic reactor	Valladão *et al*. (2011)

2.3 Limitations of Biological Treatment Methods

The complete anaerobic degradation of lipid can produce more methane yield, for instance, 1 g of glycerol trioleate ($C_{57}H_{104}O_6$), a common lipid in nature, is equivalent to 1.08 L of methane, while 1 g of glucose ($C_6H_{12}O_6$) is equivalent to only 0.37 L (Kim and Shin, 2010). Therefore, research attempts reported in literature are to utilize the FOG in wastewater effluent. Although, low-cost biotechnological applications such as solid state fermentation enable to produce high enzymatic yield, as described above (section 1.3), use of

microbes/enzymes have an issue due to accumulation of long chain fatty acid in anaerobic degradation path of FOG. The toxicity preventive measures reported in literature are limited to laboratory investigations.

Moreover, grease trap waste is a mixture of lipids, fatty acids, food particles and detergents that are discharged from food service kitchens. Therefore, it is difficult to pre-decide the levels of inhibition caused by LCFA. These inconsistencies prevent the adoption of hydrolysis of FOG as a treatment option in conventional wastewater treatment systems.

2.4 Alternative methods to manage FOG

A number of management options for FOG accumulated in grease abatement devices have been proposed viz land application (Rashid and Voroney, 2004), composting (Aikaitė-Stanaitienė *et al*., 2010), anaerobic co-digestion (Silvestre *et al*., 2011) or biodiesel production (Chung *et al*., 2010;Kobayashi *et al*., 2012). Also, comprehensive reviews of some methods such as biodiesel production (Lam *et al*., 2010) co-digestion (Long *et al*., 2012), sorption (Pintor *et al*., 2016) have been presented in recent literature.

CHAPTER 3

3 MATERIALS AND METHODS

The research methodology was developed in consecutive stages. In stage one, preliminary field visits were carried out to different food service (hotels, restaurants, and cafeteria) and food processing (bakeries and dairy) industries. Wastewater samples were collected from each location during peak hours of the production process, and wastewater characteristics were determined.

In the second stage, grease trap waste samples were collected from different hotels, restaurants, and cafeteria and physicochemical properties of FOG were determined. The same grease trap waste samples were used as the source for isolation of lipolytic fungi. Based on FOG degradation efficiency and unit enzyme activity, most effective and efficient lipolytic fungi isolates were selected for extracellular enzyme production under the optimum condition.

In the third stage, after several trials, FOG degradation was demonstrated using extracts of the lipid portion of grease trap waste (i.e., FOG). After that, raw grease trap waste (without extraction of lipid portion) was used to investigate the degradation under field conditions.

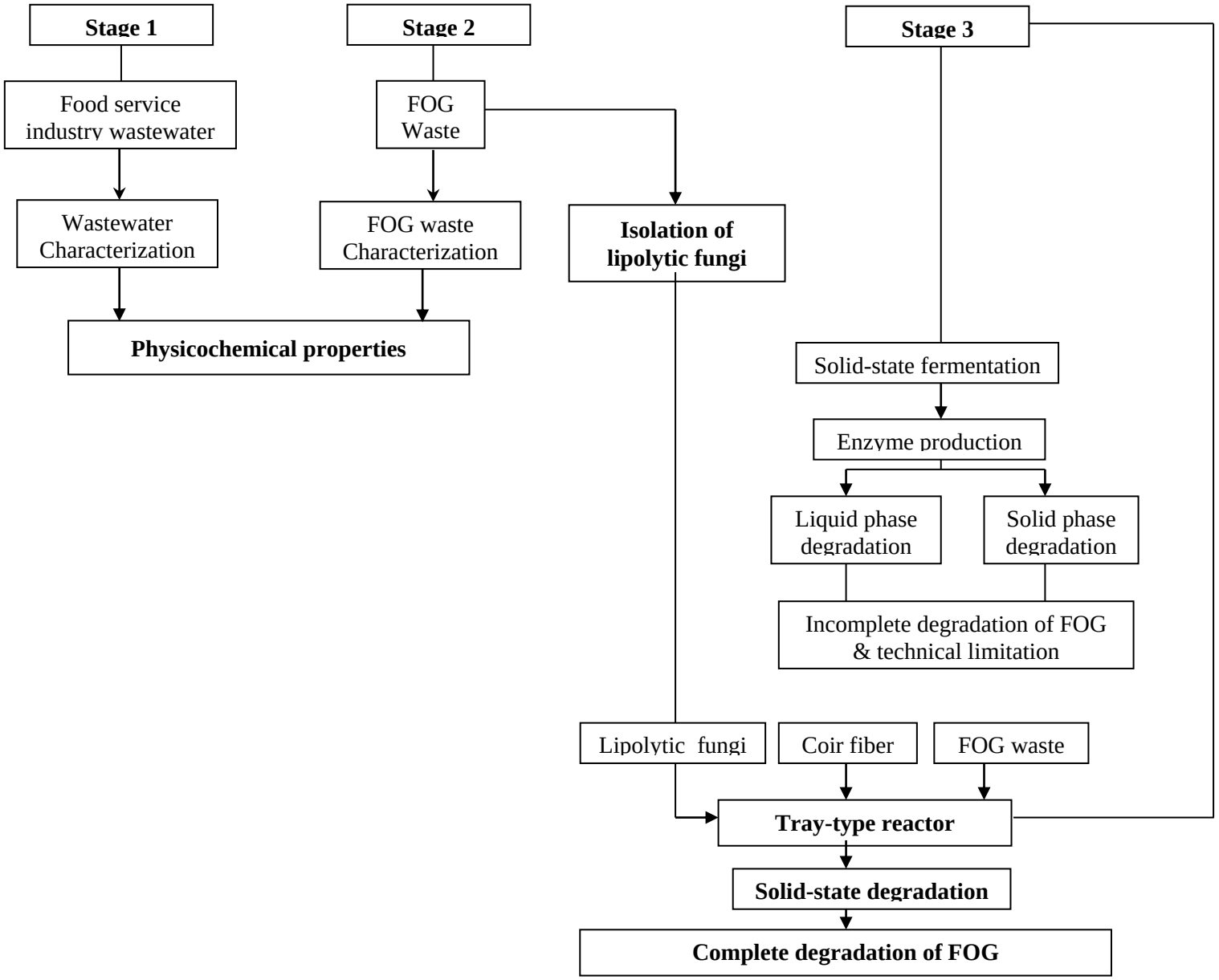


Figure 3.1: Flow chart of the methodology

3.1 Experimental set-up and analytical methods

Chemicals and glassware

In stage 1, for wastewater characterization Analytical Grade chemicals were used. In stage 2, for microbiological testing high purity (>99%) chemicals were used. In stage 3, for GC analysis, HPLC grade chemicals were used.

Equipment

A GBC 932 plus Atomic Absorption Spectrometer (AAS) operating with air/acetylene flame and air/ $N_2O$was employed to measure cation concentrations. The pH of the solution was measured with EUTEC model pH meter with a glass electrode. A mechanical shaker model (221/UA.Pm 172/35) was used for shaking. An analytical balance (model ACB 1500) was used for weighing the samples. Agilent 7890 B Gas Chromatography equipped with Mass Selective Detector (Model: 5977 A: S.N.: US 1420L216) was used for qualitative and quantitative analysis of fatty acid methyl esters.

Stage 1: Characterization of Wastewater from Food Service and Food Processing Industries

3.2 Selection of study sites

Selection of study sites was based on the production volume and the accessibility to the location for sample collection (Table 3.1). The twelve sampling locations out of eighteen locations of food service industry are of large scale and belonged to three/four/ five-star category, and only 6 sites belonged to small and medium-sized food service industries. Most of the bakeries selected for the studies are operated in large scale (7) and have their distribution channels/stalls in Colombo and metropolis. Unlike hotels and bakeries, dairies are present in few numbers in the country and have limited accessibility for sample collection due to security and hygienic reasons. Therefore, samples were collected from only two locations.

Table 3.1: FOG rich effluents collecting points

Industry	Sampling location/District	No of sampling points
Food Service Industry (Hotels, restaurants and cafeteria)	Colombo	8
	Kalutara	10
Food Processing Industry (Bakeries)	Colombo	5
	Kalutara	2
	Gampaha	3
Food Processing Industry (Dairies)	Kalutara	1
	Gampaha	1
Total		**30**

3.3 Collection and preservation of wastewater samples

The grab samples were collected on an hourly basis from commercial kitchens of each sampling point for three hour period during peak hours of the production process and brought to the Environmental Engineering Laboratory of the University of Moratuwa. Samples were collected in well-labeled polypropylene or glass bottles on an hourly basis. The collection times were usually from 0800-1500 GMT. Sample handling and preservation techniques were followed as described in Standard Methods for Water and Wastewater, 21st edition, 2005 (APHA, 2005), (Table 3.2) and *in-situ* measurements were taken for pH, temperature, conductivity and oxidation/reduction potential (ORP).

Table 3.2: Sample collection and handling

Parameter	Container	Minimum sample size (mL)	Preservation method	Maximum storage time
Fat, oil and grease	G, wide mouth bottle	500	Add HCL or H_2SO_4 to pH < 2, refrigerate	28 days
BOD	P, G	1000	Refrigerate	6 h
COD	P, G	100	Analyze as soon as possible, or H_2SO_4 to pH < 2, refrigerate	7 days
Total Nitrogen	P, G	200	Add H_2SO_4 to pH < 2, refrigerate	1 – 2 days
Total Phosphorus	P, G	100	Add H_2SO_4 to pH < 2, refrigerate	28 days

Source: APHA, 2005 Note: G – Glass; P - Polypropylene

3.4 Characterization of wastewater

In-situ measurements were taken for pH and temperature by using pH and temperature probes in pH meter. Conductivity and oxidation/reduction potential (ORP) of wastewater were measured by using a conductivity meter. The *ex-situ* measurements were taken for total FOG content, five-day Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), solids, alkalinity, total Nitrogen (TN), Total Phosphorus (TP) and salts.

3.4.1 Total FOG content

Liquid-liquid, partition gravimetric method (5520 B - APHA, 2005) was used to extract the oils/fats present in the sample. When a sample was brought into the laboratory, the sample bottle was marked at the water meniscus and acidified with 1:1 HCl to bring the pH 2 or lower. Then the sample was transferred to a separator funnel, and sample bottle was rinsed with 30 mL of n-Hexane, and solvent washing was added to a separatory funnel. The funnel was shaken for about 2 minutes, and it was kept on the holder to separate the layers. The aqueous layer was drawn into theoriginal sample container. The organic layer was drawn through a funnel containing a filter paper, and 10 g of $Na_2SO_4$was added to a clean, solvent-rinsed, pre-weighed conical flask. The extraction cycle was repeated twice with 30 mL n-Hexane. The n-Hexane was recovered by distilling the flask in a water bath at 85 °C, and n-Hexane was collected into a container in an ice bath. The flask was cooled in desiccators until a constant weight was obtained. The initial volume of the sample was determined by filling up sample bottle to the mark with water and then it was pouredinto a 1 L measuring cylinder. Fat, oil and grease content in the sample was determined in mg as follows;

$$\text{mg of fat, oil, and grease in the sample} = \frac{W_1 - W_0}{V_S} \times 1{,}000 \qquad \text{Equation (1)}$$

where W_1 is the total weight of the flask with residue (g), W_0 is the empty weight of the flask (L), and Vs is the initial sample volume (mL).

3.4.2 Five-day Biochemical Oxygen Demand (BOD_5)

The test method (5210 – B APHA, 2005) measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulphides and ferrous iron. The method consists of filling with thesample, to overflowing, an airtight

bottle of 300 mL and incubating it at 20 ^{0}C for five days. The dissolved oxygen was determined by the Azide modification of the Winkler's method before and after incubation for five days at 20 ^{0}C (4500-O C), and the BOD was computed from the difference between initial and final DO. The difference gave the BOD_5 of the sample. The pH adjustment was not made for the samples because pH ranges of samples were within the range for optimum biochemical oxidation, i.e., 6.0 to 8 (APHA, 2005).

BOD_5 calculation: For each test bottle meeting the 2.0-mg/L minimum DO depletion and the 1.0-mg/L residual DO, calculate BOD_5 as follows:

$$BOD_5 = \frac{D_1 - D_2}{P} \qquad \text{Equation (2)}$$

Where D_1 is the DO of the diluted sample immediately after preparation (mg/L), D_2 is the DO of diluted sample after 5d incubation at 20°C (mg/L) and P is the decimal volumetric fraction of sample used.

3.4.3 Chemical Oxygen Demand (COD)

Chemical Oxygen Demands (COD) were determined using open reflux method (5220 B, APHA, 2005). The organic matter present in the sample was refluxed in a medium of strong sulfuric acid solution (with silver sulphate as a catalyst) and a known amount of excess potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed, and the oxidizable matter was calculatedin terms of oxygen equivalent. Interferences from chloride were suppressed by the addition of mercuric sulphate to the reaction mixture. The chemical oxygen demand (COD) was expressed as milligrammes of oxygen absorbed from standard dichromate per litre of the sample (APHA, 2005).

Calculation:

$$COD \text{ as mg } O_2/\text{L} = \frac{(A-B)\times M \times 8{,}000}{sample\ volume,\ \text{mL}} \qquad \text{Equation (3)}$$

Where *A* is the FAS used for blank (mL), *B* is the FAS used for sample (mL), *M* is the molarity of FAS, and 8,000 is the milliequivalent weight of oxygen × 1,000 mL/L.

3.4.4 Solids

The measurements of solids were carried outusing the gravimetric procedure. The different forms of solids were determined by weighing after the appropriate handling procedures as described in APHA, 2005.

Total solids (TS) were determined by subtracting the initial weight of the clean evaporating dish as follows:

$$mg\ total\ solids/L = \frac{(A-B)\times 1000}{sample\ volume,\ \text{mL}} \qquad \text{Equation (4)}$$

Where *A* is the weight of dried residue + dish (mg), *B* is the weight of dish (mg).

Total Dissolved Solids

A well-mixed sample of 50 - 100 mL was filtered through a Whatman® GF/C glass fibre filter paper, the filtrate was evaporated to dryness in an evaporating dish and dried at 180 ± 2°C for 1 hour, then, cooled in desiccators, and weight was recorded. The drying cycle of drying, cooling, desiccating, and weighing was repeated until weight change was less than 4% of previous weight or 0.5 mg. The increase in dish weight was recorded as total dissolved solids.

$$\text{mg dissolved solids/L} = \frac{(A-B)\times 1{,}000}{sample\ volume,\ \text{mL}} \qquad \text{Equation (5)}$$

where *A* is the weight of dried residue + dish(mg) and *B* is the weight of dish (mg).

Total Suspended Solids

The pre-weighed Whatman® GF/C glass fibre filter disk was placed on the base of the Buchner funnel, and vacuum was applied. The filter disk was wetted with a small volume of reagent grade water to seal it. A well-mixed sample of 50-100 mL was filtered through filter disk, and vacuum was applied until all traces of water were removed. The filter paper was carefully removed from the base and dried at least one hour at 103 - 105 °C. Thereafter, it

was allowed to cool in a desiccator, and weight was recorded. The drying cycle of drying, cooling, desiccating, and weighing was repeated until weight change was less than 4% of previous weight or 0.5 mg. The increase in the weight of filter paperwas recorded as total suspended solids.

$$\text{mg supended solids/L} = \frac{(A-B)\times 1{,}000}{sample\ volume,\ \text{mL}} \qquad \text{Equation (6)}$$

where *A* is the weight of dried residue + dish (mg), *B* is the weight of dish (mg).

Total fixed and volatile solids

The residue from total solid was ignited to a constant weight at 550 °C. The remaining solids represent the total fixed solids while the weight losses on ignition were recorded as volatile solids (Approximation of organic matter present in the solid fraction of wastewater). The cycle of igniting, cooling, desiccating, and weighing was repeated until weight change was less than 4% of previous weight or 0.5 mg.

$$\text{mg volatile solids/L} = \frac{(A-B)\times 1{,}000}{sample\ volume,\ \text{mL}} \qquad \text{Equation (7)}$$

$$\text{mg fixed solids/L} = \frac{(B-C)\times 1{,}000}{sample\ volume,\ \text{mL}} \qquad \text{Equation (8)}$$

where *A* is the weight of residue + filter paper before ignition(mg), *B* is the weight of residue + filter paper after ignition (mg) and *C* is the weight of filter paper (mg).

3.4.5 Total Nitrogen (TN)

The persulfate method (4500-N, APHA, 2005) was used to determine the total nitrogen. All nitrogenous compounds (organic and inorganic nitrogen) were converted to nitrate at 100 to 110°C by alkaline oxidation. UV-Visible spectrophotometer was used for colorimetric determination of nitrate in the sample.

Apparatus

a. *Autoclave*, capable of developing 100 to 110°C for 30 min
b. *Glass culture tubes:* 30-mL screw-capped (polypropylene liner-less caps)

Reagents

a. *Stock nitrate solution*: Potassium nitrate (KNO_3) was dried in an oven at 105 °C for 24 h and dissolved 0.7218 g in water and diluted to 1000 mL; 1.00 mL = 100 μg NO3–N. The stock solution was preserved with 2 mL $CHCl_3$/L.
b. *Intermediate nitrate solution:* A 100 mL of stock nitrate solution was dilute to 1000 mL with water; 1.00 mL = 10.0 μg NO_3-N. The intermediate nitrate solution was preserved with 2 mL $CHCl_3$/L.
c. *Stock glutamic acid solution:* Glutamic acid was dried, $C_3H_5NH_2(COOH)_2$, in an oven at 105°C for 24 h and dissolved 1.051 g in water and diluted to 1000 mL; 1.00 mL = 100 μg N. The stock glutamic acid solution was preserved with 2 mL $CHCl_3$/L.
d. *Intermediate glutamic acid solution:* A100 mL stock glutamic acid solution was diluted to 1000 mL with water; 1.00 mL = 10.0 μg N and preserved with 2 mL $CHCl_3$/L.
e. *Digestion reagent:* A 20.1 g of potassium persulfate ($K_2S_2O_8$) having low nitrogen (<0.001% N) content and 3.0 g NaOH was dissolved in water and dilute to 1000 mL just before use.
f. *Borate buffer solution:* A61.8 g of boric acid (H_3BO_3), and 8.0 g NaOH was dissolved in water and diluted to 1000 mL.

Digestion

To a culture tube, 10.0 mL volume of sample (or standard, or a portion diluted to 10.0 mL) and 5.0 mL digestion reagent was added. It was capped tightly and the solutions were mixed by inverting the tube twice. The cap was loosened and kept in an autoclave for 30 min at 100 to 110°C and slowly cooled down to room temperature. Then, 1.0 mL borate buffer solution as added to the tube and mixed by inverting at least twice.

Blank:

A reagent blank through all steps of the procedure was carried out, and necessary corrections were applied to the results.

Calibration curve:

Calibration standards were prepared for NO_3^- in the range of 2 to 10 mg/L by diluting to required volume using the stock nitrate solution. A distilled water blank with the combined

reagent to make photometric readings for the calibration curve was used. The graph was plotted absorbance vs. nitrogen concentration to give a straight line passing through the origin.

Digestion check standard:

Glutamic acid digestion check standard was prepared by diluting, 2 mL to 100 mL volume of intermediate glutamic acid solution.

Nitrate measurement

Using colour development method (Palin test nitrates tablets), the standard curve was developed by plotting the concentration against absorbance using UV-Visible spectrophotometer at the wavelength 570 nm.

Calculation

As per to the standard curve, total nitrogen in the sample was computed by the instrument (UV-Visible spectrophotometer).

3.4.6 Total Phosphorus (TP)

Persulfate digestion method (4500-P) was used to determine the total Phosphorus in the sample by converting all forms of phosphorus to dissolved orthophosphate. UV-Visible spectrophotometer was used for colorimetric determination of dissolved orthophosphate.

Apparatus:

1) *Hotplate:* A 30 × 50 cm heating surface is adequate.
2) *Autoclave:* An autoclave capable of developing 98 to 137 kPa
3) *Glass scoop:* to hold required amounts of persulfate crystals.

Reagents:

1) *Phenolphthalein indicator aqueous solution.*
2) *Sulfuric acid solution:* Carefully added 300 mL conc. H_2SO_4 to approximately 600 mL dis*tilled water and diluted* to 1 L with distilled water.
3) *Ammonium persulfate,* $(NH_4)_2S_2O_8$, solid,
4) *Sodium hydroxide,* NaOH, 1M.

Procedure:

A 50 mL volume of thoroughly mixed sample was taken, and 0.05 mL (1drop) of phenolphthalein indicator was added. If a red colour develops, $H_2SO_4$is added to the solution drop-wise just to discharge the colour. Then 1 mL H_2SO_4 solution and 0.4 g solid $(NH_4)_2S_2O_8$ were added and heated for 30 min in an autoclave at 98 to 137 kPa. It was cooled, and 0.05 mL (1 drop) phenolphthalein was added and neutralized to a faint pink colour with NaOH. The final volume was made up to 100 mL with distilled water.

The standard series of samples were also digested according to the persulphate digestion method as described above.

Preparation of calibration curve:

A calibration curve was developed from a series of four standards within the phosphate ranges from 1 to 4 PO_4^{3-}P/ppm. A distilled water blank with the combined reagent to made photometric readings for the calibration curve was used. The graph was plotted absorbance vs. phosphate concentration to give a straight line passing through the origin.

Calculation

As per to the standard curve, total phosphorus in the sample was computed by the instrument.

3.4.7 Cations

The Flame Atomic Absorption Spectrometer (FAAS) was used to determine different cations such as calcium, magnesium, sodium and Potassium present in the sample. According to the Lambert-Beer law, the absorption is proportional to the concentration of free atoms in the flame.

$$\text{Absorbance} = \log \frac{I_o}{I_t} \, \alpha \, C \qquad \text{Equation (9)}$$

where I_o is the intensity of incident radiation emitted by the light source, I_t is the intensity of transmitted radiation (amount not adsorbed) and C is the Concentration of free atoms.

The instrument was calibrated as per to the standards of each cation that are to be measured and the sample concentrations interpolated according to the calibration curve.

(1) *Calcium stock solution:* Accurately weighed out 0.2497 g (to 0.001 g) of dried calcium carbonate ($CaCO_3$) to a watch glass and it was rinsed into a 100 mL volumetric flask

with a few millilitres of reagent grade water. Then $CaCO_3$ was dissolved in a minimum amount of 1N HCI (2.5 mL), diluted to the mark with reagent grade water to obtain 1000 μg/ mL Ca.

Atomic absorption: Lamp current – 10.0 mA; Flame type – Nitrous oxide-acetylene; Wavelength – 422.7 nm; Working range – 1 to 4 μg/ mL

(2) Magnesium stock solution: Accurately weighed out 0.100 g (to 0.001 g) of dry magnesium oxide (MgO) to a watch glass and it was rinsed into a 100 mL volumetric flask with a few millilitres of distilled water. Then MgO was dissolved in a minimum amount of 5 M HCI (2.5 mL) and diluted to the mark with reagent grade water to obtain 1000 μg/ mL Mg. Atomic absorption: Lamp current - 3.0 mA; Flame type – Air-acetylene; Wavelength – 202.6 nm; working range – 5 to 20 μg/ mL

(3) Sodium stock solution: Accurately weighed out 0.2542 g (to 0.001 g) of NaCl, to a watch glass and it was rinsed into a 100 mL volumetric flask with a few millilitres of reagent grade water and dissolved in distilled water, to obtained 1000 μg/ mL Na.

Atomic absorption: Lamp current - 5.0 mA; Flame type – Air-acetylene; Wavelength – 589.6 nm; Working range – 0.4 to 1.5 μg/ mL

(4) Potassium stock solution: Accurately weighed out 0.1906 g (to 0.001 g) of KCl, to a watch glass and it was rinsed into a 100 mL volumetric flask with a few millilitres of reagent grade water and dissolved in reagent grade water, to obtain 1000 μg/ mL K.

Atomic absorption: Lamp current - 6.0 mA; Flame type – Air-acetylene; Wave length – 769.9 nm; Working range – 1.1 to 4.4 μg/ mL.

Stage 2: Physicochemical Properties of Grease Trap Waste and Isolation of Lipolytic Fungi

Grease trap waste samples and oil-contaminated soils were collected from hotels, restaurants, cafeteria and coconut oil mills located in Colombo and its suburbs. Lipolytic fungi were isolated from grease trap waste and oil contaminated soils. The lipolytic activity of each isolate was determined by lipase unit activity. Based on their ability to prodeuce the lipase enzyme (Lipase unit activity) best five isolates were selected for further studies.

3.5 Collection and preservation of grease trap waste

Oil contaminated soil samples were collected from an industrial coconut oil mill, at a depth of 3-5 cm, from three locations of the site. Grease trap waste samples were collected at 3 points of the grease trap chamber: surface, interface (between oil and water layer) and chamber wall (scrapped). All the samples were collected insterilized sealable polythene bags and transported under cold storage to the Microbiology Laboratory, Environmental Engineering Division, Department of Civil Engineering, University of Moratuwa.

3.6 Characterization of grease trap waste

The initial water/moisture content and pH of the grease trap waste samples were determined before storing at ⁻18 °C. The samples were thawed (in a water bath at ~40 °C) and homogenized before determination of total FOG content and fatty acid profile.

3.6.1 pH and water/moisture content

The initial water/moisture content and pH of the grease trap waste samples were determined before storing at ⁻18 °C. About 20g of grease trap waste was weighed and transferred into 100 mL beaker and 40 mL distilled water was added and stirred well with a glass rod. It was allowed to stand for half an hour and the supernatant was collected to measure the pH.

A known weight of grease waste sample was taken in to clean dry dish and kept at 105 °C for 24 h and drying cycle of drying, cooling, desiccating, and weighing was repeated until weight change was less than 4% of previous weight or 0.5 mg. The moisture content of the grease waste samples wasdetermined by subtracting final weight (after 24 h of drying at 105 °C) from wet weight.

3.6.2 Total FOG content (gravimetric)

The samples were thawed (in a water bath at ~40 °C) and homogenized before determination of total FOG content and fatty acid profile. A known weight of grease trap waste was taken, and total lipid portion was extracted using the method developed by Bligh and Dyer (1959). [Refer the 2.2.4 section for detailed methodology.]

3.6.3 Fatty acid composition

Extraction of Fats, Oils and Grease (FOG)

The extraction of the lipid portion of grease trap waste is essential to the determination of the profile and subsequent quantitative analysis. The lipid extraction was carried out according to the method developed by Bligh and Dyer (1959): The grease trap waste samples were homogenized with a mixture of chloroform and methanol in a proportion such that a miscible system is formed with the water in the grease trap waste (see below section). Then, dilution was made with chloroform and water, which separates the homogenate into two layers, the chloroform layer containing all the lipids and the methanolic layer containing non-lipids. The lipid extract is obtained by isolating the chloroform layer.

Following the above method, a known weight of grease trap waste was taken and mixed with water, methanol, and chloroform at a ratio of 1:2:0.8, respectively. Subsequently, water and chloroform were added to shift the ratio to 2:2:1.8. The lower chloroform layer was transferred (using a Pasteur pipette) into a clean glass vial,and the volume was recorded. Then chloroform was evaporated, and the residue was subsequently driedunder a stream of N_2,and the final weight of the above glass vialwas recorded. Then, the extracted sample was stored in 5 mL of chloroform:methanol (10:1) mixture at ⁻18 °C until use.

Preparation of Fatty Acid Methyl Esters (FAMEs)

The fatty acid composition of the samples needs to be determined as fatty acid methyl esters (FAMEs) which are moderately polar and sufficiently volatile to be detectable by Gas Chromatography/Mass Spectrometer (GC/MS). The ester bonds of the fatty acids are hydrolyzed, and the free fatty acids that are formed in the process are converted to the corresponding fatty acid methyl esters.

In order to derive FAMEs, acid-catalyzed esterification was performed to produce FAME according to the method developed by Christie, 1993. The extracted lipid sample up to 5 mg was dissolved in toluene (1 mL) in a screw-capped test tube, and 1 % sulfuric acid in methanol (2 mL) was added. The mixture was left overnight in a water bath at 50 °C. Water

(5 mL) containing NaCl (5% w/v) was added and the esters produced were extracted with hexane (2 × 5 mL) using Pasteur pipettes. The hexane layer was washed with water (4 mL) containing $KHCO_3$ (2% w/v) and was dried over anhydrous sodium sulphate. The solution was filtered to remove the drying agent, and the solvent was removed under a stream of nitrogen to complete dryness. Then the residue was reconstituted in hexane and topped-up to a volume of 10.0 mL in a volumetric flask. A volume of 1.0 mL was taken into a vial for identification and quantification of fatty acid methyl esters (FAME) using the GC/MS.

In addition, the FAME profile of virgin cooking oils (olive oil, coconut oil, palm oil, sunflower oil, soya oil) and animal fats (butter, chicken and lard) were derivedfrom making a comparison with the fatty acid profile of the waste. The same extraction and derivatization procedure was followed for cooking oils and animal fats witha view tomaintaining consistency of experiments.

Gas Chromatography and Mass Spectrometer (GC/MS) Analysis

The gas chromatography/mass spectrometer (GC/MS) was used to identify and quantify the complete fatty acid profile present in a lipid sample. The quantification of fatty acids was made by the development of a calibration graph by using the FAME Mix, C_8-C_{24} (CRM18918 SUPELCO 100 mg, Sigma-Aldrich). Agilent 7890 B Gas Chromatography equipped with 5977 A Mass Selective Detector, splitless injector with HP-88 capillary column (length 60 m, internal diameter0.250 mm, film thickness 0.20 µm). The carrier gas was Helium at a constant flow-rate of 1 mL/min. The MS source and MS quadrupole temperatures were set at 230 °C and 150 °C, respectively. The oven settings consisted a 5 min isothermal period at 140 °C, followed by a temperature ramp to 240 °C at 4 °C/min and 10 min of hold period. The total analysis time was 40 min.

3.7 Isolation of lipolytic fungi

In order to isolate lipolytic fungi two methods were used viz enrichment culture technique and baiting technique. All the experiments related to microbiology were carried out in Microbiology Laboratory, Division of Environmental Engineering, University of Moratuwa. The cleaning of glassware and sterilization methods that have been used during experiments are given in Annex I. The details of media preparation, and aseptic techniques are given in Annex II.

3.7.1 Enrichment culture technique

The minimum salt broth (MSB) was used as the enrichment medium for fungi. A volume of 25.0 mL of MSB was autoclaved at 121˚C for 20 min, it was allowed to cool, and 2.0 mL of Ciprobid (10 mg/l) was added as an antibacterial antibiotic. A 5g wet weight each from soil samples and grease trap waste samples were added to Erlenmeyer flasks and kept under constant agitation of 100 rpm at room temperature for one hour. Thereafter, samples were incubated at 30̊C for 3-5 days and visible mycelial growths were inoculated onto fresh solid media to obtain pure cultures. The remaining portion was used to prepare spread plates on Tributyrin Agar in a tenfold dilution series up to 10^{-6} and incubated in triplicates at 30̊C for 7 days. The detection of lipase active fungi was based on the formation of clear zones/halo zones around the colonies and pure colonies of fungi were obtained by repeated sub culturing by means of streaking on Tributyrin Agar. The colonies having most conspicuous clear zones were selected for further studies. Each isolated pure fungal colony was stored in MSA slants and stored at 4̊C and sub-cultured at every six weeks.

3.7.2 Baiting technique

A 5g wet weight from soil samples was placed in a Petri dish and sterile peanuts were added and incubated at 30 ˚C for 5/7 days until fungal growth was visible. The control sample was run without sterile peanuts. Observations were noted down from day 1 to 7. The visible mycelial growths were inoculated onto fresh solid media to obtain pure cultures.

3.8 Enzyme activity

3.8.1 Determination of Lipolytic activity

Preparation of fungal inocula

A fungal spore suspension was obtained by growing a pure culture of a particular isolate on minimum salt agar and incubating at 30 ˚C for seven days. A total volume of 10 mL sterile distilled water was spread in aliquots on a culture plate,and the fungal colony surface was gently scraped using an inoculation needle. The cultures were filtered through Whatman® No. 42 filter paper into a sterile container. An aqueous spore suspension having approximately 1×10^7 spores per 1 mL was obtained using a haemocytometer for counting. Please refer Annex III for details of enumeration.

Preparation of lipase enzyme extracts

A 25 mL minimum salt liquid medium containing 1% (w/v) olive oil was prepared and inoculated with 1 mL of a spore suspension of a particular fungal isolate. A volume of 25.0 mL of sterile distilled water was inoculated in a similar manner, and treated as the control. Three replicates were tested for each fungus. All the samples were kept at 30 ˚C for 72 hours. After that, media were filtered through Whatman® No. 42 filter paper and centrifuged at 4,000 rpm for 10 minutes to remove any suspended solids and mycelial fragments. Supernatants i.e. enzyme extracts were collected, and 10 mL from each were taken for the measurement of pH values, and remaining volume (15.0 mL) was used to measure lipase activity.

Determination of lipase unit activity:

Lipase unit activity was determined in an emulsified based system having gum arabic as an emulsifier and olive oil as a substrate. A 18 mL volume of an emulsion of gum arabic (5% w:v) and olive oil (5% w:v) were prepared in 50 mM phosphate buffer solution (pH 7.0). Then 2 mL enzyme extract was added and vortex mixed. It was allowed to react for 60 min at 37 °C, and enzyme reaction was stoppedby the addition of 20 mL of a 1:1 acetone:ethanol mixture. After further agitation for 10 min for complete extraction of fatty acids, titration was performed with 0.05 M NaOH until endpoint 11.0. The control assay was run by adding the acetone:ethanol mixture before addition of the enzyme.

Lipase Unit Activity (U/min): One unit of lipase activity was defined as the amount of enzyme, which produced 1 mmol of fatty acids equivalent per minute under the assay conditions (Equation 10).

$$\text{Lipase Unit Activity} = \frac{\text{M[NaOH]} \times \text{Vol of NaOH titrated} \times 1{,}000}{\text{Time of incubation}} \qquad \text{Equation 10}$$

where M is the molarity of NaOH.

3.9 Identification of isolated fungi species

3.9.1 Macroscopic observations of fungi

Each isolate of fungi was grown on Potato Dextrose Agar (PDA) plates and incubated 30˚C for 5/7 days. The characteristics of fungi colonies such as colour (upper and lower), shape, form, elevation, etc. were noted down and tabulated.

3.9.2 Microscopic observations of fungi

In addition to PDA plates, for the microscopic observation, slide culture technique was employed (Annex III). The branching pattern of mycelia, reproductive structures including the shape of spores was observed through a light microscope (Carl Zeiss, AXIO Lab.A1).

Slide culture technique

In this technique, the fungal culture was grown directly on a small block of agar medium at the center of a glass slide placed in a damp chamber. This technique allows fungi to grow with the least disturbance to the fungal structures. The slide cultures were used to observe the development of fungal structures and particularly for observing the features of the conidial development of hyphomycetes.

Procedure

A wet filter paper was kept inside the Petri dish with a little sterile distilled water to form a damp chamber (It was re-moistened whenever necessary during incubation). A 5 mm^2 block from a fresh agar plate was cut and transferred to the centre of a glass slide by using sterile scalpel blade. Alternatively, using a sterile Pasteur pipette, add one or two drops of cooled molten PDA onto the slanted slide and let it solidify. The centre of each edge of the block was inoculated with a small quantity of the particular fungus. A sterilized coverslip (dipping in alcohol) was lowered down on to the block to complete the slide culture assembly. Then the damp chamber was kept at 30 °C for incubation. It was observed daily for signs of growth and sporulation. When the slide culture has produced sufficient growth on the glass surfaces of both the slide and the coverslip (around 5 mm of growth is usually adequate),and it was mounted for microscopic observation.

A small drop of stain (Lacto Phenol Cotton Blue) was placed on a new slide and carefully pried the coverslip away from the block (previously incubated) and lowered the coverslip on to the drop of stain. The quantity of stain used was just sufficient to reach the edges of the coverslip without excess spilling from the edges. Then, the block of agar from the slide was

removed and a drop of stain (Lacto Phenol Cotton Blue) was placed at the centre of the slide at the point from which the block was removed. A clean coverslip was lowered onto the drop of stain. In this way, two mounts were prepared from each slide culture.

3.9.3 Polymerase Chain Reaction (PCR) and DNA sequences

The best isolates of fungi (in terms of enzyme activity), were identified by sending samples to GENETEC, Sri Lanka. Then the samples were sent to Macrogen Inc, Korea for DNA sequencing (Annex IV).

Method of analysis: A 5μL of DNA was extracted from 15 mg of sample and performed Polymerase Chain Reaction (PCR) using NS1 and NS8. Three major steps were involved in a PCR. These three steps were repeated in an automated cylinder for 30 or 40 cycles, which rapidly heats and cools the test tubes containing the reaction mixture. In each step denatauration (alteration of structure), annealing (joining), and extension took place at a different temperature:

1. Denaturation: At 94 °C, the double-stranded DNA melts and opens into two pieces of single-stranded DNA.
2. Annealing: At medium temperatures, around 54 °C, the primers pair up (anneal) with the single-stranded "template" (The template is the sequence of DNA to be copied.) On the small length of double-stranded DNA (the joined primer and template), the polymerase attaches and starts copying the template.
3. Extension: At 72 °C, the polymerase and DNA building blocks complementary to the template are coupled to the primer, making a double stranded DNA molecule.

With one cycle, a single segment of double-stranded DNA template was amplified into two separate pieces of double-stranded DNA. These two pieces were then available for amplification in the next cycle. As the cycles were repeated, more and more copies are generated and the number of copies of the template was increased exponentially. Then the obtained DNA sequences were compared with the existing DNA sequences in Genbank (http://ncbi.nlm.nih.gov.blast).

Stage 3: Biodegradation of Fat, Oil and Grease

The best five isolates were used for lipase enzyme production under solid-state fermentation. The produced enzymes (from the solid state fermentation) were used to determine the lipid degradation rates of different sources of oils and fats such as vegetable oils (olive, palm and sunflower) and animal fats (lard and chicken). The lipid degradation rate of grease trap waste was determined by separating the lipid portion (i.e., FOG) from the waste by applying heat (100 °C) in a water bath. The lipid degradation rates of FOG (separated from grease trap waste) were determined in two different ways: (i) use of emulsified FOG (liquid phase) (ii) use of non-emulsified FOG (solid phase).

3.10 Solid-state fermentation

3.10.1 Composition and the elemental ratio of the fermentation medium

The basal fermentation medium was rice bran, ground and sieved to provide particle sizes around 415 µm. Coir fibre sieved to have particle size < 2mm was used as a bulking agent for the fermentation medium. The composition of fermentation medium and the elemental ratio of the fermentation medium is given in Tables 3.3 and 3.4.

Table 3.3: The composition of fermentation medium

Component	**Weight (g/g dry weight of substrate)**	**Weight percentage (%)**
Rice Bran	10	2 (w/v)
Coir fiber	1	1.5
C source: Glucose	0.15	1.5
Inductor: Olive oil	0.15	0.75
N Source:$(NH_4)_2SO_4$	0.075	
P & K Source:		
K_2HPO_4	0.005	0.05
KH_2PO_4	0.005	0.05
Salts:		
Mg SO_4	0.0045	0.045
$CaCl_2$	0.00375	0.0375
Moisture (rice bran)	-	30

Table 3.4: The elemental ratio of the fermentation medium

Element	C	N	P	K
Amount (g)	6.775	1.186	0.102	0.104
Ratio	102	18	2	2

3.11 Determination of lipid degradation rates

The lipid degradation rate was determined by subtracting remaining oils/fats weight from the initial weight of particular oils/fats in the medium. The oils/fats recovery percentage of weights during the extraction process was also calculated in control samples. For the grease trap waste, the lipid portion (i.e., FOG) was separated from the waste by applying heat (100 °C) in a water bath. About 80% of FOG samples had a melting point at 80 - 100 °C and therefore retain in solid form at room temperature. Then the upper layer was scrapped and the enzymatic degradation of FOG with time was observed.

3.11.1 Commercial grade oils and fats

The enzyme extracts of best isolates were tested for their capability of degrading different oils/fats. An aqueous medium of 20 mL volume, supplemented with 5% (v/w) of vegetable oil (olive, palm and sunflower) or animal fats (lard and chicken) and 10% (v/w) of enzyme extract were vortex-mixed under sterile conditions in 50 mL containers. For each type of oil and fat, six sets of 50 mL containers were prepared as described above and then containers were kept on a rotary shaker under 100 rpm at 30 °C for 84 h. During degradation, samples (50 mL containers) were taken out at predetermined time intervals for the analysis of oil/fat degradation rate and lipase activity. The degraded products of vegetable oils and animal fats were determined using Gas Chromatogram and Mass Spectrometer (GC/MS). The residual oils and fats were extracted from the aqueous medium according to the method developed by Bligh and Dyer (1959). The degradation rate of the oils/fats was calculated by comparing the content of residual oils/fats in experimental and blank flasks. All experiments were carried out in triplicate.

3.11.2 Grease trap waste (use of emulsified FOG - liquid phase degradation)

Since the FOG waste was in a completely solid state, the experiments had to carry out with an emulsified medium to have a better contact surface with the enzyme. As described above, for each waste type six sets of 50 mL containers were prepared in an emulsified medium and kept in a rotary shaker under 100 rpm at 30 °C for 84 h. During degradation, samples (50 mL

containers) were taken out at predetermined time intervals for the analysis of oils/fats degradation rate and lipase activity. The composition of the emulsified medium: an aqueous medium of 20 mL volume, supplemented with 5 % (w/w) of FOG (separated from grease trap waste), 5 % (v/w) of gum arabic and 10% (v/w) of enzyme extract. The studies carried out without emulsification did not give betterresults.

3.11.3 Grease trap waste (use of non-emulsified FOG - solid phase degradation).

Although emulsified FOG degradation by lipase enzyme in liquid phase has shown promising results, authors have realized that 1:1–1.5 (substrate: emulsifier) emulsification is required and pH drop during enzymatic hydrolysis was observeddue to to the accumulation of long-chain fatty acids. Therefore, pH adjustment was vital for better lipase activity in continuous operation. Therefore, the experimental set-up was changed, and FOG was degraded in solid phase using minimal salt agar medium.

The composition of 100.0 mL of the minimal salt agar medium is FOG: 100 µL (1% v/w), K_2HPO_4: 0.7 g, KH_2PO_4: 0.2 g, Trisodium Citrate ($Na_3C_6H_5O_7$): 0.05 g, $MgSO_4$: 0.01 g, $(NH_4)_2SO_4$: 0.15 g and Agar: 1.5 g. Distilled water was added up to 100.0 mL, and growth medium was autoclaved at 121 °C, 1 bar pressure for 15 minutes and left to cool down to about 80 °C in a bio-safety cabinet. About 12–15 mL of autoclaved medium was poured into a Petri dish and left it to solidify completely. Then the Petri dish was inoculated with lipolytic fungal isolates, by taking a fraction (approximately 0.5 cm^2) from a pure culture of the respective mycelium under aseptic conditions and placing it at the centre of the Petri dish. Each inoculation was run in triplicate and control sample (without inoculation) was also tested. Then all the Petri dishes were incubated at 30 °C for 72 hours. Thereafter, the remaining FOG was extracted according to the method developed by Bligh and Dyer (1959), and the weights were recorded.

3.12 Solid-state degradation of FOG by bioaugmentation

Naturally, fungi are in favor of growing on relatively low moisture conditions with sufficient aeration. Solid- state degradation (SSD) nearly resembles natural growth conditions for fungi. In the second stage, SSD was performed with raw grease trap waste (without extracting FOG) and without adjusting the pH (Table 3.5). Low moisture conditions were obtained by drying and sufficient aeration was provided by mixing the waste with coir fiber which is a low cost bulking agent.

Inoculum Preparation for fungi: A fungal spore suspension was obtained by growing a pure culture of particular isolate on minimum salt agar and incubating at 30 ˚C for seven days. A total volume of 10 mL sterile distilled water was spread in aliquots on a culture plate and the fungal colony surface was gently scraped using an inoculation needle. The cultures were filtered through Whatman® No. 42 filter paper (2.5 µm) into a sterile container. An aqueous spore suspension having approximately 1 x 10^7 spores per 1 mL was obtained using a haemocytometer.

Solid substrate: The raw grease trap waste was used as a solid substrate and it was a slurry mixture of having moisture/water between 52 – 93 wt %. Though, the germination of spores occurred at upper water activity levels (~1), the presence of high moisture contents, in grease waste samples made a difficulty in mixing with bulking agent and prevent the uniform distribution of spores within the reactor and resulted with poor FOG degradation efficiency. Therefore, in order to facilitate proper aeration condition by mixing with coir fiber and uniform distribution of spores for optimum FOG degradation was determined at different initial moisture levels by drying grease trap waste at 70 °C.

As described above, the initial and final FOG content of the waste sample was determined according to the method proposed by Bligh and Dyer for total lipid extraction.

Degradation of grease trap waste: Solid state degradation was performed with grease trap waste (previously dried to have a moisture about 35%) by mixing with coir fiber as a bulking agent in a tray type reactor (120 mm × 100 mm × 25 mm) (Figure 3.1). In most fermentation applications (enzyme production), generally 10^7 spores/mL is used for 1 g of substrate. In this present study grease trap waste had different proportions of FOG. Therefore, in order to maintain consistency of experiments 10 mg of FOG for 1 g dry weight of substrate was used for inoculation by spore suspension of fungi having approximately 1×10^7 spores/mL (Table 3.5). Once the tray type reactor set up is compiled, the high relative humidity (moisture) level in the reactor need to be increased for germination of spores.

Table 3.5: Composition of solid state degradation medium

Parameter	Conditions	
	1. With extracted FOG	2. Raw grease trap waste
pH	Adjusted to 7± 0.1	4.5-6.5
Moisture content of the substrate	< 10 %	25-35%
FOG content in the substrate	100 µL (1% v/w)	10 mg/g dry weight
Inoculation	A 0.5 cm^2 fraction from a pure culture	1×10^7 spores/mL
Bulking agent	None	1 % w/v coir fiber
Temperature	30 °C	30 °C

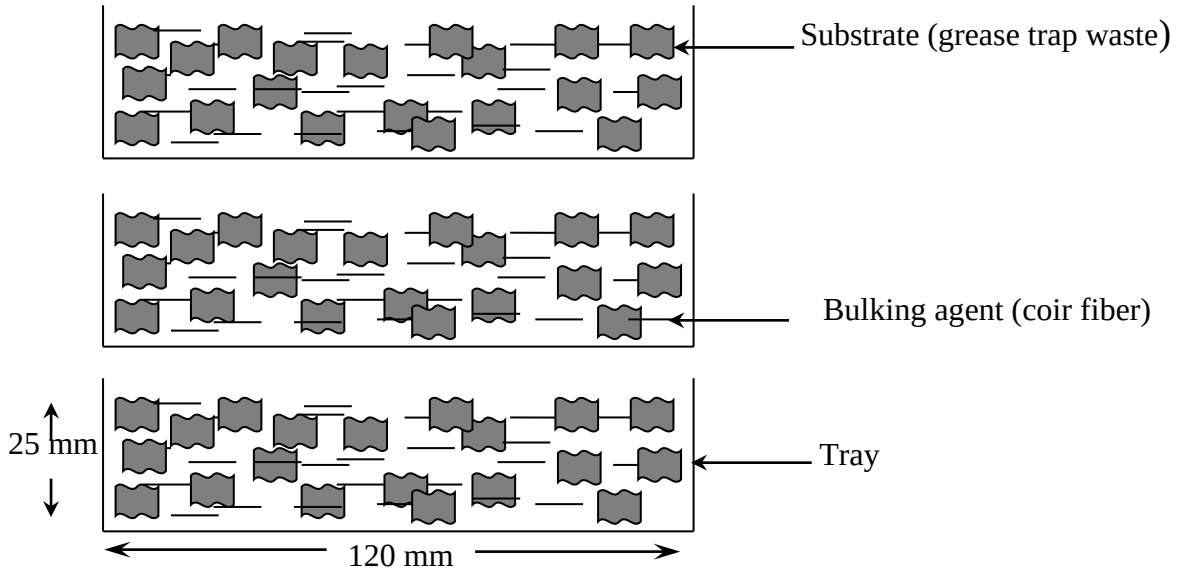


Figure 3.2: Schematic diagram of the tray-type reactor

The presence of liquid water is essential for the germination of germination and fungal hypae to grow. Thereafter, the moisture content in the tray type reactor was increased up to 65% by adding 1.9 mL of distilled water per gram dry weight of solid substrate. The medium was incubated at 30°C without pH adjustment (Figure 3.1).

4 RESULTS AND DISCUSSION

The physical, chemical and biological characteristics of wastewater may vary throughout the day and widely depend on the function and activity of the industry. Moreover, flow rate values and wastewater characteristics may vary over a period of a year. Because of this variation, it is often difficult to define typical operating conditions for industrial activities.

For the wastewater to be discharged to the common collection system of a municipal wastewater treatment facility, it is necessary to characterize the wastewater adequately to identify the ranges of constituent concentrations and mass loading. Such characterization is also needed to determine whether pretreatment is required before, the wastewater is discharged into the common treatment system.

During field visits, individual wastewater treatment plants could be observed with most of the large-scale hotels and bakeries while small and medium-scale hotels and bakeries, do not have a treatment facility. They discharge the wastewater to the sewer. Almost all small and medium-scale industries have installed a grease trap before the discharge point.

The wastewater characteristics of hotels, restaurant, bakery and dairy industries, have illustrated and compared using graphs. The tables have been used to show ratios between the various constituents in wastewater, which has a significant influence on the selection and functioning of wastewater treatment processes. Research findings are presented by following the same order as described in the methodology.

Stage 1: Characterization of Wastewater from Food Service and Food Processing Industries

4.1 Wastewater characteristics

4.1.1 pH, temperature and conductivity

The in-situ measurements of pH, temperature and conductivity are highly variable (Figure 4.1).

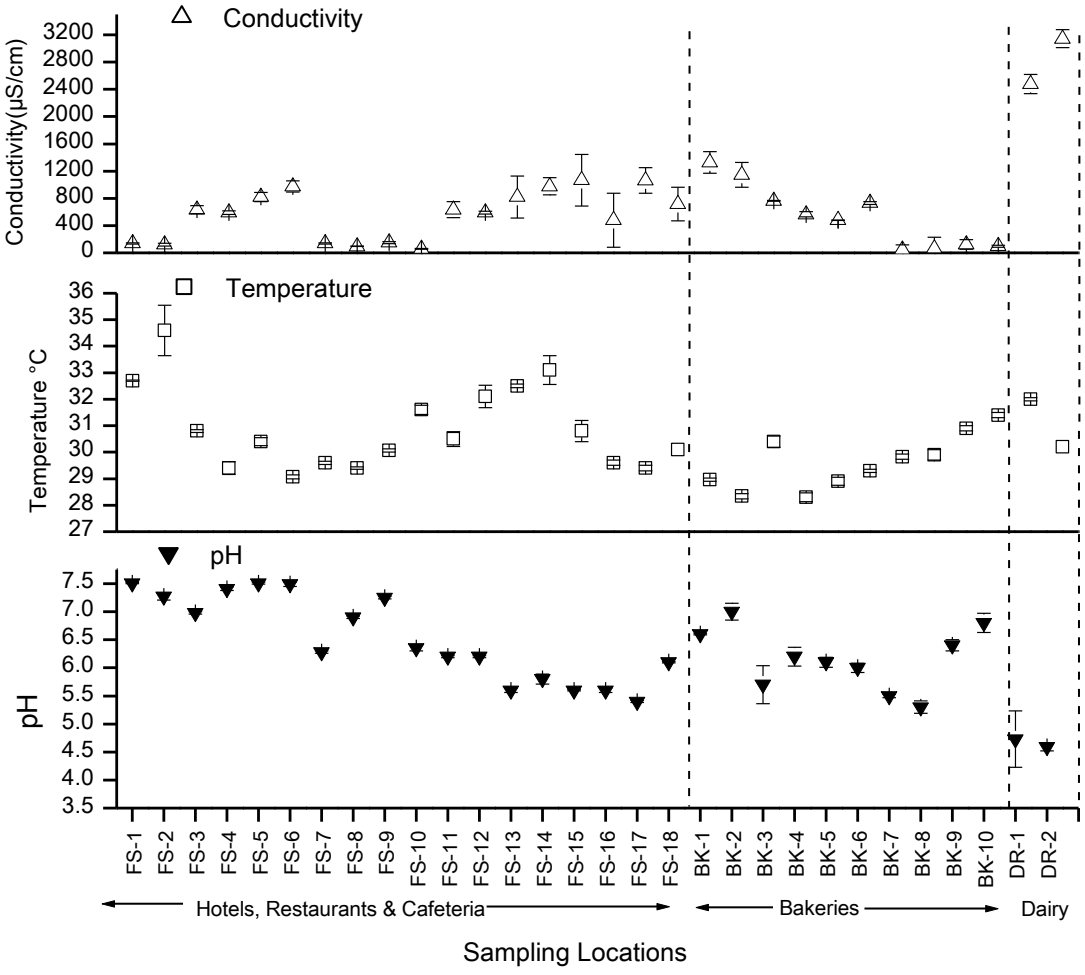


Figure 4.1: In-situ measurements of wastewater samples: pH, temperature and conductivity

Note: FS (string) - Food Service Industries; BK (string) - Bakeries; DR (string) - Dairy
1to15 (digit) - Location Number; Error bars – standard deviation

However, in food service industry (hotels, restaurants and cafeteria) and food processing industry (bakeries), pH was not in the alkali range and retained in between 5 to 7.5 except for

those of dairy industry. The temperature varied between 28 to 35 °C, and the conductivity ranged from 45 to 1300 μS/cm for all three industries.

4.1.2 Total FOG content

The fats, oils and grease (FOG) content in the wastewater samples is shown in Figure 4.2. The average FOG content in the food service industry was 800 mg/L while lowest value (28 mg/L) was recorded from the small cafeteria and the highest value (2,450 mg/L) was recorded from a star grade hotel. The average FOG content in bakeries was recorded as 2,957 mg/L, and the highest recorded value was 15,482 mg/L. Although only two samples collected from the dairy industry, and as expected, the FOG content was high in both samples (Figure 4.2).

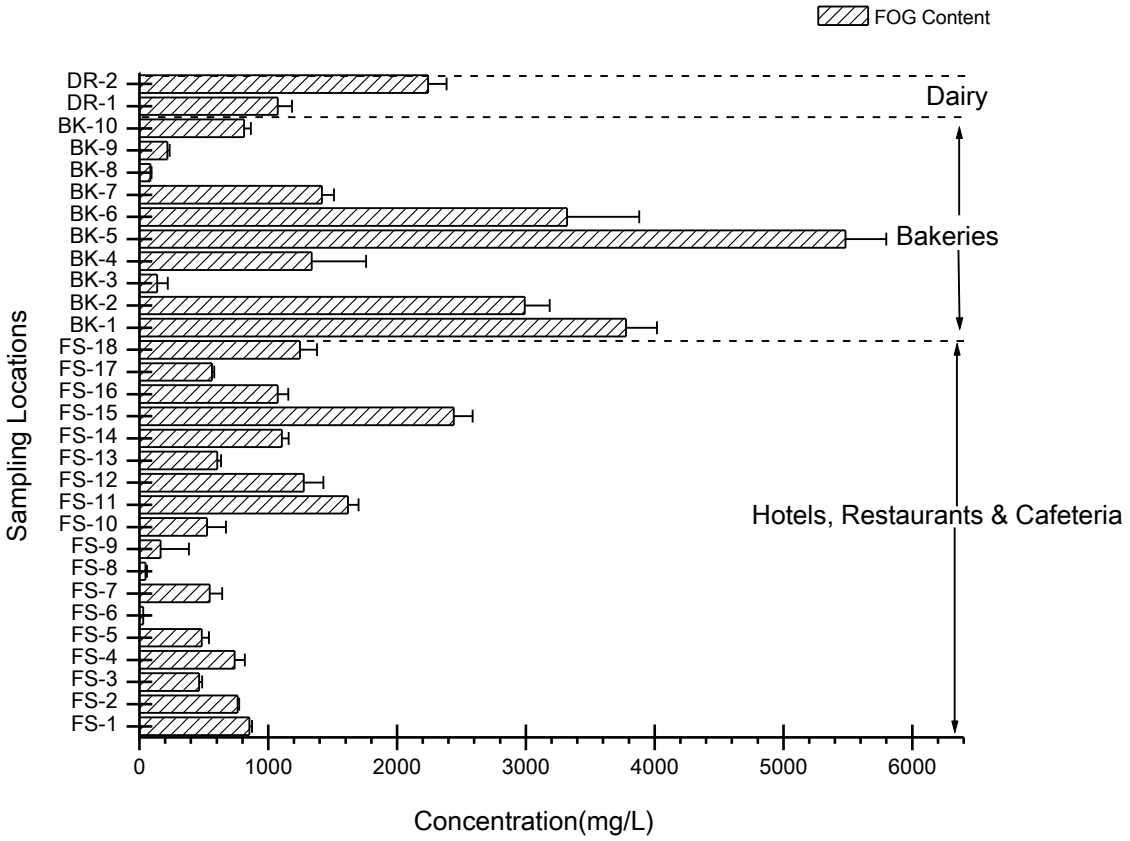


Figure 4.2: Total FOG content of wastewater samples

Note: FS (string) - Food Service Industries; BK (string) – Bakeries; DR (string) – Dairy; 1to15 (digit) – Location Number; Error bars – standard deviation

4.1.3 BOD and COD

The 5-day Biochemical Oxygen Demand (BOD_5) variation of the effluent from food service industry ranged from 421 mg/L to 2,147 mg/L while in bakeries BOD_5 ranged from 720

mg/L to 6,300 mg/L. Most of the higher values for BOD_5 was obtained during the cleaning of the process vessels. For the dairy industry, the recorded values for the BOD_5 were at the higher levels of above 3,500 mg/L. The corresponding Chemical Oxygen Demand (COD) ranged from 720 mg/L to 3,320 mg/L for food service industries and for the bakeries corresponding COD ranged from 1,600 mg/L to 11,850 mg/L (Figure 4.3).

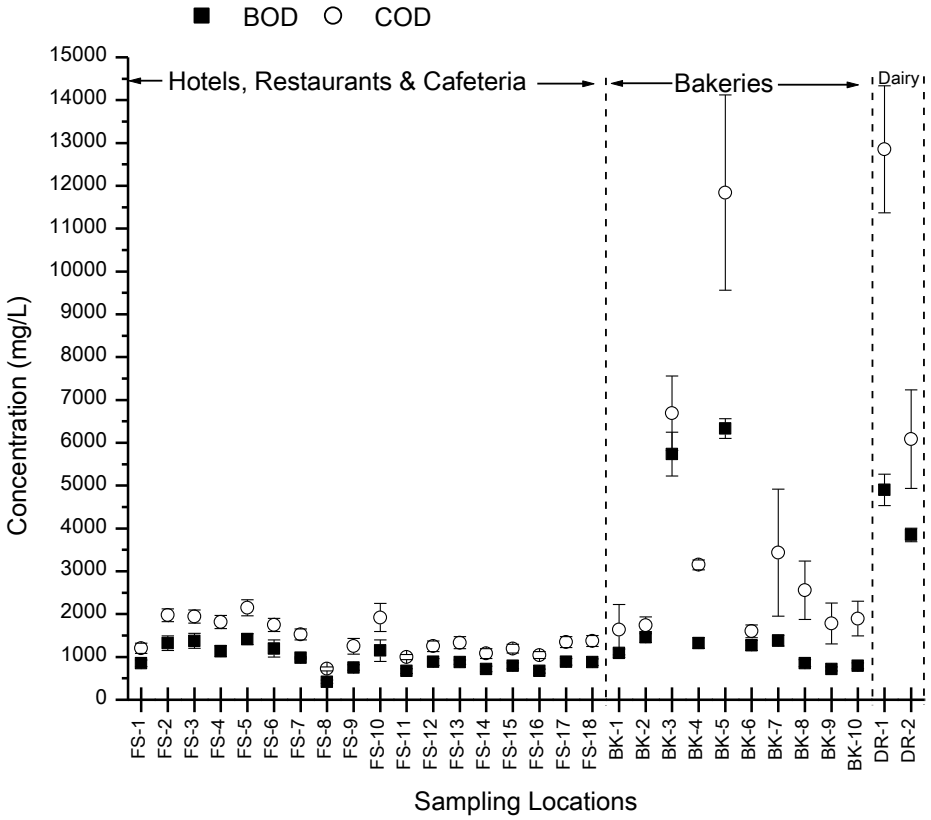


Figure 4.3: BOD_5 and COD levels in wastewater samples

Note: FS (string) - Food Service Industries; BK (string) – Bakeries; DR (string) – Dairy; 1to15 (digit) – Location Number; Error bars – standard deviation

The average BOD_5, COD and COD/BOD_5 ratio values for the food service industries and bakeries are shown in Table 4.1. The BOD and COD levels within and in between the two industries at the $P < 0.05$, means were significantly different. It should be noted, that the wastewater characteristics of food service and bakeries were determined by collecting samples from the kitchen effluent. The dairy industry has a massive production process, comparative to the kitchen operation in a hotel or bakery. Also, wastewater is discharged from combined channels. Therefore, the obtained values for certain parameters such as BOD_5, COD, conductivity, solids, nutrients (Nitrogen and Phosphorus) are remarkably high

in the dairy industry. Although, the total FOG content is relatively low in the dairy industry; the elevated solid content in the wastewater may have a direct impact on elevated BOD and COD levels.

Table 4.1: Average BOD_5 and COD Values in food service and food processing industries

Parameter	Unit	Foodservice Industry (Hotels, Restaurants and Cafeteria)	Food Processing Industry (Bakeries)
BOD_5	mg/L	998	2,095
COD	mg/L	1,515	3,632
COD/BOD_5		1.5	1.7

4.1.4 Solids

The most important physical characteristics of wastewater are total solid content (Metcalf and Eddy 2003). Total solids comprise of two fractions; total dissolved solids and total suspended solids. The dissolved and suspended solids can further divideinto volatile and fixed solids. VSS is the volatile portion of suspended organic particles. FSS are inorganic particles suspended in the liquid; such as un-dissolved salt crystals and silt particles. TDS represent the salt content in the wastewater sample. The proportions of different solid fractions in wastewater samples are shown in Figure 4.4. The total solid content in the food service industry varied from 700 to 5000 mg/L while bakery has total solid content ranged from 1800 to 7000 mg/L. However, the highest total solid content was recorded from the dairy which was around 14,600 mg/L. The total dissolved solids content in food service, bakeries and dairy are higher than the total suspended solids. In general, the total dissolved solids concentration is the sum of the cations and anions in the water. However, the correlation coefficient was 0.6 between total dissolved solids and conductivity.

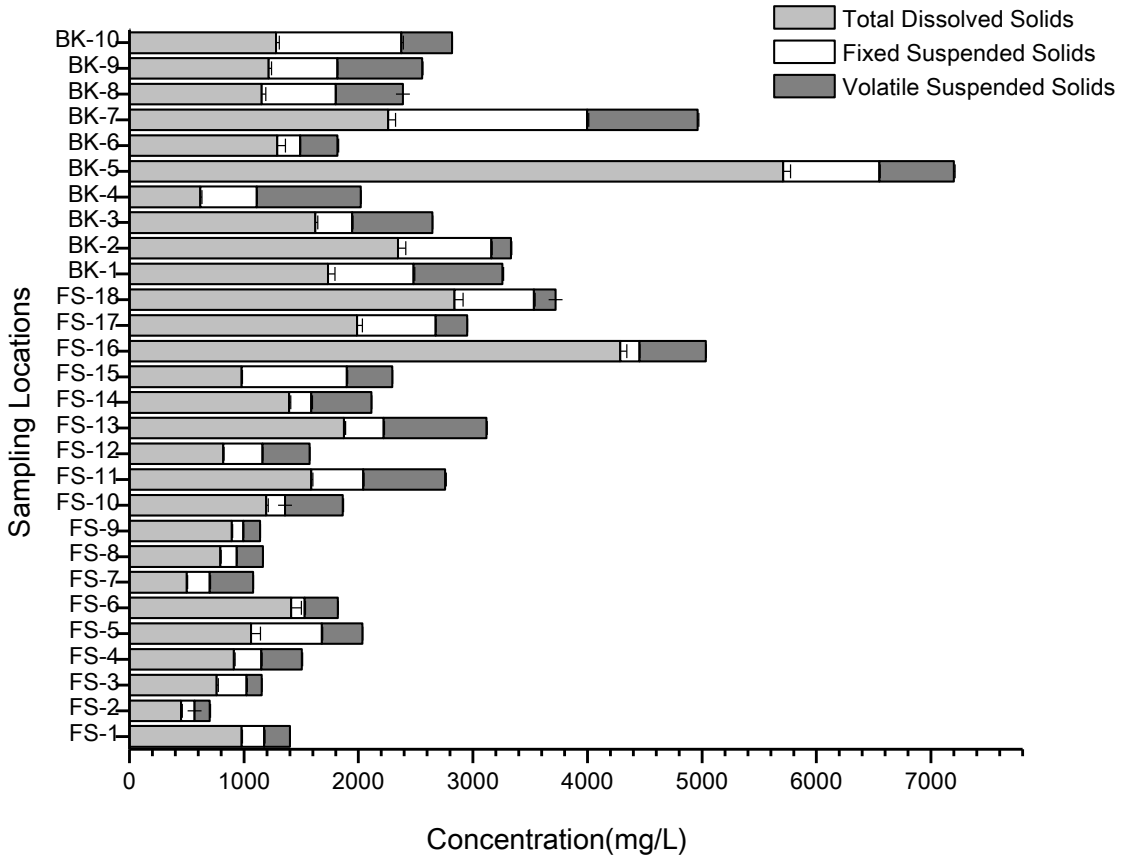


Figure 4.4: Solid composition in wastewater samples

Note: FS (string) - Food Service Industries; BK (string) - Bakeries; 1to15 (digit) - Location Number; Error bars – standard deviation

4.1.5 Total Nitrogen (TN) and Total Phosphorus (TP)

Wastewater from food service industry has an average total nitrogen content of 3.7 mg/L and total phosphorus content of 5 mg/L. While bakery industry has an average total nitrogen content of 3 mg/L and total phosphorus content of 6 mg/L.

4.1.6 Cations

Na, K and Ca content in wastewater from both industries are shown in Figure 4.5. Food additives and flavors with Na and K salts may have contributed to elevated Na and K levels in wastewater.

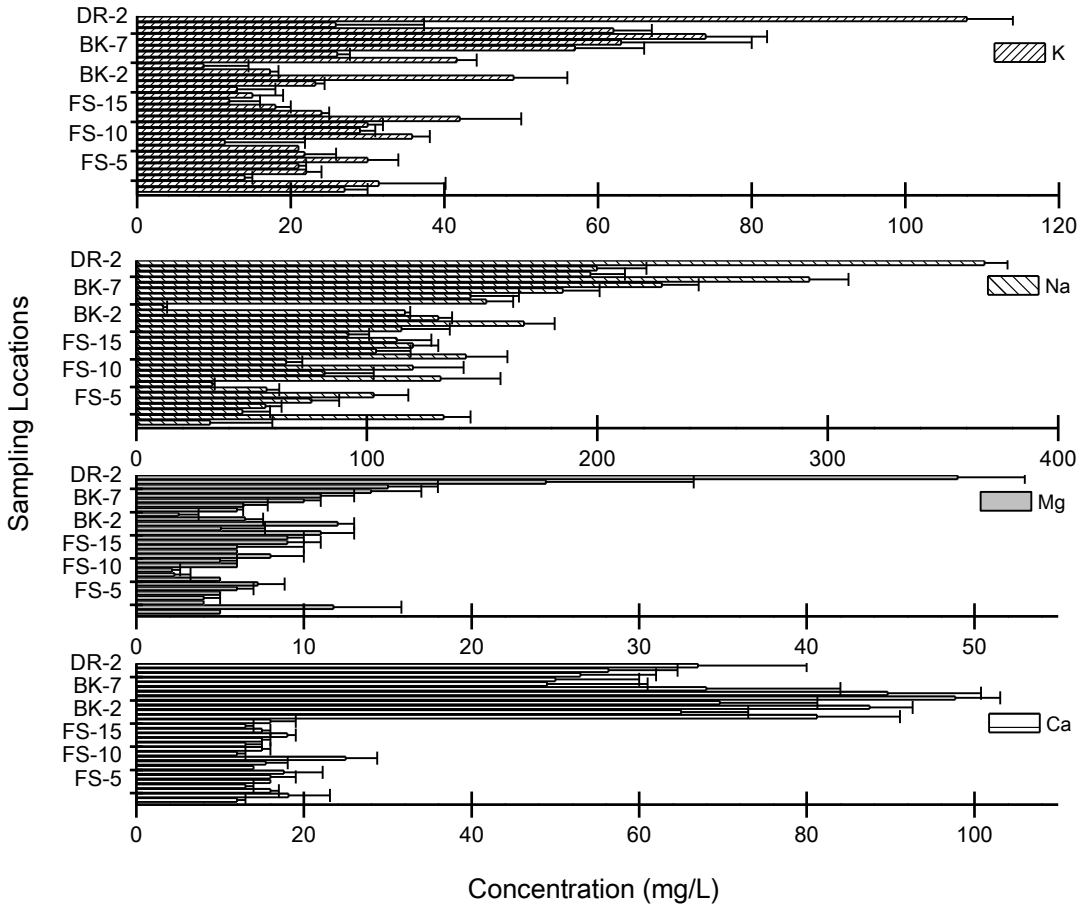


Figure 4.5: Different cation levels in wastewater samples

Note: FS (string) - Food Service Industries; BK (string) - Bakeries;1to15 (digit) - Location Number; Error bars – standard deviation

4.1.7 Ratios between constituents

Wastewater with high COD to BOD ratio indicates that a substantial part of the organic matter is difficult to degrade biologically. Presence of high volatile component (VSS to TSS ratio) in suspended solids implies that it can be successfully digested under anaerobic conditions (Table 4.2). Thus, wastewater components are subject to dilution, which however will not change the ratios between the components.

Table 4.2: Different ratios between constituents in wastewater

Wastewater Source	Ratio		
	High	**Medium**	**Low**
		COD/BOD	
Municipal wastewater (Metcalf and Eddy, 2003)	2.5 – 3.5	2.0 – 2.5	1.5 – 2.0
Hotels and Restaurants wastewater (Present Study)			1.4 – 1.7
Bakery wastewater (Present Study)	1.2 – 2.9		
		VSS/TSS	
Municipal wastewater (Metcalf and Eddy, 2003)	0.8 – 0.9	0.6 – 0.8	0.4 – 0.6
Hotels and Restaurants wastewater (Present Study)			0.2 – 0.8
Bakery wastewater (Present Study)			0.2 – 0.7

Stage 2: Physicochemical Properties of Grease Trap Waste and Production of Lipase from Lipolytic Fungi

In this section, the physicochemical properties of grease trap waste samples including pH and moisture levels are presented. A comparison was made between the fatty acid profiles of commercial grade cooking oils and fats and grease trap waste. The enzyme activity (produced by solid state fermentation) of each fungi isolate (identified by DNA sequencing) are also presented in this section.

4.2 Physicochemical properties of grease trap waste

4.2.1 pH and moisture

The moisture content of grease trap waste samples varied between 52–93% (w/w) and the FOG content varied between 16–37 % (w/w) (Table 4.3). The pH of the waste samples varied between 4.5–6.5 (Table 4.3).

Table 4.3: Physicochemical characteristics of grease trap waste from different locations

Grease trap waste from different locations		FOG Characteristics (Mean ± SD; n = 3)		
		pH	Moisture content (wt %)	Total FOG content (wt %)
Small and Medium-sized Hotels	W-1	6.1 ± 0.2	78.5 ± 3.5	34.2 ± 2.1
	W-2	5.9± 0.2	93.2 ± 1.5	26.5 ± 1.9
	W-3	4.8 ± 0.2	86.5 ± 6.6	29.4 ± 1.5
	W-4	6.5± 0.1	90.8 ± 2.1	16.5 ± 1.4
	W-5	4.7 ± 0.2	88.4 ± 4.1	35.4 ± 2.7
Large-sized Hotels	W-6	5.6 ± 0.1	85.2 ± 2.6	34.1 ± 2.0
	W-7	5.4 ± 0.1	80.9 ± 5.1	16.3 ± 1.2
	W-8	5.2 ± 0.2	68.9 ± 4.7	37.8 ± 1.8
	W-9	5.3 ± 0.1	74.5 ± 3.7	26.2 ± 2.2
	W-10	5.2± 0.2	52.2 ± 1.2	37.1 ± 3.1
	W-11	6.1 ± 0.1	88.6 ± 4.1	18.7 ± 1.3
	W-12	4.5 ± 0.2	90.8 ± 2.1	21.6 ± 1.4
	W-13	5.3 ± 0.2	84.6 ± 3.7	28.4 ± 2.2
	W-14	6.1± 0.2	81.7 ± 3.1	19.8 ± 1.6
	W-15	5.5 ± 0.1	79.4 ± 2.8	31.8 ± 3.1

Note:- W: grease trap waste; 1 to 15: location number

4.2.2 Fatty acid composition of grease trap waste, commercial grade oils and fats

The main components of cooking oils and animal fats are esters of fatty acids attached to a glycerol molecule. Normally, triglycerides of cooking oils and animal fats consist of different fatty acids and thereby have different physical and chemical properties. In the present study, on average, palmitic acid 49.5% (w/w) and oleic acid 33% (w/w) were observed to be the most abundant fatty acids present in grease trap waste. Myristic, stearic and linoleic acids had contributed only less than 10% (w/w) to the grease trap waste composition (Table 4.4, Figure 4.6). Analysis of commercial grade oils: showed that palmitic acid content was more than 40% (w/w) in palm oil, whereas in olive oil, coconut oil, soya oil and sunflower oil, the palmitic acid content was less than 12% (w/w). Analysis of animal fats showed that palmitic acid content in butter, was nearly 40% (w/w) while chicken fats contained 29.5% (w/w) and lard contained 27.5% (w/w) (Table 4.4). Oleic acid present at a higher percentage of commercial grade oils, with more than 70% (w/w) in olive oil, and about 40% (w/w) in palm oil. In animal fats (chicken and lard), the oleic content was more than 35% (w/w) (Table 4.4). The details of chromatogram analysis are given in Annex V.

Oil heated during the frying process may reach temperatures between 150 - 190 °C (Choe and Min, 2007) for a relatively long period of time. Also, the use of same oil repeatedly for economic reasons may cause various physical and chemical changes in the oil. Some physical changes include an increase in viscosity and specific heat and change in surface tension and color (Cvengroš and Cvengrošová, 2004). Besides, oils are subjected to thermolytic, oxidative and hydrolytic reactions and result in the formation of many undesired harmful compounds (Choe and Min, 2007),and changes in fatty profile have been cited (Kowalski, 2007; Kumar *et al*., 2012).

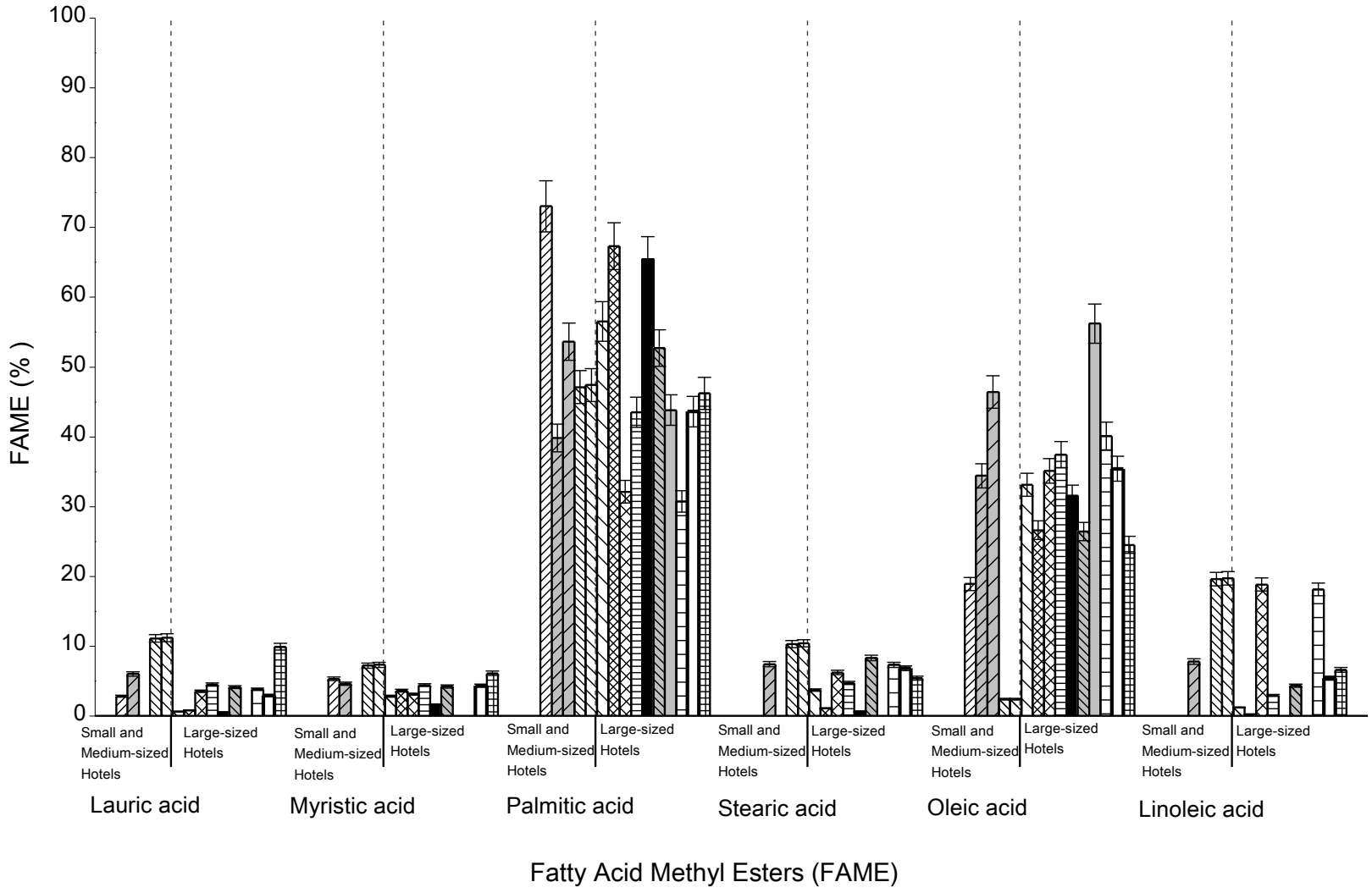


Figure 4.6: Relative fatty acid percentage (w/w) of grease trap waste (Mean ± SD; n = 3)

Note:- Large-sized hotels: number of rooms > 50; Small and Medium-sized hotels: number of rooms < 50. ; Error bars – standard deviation

Table 4.4: Fatty acid composition of commercial grade cooking oils and animal fats.

Fatty acid	Fatty acid composition as a percentage of weight (w/w) (Mean ± SD)								
	Olive oil (n=3)	**Coconut oil (n=3)**	**Palm oil (n=3)**	**Soya oil (n=3)**	**Sunflower oil (n=3)**	**Butter fat (n=3)**	**Chicken fat (n=3)**	**Lard (n=3)**	**Oil/grease trap waste (n=15)**
Caprylic	0.0*	2.5± 1.0	0.0*	0.0*	0.0*	0.3± 0.0	0.0*	0.4 ± 0.2	0.0 ± 0.0
Capric	0.0*	4.6 ± 0.7	0.1 ± 0.0	0.0*	0.0*	1.1 ± 0.1	0.0*	0.5 ± 0.3	0.1 ± 0.0
Lauric	0.0*	47.1 ± 2.4	1.0 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	1.9 ± 0.2	1.5 ± 0.1	5.8 ± 0.1	4.1± 3.1
Myristic	0.0*	20.2 ± 1.8	1.4 ± 0.1	0.1 ± 0.0	0.5 ± 0.0	11.8 ± 0.5	1.7 ± 0.1	6.1 ± 0.2	3.6± 1.4
Palmitic	12.0 ± 0.5	12.2 ± 1.4	42.2 ± 2.1	11.3 ± 0.6	7.5 ± 0.3	39.3 ± 2.0	29.5 ± 1.4	27.5 ± 1.7	49.5± 12.0
Palmitoleic	1.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	4.0 ± 0.1	2.6 ± 0.4	0.6 ± 0.1
Stearic	3.7 ± 0.2	3.5 ± 0.2	5.0 ± 0.2	3.1 ± 0.1	3.5 ± 0.7	18.2 ± 0.9	6.9 ± 0.3	9.8 ± 0.6	4.8± 1.7
Oleic	78.4 ± 3.9	7.1 ± 0.2	40.0 ± 1.9	24.9 ± 1.1	21.5 ± 1.0	25.8 ± 1.2	35.6 ± 1.7	36.5 ± 1.6	33.0± 9.2
Linoleic	4.7± 0.2	2.7 ± 0.1	10.1 ± 0.5	60.0 ± 2.8	66.2 ± 3.2	0.9 ± 0.1	20.9 ± 1.0	10.9 ± 0.4	7.0± 2.5
Arachidic	0.1 ± 0.0	0.0*	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0*	0.0*	0.0*
Linolenic	0.0*	0.0*	0.0*	4.8 ± 1.2	0.1 ± 0.0	0.8 ± 0.0	1.5 ± 0.1	0.4 ± 0.0	0.0*
Behenic	0.0*	0.0*	0.0*	0.3 ± 0.2	0.5 ± 0.0	0.0*	0.0*	0.0*	0.0*
Erucic	0.0*	0.0*	0.0*	0.0*	0.0*	0.0*	0.0*	0.0*	0.0*
Lignoceric	0.0*	0.0*	0.0*	0.1 ± 0.1	0.1 ± 0.0	0.0*	0.0*	0.0*	0.0*
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Note: *Below the limit of detection(The average values observed for grease trap waste samples are shown for comparison purposes).

The physicochemical properties of grease trap waste depend on the type of restaurant, grease trap configuration such as size, inlet/outlet piping, number of baffles (Long *et al*., 2012). Some physicochemical properties of grease trap waste were investigated in the present study (Table 4.3 and Table 4.4). As expected, the average pH of the grease trap waste was slightly acidic (pH 5.5) (Kang *et al*., 2011; Nanayakkara and Witharana 2015) and fatty acid composition was dominated by palmitic acid (~50%) and oleic acid (~33%). However, in reported literature, oleic acid is known to be the abundant fatty acid in food industry wastewater (Kiepper *et al*., 2001). This is perhaps due to a growing interest in palm oil in Southern Asia while in Mediterranean countries olive oil production is high (Pintor *et al*., 2016). Nevertheless, during field visits and discussions with food service industry personnel, it was noted that palm oil (refined, bleached and deodorized) is used as the main oil source for cooking applications. Minor amounts of olive oil are used in the preparation of salads, but the possibility of its presence in wastewater is low. Animal fats, especially chicken fat and lard may contribute to elevating the levels of palmitic and oleic acid content and may enter the wastewater stream during food preparation (roasting, grilling, and frying) and cleaning of ovens, pots, pans and other utensils (Table 4.4, Figure 4.6).

4.3 Isolation and identification of lipolytic fungi

The initial identification (up to the genus level) of isolated fungi was based on macroscopic and microscopic observations (Tables4.5 and 4.6). The best five isolates were selected based on their enzyme activity and identified them to the species level using molecular techniques: Sequencing the ITS 1 and 4 regions. Altogether, eight fungal species were isolated:four species from soil samples and the four species from grease trap waste samples. *Geotrichum candidum* was able to be isolated by using baiting technique. The observation made during the study was given in Figure 4.7.

Day 1

Soil sample contaminated with oil Soil sample mixed with peanuts

Day 3

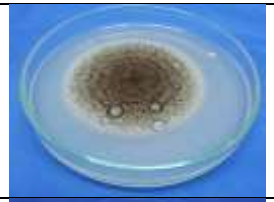
Soil sample contaminated with oil Soil sample mixed with peanuts

Day 6

Soil sample contaminated with oil Soil sample mixed with peanuts

Figure 4.7: Isolation of fungi using baiting technique

Table 4.5: Macroscopic observations of fungi

Sample code	Colony appearance	Form	Elevation	Texture	Margin	Colour		Diameter / mm (at day 7)
						Surface	Reverse	
F1 *Aspergillus niger* Soil		Circular	Flat	Wool-like	Entire	Black	Pale yellow	41 – 42
F2 *Geotrichum candidum* Soil		Circular	Flat	Cotton-like	Entire	White	Pale yellow	62 – 65
F3 *Aspergillus fumigatus* Grease trap waste		Irregular	Flat	Wool-like	Undulate	White	Pale yellow	48 – 53
F4 *Fusarium proliferatum* Grease trap waste		Circular	Raised	Wool-like	Entire	White	Pale yellow	65 - 65

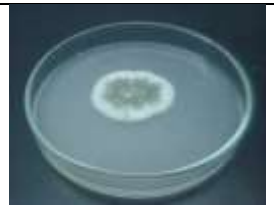
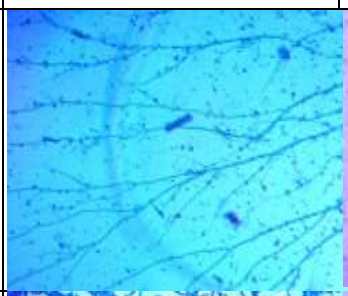
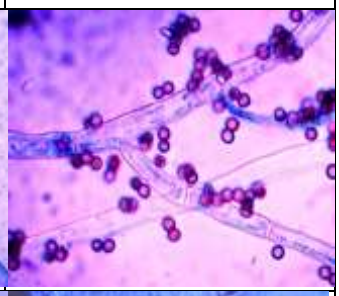
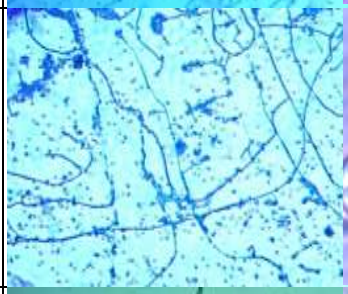
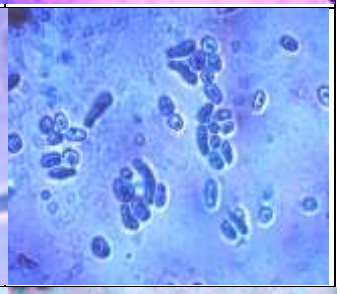
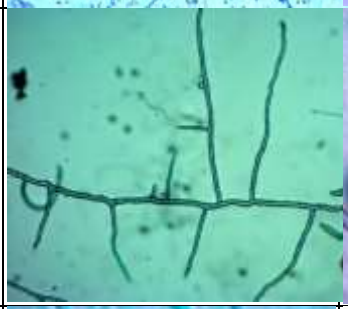
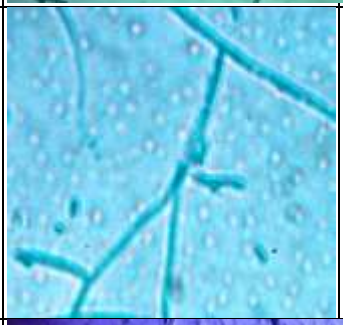
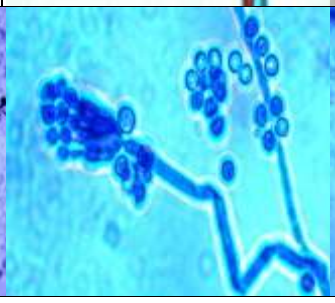
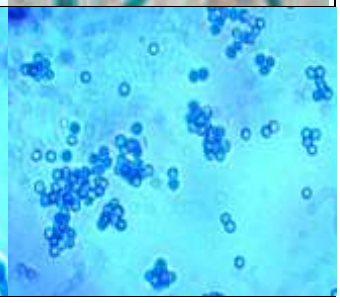
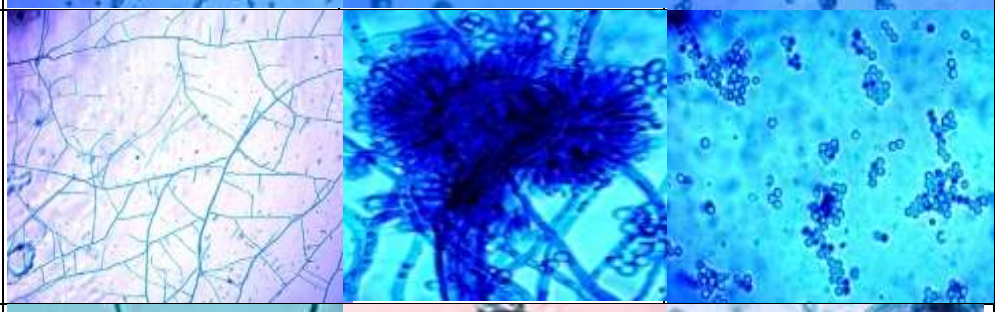
F5 *Penicillium citrinum* Grease trap waste		Circular	Flat	Velvet-like	Entire	Green, white border	Pale yellow	36 - 38
F6 *Penicillium* sp Soil		Circular	Flat	Velvet-like	Entire	Green, white border	Pale yellow	30 - 35
F7 *Aspergillus sp.* Soil		Circular	Raised	Wool-like	Entire	White and yellow	Pale yellow	42 – 46
F8 *Rhizopus* sp Grease trap waste		Irregular	Flat	Wool-like	Undulate	White	Pale yellow	40 - 54

Table 4.6: Microscopic observations of fungi

Sample code	Mycelium (100×)	Reproductive structures (1000×)	Spores (1000×)
F1 *Aspergillus niger*			
F2 *Geotrichum candidum*			
F3 *Aspergillus fumigatus*			
F4 *Fusarium proliferatum*			
F5 *Penicillium citrinum*			

F6 *Penicillium sp*	
F7 *Aspergillus sp.*	
F8 *Rhizopus sp*	

4.4 Production of enzymes

The present study was able to produce enzymes using solid state fermentation (SSF) with higher lipase unit activity, ranging from 9–22 (U)/g dry weight (Table 4.7).

Table 4.7: Lipase activity of the isolated fungi

Sample code	Sample code	Enzyme activity (U) /g dry weight ± SD	Final pH of the medium ± SD
F1	*Aspergillus niger*	15.42 ± 2.7	3.8 ± 0.5
F2	*Geotrichum candidum*	11.62 ± 2.1	4.1 ± 0.2
F3	*Aspergillus fumigatus*	9.47 ± 1.4	4.5 ± 0.2
F4	*Fusarium proliferatum*	18.29 ± 3.2	3.4 ± 0.3
F5	*Penicillium citrinum*	21.75 ± 3.1	3.1 ± 0.5

Aspergillus niger has been used to produce lipolytic enzymes by mixing agro-industrial waste such as rice, wheat and soya bean bran (Costa *et al*., 2017). Different compositions of agro-industrial waste and nutrient supplements (glucose, glycerol or soybean oil) were tested, and

highest lipolytic activity values were recorded for the mixture of rice bran and glycerol (19.844 U·g-1) while activity of 13.267 U·g-1 was recorded when using rice bran alone. Mukhtar *et al*.(2015) were able to isolate seven different lipolytic fungal strains from soil samples, among them *Aspergillus niger* has given the best reproducible results. The recorded maximum lipase activity was 12 ± 0.059 U/mL. For this study, olive oil was used as substrate and the medium was moistened with a diluents containing (g/mL): MgSO4.7H2O, 0.05; KH2PO4, 0.1; KCl, 0.05; FeSO4, 0.001; glucose, 0.8 and peptone, 2.0 (pH 6.5). Similarly, Treichel *et al*. (2016) produced lipase using canola cake as substrate. They optimized the fermentation process, by adding nitrogen supplement and adjusting the medium moisture. In addition, for enzyme extraction process effects of temperature and pH were determined. The produced enzyme, i.e., lipase was characterized in terms of hydrolytic and esterification activity, specificity and stability at 10 and -1°C. The recorded the best condition for the lipase production was 60 wt% of moisture and 2 wt% of urea as a supplemental source of nitrogen, The coconut and soybean oil showed the best hydrolytic activities of 90 U/g and 74 U/g, respectively.

Castro *et al*. (2016) optimized the production of lipase from *Geotrichum candidum* using submerged fermentation. Cottonseed oil, a low-cost by-product of cotton processing, was used as both an inducer and a carbon source. A maximum lipase activity of 27.17 IU mL-1 was achieved after 30h fermentation in a 5L stirred tank bioreactor under optimal conditions: 2.3% (m/v) of casein peptone, 0.8% (v/v) of cottonseed oil and 0.05% (m/v) of $MgSO_4$ and $NaNO_3$. The lipase purification was done in a single step by immobilizing on PHB particles. Moreover, the produced lipase showed high specificity to hydrolyze long-chain fatty acids with cis-9 double bonds, such as oleic and linoleic acids. It has been proven in the literature that the *Aspergillus* spp. have a good potential in producing lipase in combination with new technology. A lipase gene from *Aspergillus fumigatus*, was cloned in *Escherichia coli*. The recombinant lipase exhibited a very high specific activity of 1.00×10^3 U mg^{-1} on a standard substrate of *p*-nitrophenyl acetate. The recombinant lipase (AFL1-1) is an alkaline enzyme which was stable in the pH range 6.0~8.5 for 16 h (at 4 °C) and at 30~50 °C for 1 h.

The production and optimization of lipase from *Geotrichum candidum*were carried out in soybean molasses by submerged fermentation. The fermentation process was optimized by changing molasses concentration, pH and temperature. The highest enzyme activity 11.48 U/mL was recorded after 24 h at the molasses concentration of 200 g/L, the

temperature at 27 ± 1 °C and pH 3.5 (Júnior *et al*., 2016). Although experimental condition differed, a similar observation was recorded from the present study too.

Prazeres *et al*.(2006), observed the effect of different commercial detergents and surfactants on the enzymatic activity of lipase from *Fusarium* spp. They have demonstrated that the enzyme was compatible with various ionic and non-ionic surfactants as well as commercial detergents. This ability of an enzyme is vital to stand in actual waste treatment facility because the FOG waste is contaminated with commercial enzymes and other chemical emulsifiers. However, the best assay conditions observed for this lipase from *Fusarium oxysporum* were pH 8.0 and a temperature of 50ºC. The enzyme was stable at alkaline pH and remained 93% of residual activity during 1 h incubation at 60ºC. The highest lipase activity was measured with triglycerides of middle and long chain fatty acids (C8–C18). In this present study also, *Fusarium proliferatum* was able to degrade middle and long chain fatty acids at an enzyme activity of 18.29 ± 3.2.

The enzyme activity of *Penicillium citrinum* was optimized with dextrose and peptone as carbon and nitrogen sources at the medium pH of 7 and incubation temperature of 35 °C. The enzyme was tested with about 20 % effluents from ayurvedic pharmaceuticals and minimal medium and it could achieve maximum lipase activity of 266 U/mL using *Penicillium citrinum* (Kumar *et al*., 2016).

Annibale *et al*.(2006), carried out a study using olive-mill wastewater to investigate the possible growth medium for the microbial production of extra-cellular lipase. To this end, they screened the strains of *Geotrichum candidum*, *Aspergillus niger*,and*Penicillium citrinum*. All species were able to grow on the undiluted olive-mill wastewater, producing extra-cellular lipase activity.

The similar results have been obtained by other workers for lipolytic activity (Gombert *et al*., 1999; Falony *et al*., 2006; Valladao *et al*., 2011) for above isolates.

4.5 Limitation of using enzymes (produced by SSF)

1. Purification and Storage of enzyme

With the advances in industrial biotechnology, lipase and other hydrolytic enzymes have been produced more economically by solid-state fermentation (SSF). In addition, some researchers have focused on advanced methods such as gene cloning and enzyme purification methods to yield high values for enzyme activity. The present study was also able to produce

enzymes from five fungal isolates with higher lipase unit activity, without purification, ranging from 9–22 (U)/g dry weight (Table 4.7).

The basic structure of an enzyme (lipase) consists of protein. Proteins can lose activity as a result of proteolysis, aggregation and suboptimal buffer conditions. Therefore, enzymes need to be stored under certain conditions to retain their original structural integrity and/or activity. In general, the stability of an enzyme is less than 24 hrs (at room temperature) and need to store at low temperatures, usually below 4 °C. The typical shelf life under above condition is about one month.

The practical application of enzymes in wastewater treatment, need to have high volumetric production of enzymes. Meantime, as mentioned above, suitable conditions need to be employed for the enzyme storage. Therefore, the virtual application of enzymes depends on the cost that needs to spend for production, purification and storage.

2. The interaction between enzyme and waste (FOG)

Despite the fact that the production of an enzyme by solid-state fermentation is an economical option, proper interaction between FOG waste (solid phase) and enzyme (liquid phase) couldn't be expected, because FOG exists in solid/semi-solid form at room temperature. Therefore, once FOG is added to the liquid medium (enzyme), it may form irregular clusters, which make the lesser availability of surface for enzymatic attack. Therefore, to have a complete degradation of lipids, it may need more time (Figure 4.8). Other authors have reported the similar results, (Al-Darbi *et al*., 2005; El-Bestawy *et al*., 2005; Čipinytė *et al*., 2009) who have noted the biodegradation rate depends on the physical state of lipids.

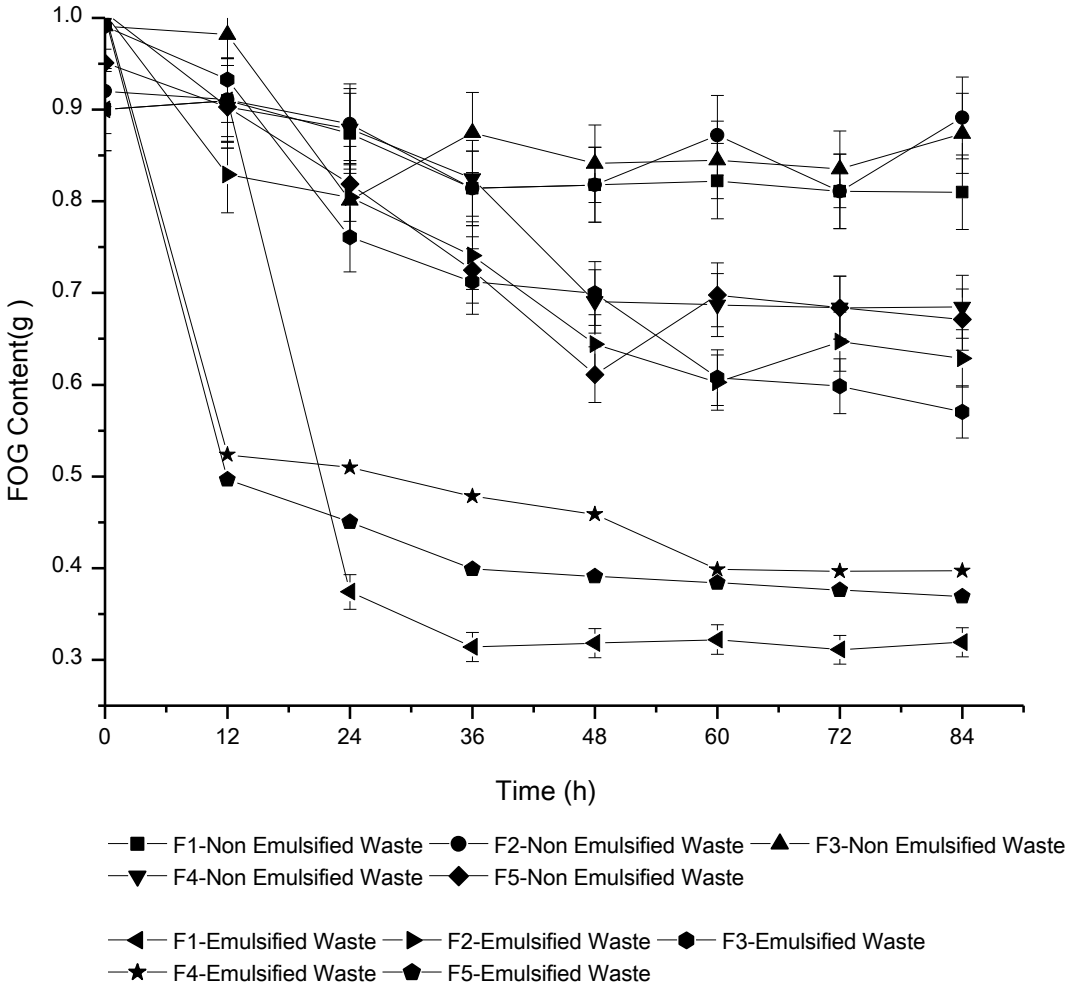


Figure 4.8: Fat, oil and grease degradation with time by fungal isolates for emulsified and non-emulsified grease trap waste

About 80% of FOG samples had melting points between 80–100 °C and therefore retained in solid form at room temperature. Therefore, to facilitate better contact surfaces for enzymes, emulsification was done using gum Arabic up to 5–15 % (w/v) for the 5 % (w/w) FOG samples and satisfactory degradation rates were achieved (Figure 4.8).

Although, the emulsification of FOG was able to achieve better degradation rates; it may need to incur additional cost for emulsification process. Therefore, it is worth to mention that the use of enzyme extracts together with emulsifier for degradation of FOG in grease trap waste may not be an option.

3. Post-operation issues

In wastewater treatment, enzymes/ patented biological formulations have been used to degrade the FOG. These enzymes/ patented biological formulations catalyze the initial hydrolysis step of FOG to form long-chain fatty acids (LCFA) and glycerol. The addition of lipase enzyme into wastewater accelerates the accumulation of LCFA in the system. Then, accumulated LCFA may lead to decrease the pH in the medium. Lowering of pH would affect the whole treatment process (Please refer section 2.3).

In this study, solid phase degradation is proposed as an economically viable solution to degrade FOG at no cost for enzyme production, purificationand emulsification. Fungi naturally prefer to grow in the heterogenic environment at low moisture condition. The grease trap waste is already a mixture of different types of oils/fats, fatty acids, triglycerides, detergents etc. (heterogenic). In addition, most of the separated FOG was at the solid form at room temperature (low moisture). Considering these two factors, experimental set up was designed to facilitate the natural growth conditions of fungi by using FOG as the main carbon source (Please refer section 3.2).

Stage 3: Biodegradation of Fat, Oil and Grease

4.6 Solid State Degradation of extracted FOG from grease trap waste

The extracted FOG (Figure 4.9) was provided as solid substrate at low moisture levels in a Petri dish to be degraded by lipolytic fungi.

Raw grease trap waste FOG extracted from raw grease trap waste

Figure 4.9: FOG waste before extraction and after extraction

The FOG degradation efficiency was determined by taking the initial and final weight of FOG after 72 h (3 days) of incubation time. Detailed studies conducted using the five isolates have shown that the degradation of animal fats by fungi was less efficient (Table 4.8) than degradation of cooking oils.

Table 4.8: FOG degradation efficiency as a percentage of weight reduction by fungi isolates within 72 h

Oil/fat type	Degradation efficiency by fungi isolates (Mean ± SD; n = 3)				
	F1	**F2**	**F3**	**F4**	**F5**
Olive oil	49.5 ± 3.2	31.4 ± 2.5	31.4 ± 3.8	43.3 ± 5.4	58.1 ± 2.4
Coconut oil	5.4 ± 0.8	4.7 ± 1.2	7.5 ± 1.6	92.0 ± 1.2	64.5 ± 1.7
Palm oil	48.5 ± 1.7	31.4 ± 1.5	19.5 ± 2.5	54.5 ± 2.8	78.1 ± 1.5
Soya oil	5.3 ± 1.2	15.1 ± 1.4	<1	33.2 ± 2.1	35.47 ± 2.4
Sunflower oil	13.4 ± 1.5	31.4 ± 1.2	<1	93.1 ± 6.4	78.9 ± 3.8
Butter fat	<1	<1	<1	10.1 ± 4.1	9.0 ± 2.1
Chicken fat	<1	16.4 ±1.2	<1	98.0 ± 1.4	96.16 ± 2.4
Lard	<1	9.8 ± 1.2	<1	92.4 ± 2.1	89.12 ± 5.1

Note: **F1**: *Aspergillus nige,* **F2**: *Geotrichum candidum,* **F3**: *Aspergillus fumigates,* **F4**: *Fusarium proliferatum,* **F5**: *Penicillium citrinum*

Nevertheless, three fungal isolates (*Aspergillus niger*, *Fusarium proliferatum* and *Penicillium citrinum*) have shown significantly high degradation efficiencies (at the $P < 0.05$) for cooking oils and animal fats. For each waste type, more than 80% of degradation efficiencies were recorded by above three fungi isolates within 72 h (Table 4.8).

4.7 Solid State Degradation of raw grease trap waste (without extracting FOG)

The raw grease trap waste was provided as solid substrate (after drying to have relatively low moisture condition. The recorded optimum initial moisture content is between 25 – 30 % of initial weight (Figure 4.10).

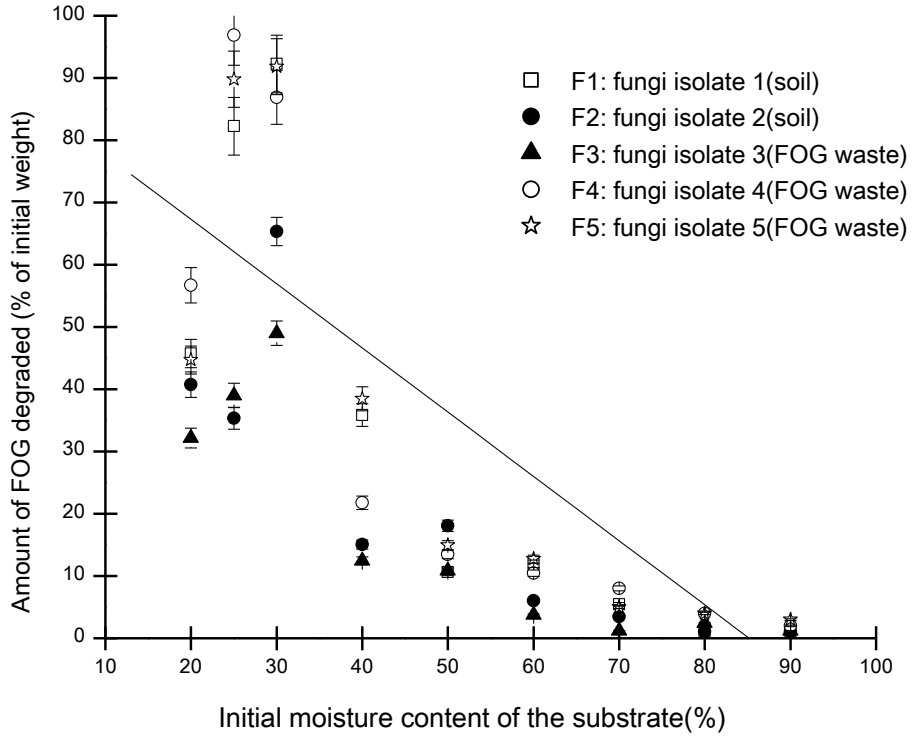


Figure 4.10: FOG degradation efficiency at different initial moisture levels (at pH 7)
Note: Error bars – standard deviation

In this study, the recorded optimum pH for FOG degradation was 6 –7 at 30 °C for selected fungi isolates (Figure 4.11).

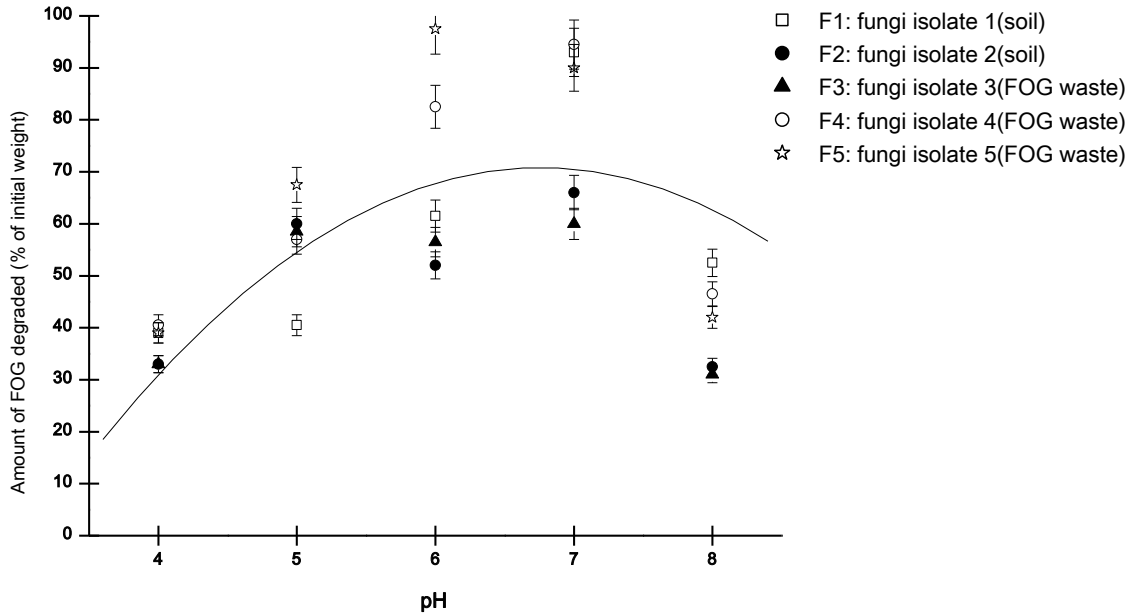


Figure 4.11: FOG degradation efficiency at different pH levels (Moisture content ~ 30%)
Note: Error bars – standard deviation

However, in literature lipolytic fungi have shown optimum FOG degradation for different pH values: at pH 2.5 and at 45 °C (Mahadik *et al*., 2002); at pH 5 and at 37–40 °C (Kirsh, 1935); at pH 5 and at 30 °C for synthetic substrate (triacylglycerols).For natural substrate (oil) at pH 7 and at 30 °C (Hee–Yeon *et al*., 2007); at pH 8.5 and at 40 °C (Ülker *et al*., 2011); at pH 7–9 and at 30–45 °C (Mahmoud *et al*., 2015). Although the recorded pH of grease trap waste varied from 4.5–6.5, it has been in a compatible range with other reported pH values for FOG degradation. Therefore, the pH of the grease trap waste was not adjusted to the optimum of 6–7. Also, adjustment of pH of the grease trap waste would entail additional expenditure, which will make the overall cost of wastewater treatment higher.

Maintaining the moisture content at the required level in the reactor has become a critical factor for better degradation of grease trap waste. The observed optimum moisture content for degradation of grease trap waste was 65% (Figure.12). In the reported literature, almost similar moisture levels were observed as optimum moisture content for lipolytic fungi, which was 65% (Falony *et al*., 2006; Kotogan *et al*., 2014) 50–55% Raimbault and Alazard, 1980).

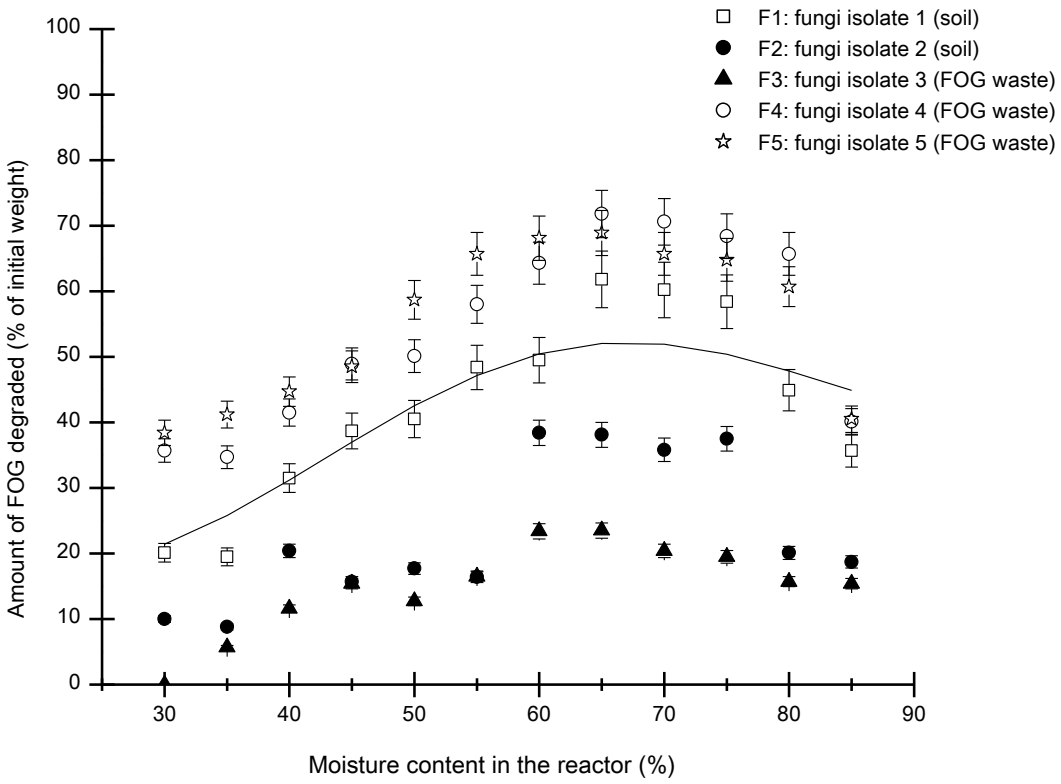


Figure 4.12: Grease trap degradation efficiency at different moisture levels in the reactor

As described in the methodology, the developed new approach to degrade the raw grease trap waste from the food service industry is based on bioaugmentation. In this approach, fungi are grown on solid surfaces (in a tray type reactor) which allowed them to degrade the FOG and other organic components present in the waste. Fatty acids and other hydrolyzed products that are produced such as glucose and amino acids may be used to fulfill their nutrient requirement, metabolism, and growth. Fungi may reach the end of their life cycle in the reactor by forming spores due to the limitation of foods. This degraded residue can be used as an inoculum for the second set of grease trap waste. Consequently, once the degradation cycle is started, putting them into practice would be easier because no continuous inoculation is needed. About 50% degradation efficiencies were achieved for SSD of raw grease trap waste (Figure 4.13).

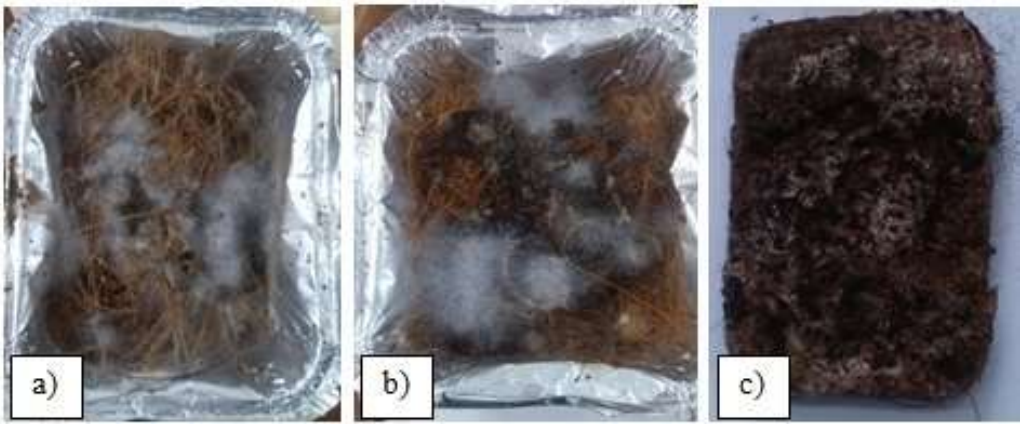


Figure 4.13: Solid State Degradation of raw grease trap waste: a) After 3 days b) After 5 days c) After 7 days

The solid-state degradation of FOG (as extracted grease trap waste) was performed under optimum condition (pH of the medium: 7; Initial moisture content 25-35%). The observed results indicated that higher amount of FOG can be degraded under optimum condition, for instance F1, F4 and F5 species were able to degrade more than 80% of FOG (from initial weight w/w) except for few locations. However, the observed degradation efficiencies for raw grease trap waste (without extraction of FOG) were less than the values those obtained under optimum conditions for FOG degradation (Figures 4.14 & 4.15). This may perhaps due to the pH of raw grease trap waste was varied between 4.5–6.5 (the optimum pH for the FOG degradation is 7, Figure 4.11). The results indicated that at least three fungal isolates (F1, F4 and F5) used in the present study were able to reach a degradation efficiency of around 50% for raw grease trap waste (Figure 4.15).

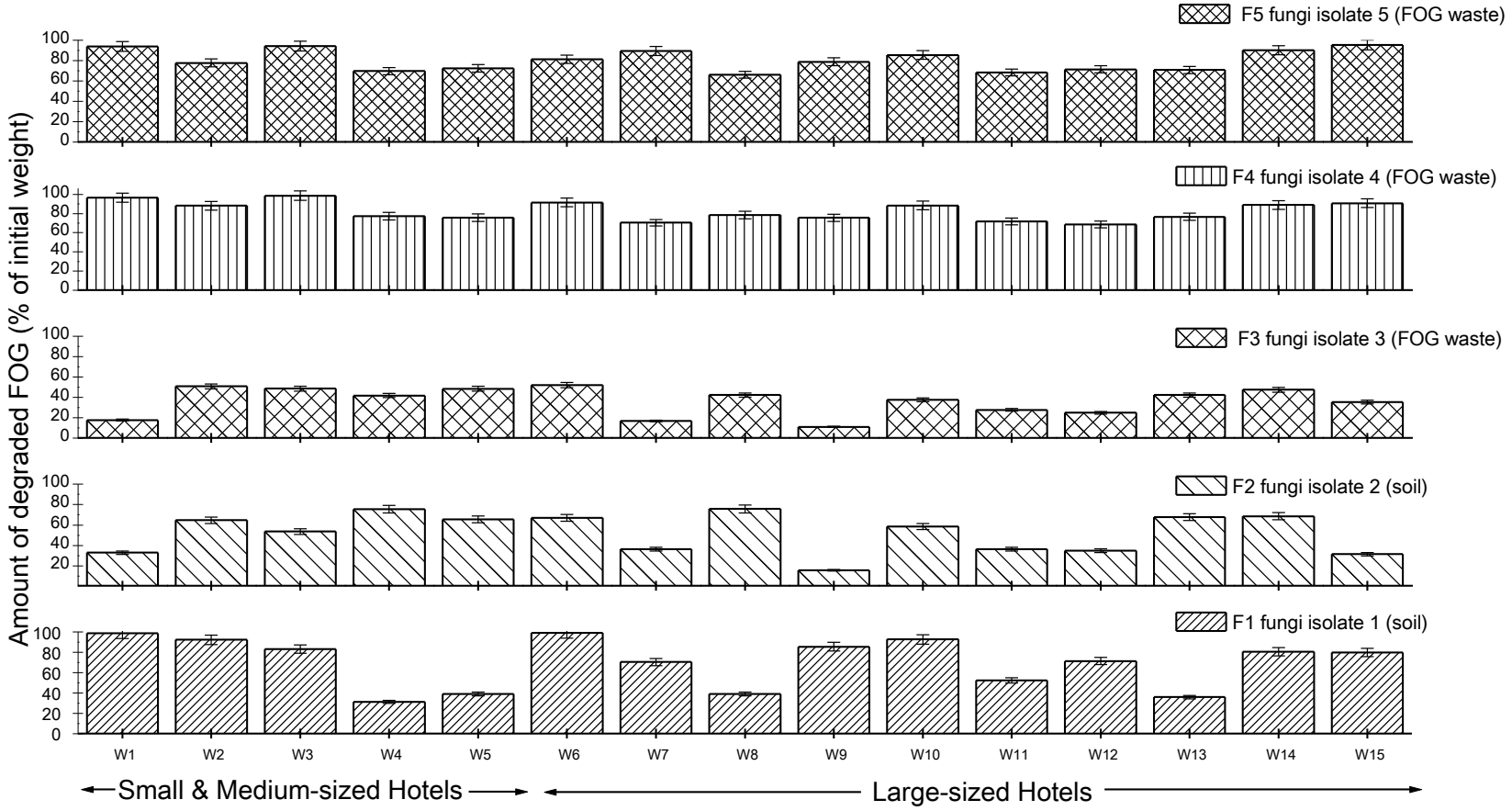


Figure 4.14: FOG degradation efficiency of selected fungi isolates on extracted grease trap waste
(Initial oil/fat content: weight of the 100µL volume of oil/fat, incubation time: 72 h, temperature: 30°C.)
Note:- W: grease trap waste; 1 to 15: location number; Large-sized hotels: number of rooms > 50; Small and Medium-sized hotels: number of rooms < 50.

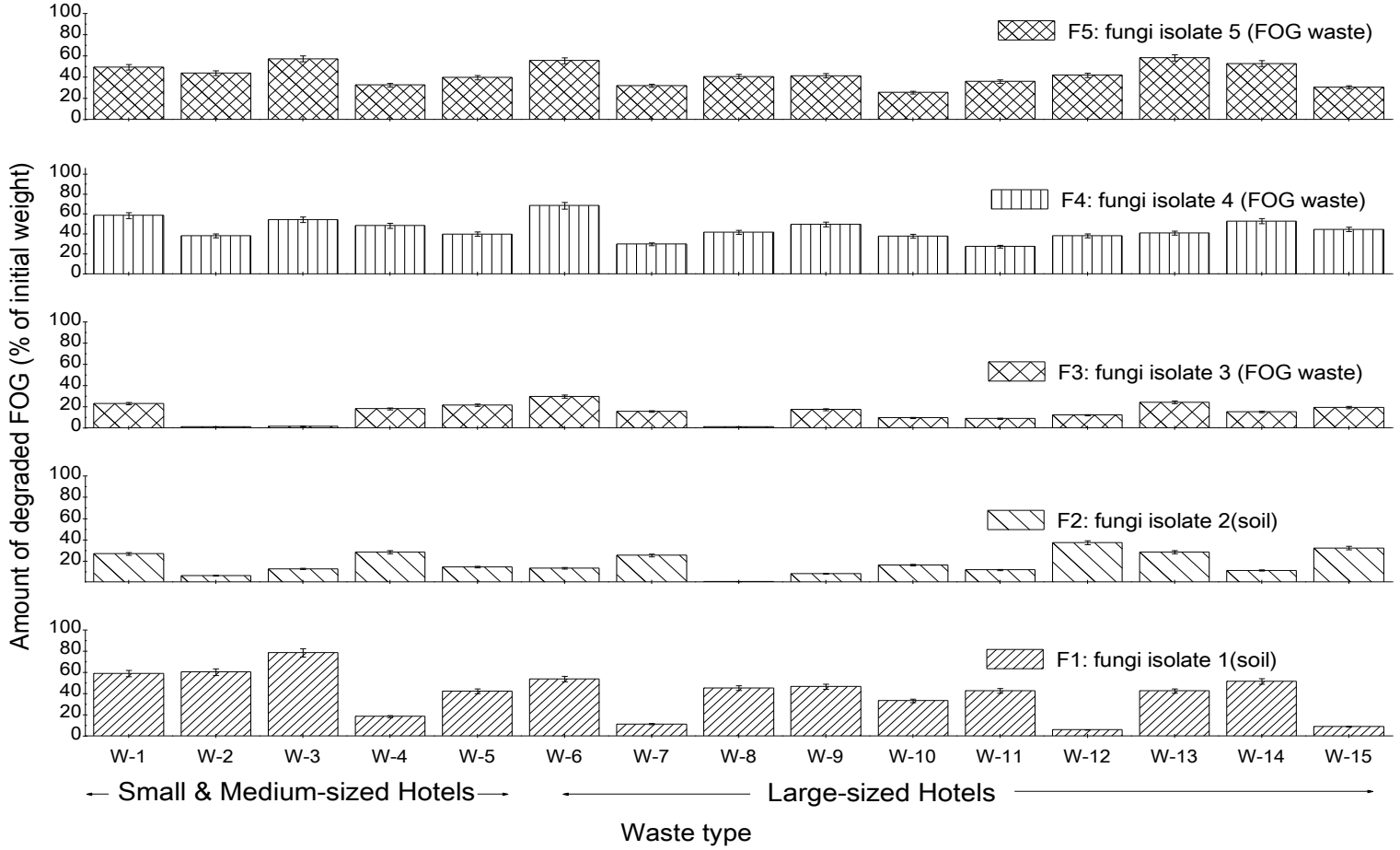


Figure 4.15: FOG degradation efficiency of selected fungi isolates on raw grease trap waste
(Initial FOG content: 10 mg/g dry weight, incubation time: 72 h, temperature: room temperature)
Note:- W: grease trap waste; 1 to 15: location number; Large-sized hotels: number of rooms > 50; Small and Medium-sized hotels: number of rooms < 50.

Table 4.9: Optimum condition for scale-up implementation

Parameter	Process Conditions	
	Start-up Process	**Continuation**
Carbon source	Grease trap waste	Grease trap waste
pH	Adjusted to 7.0 ± 0.1	No need to adjust the pH, default grease trap waste is acceptable (4.5–6.5)
Initial moisture: substrate	< 35 %	< 35%
Reactor type	Tray type reactor (1 m ×1 m × 0.5 m)	Few tray type reactors (1 m ×1 m × 0.5 m)
Reactor moisture/humidity	~ 65%	~ 65%
FOG content in the substrate	10 mg/g dry weight	100 mg/g dry weight
Bulking agent	1 % w/v coir fiber	1 % w/v coir fiber
Inoculation	1×10^7 spores/mL	Degraded residue from start-up process
Temperature	Room temperature	Room temperature

The developed methodology provide substantiation that there is a significant potential to implement this low-cost biotechnological application in pilot/full scale due to its high degradation efficiency. The optimum conditions for scale-up criteria that could be used to implement in pilot/ full-scale operations are given in Table 4.9. However, as spore inhalation of fungi can lead to allergic reactions, the use of personal protective wear is recommended.

CHAPTER 5

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The study showed that there is a significant potential for implementation of this developed methodology as a biotechnological application to degrade FOG in the foodservice industry. Based on the objectives of the study following conclusions can be made:

- Wastewater characteristics (total FOG content, BOD and COD levels, nutrient levels (total Nitrogen and total Phosphorus), solid levels) among three industries viz. hotels/restaurant, bakery and dairy were significantly different ($P < 0.05$).
- The most abundant fatty acids present in grease trap waste are palmitic acid 49.5% (w/w) and oleic acid 33% (w/w). Analysis of commercial grade oils showed that palmitic acid content was more than 40% (w/w) in palm oil while oleic acid present at a higher percentage in olive oil which is about 70% (w/w), and about 40% (w/w) of olive oil is present in palm oil. In animal fats (chicken and lard), the oleic content was more than 35% (w/w).
- Altogether, eight fungi species were isolated, which have the capability of producing lipase enzyme. Among them, four fungal species were isolated from soil samples, and four fungi species were isolated from grease trap waste samples. The best-isolated fungi species were *Aspergillus niger, Geotrichum candidum, Aspergillus fumigates, Fusarium proliferatum* and *Penicillium citrinum.*
- The optimum elemental ratio for C:N:P:K is 102:18:2:2 for solid state fermentation. The present study was able to produce enzymes using above fungi species under solid-state fermentation (SSF) with higher lipase unit activity, ranging from 9–22 (U)/g dry weight.
- The optimum conditions to degrade grease trap waste by solid-state degradation: initial moisture content of grease trap waste should be 25–35% of

weight; grease trap waste should be between pH 6 7; the reactor moisture condition for continuation of degradation should be maintained around 65%.

- Solid-state degradation can be used to degrade grease trap waste rapidly using isolated fungi. The recorded FOG degradation efficiencies around 50% within 72 h with raw grease trap waste without adjusting the pH.
- The raw grease trap waste (after drying to have moisture content between 25–35%) can be used as a solid substrate. The degraded residue can be used as an inoculum for the second set of grease trap waste. Therefore, once the degradation cycle is started, continuous inoculation for the rest of the process is not needed.

5.2 Recommendations

The research findings often encourage future research directives on followings:

- Standard methods for screening and isolation predominantly recommend using olive oil as the main carbon source. In the present study, lipolytic microorganisms were isolated from grease trap waste and oil contaminated the oil**.** The screening and isolation process of fungi could be optimized by using locally available fat or oil sources. Therefore, it is recommended to test the enzyme activity or lipid degradation rate with the original fat/oil source (where they have been isolated).
- Development of cost-effective methods for drying of grease trap waste in order to retain the required moisture level in the substrate for start-up the degradation cycle is recommended.
- The research on the use of microbial consortia to degrade the grease trap waste is also recommended.

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LIST OF ANNEXES

Annex I: Cleaning of glassware and sterilization methods

Cleaning of glassware

General

Generally, glassware is cleaned by several cycles of washing/rinsing: (i) cleaning with detergent and tap water; (ii) rinsing with weak acid (1% HCl) (iii) couple of rinses with distilled water and followed by final rinses with deionized water. In addition, glassware is rinsed withan appropriate solvent just before use. Whenever the thorough cleaning is not possible immediately, glassware is soaked in water or put into acid water (a 1% solution of hydrochloric or nitric acid) until it clean.

The common solvent used for cleaning/rinsing of glassware

1. **Strong Acids**. e.g., concentrated HCl or H_2SO_4. wash Under the fume hood.
    1. Carefully rinse the glassware with copious volumes of tap water.
    2. Rinse 3-4 times with deionized water. Then let it dry.
2. **Strong Bases**, e.g., 6M NaOH or concentrated NH_4OH. Wash Under the fume hood.
    1. Carefully rinse the glassware with copious volumes of tap water.
    2. Rinse 3-4 times with deionized water, then let it dry.
3. **Weak Acids**, e.g., acetic acid solutions or dilutions of strong acids such as 0.1M or 1M HCl or H_2SO_4.
    1. Rinse 3-4 times with deionized water and then let it dry.
4. **Weak Bases**, e.g., 0.1M and 1M NaOH and NH_4OH.Rinse thoroughly with tap water to remove the base.
    1. Then rinse 3-4 times with deionized water and let it dry.

Cleaning/rinsing of glassware used for GC/MS analysis

1. Rinse the glassware with the appropriate solvent (e.g., Acetone, Hexane, Ethanol)
2. Use deionized water for water-soluble contents.

3. Use ethanol for ethanol-soluble contents, followed by rinses in deionized water.
4. Rinse with other solvents as needed, followed by ethanol and finally deionized water.
5. If the glassware requires scrubbing, scrub with a brush using hot soapy water, rinse thoroughly with tap water, followed by rinses with deionized water

Cleaning with soap and water

1. Soak the glassware in soap solution for at least 10 to 15 minutes or leave overnight.
2. Scrub with brush or cloth or sponge
3. Rinse thoroughly with tap water.
4. Again rinse with distilled or deionized water.

Sterilization Methods

The sterilization was done by following methods:

Wet heat (autoclaving)

The method using pressurized steam to heat the material to sterilization. This is a very effective method, which kills all microbes, spores,and viruses. Although for some specific bugs, especially high temperatures or incubation times are required. Autoclaving kills microbes by hydrolysis and coagulation of cellular proteins, which is efficiently achieved by intense heat in the presence of water. The intense heat comes from the steam. The pressurized steam has a high latent heat; at 100 °C,it holds 7 times more heat than water at the same temperature. This heat is liberated upon contact with the cooler surface of the material to be sterilized, allowing rapid delivery of heat and good penetration of dense materials. All objects should be sterilized at 121°C and 15 psi for 15-20 min. Cotton plugs, gauze, plastic caps, glassware, filters, pipettes, water, and nutrient media can all be sterilized by autoclaving.

Dry heat (baking)

Dry heat tends to kill microbes by oxidation of cellular components where heat is transferred by convection, conduction or radiation. This requires more energy than protein hydrolysis,so higher temperatures are required for efficient sterilization by dry heat. This method is used to

sterilize glassware such as bottles, Petri dishes,and test tubes, dry heat is required,and this is carried out in a hot air oven. The temperature of 100 °C for one hour can destroy the non-sporing organism. Fungal spores need 115 °C for one hour. While for other all bacteria 160 °C temperature is needed for one hour. The contents must not be removed from the oven immediately as a slow cooling period is necessary, ideally, when the temperature has reduced down to 50°c, but no less. The reason for the gradual cooling period is to avoid the cracking of glassware as well as preventing air (that could potentially contain contaminating organisms) entering the oven.

Glass pipettes (graduated and Pasteur) with ends plugged to a depth of 20 mm with non absorbent cotton wool, nylon or glass syringes (polypropylene or other types of plastic are not suitable), metal needles, lancets and forceps, dry swabs in glass tubes, glass or aluminum Petri dishes, glass test tubes with aluminum tops or non-absorbent cotton wool plugged, bottles with aluminum tops with silicone linings, glass flasks or cylinders covered with aluminum foil etc were sterilized using dry heat method.

Solvents

Ethanol was commonly used as a disinfectant, which diluted to 90% in water to be effective.

Flaming

Inoculating loops or wires, points of forceps and searing spatulas are held in a Bunsen flame till they become red hot, for sterilizing them. If the loops contain infective proteinaceous material, they should be first dipped in chemical disinfectants before flaming to prevent spattering. Scalpels, needles, mouths of culture tubes, glass slides, cover slips, etc. could be passed a few times through the Bunsen flame without allowing them to become red hot. The bacteria get destroyed.

Radiation

UV radiation was not commonly used, however, UV radiation was used (in Laminar flow cabinet) for sterilization.

Annex II: Details of media preparation, sterilization and aseptic techniques

Media preparation methods

1. Enrichment media- R_2B Broth for 1L

Compound	Amount
Peptone	0.375g
$MgSO_4.7H_2O$	0.5g
Sodium pyruvate	0.3g
Casin acid hydrolysate	0.5g
K_2HPO_4	0.3g
Yeast extract	0.5g
Soluble starch	0.5g
Glucose	0.5g
pH	7.2

The enrichment broth will be prepared by dissolving respective amounts of above compounds in aseptic container/1L and pH will be adjusted to 7.2 by adding 0.1N HCL/0.1N NaOH.

2. Minimal Salt agar

The composition of 100.0 mL of the minimal salt agar medium is

Compound	Amount (g)
K_2HPO_4	0.7 g
K_2HPO_4	0.7 g
KH_2PO_4:	0.2 g
Trisodium Citrate ($Na_3C_6H_5O_7$)	0.05 g
$MgSO_4$	0.01 g,
$(NH_4)_2SO_4$	0.15 g
Agar	1.5 g.

Distilled water was added up to 100.0 mL and the pH of the medium was adjusted to 7 ± 0.1.

3. Tributyrin agar

The composition of 100 mL of Tributyrin Agar

Compound	Amount
Peptone	0.5% (w/v)
Yeast extract	0.3% (w/v)
Tributyrin	1% (v/v)
Agar	2%
pH	7

4. PDA (Potatoes Dextrose Agar)

Potato Dextrose Agar medium (per 1000 mL of distilled water), pH 6-6.5

Chemicals	Amounts /g
Potatoes (small cubes)	200
Dextrose	20
Agar	15

Sterilization method and dispensing sterile agar media

1. Plug the mouth and neck of the flask and test tubes (which contain the media) with cotton wool and cover with aluminum foil. Loosen the screw caps one-quarter turn.
2. Label the flask and the test tubes indicating the date, group, medium type,etc. and place them in an autoclave machine for sterilization (121°C and 15 psi for 15-20 min).
3. Do not shake the media immediately after removing from the autoclave, as the superheated media may boil over if agitated.
4. Place the 2 pours and kept in a 50 ºC water bath.
5. Place the 2 slants in a slanted tray or other device where they will tilt at an angle.
6. Take two sterile Petri dishes and label the underside of the plate with the name, date,and type of medium.
7. Remove a tube from the water bath, once it is cooled to about 50 ºC, wipe the excess moisture on the bottom of the tube, remove the cotton wool plug and flame the neck of the test tube.

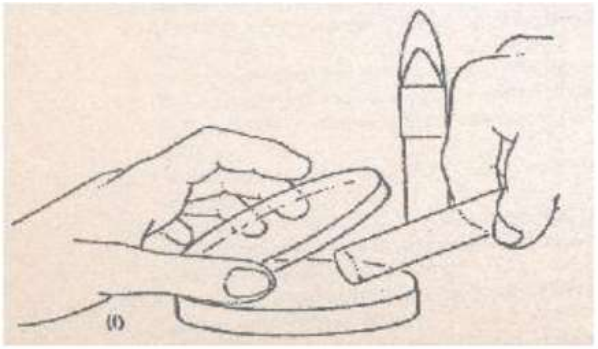


Fig 1

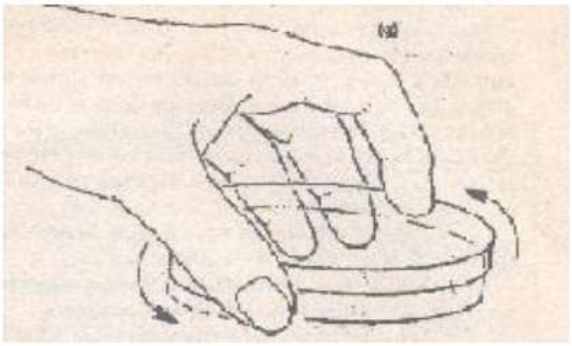


Fig 2

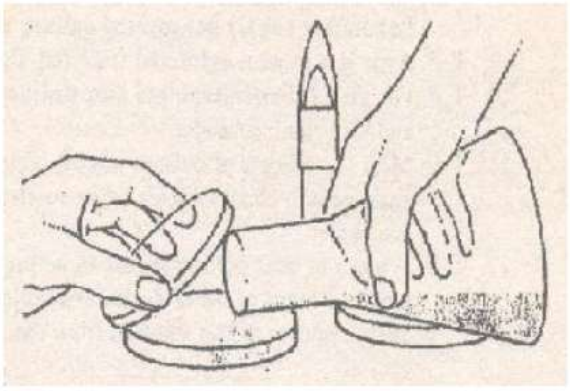


Fig 3

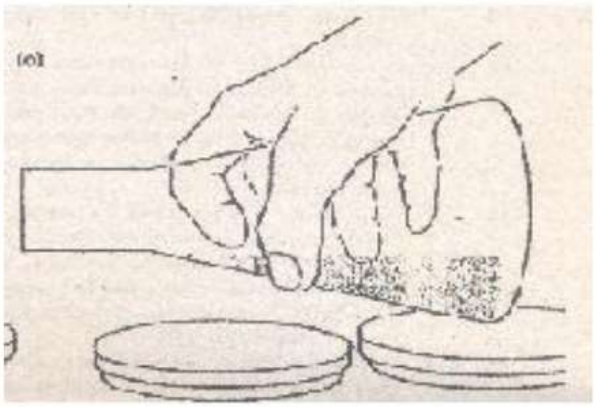


Fig 4

8. While working near the flame, carefully pour the contents of the tube into the bottom of the above prepared Petri plate, using the lid of the plate to shield the surface of the dish from your breath (Fig 1). Gently tilt and rotate the dish so that the medium completely covers the surface (Fig 2). Avoid splashing.
9. Similarly, label 4-5 sterile Petri plates. Obtain a flask of Nutrient Agar cooled to pouring temperature and wipe the moisture on the bottom of the flask. Flame the mouth of the flask after careful removal of the cotton plug.

10. Pour the plates (Fig 3) keeping the flask in a tilted position (Fig 4) until all the plates are poured. If you are right-handed fill the plates from left to right.
11. When all plates and slants have solidified, invert the plates (except the plates containing semi-solid media) and keep the slants in an upright position. Tighten screw caps.

Aseptic transfer techniques

Microorganisms are transferred from one culture to another, and the process is called subculturing. Microorganisms are always present in air, on laboratory surfaces,etc. These microorganisms can serve as a source of external contamination that interferes with experimental results. Therefore, it is essential to use proper techniques during subculturing/transferring/inoculating. An inoculating needle or loop must always be sterilized by holding it in the hottest portion of the Bunsen burner flame, the inner blue cone until it becomes red hot. Then the upper portion of the handle is rapidly passed through the flame. Once flamed, the loop is never put down. Hold in hand to cool for 10 - 20 seconds.

Procedure:

1. Obtain a bacterial agar slant culture and 2 tubes of sterile Nutrient Broth (use 1 tube as a control).
2. Hold the inoculating needle from your dominant hand, flame the wire from its handle connection to its tip. Keep the needle in the air (near the flame) to be cooled.
3. The stock culture tubes and the tube to be inoculated are held in the palm of the other hand and secured with the thumb. The two tubes are separated to form a V in hand (Fig. 2).
4. The tubes are uncapped by grasping the first cap with the little finger and the 2nd cap with the next finger and lifting the closures upwards. Once removed these caps must be kept in the hand that holds the sterile inoculating needle or the loop (Inner aspects of the caps should be pointing away from the palm of the hand).
5. Without gouging, the agar, remove a barely visible bit of bacterial growth from the surface of the slant and inoculate it into one of the sterile broths. Shake the loop/needle to dissolve the organism.
6. Flame the neck of the tube and replace the cap on the same tube from which it was removed. Flame the needle before keeping in place as shown in Fig. 1.
7. Now transfer a loopful of inoculum to the other sterile Nutrient Broth following the same procedure.
8. Incubate your culture broths at room temperature for no more than 48 h.
9. Using the same aseptic transfer techniques, microorganisms can be transferred to a Petri dish, agar slant and an agar deep.

Alcohol flaming forceps for sterile transfer

In this procedure, burn the alcohol off the tips of forceps 3 times, and then use the forceps to transfer the sterile paper disks into a sterile broth.

Procedure:

1. Hold the forceps horizontally and apply a small amount of alcohol to the tips using a dropper; pass the tips through and out of the flame. Allow the alcohol to burn off the forceps (Fig 5).
2. Repeat the procedure two more times (Warning: Never dip the hot forceps into alcohol).
3. Incubate this at the room temperature for no more than 48 h.

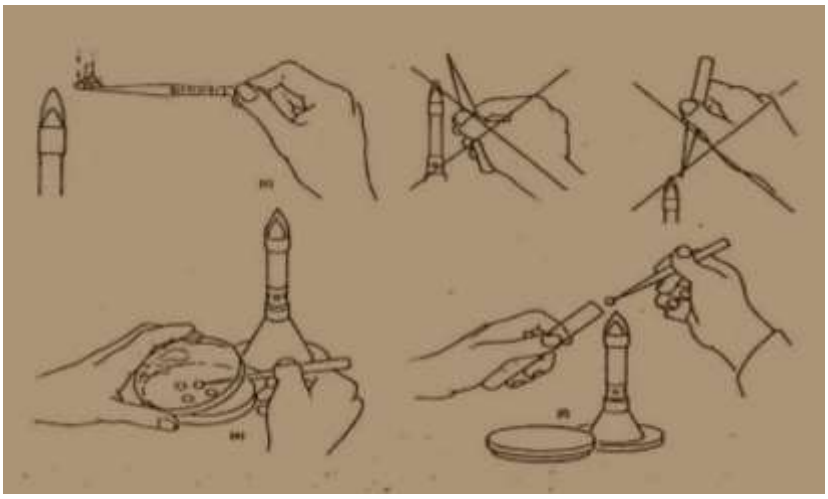


Fig 5: Processes utilized in alcohol flaming forceps for sterile transfer

Annex III: Observation of fungi,staining,and enumeration

Observation of Fungi

1. Adhesive Tape Method

Adhesive tape method is a simple, quick and easy method of observing fungi, with minimum disturbance to the fungal structure.

Materials

- Sporulating fungal culture plate / infected plant material.
- Adhesive tape
- Glass Slide
- Lacto Phenol Cotton Blue stain

2. Slide Culture Technique

A fungal culture grown directly onto the glass surface of a slide and coverslip such that it can be mounted for observation with minimal disturbance of fungal structures. The technique involves inoculating the required fungus on to a small agar block mounted at the centre of a slide and incubated inside a damp chamber. Slide cultures were used to observe the development of fungal structures and particularly for observing the fundamental taxonomic feature of the conidial development of hyphomycetes. The usual slide preparation technique of lifting fungal material from an agar plate with a needle, mounting on a slide and clearing, disturbs delicate features such as chains of spores and can obscure details of a fine structure such as conidiogenous cells, phialides,and denticles.

In slide culture, the very little nutrient is available to inoculum,and in response to difficult growing conditions, sporulation occurs readily on the glass. Older hyphae sporulate first,and as new hyphae grow, further spores develop thus allowing the successions of development features to be followed.

Materials

- A sterile Petri dish containing filter paper, Z or V-shaped glass rod and a glass slide placed on the rod
- Sterile distilled water.
- Fresh PDA plate
- Lacto Phenol Cotton Blue
- Scalpel
- A fungal culture
- Slides & coverslips
- Alcohol

Procedure

1. Wet the filter paper inside the Petri dish with a little sterile distilled water to form a damp chamber (Re-moisten if necessary during incubation).

2. Using sterile scalpel blade, cut 5 mm^2 blocks of the medium from a fresh agar plate and transfer one block to the centre of the slide inside the damp chamber. Alternatively, using a sterile Pasteur pipette, add one or two drops of cooled molten PDA onto the slide and let it solidify.

3. Inoculate the centre of each face of the block with a small quantity of the required fungus.

4. Sterilize a coverslip by dipping in alcohol and flaming and lower on to the block to complete the slide culture assembly.

5. Incubate the slide culture at 25 0C or allow growing on the laboratory bench, observing daily for signs of growth, sporulation if applicable. In certain fungi, colour changes occur when sporulating.

6. When the slide culture has produced sufficient growth on the glass surfaces of both the slide and the coverslip (around 5 mm of growth is usually adequate), the slide culture is ready for mounting.

7. Place a small drop of stain on a fresh slide and carefully pries the coverslip away from the block.

8. Lower the coverslip very carefully on to the drop of stain, taking care not to trap any air bubbles. The quantity of stain used should be just sufficient to reach the edges of the coverslip without excess spilling from the edges.

9. If any air trapped, leave the slide unsealed on a hot plate to release the air, then seal with a suitable sealant.

10. Remove the block of agar from the slide and place a drop of stain in the centre of the slide at the point from which the block was removed,

11. Carefully lower a clean slip onto the drop of stain and seal as described above. In this way, two mounts are prepared from each slide culture.

Enumeration of microbial cells using Haemocytometer

The depth of the chamber is 0.1 mm, the grid can be seen under the microscope. There are 25 large squares. Each of these large squares is further divided into 16 small squares.

- The area of a large square is 1/25 mm^2
- As the depth of the chamber is 0.1 mm
- The volume of a large square is 0.1 × 1/25 mm^2

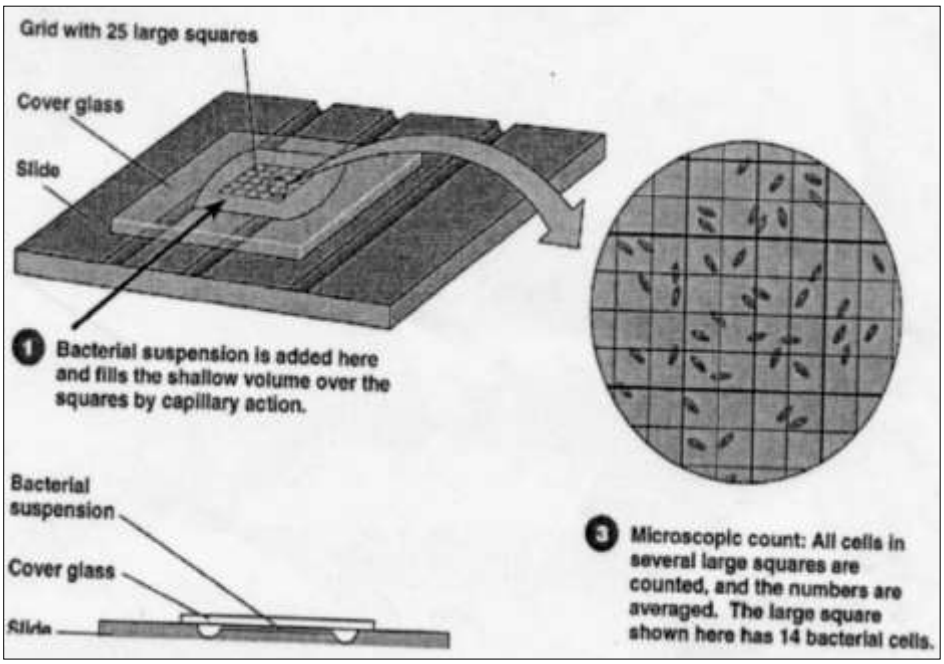


Fig 1: Schematic diagram of Haemocytometer

Preparation of a dilution series

- Label seven test tubes containing 9 mL of sterile saline solution with the dilutions from 10-1 to 10-6 and control.
- Under aseptic conditions pipette out 1 mL of the spore suspension into the first test tube containing 9 mL of sterilized saline solution. Mix well.
- Pipette out 1 mL of the diluted suspension from the first test tube into the second test tube containing 9 mL of sterile saline solution. Mix well.
- Repeat the same procedure to prepare a serial dilution of the provided suspension.
- From each diluted sample transfer a small volume of the suspension onto the Haemacytometer using a Pasteur pipette, and cover with a coverslip.
- Observe the Haemacytometer under the microscope. Count the number of spores present in several large squares.

- Calculate the average number of spores per large square by dividing the total number of cells by the number of large squares counted.
- Using the average number of spores per large square and the volume of a large square, calculate the number of spores present in 1 mL of sample.
- Express the concentration of spores in the suspension as the number of spores per mL (1 mL = 10^3 mm^3)

Annex IV: DNA sequencing report

Aspergillus fumigates

BLAST ® » blastn suite » RID-YEY6PNWJ015

BLAST Results

Job title: Nucleotide Sequence (696 letters)

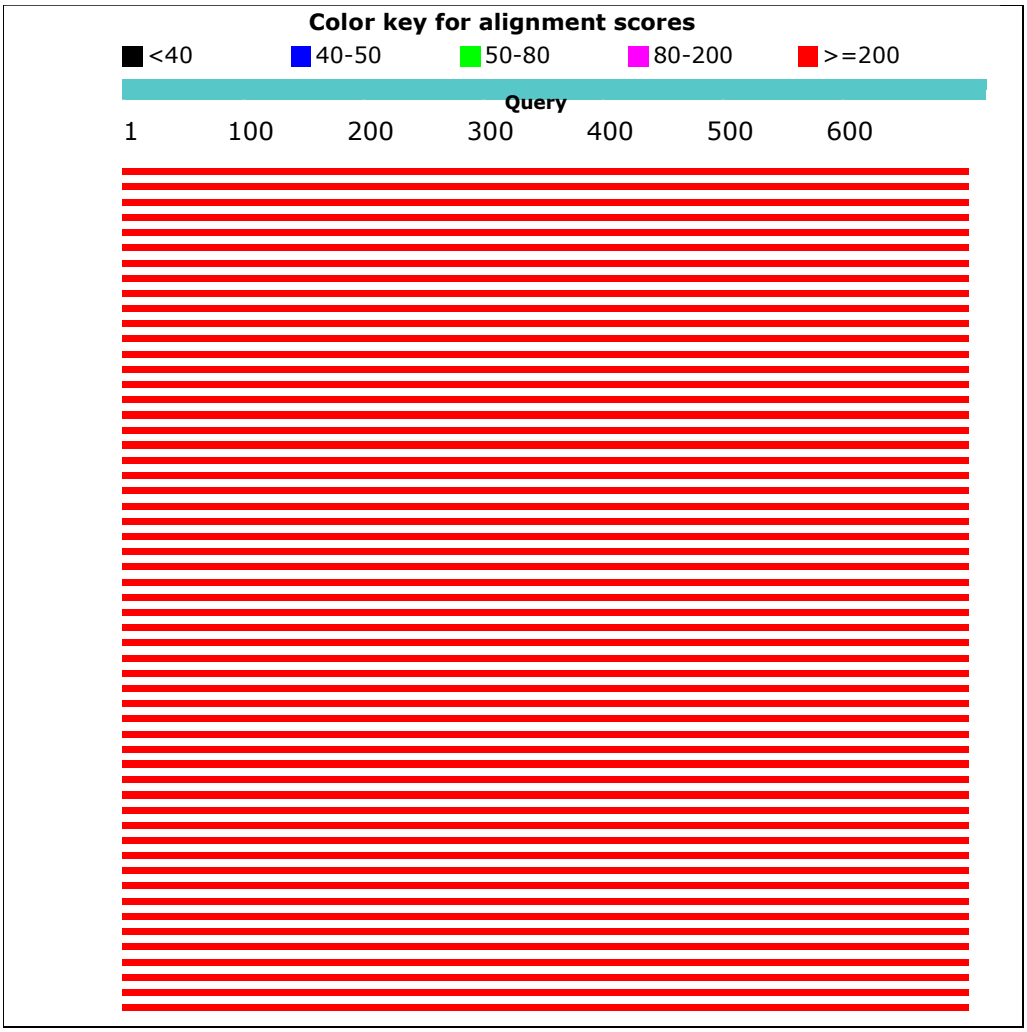
RID YEY6PNWJ015 (Expires on 10-19 23:52 pm)

Query ID lcl|Query_114859
Description None
Molecule type nucleic acid
Query Length 696

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.7.0+

Graphic Summary

Distribution of the top 100 Blast Hits on 100 subject sequences



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Fungal sp. E41 KL-2014 18S ribosomal RNA gene, partial sequence	1262	1262	100%	0.0	99%	KJ747403.1
Fungal sp. ZJ24 18S ribosomal RNA gene, partial sequence	1258	1258	100%	0.0	99%	KT582270.1
Fungal sp. ZJ20 18S ribosomal RNA gene, partial sequence	1258	1258	100%	0.0	99%	KT582263.1
Aspergillus fumigatus strain VV11 18S ribosomal RNA gene, partial sequence	1258	1258	100%	0.0	99%	KT031993.1
Aspergillus nomius strain H5 18S ribosomal RNA gene, partial sequence	1253	1253	100%	0.0	99%	JF416646.1
Aspergillus sp. ZL-2008 18S ribosomal RNA gene, partial sequence	1253	1253	100%	0.0	99%	EU770325.1
Aspergillus nomius gene for 18S rRNA	1253	1253	100%	0.0	99%	AB008404.1
Fennellia flavipes gene for 18S rRNA, partial sequence	1253	1253	100%	0.0	99%	AB008400.1
Aspergillus sp. clone EF019 small subunit ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	MG015948.1
Aspergillus flavus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: PSU2	1247	1247	100%	0.0	99%	LC127086.1
Aspergillus sp. SHP19 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KX186570.1
Aspergillus fumigatus strain YuZhu2 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KU512836.1
Aspergillus sp. X22 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KU350749.1
Aspergillus sp. N1-14' 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KT935264.1
Fungal sp. ZJ41 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KT582241.1
Aspergillus terreus strain ASPII 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KU131580.1
Aspergillus flavus strain EGYII 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KU060796.1
Aspergillus sp. ISSFT-021 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KT832787.1
Uncultured Aspergillus clone GRP13 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KR233004.1
Aspergillus fumigatus isolate HWT5 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KR135118.1
Fungal sp. isolate nussu_AT 18S small subunit ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KT714191.1
Fungal sp. isolate nussu_AF 18S small subunit ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KT714190.1
Aspergillus fumigatus 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KR019681.1
Aspergillus terreus isolate ML3-1 18S ribosomal RNA gene, partial sequence	1247	1247	100%	0.0	99%	KM874778.1

Aspergillus niger

BLAST ® » blastn suite » RID-YEWJERVD014

BLAST Results

Job title: Nucleotide Sequence (629 letters)

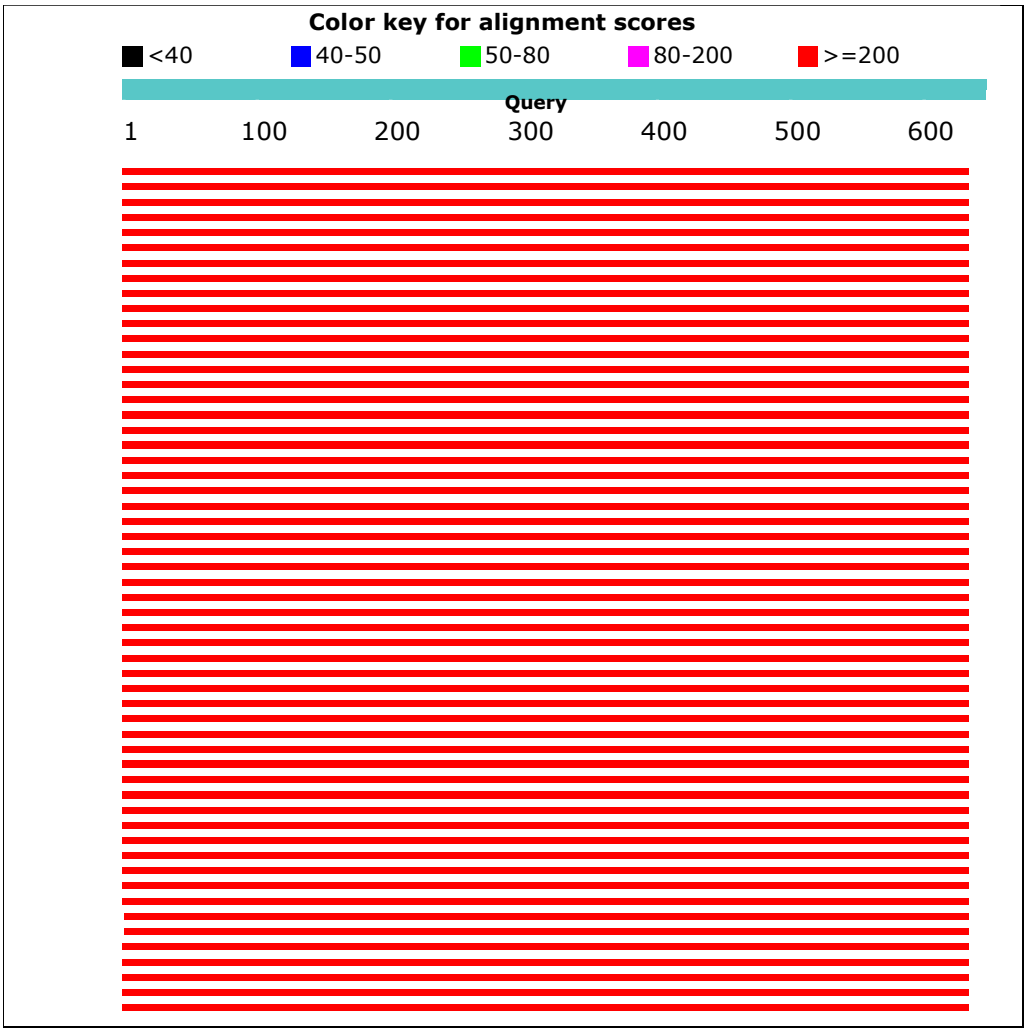
RID YEWJERVD014 (Expires on 10-19 23:25 pm)

Query ID lcl|Query_238819
Description None
Molecule type nucleic acid
Query Length 629

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.7.0+

Graphic Summary

Distribution of the top 101 Blast Hits on 100 subject sequences



Descriptions

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Aspergillus niger strain EG6 18S ribosomal RNA gene, partial sequence	1134	1134	100%	0.0	99%	KX457673.1
Aspergillus niger clone EF026 small subunit ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	MG015949.1
Aspergillus niger strain ANTS 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KY825168.1
Marsdenia tenacissima strain TZ-13 small subunit ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KY427888.1
Aspergillus sp. J10 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KU170626.1
Aspergillus niger strain ISSFR-019 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KT832786.1
Aspergillus niger strain ISSFR-018 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KT832785.1
Aspergillus terreus strain ISSFR-002 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KT832781.1
Aspergillus niger strain ISSFR-001 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KT832780.1
Eurotiales sp. Rco001EF4 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KP963611.1
Aspergillus niger strain AN0512 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KP036601.1
Aspergillus niger strain DEF7 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KJ652025.1
Aspergillus niger strain CWJ-2 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KJ699094.1
Aspergillus awamori strain K-03 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KF922319.1
Aspergillus niger strain W1102 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KF758784.1
Aspergillus niger strain IS513 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	KF164284.1
Aspergillus niger strain A041 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JX393054.1
Aspergillus niger strain HKS11 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JX112703.1
Aspergillus awamori strain FCYN 206 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JQ728449.1
Uncultured eukaryote clone MEU7-70 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JQ693066.1
Aspergillus terreus strain IG4 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JQ012802.1
Aspergillus awamori strain IG3 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JQ012801.1
Aspergillus fumigatus strain IG2 18S ribosomal RNA gene, partial sequence	1133	1133	100%	0.0	99%	JQ012800.1

Fusarium proliferatum

BLAST ® » blastn suite » RID-YEYKB8N4015

BLAST Results

Job title: Nucleotide Sequence (695 letters)

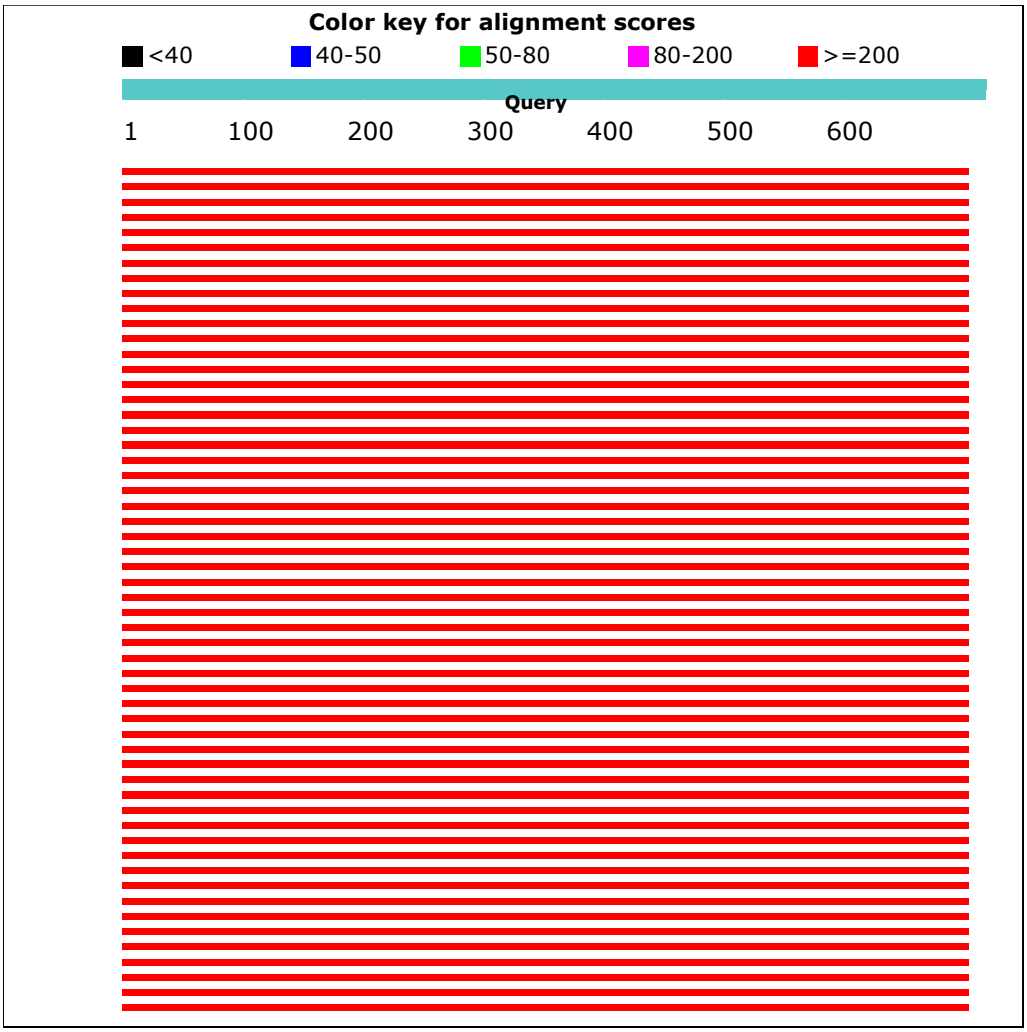
RID YEYKB8N4015 (Expires on 10-19 23:59 pm)

Query ID lcl|Query_205749
Description None
Molecule type nucleic acid
Query Length 695

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.7.0+

Graphic Summary

Distribution of the top 278 Blast Hits on 100 subject sequences



Descriptions

Sequences producing significant alignments:

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Fusarium proliferatum partial 18S rRNA gene for 18S ribosomal RNA, strain ITEM2400	1267	1267	100%	0.0	99%	LT841264.1
Fusarium proliferatum partial 18S rRNA gene for 18S ribosomal RNA, strain ITEM2287	1267	1267	100%	0.0	99%	LT841250.1
Fusarium oxysporum f. sp. dianthi partial 18S rRNA gene for 18S ribosomal RNA, strain Fod008	1267	1267	100%	0.0	99%	LT841236.1
Fusarium oxysporum f. sp. dianthi partial 18S rRNA gene for 18S ribosomal RNA, strain Fod001	1267	1267	100%	0.0	99%	LT841222.1
Fusarium oxysporum f. sp. cumini partial 18S rRNA gene for 18S ribosomal RNA, strain F11	1267	1267	100%	0.0	99%	LT841208.1
Fusarium verticillioides partial 18S rRNA gene, isolate TA_doublecycles	1267	1267	100%	0.0	99%	LT853257.1
Fusarium verticillioides partial 18S rRNA gene, isolate TA_pink1	1267	1267	100%	0.0	99%	LT853256.1
Gibberella sp. strain Z5 18S ribosomal RNA gene, partial sequence	1267	1267	100%	0.0	99%	KY368615.1
Fusarium verticillioides 7600 18S ribosomal RNA (FVEG_17767), partial rRNA	1267	1267	100%	0.0	99%	XR_001989355.1
Fusarium oxysporum f. sp. lycopersici 4287 18S ribosomal RNA rRNA	1267	1267	100%	0.0	99%	XR_001936476.1
Fusarium oxysporum f. sp. lycopersici 4287 18S ribosomal RNA rRNA	1267	1267	100%	0.0	99%	XR_001936474.1
Fusarium oxysporum f. sp. lycopersici 4287 18S ribosomal RNA rRNA	1267	1267	100%	0.0	99%	XR_001936464.1
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Fusarium oxysporum f. sp. lycopersici 4287 18S ribosomal RNA rRNA	1267	1267	100%	0.0	99%	XR_001936454.1

Geotrichum candidum

BLAST ® » blastn suite » RID-YEX0S9ZP015

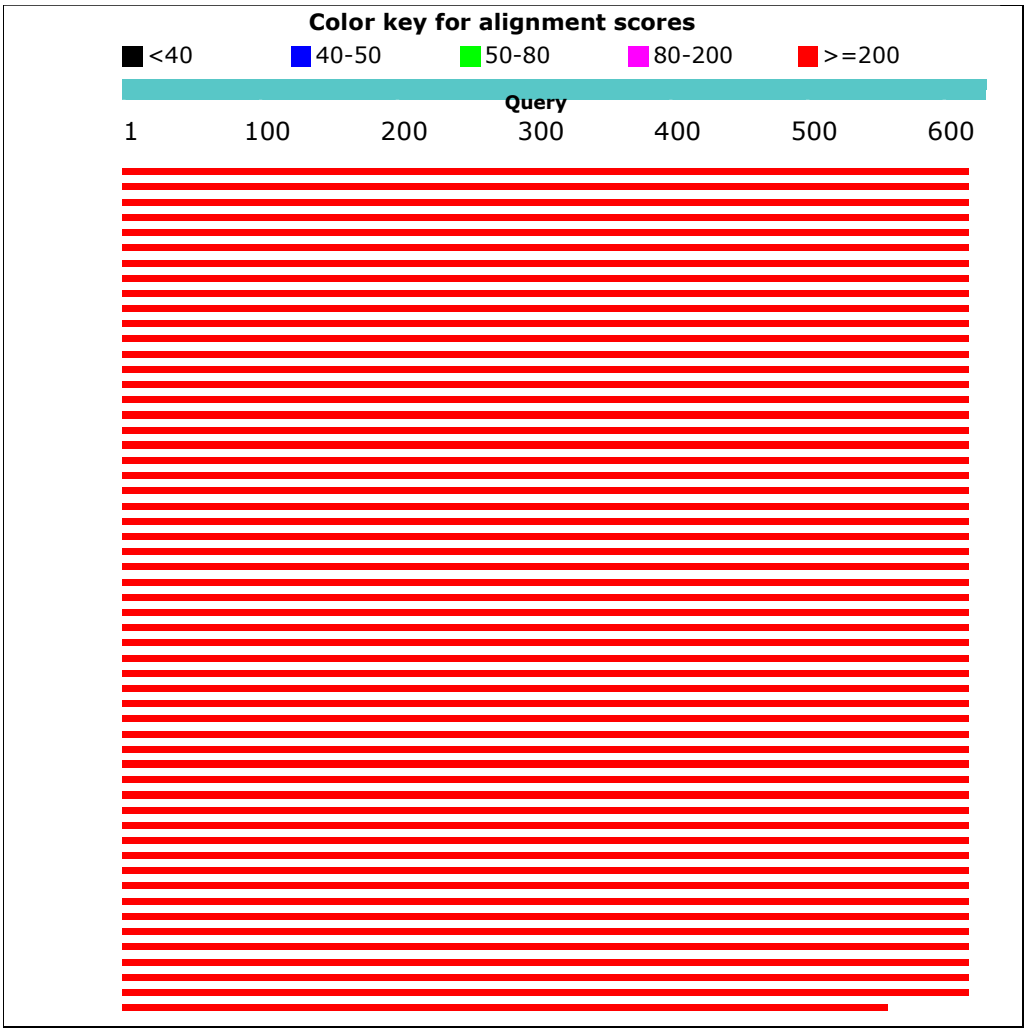
BLAST Results

Job title: Nucleotide Sequence (610 letters)

RID	YEX0S9ZP015 (Expires on 10-19 23:32 pm)		
Query ID	lcl|Query_151125	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.7.0+
Query Length	610		

Graphic Summary

Distribution of the top 103 Blast Hits on 100 subject sequences



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Geotrichum candidum voucher Oyster small subunit ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	KY977411.1
Geotrichum candidum strain AJP1 18S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	KY091650.1
Geotrichum candidum strain TZ-9 18S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	KU899094.1
Geotrichum candidum clone AS F-1 18S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	KJ543497.1
Galactomyces geotrichum strain LMA-70 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	JQ668740.1
Galactomyces geotrichum strain LMA-21 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	JQ668739.1
Galactomyces geotrichum strain SK15 18S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	GU561987.1
Galactomyces geotrichum strain PAMF4 18S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	JN256015.1
Galactomyces geotrichum strain PAMF3 18S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	JN252305.1
Galactomyces geotrichum strain LMA-75 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	1122	1122	100%	0.0	99%	JF262191.1
Galactomyces geotrichum strain RCPA U/N 127763 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1122	1122	100%	0.0	99%	JF907594.1
Galactomyces geotrichum gene for 18S ribosomal RNA, partial sequence	1122	1122	100%	0.0	99%	AB162430.1
Geotrichum candidum DNA for 18S ribosomal RNA, strain IFO 4599	1122	1122	100%	0.0	99%	AB000652.1
Geotrichum candidum strain TZ-6 18S ribosomal RNA gene, partial sequence	1116	1116	100%	0.0	99%	KU899093.1
Uncultured eukaryote partial 18S rRNA gene, clone 4A_29_M13-21F	1116	1116	100%	0.0	99%	LN897860.1
Geotrichum candidum strain GC 18S ribosomal RNA gene, partial sequence	1116	1116	100%	0.0	99%	KJ814246.1

Penicillium citrinum

BLAST ® » blastn suite » RID-YEXK7G4S015

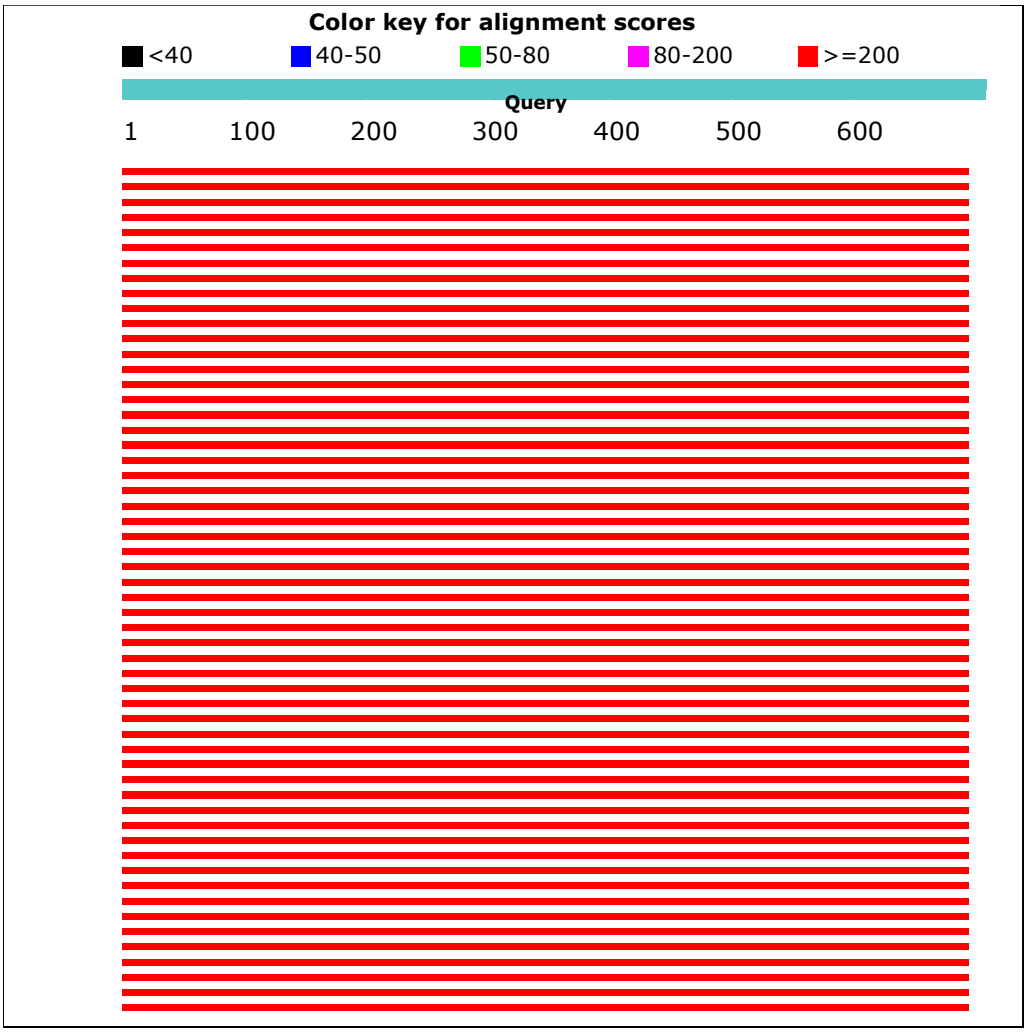
BLAST Results

Job title: Nucleotide Sequence (686 letters)

RID	YEXK7G4S015 (Expires on 10-19 23:42 pm)		
Query ID	lcl|Query_54475	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.7.0+
Query Length	686		

Graphic Summary

Distribution of the top 100 Blast Hits on 100 subject sequences

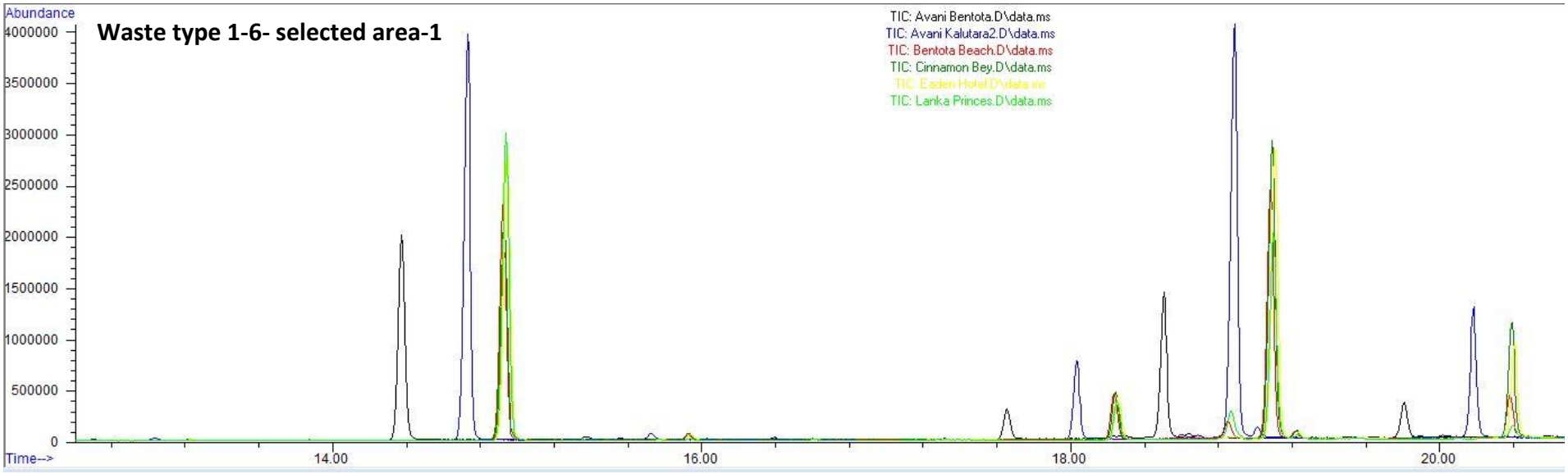


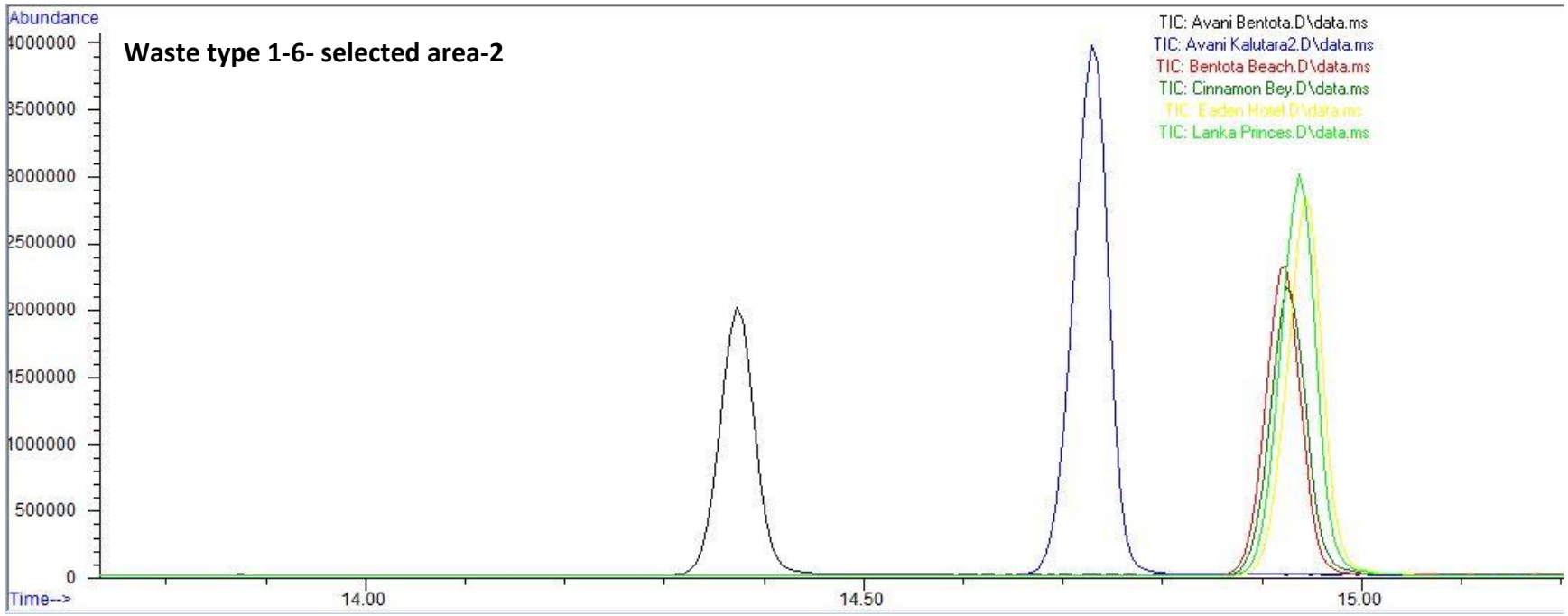
Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Penicillium citrinum genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: PSU3	1238	1238	100%	0.0	99%	LC127087.1
Penicillium sp. yz11-22N2 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KU182953.1
Penicillium sp. Y28 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KP872503.1
Penicillium citrinum strain MF410 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KM096252.1
Penicillium citrinum strain MF408 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KM096251.1
Penicillium citrinum strain MF399 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KM096250.1
Penicillium citrinum strain MF396 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KM096249.1
Penicillium citrinum strain TG2 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KC960012.1
Penicillium sp. WC-29-5 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KJ138167.1
Penicillium citrinum strain Salicorn 46 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	KF758801.1
Penicillium griseofulvum isolate Pen0 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	FJ717697.1
Penicillium sp. CPCC 480465 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	EU827607.1
Penicillium sp. CPCC 480032 18S ribosomal RNA gene, partial sequence	1238	1238	100%	0.0	99%	EU835655.1
Penicillium sp. MI 31 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence	1238	1238	100%	0.0	99%	AB245443.1
Penicillium decumbens 16S ribosomal RNA gene, partial sequence	1234	1234	100%	0.0	99%	KC842215.1
Penicillium sp. KF612 18S ribosomal RNA gene, partial sequence	1232	1232	100%	0.0	99%	KM096170.1
Penicillium decumbens genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA, ITS2, isolate MMH 89-p1	1230	1230	100%	0.0	99%	FR774046.1
Penicillium sp. strain NSY15 18S ribosomal RNA gene, partial sequence	1227	1227	100%	0.0	99%	KX906964.1
Penicillium cyaneum strain XMU01 18S ribosomal RNA gene, partial sequence	1227	1227	100%	0.0	99%	MF198446.1
Penicillium sp. strain R57 18S ribosomal RNA gene, partial sequence	1227	1227	100%	0.0	99%	MF040753.1
Penicillium sp. strain OUCMDZ-4736 small subunit ribosomal RNA gene, partial sequence	1227	1227	100%	0.0	99%	KY848519.1
Penicillium sp. strain Z3 18S ribosomal RNA gene, partial sequence	1227	1227	100%	0.0	99%	KY368614.1
Uncultured fungus clone TF-1 18S ribosomal RNA gene, partial sequence	1227	1227	100%	0.0	99%	KU359431.1

Annex V: Details of chromatogram analysis


Waste type 1-6- selected area-1
Abundance
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Time-->
14.00
16.00
18.00
20.00
TIC: Avani Bentota.D\data.ms
TIC: Avani Kalutara2.D\data.ms
TIC: Bentota Beach.D\data.ms
TIC: Cinnamon Bey.D\data.ms
TIC: Eaden Hotel.D\data.ms
TIC: Lanka Princes.D\data.ms


Abundance
Waste type 1-6- selected area-2
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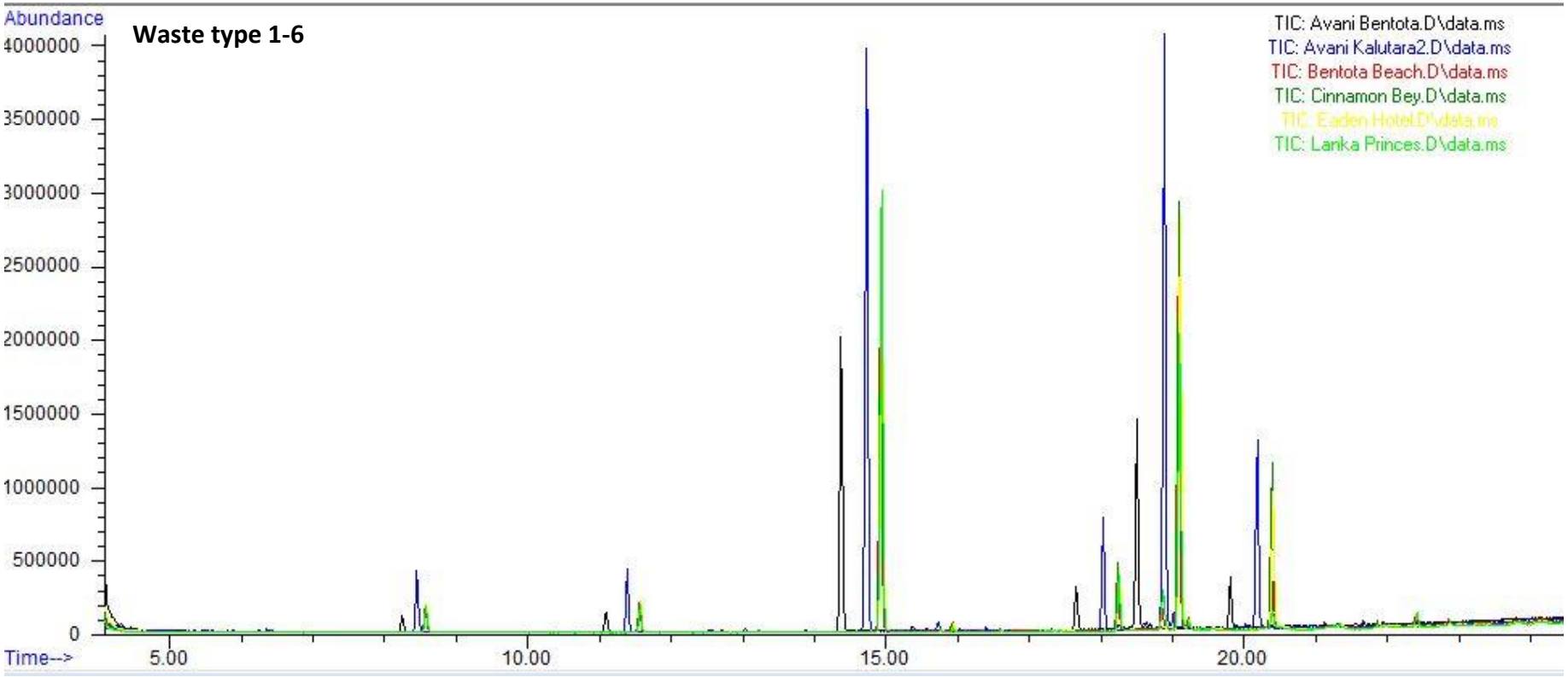
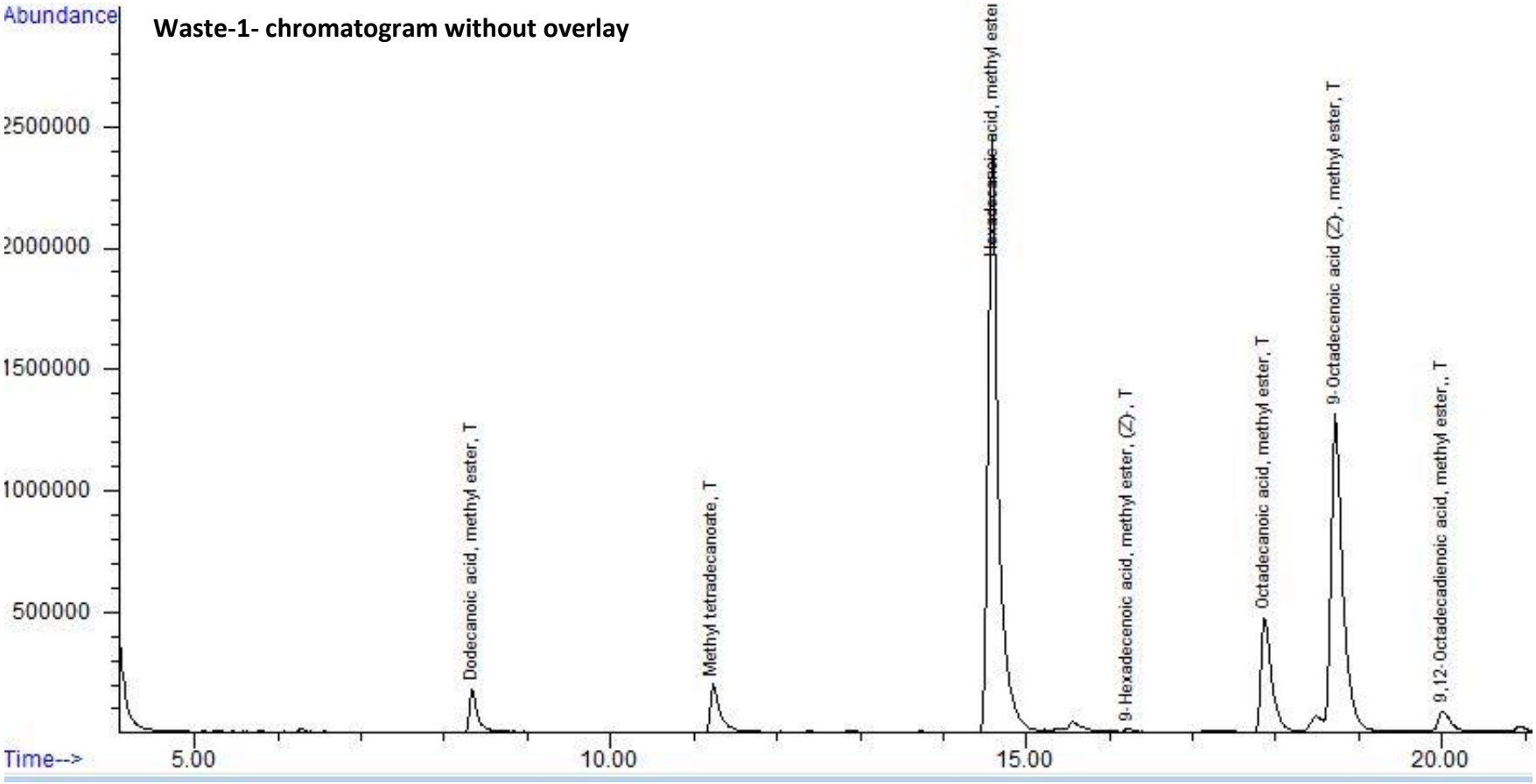
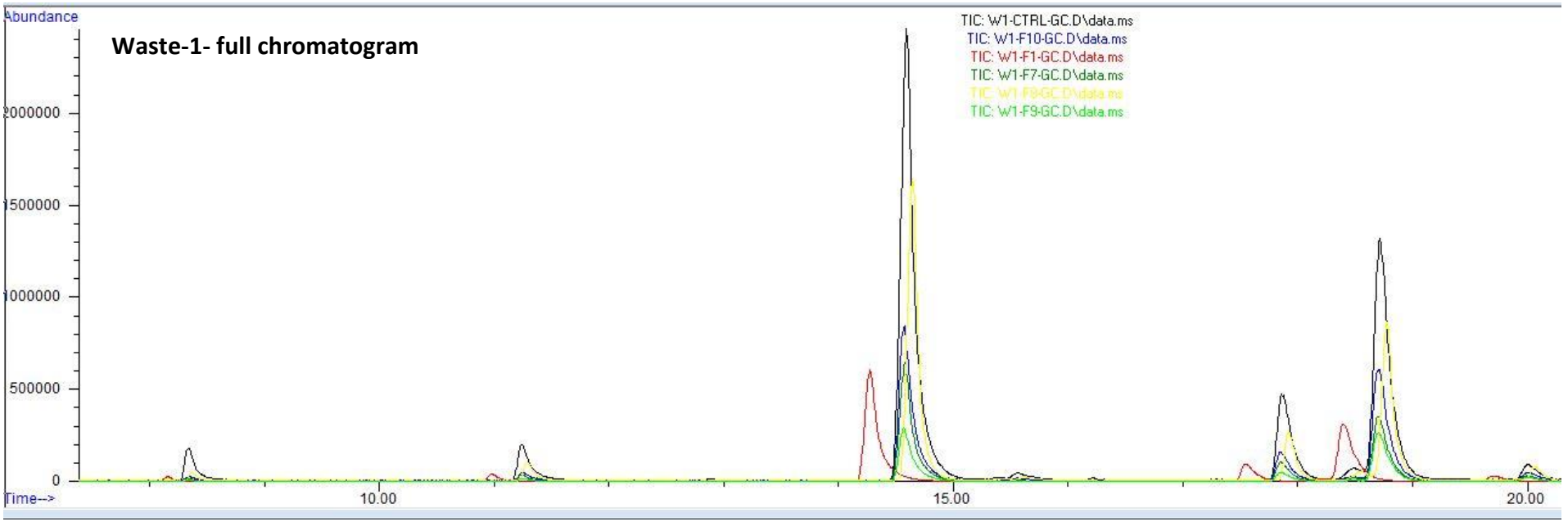
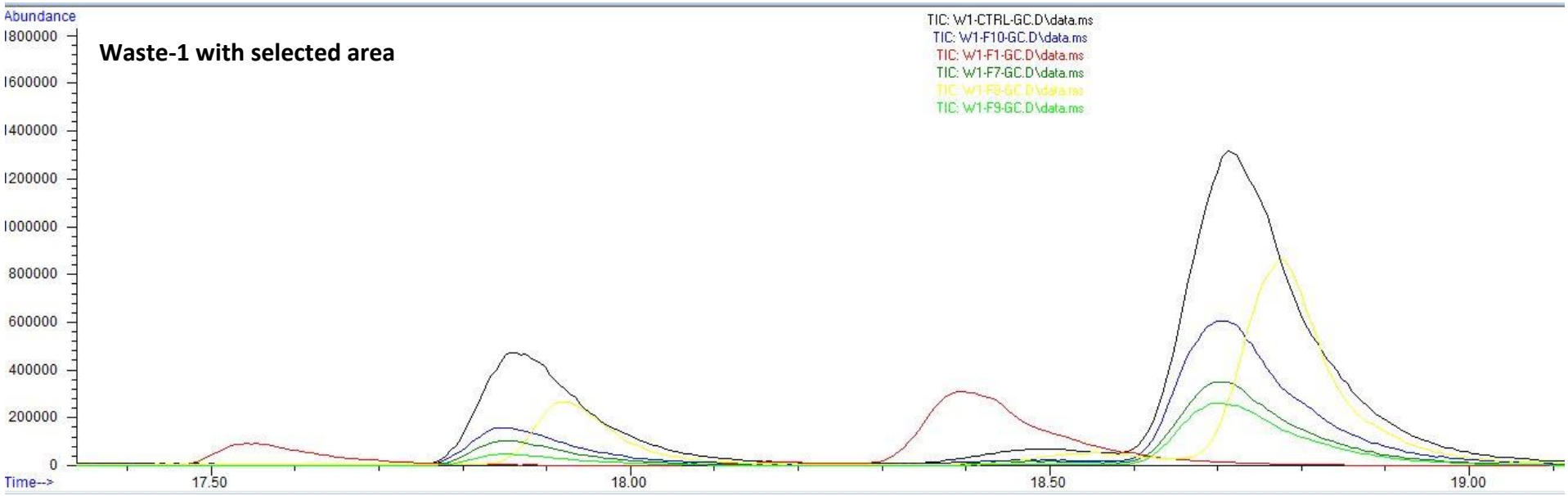
TIC: Avani Kalutara2.D\data.ms
TIC: Bentota Beach.D\data.ms
TIC: Cinnamon Bey.D\data.ms
TIC: Lanka Princes.D\data.ms
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Time-->
14.00
14.50
15.00


Abundance
Waste type 1-6
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Time-->
5.00
10.00
15.00
20.00
TIC: Avani Bentota.D\data.ms
TIC: Avani Kalutara2.D\data.ms
TIC: Bentota Beach.D\data.ms
TIC: Cinnamon Bey.D\data.ms
TIC: Eaden Hotel.D\data.ms
TIC: Lanka Princes.D\data.ms

Waste-1- chromatogram without overlay

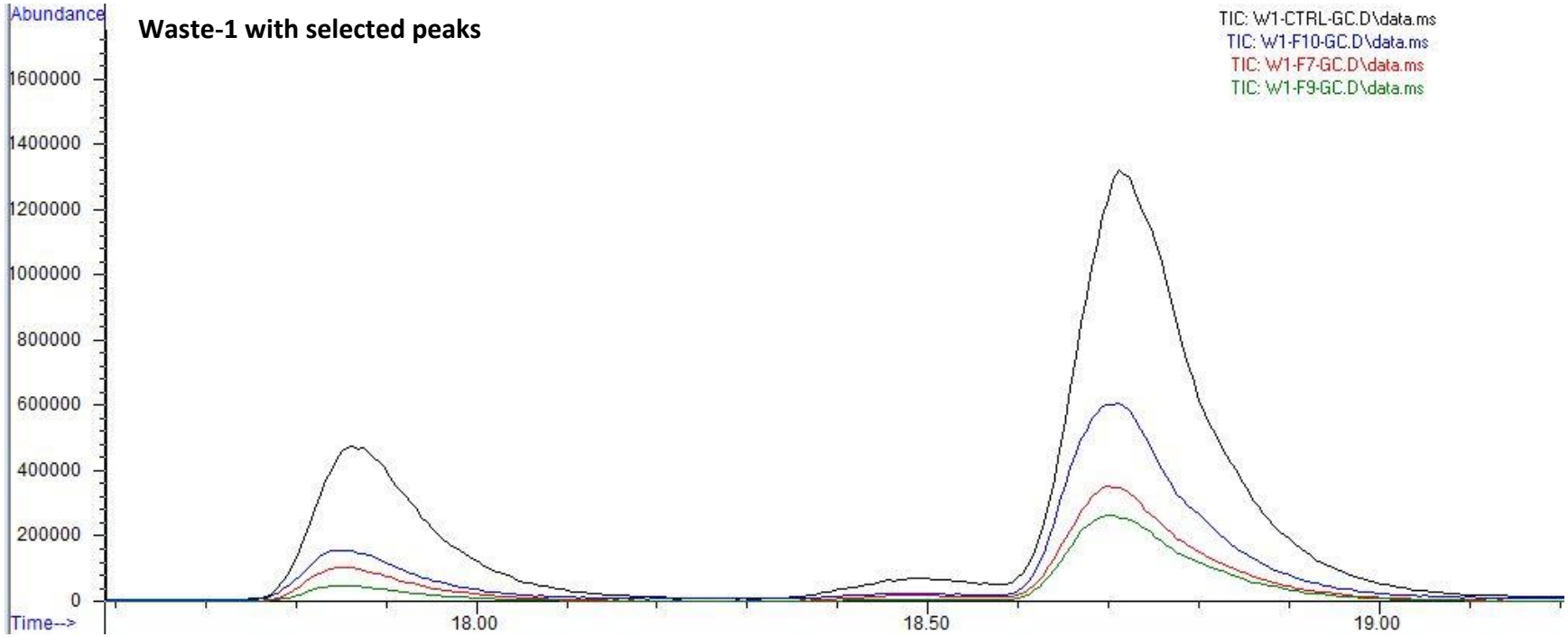



Abundance
Waste-1- full chromatogram
TIC: W1-CTRL-GC.D\data.ms
TIC: W1-F10-GC.D\data.ms
TIC: W1-F1-GC.D\data.ms
TIC: W1-F7-GC.D\data.ms
TIC: W1-F8-GC.D\data.ms
TIC: W1-F9-GC.D\data.ms
2000000
1500000
1000000
500000
0
Time-->
10.00
15.00
20.00

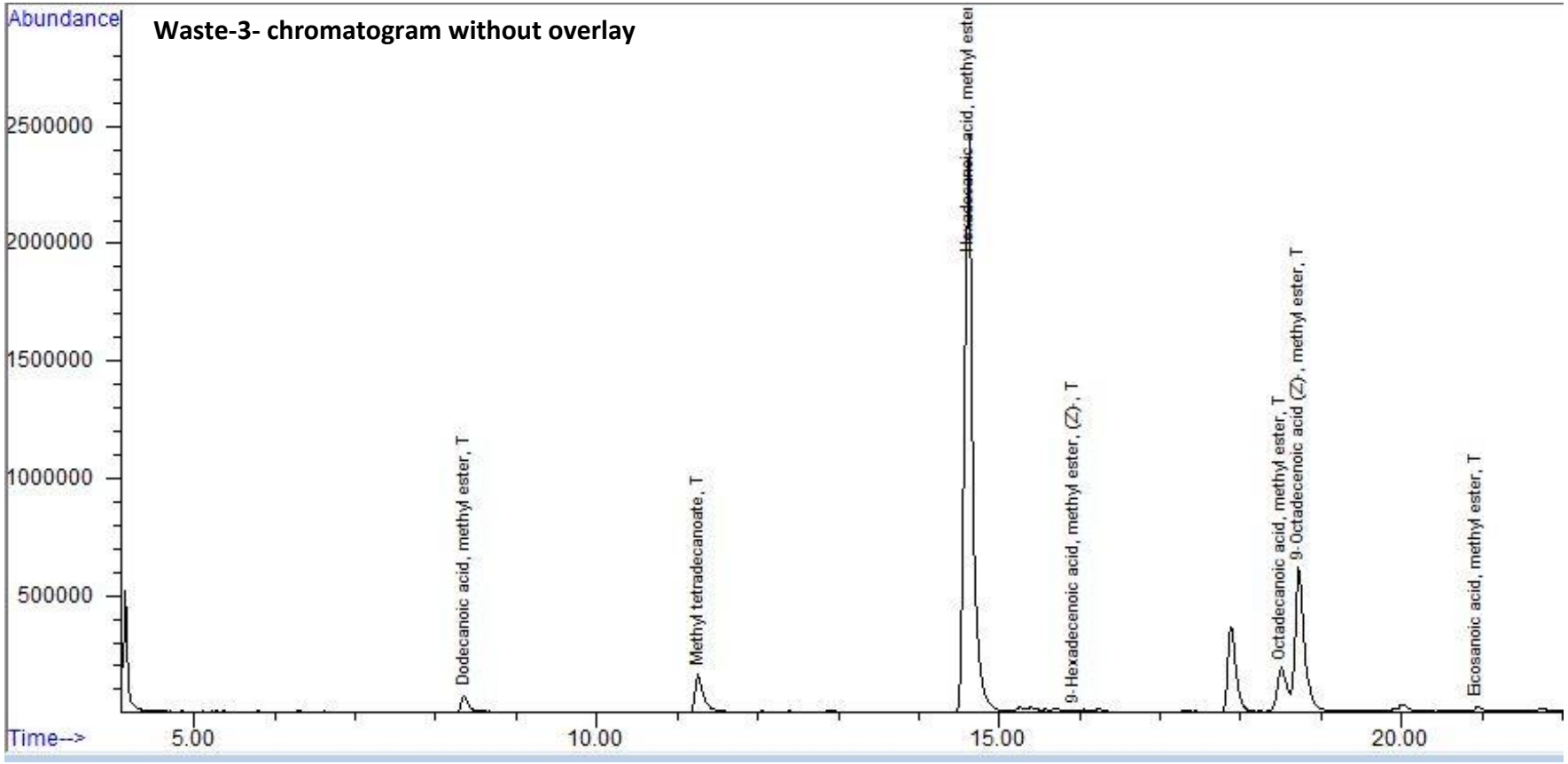


Waste-1 with selected area

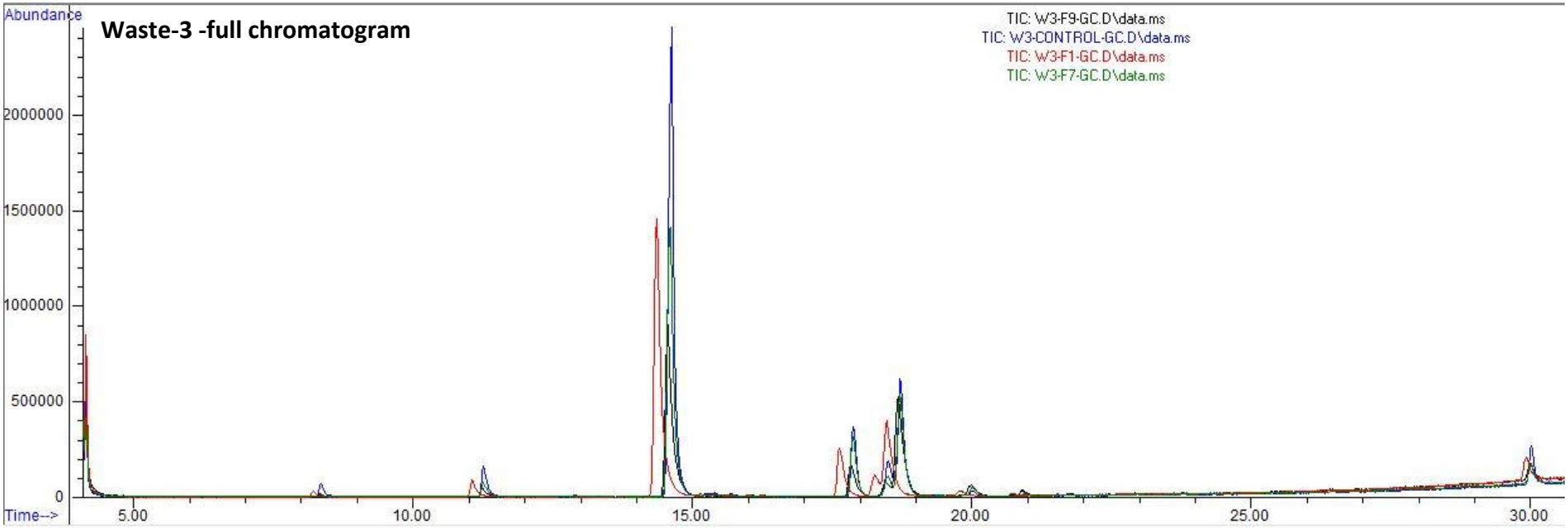
Waste-1 with selected peaks

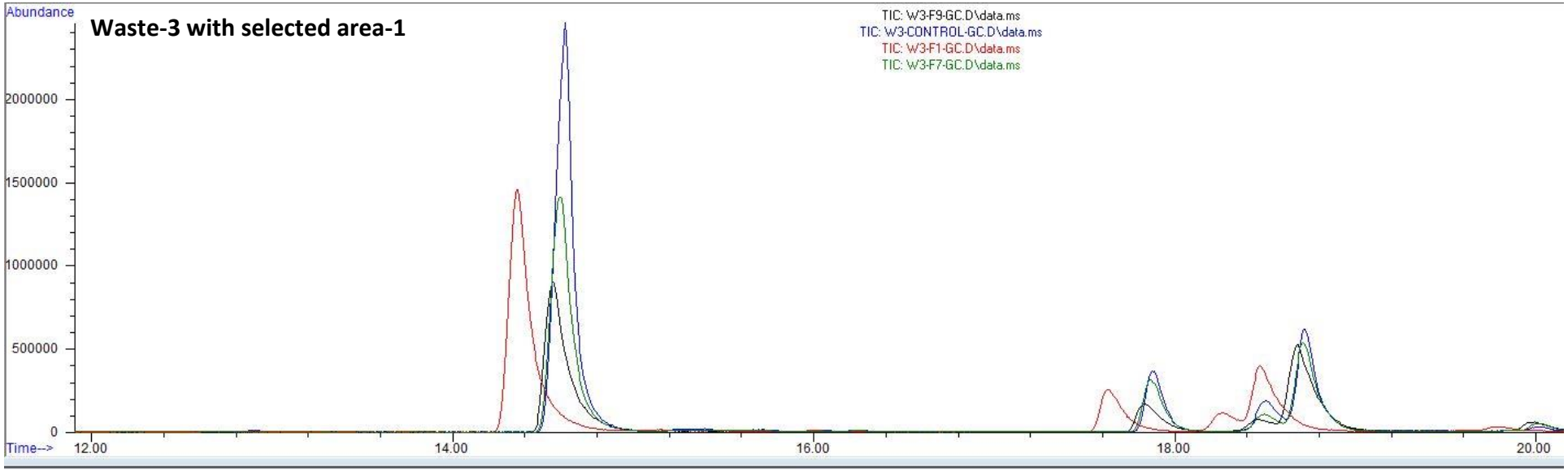


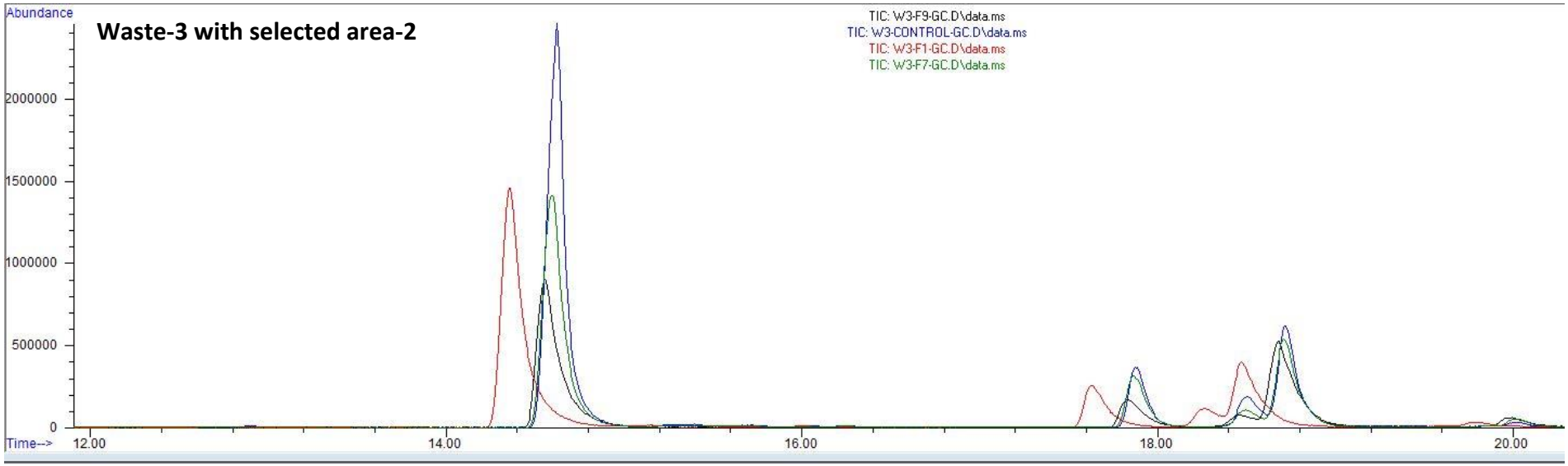
Waste-3- chromatogram without overlay



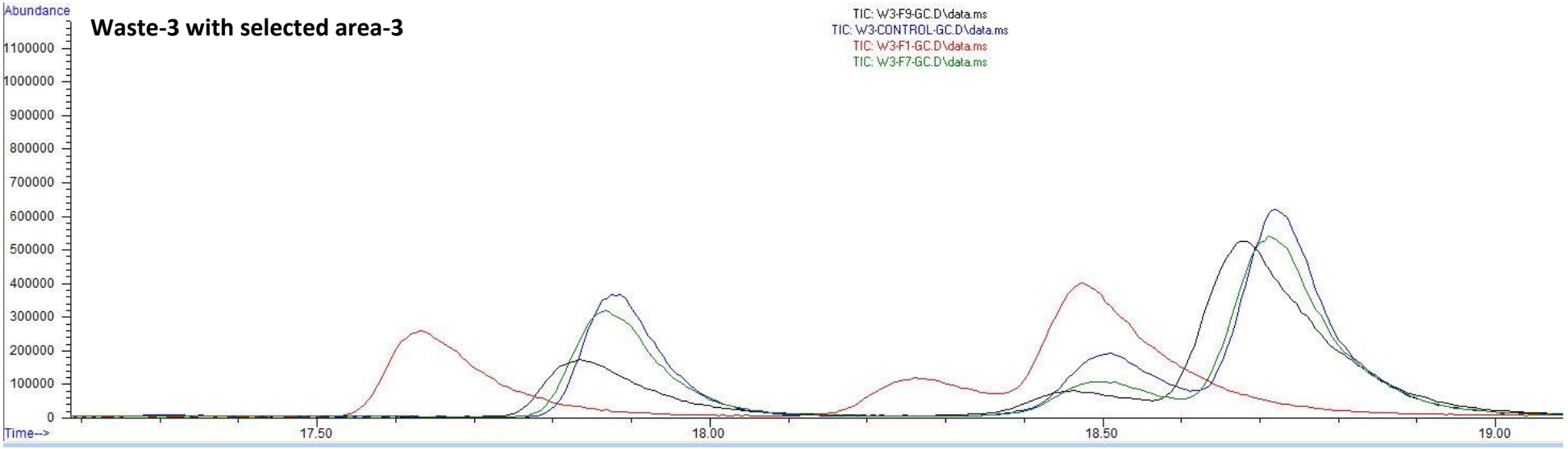
Waste-3 -full chromatogram

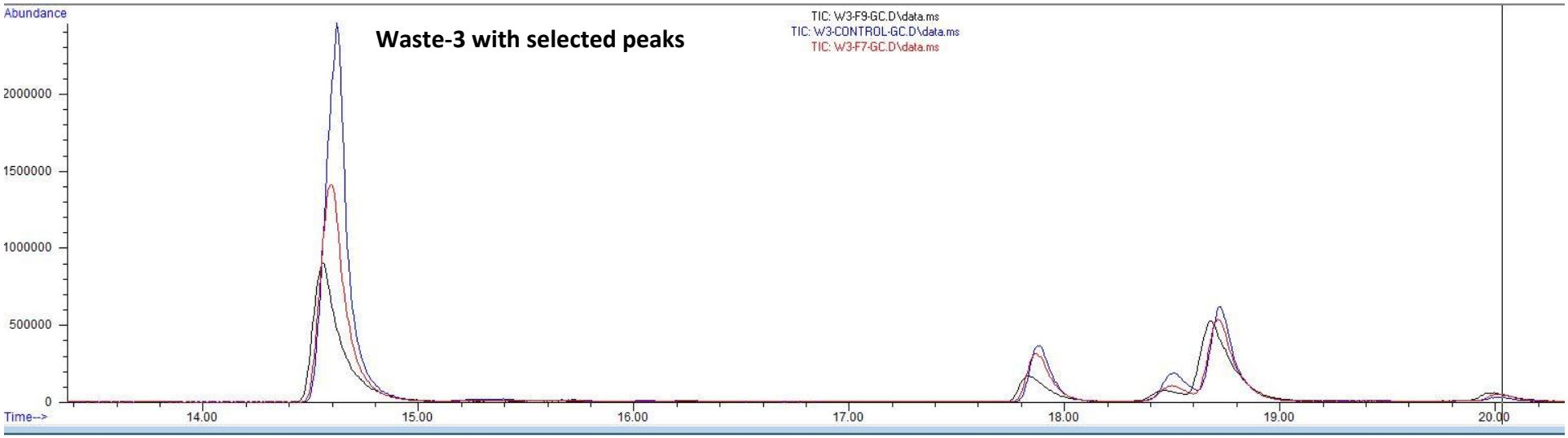



Abundance
Waste-3 with selected area-1
TIC: W3-F9-GC.D\data.ms
TIC: W3-CONTROL-GC.D\data.ms
TIC: W3-F1-GC.D\data.ms
TIC: W3-F7-GC.D\data.ms
2000000
1500000
1000000
500000
0
Time-->
12.00
14.00
16.00
18.00
20.00

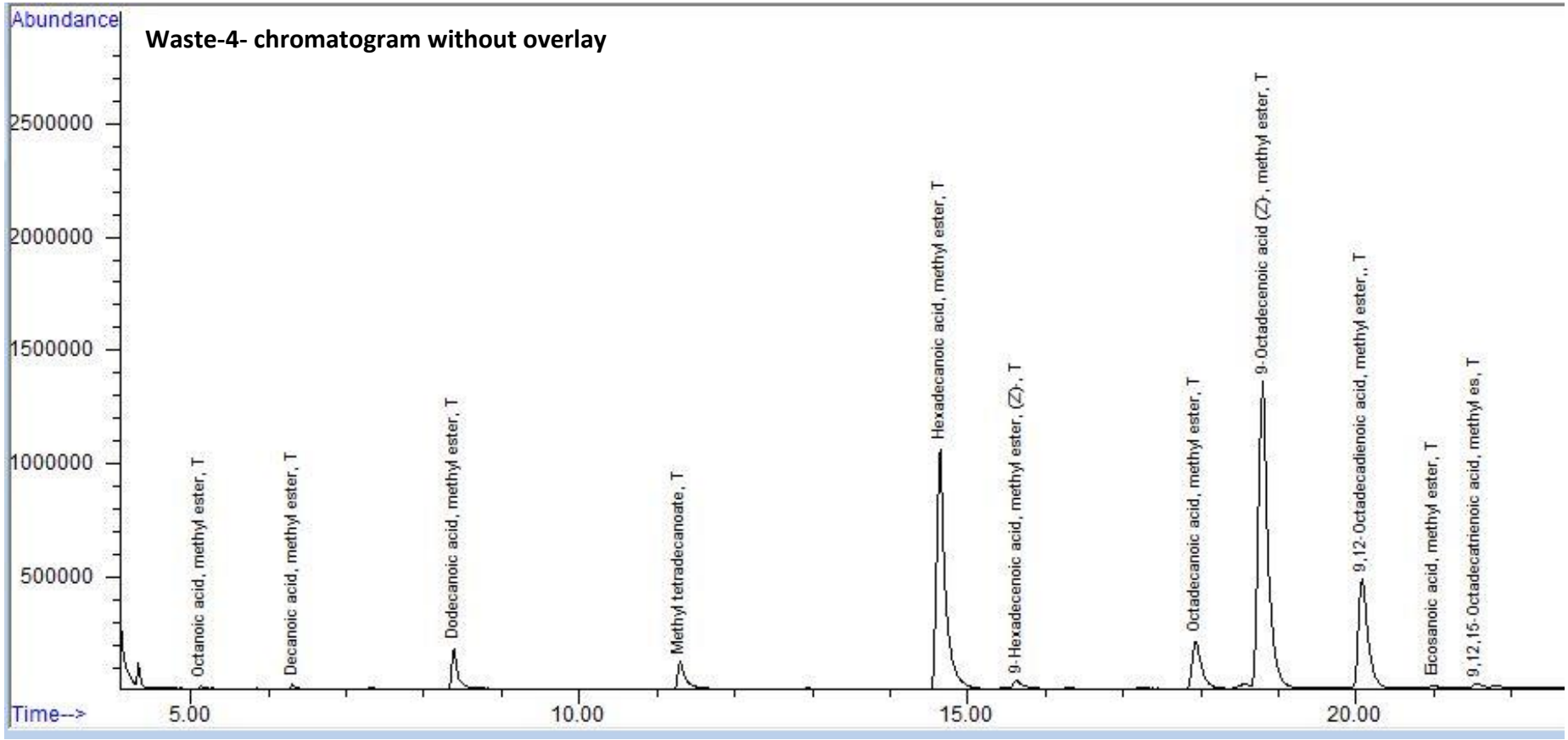

Waste-3 with selected area-2
Abundance
TIC: W3-F9-GC.D\data.ms
TIC: W3-CONTROL-GC.D\data.ms
TIC: W3-F1-GC.D\data.ms
TIC: W3-F7-GC.D\data.ms
2000000
1500000
1000000
500000
0
Time-->
12.00
14.00
16.00
18.00
20.00

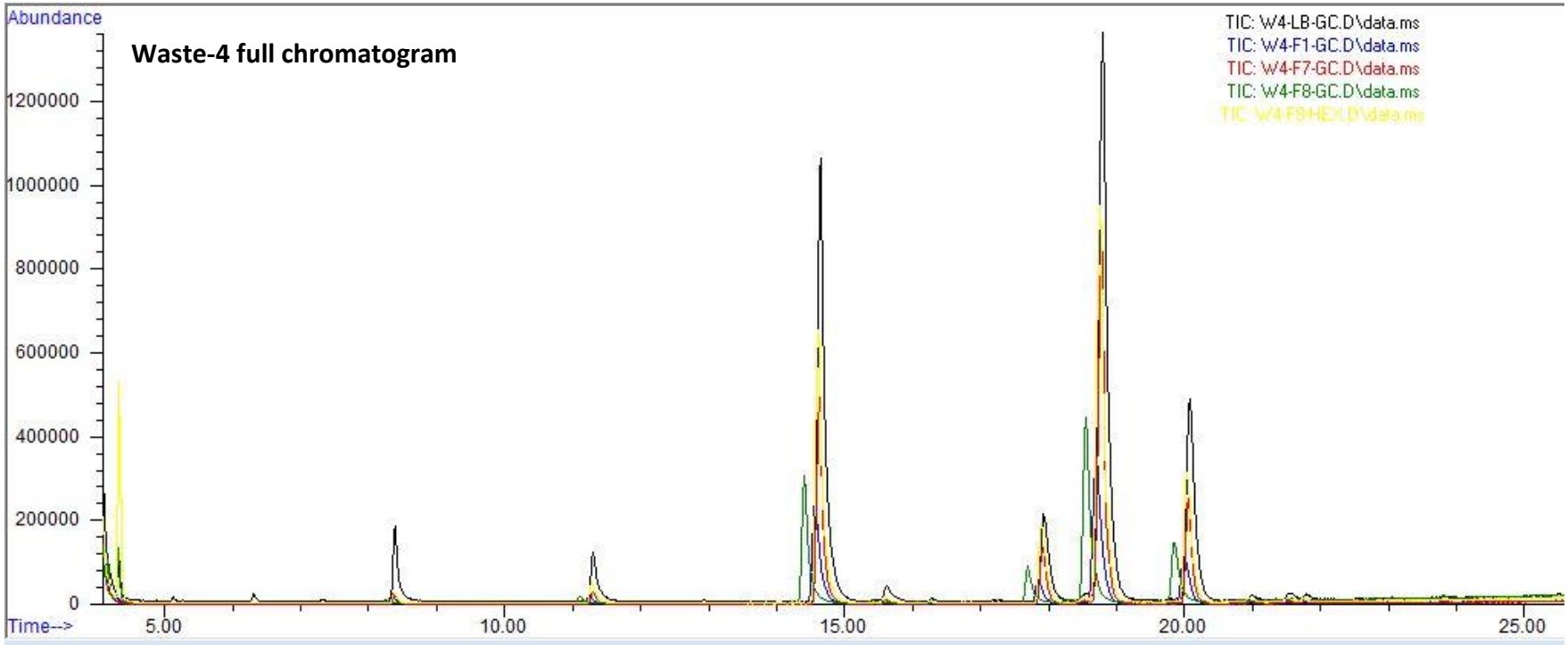
Waste-3 with selected area-3




Abundance
Waste-3 with selected peaks
TIC: W3-F9-GC.D\data.ms
TIC: W3-CONTROL-GC.D\data.ms
TIC: W3-F7-GC.D\data.ms
2000000
1500000
1000000
500000
0
Time-->
14.00
15.00
16.00
17.00
18.00
19.00
20.00

Waste-4- chromatogram without overlay




Abundance
Waste-4 full chromatogram
TIC: W4-LB-GC.D\data.ms
TIC: W4-F1-GC.D\data.ms
TIC: W4-F7-GC.D\data.ms
TIC: W4-F8-GC.D\data.ms
TIC: W4-F9HEX.D\data.ms
1200000
1000000
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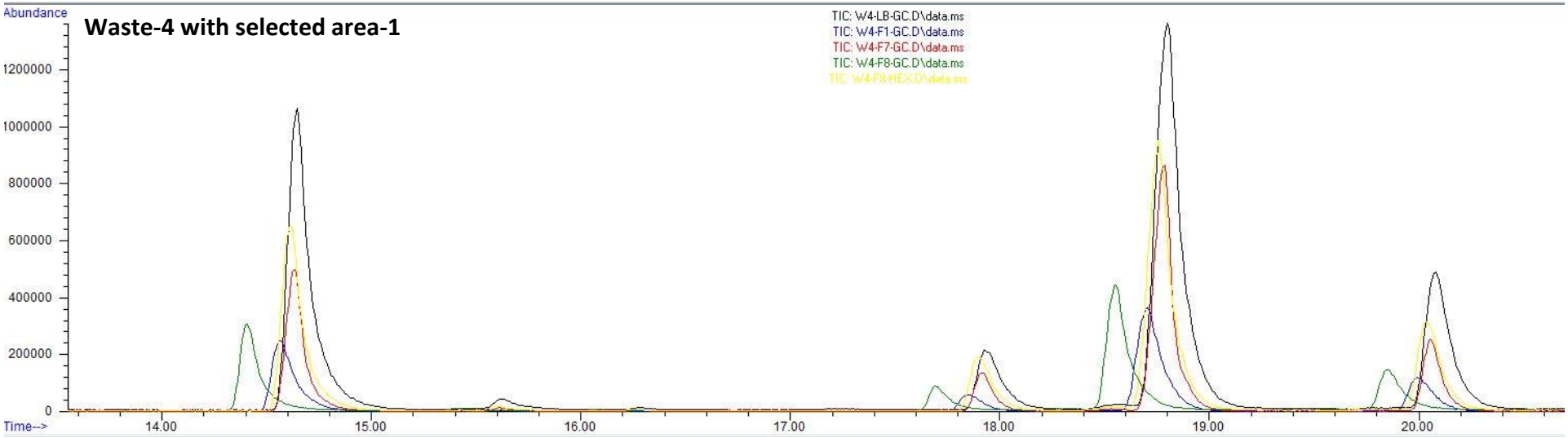
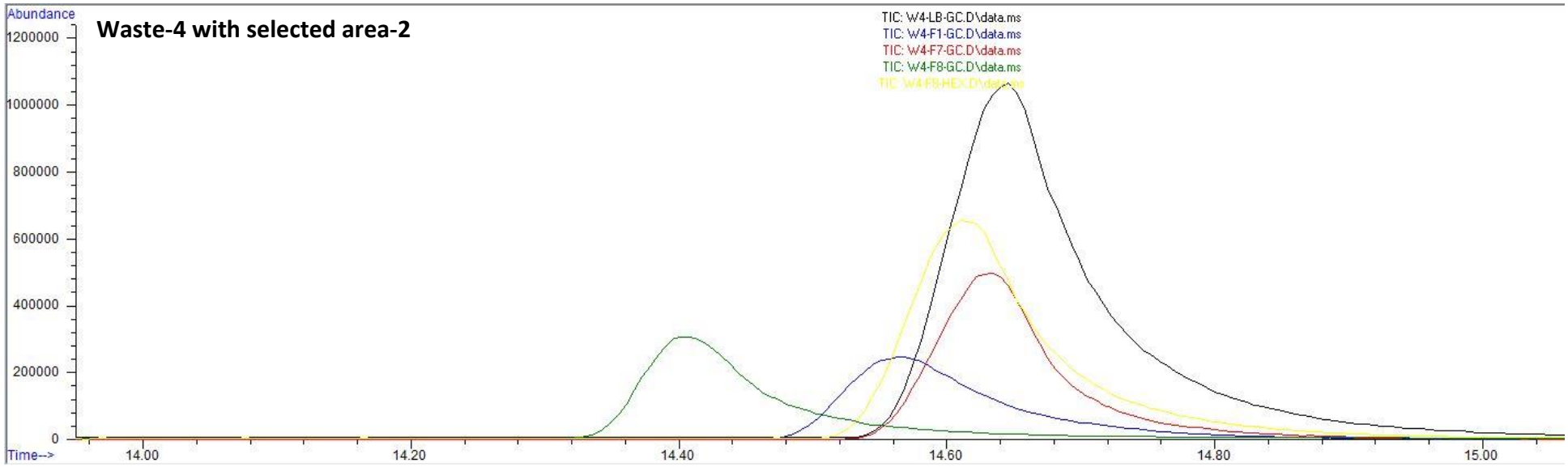
0
Time-->
5.00
10.00
15.00
20.00
25.00


Waste-4 with selected area-1
Abundance
TIC: W4-LB-GC.D\data.ms
TIC: W4-F1-GC.D\data.ms
TIC: W4-F7-GC.D\data.ms
TIC: W4-F8-GC.D\data.ms
1200000
1000000
800000
600000
400000
200000
0
Time-->
14.00
15.00
16.00
17.00
18.00
19.00
20.00


Waste-4 with selected area-2
Abundance
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1000000
800000
600000
400000
200000
0
Time-->
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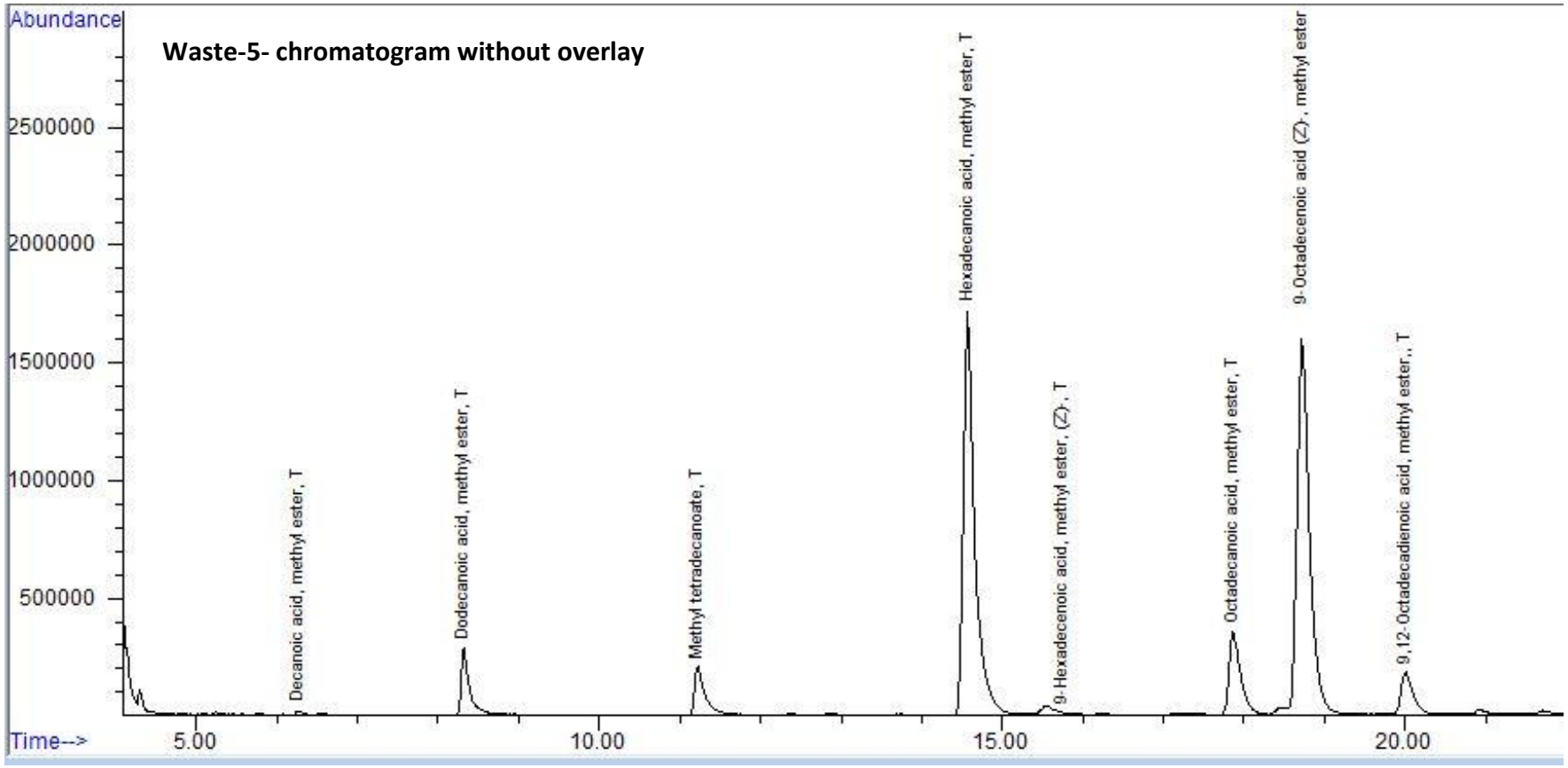
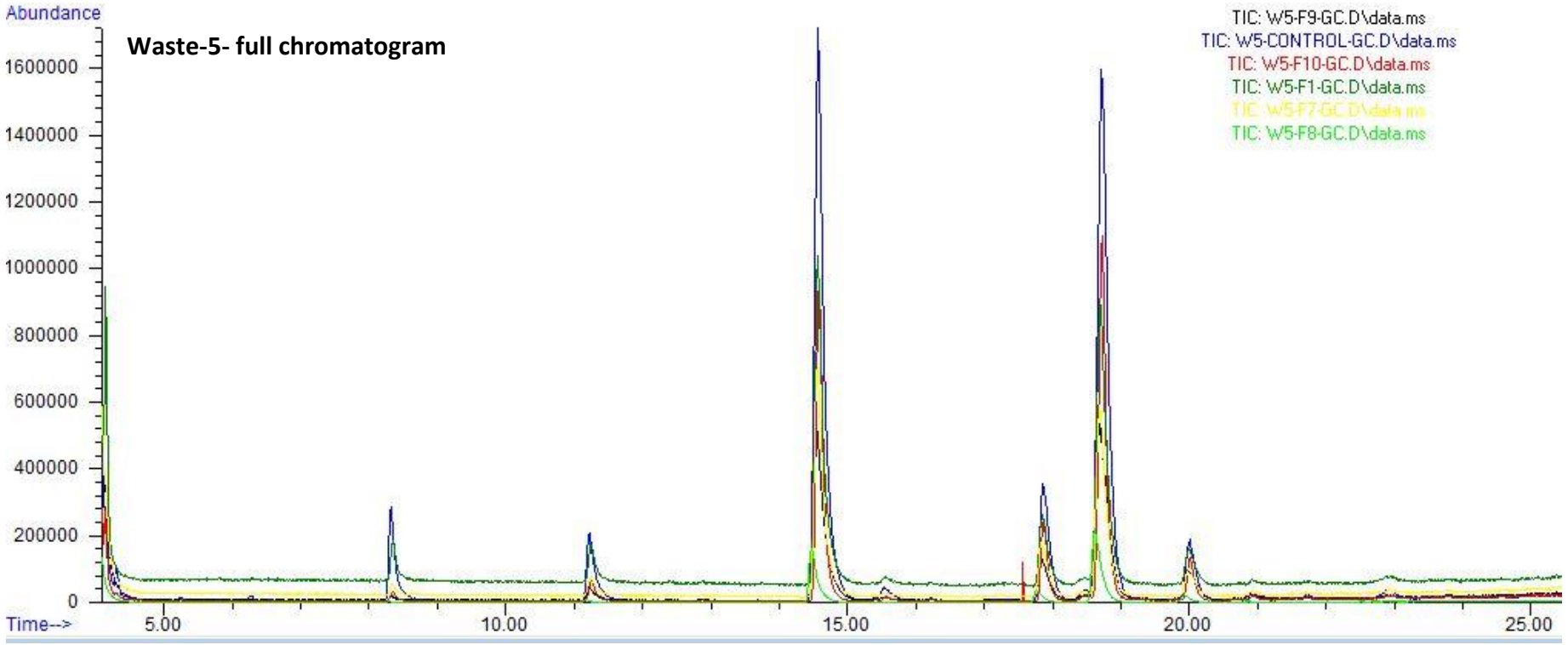
14.20
14.40
14.60
14.80
15.00
TIC: W4-LB-GC.D\data.ms
TIC: W4-F1-GC.D\data.ms
TIC: W4-F7-GC.D\data.ms
TIC: W4-F8-GC.D\data.ms

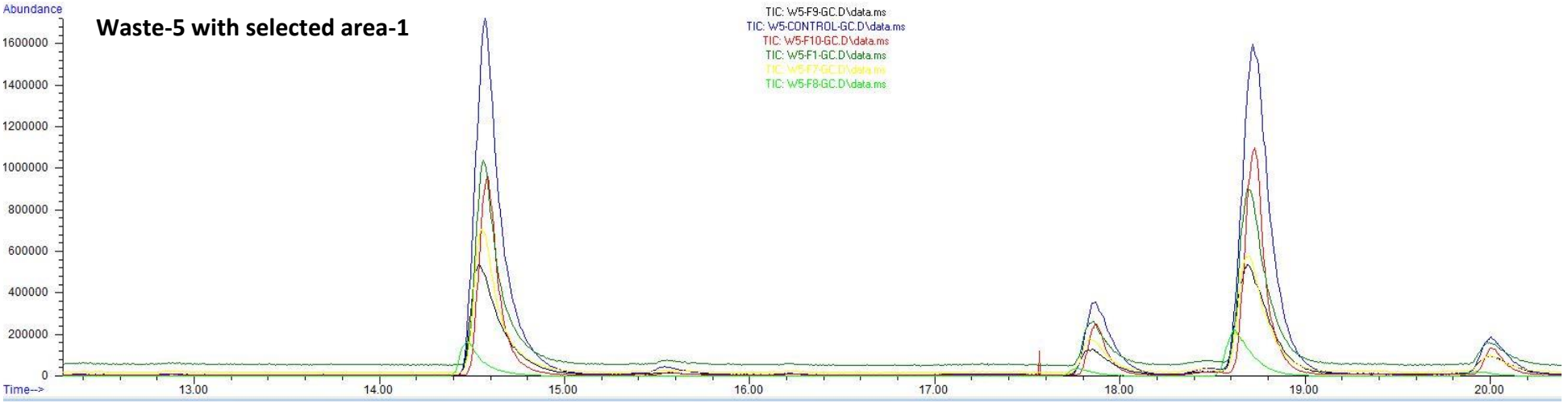
Waste-5- chromatogram without overlay



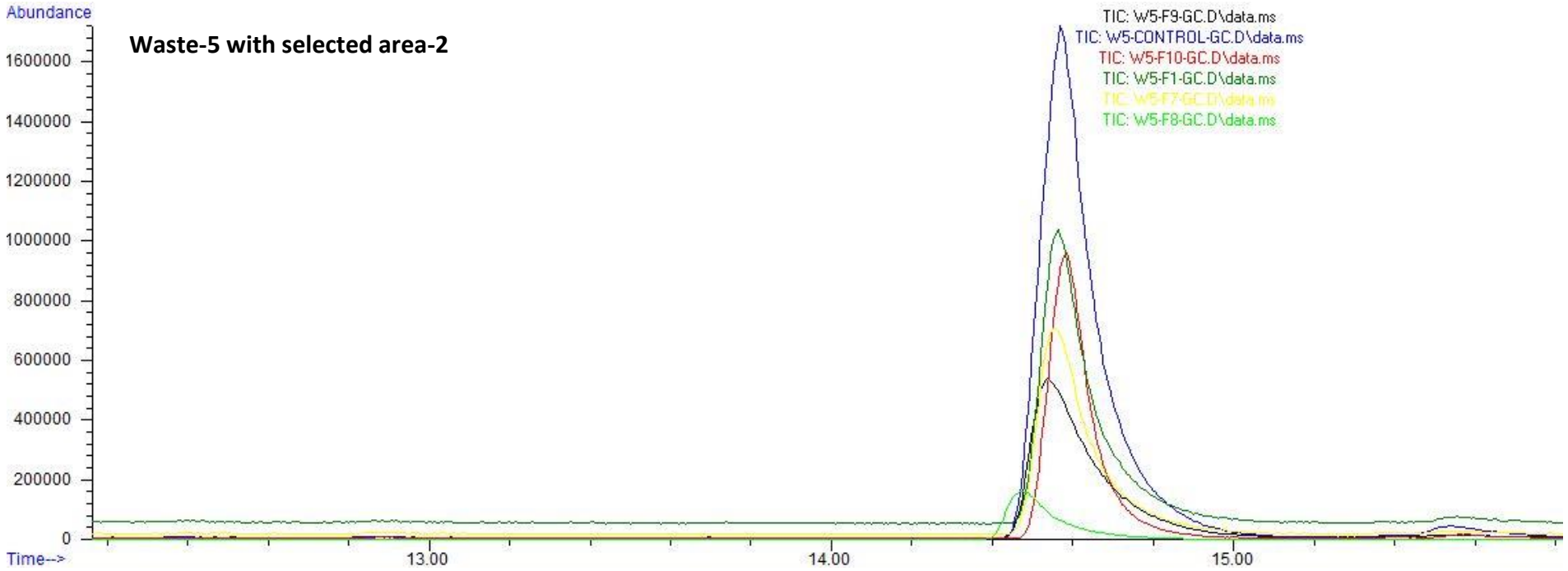
Waste-5- full chromatogram



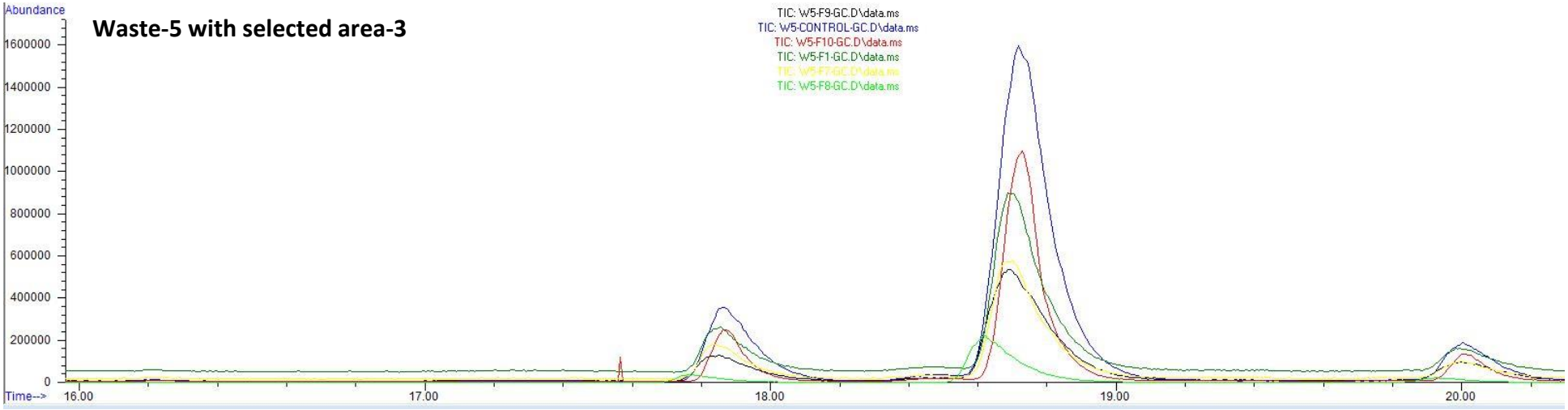
Waste-5 with selected area-1



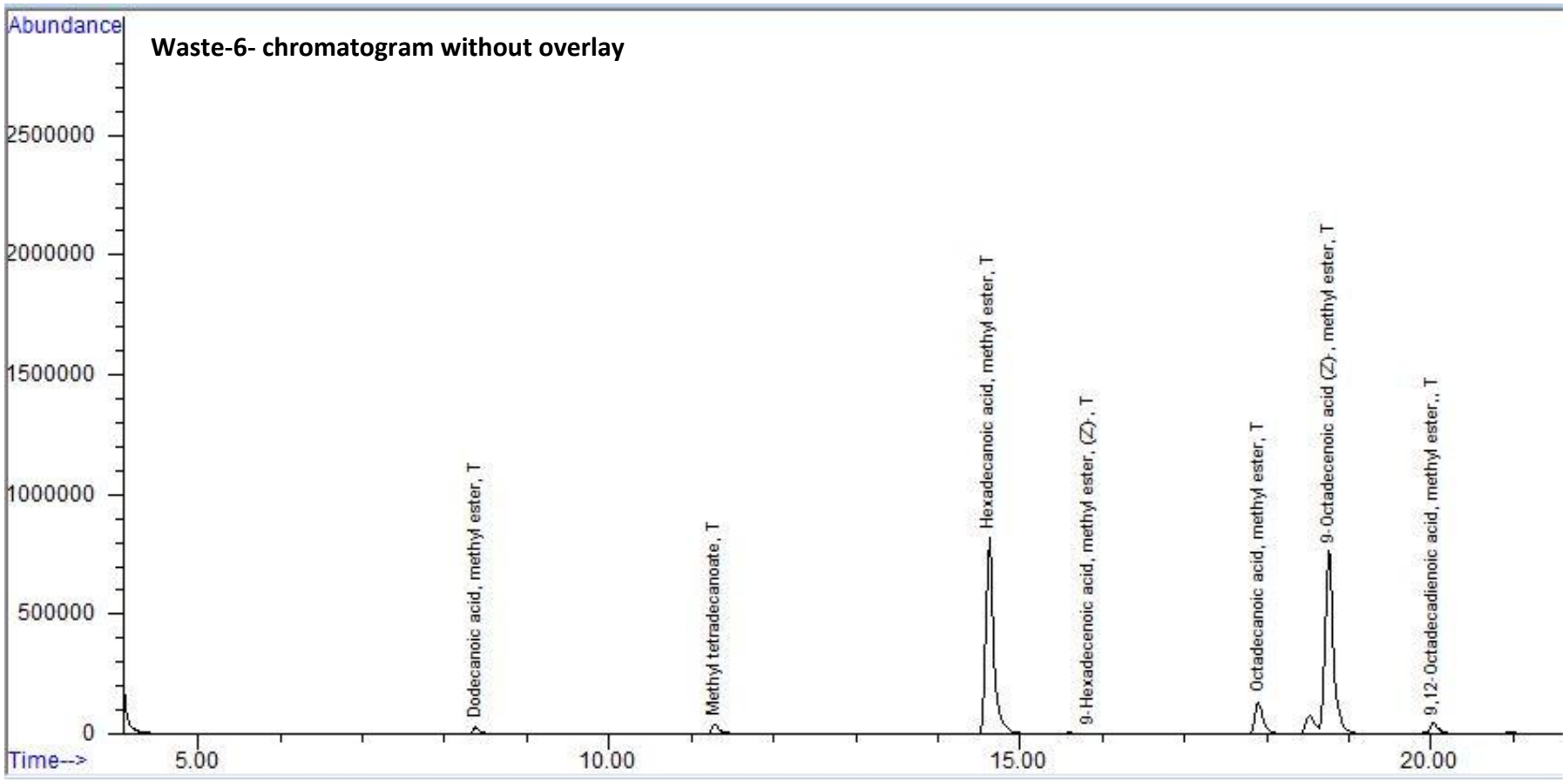
Waste-5 with selected area-2

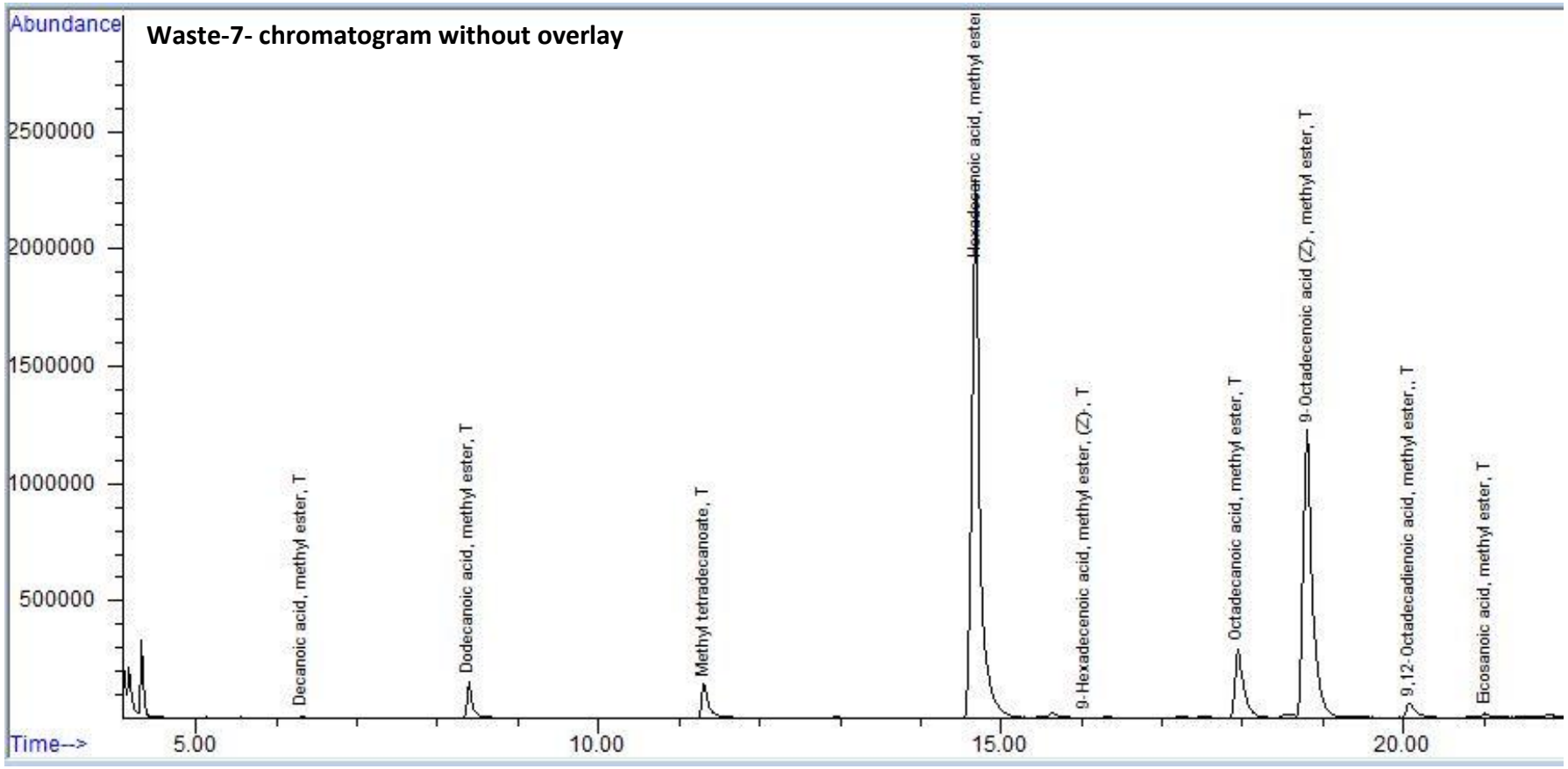


Waste-5 with selected area-3



Waste-6- chromatogram without overlay




Waste-7- chromatogram without overlay
Abundance
2500000
2000000
1500000
1000000
500000
Time-->
5.00
10.00
15.00
20.00
Decanoic acid, methyl ester, T
Dodecanoic acid, methyl ester, T
Methyl tetradecanoate, T
Hexadecanoic acid, methyl ester
9-Hexadecenoic acid, methyl ester, (Z)-, T
Octadecanoic acid, methyl ester, T
9-Octadecenoic acid (Z)-, methyl ester, T
9,12-Octadecadienoic acid, methyl ester,, T
Eicosanoic acid, methyl ester, T