



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



**EVALUATION OF SPENT COFFEE
GROUNDS AS FEEDSTOCK FOR ENZYME
PRODUCTION**

ELİF NUR AVCI

MASTER THESIS

Department of Bioengineering

Thesis Supervisor

Prof. Dr. Dilek KAZAN

Thesis Co-Supervisor

Dr. Orkun PİNAR

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ÖZET

Tez Başlığı: KULLANILMIŞ KAHVE TELVELERİNİN ENZİM ÜRETİMİNDE HAMMADDE OLARAK DEĞERLENDİRİLMESİ

Biyoproseslerin en önemli maliyetini, kullanılan karbon kaynakları oluşturmaktadır. Bu tez kapsamında, operasyonel maliyeti düşürmek ve sürdürülebilir bir biyoproses geliştirmek amacı ile kahve telvesi atıklarının mikrobiyal lipaz enzimi üretiminde karbon kaynağı olarak kullanımı hedeflenmiştir. Bu kapsamda altında, kahve telvesi atığının toplandıktan sonra yaklaşık olarak %60'ının sudan oluştuğu, kurutulmuş 1 gram kahve telvesi atığından heksan:izopropanol (1:1) çözeltisi kullanılarak 0,2 mL kahve yağı; %3 H₂SO₄ (v/v) çözeltisi kullanılarak ise %39,11±1,8 (w/w) indirgen şeker ve % 9,95 ± 0,3 gallik asit eşdeğeri elde edildiği tespit edilmiştir. Kahve telvesi atığının içeriği belirlendikten sonra, daha önce lipaz üreticisi bir maya olarak bilinen *C. diffluens* D44 ile kahve telvesi atıklarından lipaz üretimi koşulları Design Expert programında Box-Behnken tasarımı kullanılarak optimize edilmiştir. Yapılan çalışmalar sonucunda, en yüksek lipaz enzim aktivitesi, 29,4 °C'de, pH'ı 8,3 olan ve %8,7 (w/v) kullanılmış kahve telvesi içeren bir ortam ile 14,6 U/mL olarak bulunmuştur. Üretilen D44 lipaz enziminin optimum sıcaklığı ve pH'ı ise sırasıyla 37 °C ve 8,0 olarak belirlendi. Buna ek olarak, üretim sonucunda elde edilen enzim içeren üst faz sıvısına GC-FID analizi yapıldı ve ortamdaki yağ asitlerinin bileşimi %31,05 linoleik asit, %28,67 palmitik asit, %20,44 heptadekanoik asit ve %19,84 oleik asit olarak belirlendi. Ayrıca, kahve telvesi atıklarının bakteriler tarafından kullanımını incelemek için İstanbul'un Kadıköy bölgesindeki toprak örneğinden *B. subtilis* BT2 izole edilerek, %1 (w/v) kahve telvesi içeren besi ortamından lipaz üretimi gerçekleştirildi. Yapılan üretim sonucunda, üretilen bakteriyel lipaz aktivitesi 40.426 U/mL olarak bulundu. Üretim ortamına %0,1 Tween80 (v/v) eklenmesi ile üretilen lipaz aktivitesi yaklaşık %10 arttı ve lipaz aktivitesi 44.711 U/mL olarak belirlendi. Üretilen bakteriyel lipaz enziminin optimum sıcaklığı ve pH'ı ise sırasıyla 37 °C ve 8,0 olarak belirlendi. Buna ek olarak, kahve telvesi atığından ekstrakte edilen kahve yağı ve şekeri kullanılarak, BT2 lipazının katalizi ile şeker-yağ asidi esteri üretimi yapılarak, üretilen şeker-yağ asidi esterleri FTIR analizi ile belirlendi. Son olarak, maya ve bakteri tarafından lipaz enzimi üretiminde kullanılan kahve telvesi atığının tekrar kullanılabilirliğini araştırmak için kullanılan kahve telvesi atığı örneklerinin SEM görüntüsü çekildi ve yapısal değişimleri incelendi.

ABSTRACT

Thesis Title: EVALUATION OF SPENT COFFEE GROUNDS AS FEEDSTOCK FOR ENZYME PRODUCTION

The most significant cost of bioprocesses is the carbon sources used. In this thesis, it was aimed to use SCG as a carbon source in the production of microbial lipase enzyme in order to reduce operational costs and develop a sustainable bioprocess. Under this scope, it has been determined that approximately 60% of the SCG consists of water after collection. In addition, 0.2 mL of coffee oil was extracted from 1 gram of dried SCG by using hexane: isopropanol (1:1) solution and $39.11 \pm 1.8\%$ (w/w) reducing sugar and $9.95\% \pm 0.3$ GAE were obtained by using 3% H₂SO₄ (v/v) solution. After the content of the SCG was determined, the conditions for lipase production from SCG by *C. diffluens* D44, previously known as a lipase producing yeast, were optimized in the Design Expert program using the Box-Behnken design. As a result of the studies, the highest lipase enzyme activity was found to be 14.6 U/mL at 29.4 °C with a pH of 8.3 and a medium containing 8.7% (w/v) SCG. The optimum temperature and pH of the produced D44 lipase enzyme from SCG were determined as 37 °C and 8.0, respectively. Besides, GC-FID analysis was performed on the enzyme-containing upper phase liquid obtained as a result of production. The composition of fatty acids in the medium was determined as 31.05% linoleic acid, 28.67% palmitic acid, 20.44% heptadecanoic acid and 19.84% oleic acid. Furthermore, *B. subtilis* BT2 was isolated from the soil sample in Kadıköy, Istanbul to evaluate the use of SCG by bacteria and lipase was produced from a nutrient medium containing 1% (w/v) SCG. As a result of the production, the bacterial lipase activity produced was found to be 40.426 U/mL. Adding 0.1% Tween80 (v/v) to the production medium increased the bacterial lipase activity produced from SCG by approximately 10%, resulting in 44,711 U/mL. The optimum temperature and pH of the produced bacterial lipase enzyme were determined as 37 °C and 8.0, respectively. In addition, using coffee oil and sugar extracted from SCG, sugar-fatty acid ester production by BT2 lipase catalysis was determined by FTIR analysis. Finally, SEM images of the SCG samples used to investigate the reusability of SCG used in lipase enzyme production by yeast and bacteria were taken and their structural changes were examined.

SYMBOLS

%	:	Percentage
°C	:	Celsius
A	:	Absorbance
g	:	Grams
hr	:	Hour
L	:	Liter
M	:	Molar
mL	:	Milliliter
mM	:	Millimolar
mg	:	Milligram
min	:	Minute
nm	:	Nanometer
rpm	:	Revolution per minute
v	:	Volume
V	:	Volt
w	:	Weight
U	:	Unit
μg	:	Microgram
μL	:	Microliter
μmol	:	Micromole

ABBREVIATIONS

ANOVA		Analysis of variance
BSA	:	Bovine Serum Albumin
<i>B. subtilis</i>	:	<i>Bacillus subtilis</i>
<i>C. diffluens</i>	:	<i>Cryptococcus diffluens</i>
df		Total degrees of freedom
DNS	:	3,5-dinitrosalicylic acid
GAE		Gallic acid equivalence
MSW		Municipal solid waste
MW	:	Molecular weight
NB		Nutrient broth
OD	:	Optical density
p-NP	:	p-nitrophenol
p-NPP	:	p-nitrophenyl palmitate
RSM		Response Surface Methodology
SEM		Scanning Electron Microscopy
SCG	:	Spent coffee grounds

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1. INTRODUCTION

Rapid urbanisation and population growth in parallel with technological advancements and industrialisation are putting growing stress on the environment, both in our country and throughout the world (Goldstone, 2002). In this process, while the development of production and marketing activities necessitates a more intense use of natural resources, the waste generated as a result of the overuse of resources has grown to the point where these wastes endanger the environment and human health (Kaçtıoğlu and Şengül, 2010). Moreover, a high amount of solid waste has been generated due to this urbanization that many local governments are facing unprecedented hurdles in handling it, including issues with collection and disposal (Shekdar, 2009; Park and Lah, 2018). Additionally, because of changes in consumption patterns, increased consumerism, and the lack of waste treatment facilities, these problems have grown.

Although it is not stated in history books, the first invention of mankind is the generation of waste. Waste is defined as a material with negative economic value, which may be considered as useless or harmful (Çevre Kanunu, 1983). In fact, this definition has changed in the last decade and waste has been evaluated as an economically valuable material. Today, waste has been adopted in many societies both as raw material and as surface mining. It is also described as waste arising from public and industrial (untreated waste), commercial and institutional based, excluding general purpose and hazardous waste, constructive and destruction waste, and liquid waste such as water, wastewater, and industrial processes (Gautham et al., 2010; Ahmed et al., 2021).

Waste can be classified as solid, liquid, and gaseous waste, in general, based on various factors such as consumption, production, chemical, and physical properties (**Figure 1.1**) (Marshall and Farahbakhsh, 2013). Among liquid and gaseous waste, many processes in the procurement of raw materials, production, and product consumption create solid waste. As specified by the United Nations Environment Program (UNEP), solid waste is a substance that the owner does not want, does not need, does not use, and must be treated and disposed of (Öztürk, 2010). In other words, solid waste refers to the solid materials that must be disposed of on a regular basis in terms of undesirable human and environmental health by the manufacturer (White et al., 1995). Regardless of its origin

(domestic, commercial, or industrial), waste can be expressed as the loss of usefulness after the use of raw materials, fuel and water, and thus losing its financial value for the person (Read, 1999). Large amounts are produced by agricultural and mining operations, as well as leftovers from wastewater treatment, and electrical power generation.

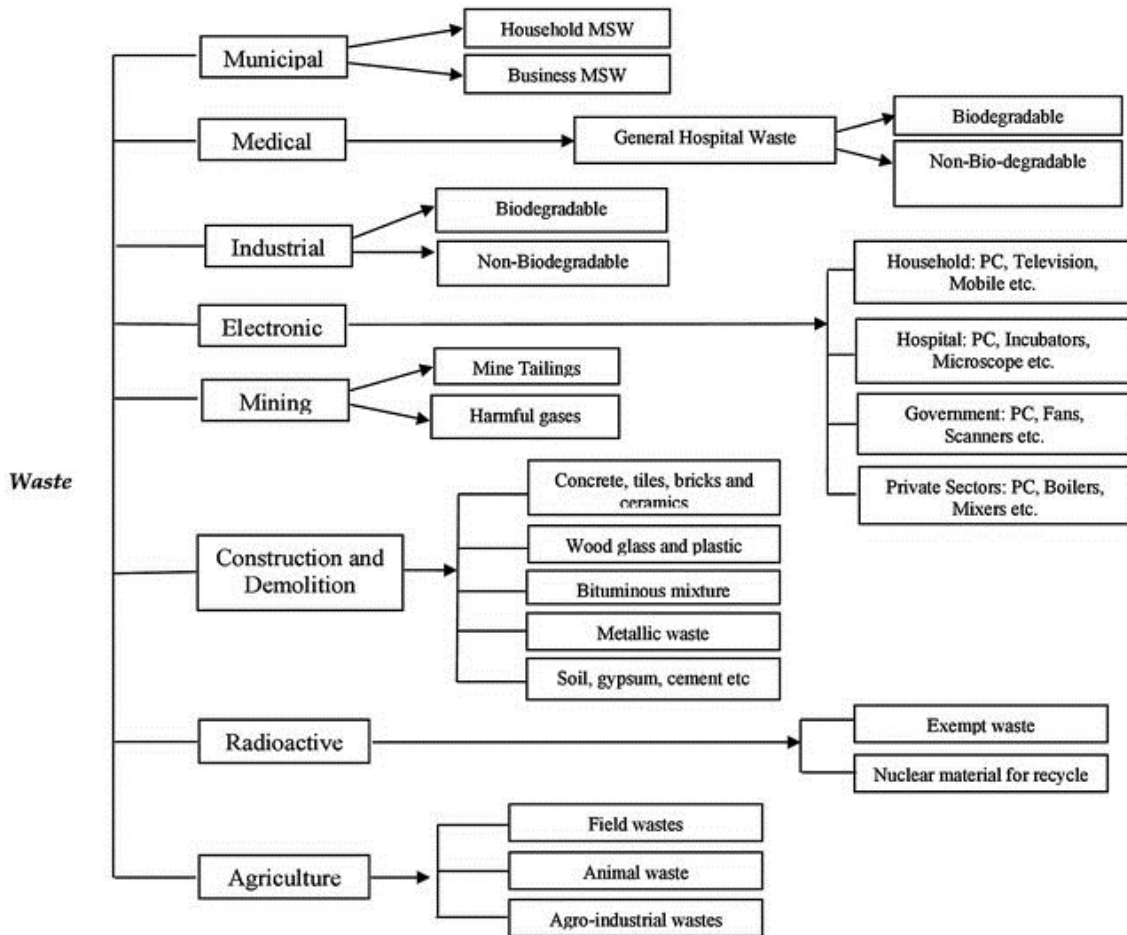


Figure 1.1. The classification of waste (Lone et al., 2020)

As it is well known, solid waste can adversely affect the environment and human health. Considering human health, the disease is caused by contagious substances in their content (Güler and Çobanoğlu, 1996; Tokgöz and Sarmaşık, 1982). While diseases such as leprosy, plague, cholera, dysentery, tuberculosis, rabies, and malaria, which can be transmitted directly or through intermediate animals, are examples of biological negativities, leachate and gases generated in landfills cause chemical and biological adverse effects, and waste left irresponsibly to the environment can cause physical harm to humans. The relationship between inadequate cleaning and waste management

practices, the environment, and human health problems is mostly observed in underdeveloped and/or developing countries (Palabıyık, 2001; Omang et al., 2021).

Solid waste management was defined by Tchobanoglous et al. (1993) as the governance of solid waste formation, piling up, mobilization and transport, processing, and effacing according to the best guidelines of community health, national economy, engineering, preservation, landscaping, and further environmental considerations, while also being responsive to public attitudes.

As a management problem, although 10-40% of local government budgets were allocated to solid waste management services, which are an important part of urban environmental management, especially in underdeveloped and/or developing countries, services cannot be provided at the desired level (Bartone, 1991).

Addressing solid waste, the management problem related to collection, transportation, and removal services together with rapid urbanization and industrialization must be developed, and the responsible actors, institutions, and organizations must be strengthened depending on the application of new managerial approaches and models. (Lissah et al., 2021) There are three sorts of waste operations: storage, collection and transportation, and lastly treatment and disposal. For storage, tanks and containers are extremely handy for storing waste once it has been created and, the containers may be simple or lined with various metals depending on the sort of waste. This may be determined by the compatibility of the waste materials. For the collection and delivery of materials to the endpoint, the process differs depending on the kind of waste, since some must be assorted before treatment or adequately packaged before transporting. Depending on the requirements, treatment and disposal may take place on-site or off-site based on the degree of treatment and availability, disposal methodologies, and cost efficiencies. Since biodegradable and nonbiodegradable compounds will be treated in a different manner, biochemical approaches are associated with anaerobic breakdown, while thermal processes include pyrolysis, incineration, or gasification. (Mousavi et al., 2022) Treatments of waste for energy production now provide the choice of numerous advantages, including economic stability and proper disposal (JeyaSundar et al., 2020).

Considering all these explanations, it becomes clear that the waste problem is not only an environmental or a public one, but also an issue that should be given great importance in terms of administrative circles, policy practitioners, and executives.

Solid waste is one of the primary environmental problems in our country, as in the world. Since the most important step is to find local solutions to solve waste problems, the zero-waste project, which is one of the most important duties of local institutions, was initiated for the collection, mobilization, and effacing of solid waste.

1.1. Classification of Municipal Solid Waste

Municipal solid waste (MSW), among the most serious environmental issues, is an expression commonly meant describe to the nonhomogeneous collection of waste generated in municipal areas (Moreno et al., 2013). Since waste management is typically in the control of municipalities, they must find and provide an effective and efficient solution for the residents. Nonetheless, they are frequently confronted with several issues in handling the MSW because of a scantness of financial capacity, as well as a lack of structure and complexity (Sujauddin et al., 2008). The content of MSW diversifies greatly according to location and nation and this diversity is mostly determined by lifestyle, economic position, rules of management of waste, and industrial configurations. The amount and content of MSW are crucial in designating the method of handling and management. Therefore, the existence of possible dangerous ingredients in the ash should be carefully evaluated (Manegdeg et al., 2021). In this regard, the waste composition will give useful information on the material's usability for composting or fuel generation via biological conversion (Bagawan et al., 2021). Such information is also critical and valuable for establishing municipal solid waste reuse facilities for conversion to the electricity. For that reason, scientists and engineers may designate the utility of MSW as a fuel originated from its elemental composition and calorific value. Meantime, such data will ease the prediction of the composition of gaseous emissions. Following that, MSW is exposed to different energy transformation processes such as incineration and gasification.

Municipal waste is often derived from several sources where various human activities occur. Several studies have found that the bulk of MSW created in growing nations

comes from households (55–80%), followed by trading industries (10–30%). The MSW of trading industries is made up of different amounts created from different sources including industries, streets, and different institutions (Nabegu, 2010).

In general, solid waste such as food waste, yard trash, wood, plastics, papers, metals, leather, rubbers, inert materials, batteries, textiles, and other types of items are quite diverse (Abdel-Shafy and Mansour, 2018). As a result, their physico-chemical properties vary based on the origin of the waste. The non-homogeneity of the produced solid waste is the principal impediment to the categorization and usage of materials. The nature of this varies from area to area which means it is either undesirable or unwanted material. Municipal solid waste is also divided into organic and inorganic waste. Organic waste constitutes a significant portion of domestic solid waste (Shyamala et al., 2012), while inorganic waste includes different items such as metals, plastics, and other non-biodegradable materials. From a toxic point of view, some solid waste such as pesticides, medical waste, e-waste, herbicides, fertilizers, paints, etc. are classified as dangerous goods and are specially not mixed with general urban waste, and it is recommended to dispose of it in a proper way. Compared with developed countries, solid waste of growing countries is characterized by a high content of organic matter (Porta et al., 2009; Ziraba et al., 2016).

Since organic waste is used within the scope of this thesis, they are explained in detail in the following section.

1.1.1. Types of organic waste

The most common sources of organic waste are the one produced by household activities, agriculture, and industrial processes (Hara et al., 2010). These types of waste are in many forms, including food waste, green waste, wastepaper, fertilizer, human waste, sewage, and meat processing plant waste. Although most soil organic waste adds minerals and nutrients to plant growth and soil fertility, improper disposal can induce serious detriment to the environment (Singh et al., 2017). Income level, lifestyle, and urbanization level are the factors affecting waste generation. Income level also affects the type of waste generated. In the low-income group, the amount of organic waste is higher, and the percentage of paper and plastic (packaging waste) is around 13% of overall waste at the

low-income level, while this value is very high in developed countries. When the global solid waste composition was examined, 46% of domestic solid waste is organic, 17% is paper, 10% is plastic, 5% is glass, 4% is metal, and 18% is the rest. Therefore, it was seen that organic waste covers the largest area with 46% of total solid waste (Hoornweg and Bhada-Tata, 2012). Since most of the organic waste is biodegradable, they can be consumed by living sources such as microorganisms, plants, and animals, and can be broken down into simpler organic molecules.

Generally, the organic constituents of MSW that generate a significant portion of domestic solid waste, might be categorized into three sub-groups: fermentable, non-fermentable, and putrescible. Putrescible waste such as foodstuff are deteriorated quickly while producing unpleasant odors such as food waste (Ziraba et al., 2016). Fermentable waste is also broken down quickly but do not produce the foul aromas associated with putrescible waste such as crops. The degradation of non-fermentable waste such as wood take for a long time.

As the United States Environmental Protection Agency (EPA) stated in 2018, 21.6% of the total municipal solid waste was food waste which was estimated as 63.1 million tons ("Food: Material-Specific Data | US EPA", 2022). It is very important to bring food waste into the economy to protect air, soil, and water and prevent environmental damage. Therefore, within the scope of these thesis, we focused on food waste.

1.1.2. Food waste

Food waste is a phenomenon with significant economic value and, above all, social consequences. The inefficient end-use of food waste creates significant environmental drawbacks and the amount of food wasted has increased over time (Papargyropoulou et al., 2014; Thyberg et al., 2015). Food is wasted throughout the entire food chain, from primary production, food processing, and manufacturing, through wholesale and retail to catering establishments and households (Jin et al., 2018; Principato et al., 2021). There is considerable variation in the volume of waste, and the last part of the food chain (household) is the largest source of food waste in the food chain (Griffin et al., 2008). Food waste is also animal, vegetable, or vegetable residue, known as waste, generated during the handling, preparation, cooking, and consumption of food (Parfitt et al., 2010).

According to the U.N. Food and Agriculture Organization (FAO), 33% of produced total food for human use is squandered. Every year, an average of 1300 million tons of food, equivalent to 30% of food production, was wasted all over the world. The 2020 study from the Turkey Waste Prevention Foundation estimates that 26 million tons of food were wasted annually on average in Turkey, out of a global total of 1.3 billion tons. These meals were thrown out every year at a cost of \$1 trillion globally (Turkey Waste Prevention Foundation (TİSVA), 2020).

Food waste also causes public problems, including food shortages, and reduced food availability. Food waste and loss not only jeopardize the long-term viability of our food systems but also may have a detrimental influence on food reliability and protection, as well as contribute to rising food prices. When food is thrown away, all the sources necessary to make it are squandered, including water, space, electricity, labor, and money. Furthermore, dumping food waste and trash in landfills produces greenhouse gas emissions, which accelerate the climate crisis. Estimates show that 8-10% of global greenhouse gas emissions were relevant to food that is not consumed.

Our food production cannot be robust unless they are sustainable, that is why we must prioritize the implementation of integrated ways to decrease food waste. Global and regional practices are necessary to maximize the utilization of the foods that are produced. Therefore, technology, innovative solutions (such as e-commerce portals for sales and flexible mobile food processing equipment), new methods of manufacturing, and excellent procedures to monitor food stability, and reduce food wastage are critical to executing this radical change. According to Sustainable Development Goal 12 (SDG), there seems to be an urgent need to enhance action to decrease food wastage. For that reason, FAO and Turkey's Ministry of Agriculture and Forestry are collaborating on a countrywide program dubbed "Save Your Food" to reduce food wastage. The campaign displays a number of measures to be addressed by a broad range of stakeholders, including the public and commercial sectors, and academics based on Turkey's first National Strategy and Action Plan on the Prevention and Reduction of Food Waste. Knowledge transfer among food chain actors, primarily end consumers and waste management specialists, accounts for a large share of activities aimed to reduce food waste throughout the food supply chain. To support to progress on the issues, FAO has

begun educating diverse stakeholders, including but not limited to food merchants, suppliers, and entrepreneurs, on hindering and management of food waste.

As for the food management system, the production of value-added products from food waste is very substantial to diminish the negative effect of food waste on human health, the environmental, and the national economy (Salihoglu et al., 2018). Since this waste consists of bioactive compounds such as polyphenols and essential oils alongside nutritional fibers that may be lower back as valuable products, they offer monetary advantages for the food, cosmetic and pharmaceutical industries. Therefore, purchasers pick herbal and fiber-wealthy supplements which are very treasured merchandise for the food industry (Elleuch et al., 2011). The insoluble fraction of dietary fiber including contains cellulose, lignin, hemicellulose, pectin, gum, and other carbohydrates is associated with intestinal regulation, and the soluble fraction is associated with lower cholesterol levels, and intestinal glucose absorption.

In recent years, the food industry has focused on waste valuation to prevent both the loss of these precious substances and the environmental problems caused by these wastes (Aksit and Gencelep, 2021). In the present work, spent coffee ground was evaluated for the production of value-added products. Spent coffee grounds are among the putrescible organic waste which is rich in protein, carbohydrates, fats, and polyphenols. In the following parts, it is explained in detail.

1.1.3. Spent coffee grounds (SCG)

Being grown in more than 80 countries globally, coffee is one of the biggest commodities merchandised in the global market because of its invigorating properties. According to the report of the International Coffee Organization (ICO), the annual coffee consumption in the world is approximately 10 million tons while 250 thousand tons have been reported in Turkey (International Coffee Organization, 2020). Therefore, the coffee industry generates large amounts of waste each year as SCG.

SCG is an insoluble residue that forms after coffee beans are milled and brewed. It was reported that during the manufacture of 1 kilogram of soluble coffee, around 0.650 tons of SCG is created by using 1000 kilograms of green coffee beans. In addition, 2000 grams of wet SCG is recovered by the manufacturing of 1000 grams of instant coffee

approximately (Murthy and Naidu, 2012). Besides these, around half of SCG is produced via instant coffee processing in industrial facilities and cafes, with the rest coming from residential use (Scully et al., 2016). SCG includes caffeine, polyphenols, and tannins, making it poisonous and causing massive pollution if released into the environment (Cruz et al., 2012; Scully et al., 2016). If these types of waste are not recycled, they are thrown away and sent to landfills (Santos et al., 2017) Since a large amount of oxygen is required for the decomposition of SCG in nature, throwing SCG to the environment poses a risk due to its decay. Every ton of coffee waste thrown causes to generate 2470 m³ of CO₂ and 340 m³ of CH₄ emissions when it decomposes. In addition, coffee waste, which has high disposal costs, pollutes groundwater due to its rich oil content (Corro et al., 2014).

When it comes to converting spent coffee grounds to products, it's crucial to know what their composition are. It should be emphasized that, like with other biological feedstocks, the content of spent coffee grounds differs exceedingly based on a variety of parameters including brewing technique, growth circumstances, and coffee type. Most spent coffee grounds, on the other hand, have a similar composition. Polysaccharides, specifically cellulose and hemicellulose, make up the majority of the dry mass of spent coffee grounds, accounting for around half of the total dry mass (Ballesteros et al., 2014). In the previous studies conducted, it was reported that the spent coffee grounds consisted 36.7% of hemicellulose, 21.2% of mannan, 13.8% of galactan, 13.6% of protein, 8.6% of cellulose, 2.2% of acetyl, and 1.7% of arabinan (Mussatto et al., 2011). In addition, it was reported that espresso included 44.2% of linoleic acid, 32.8% palmitic acid, 10.3% oleic acid, and the remaining 7.1% stearic acid (Cruz et al., 2012). Additionally, the micronutrients in spent coffee grounds mainly include K⁺, Ca⁺², Mg⁺², S⁻², P⁻³, Fe⁺, Mn⁺², B⁻³, and Cu⁺ (Dattatraya Saratale et al., 2020).

1.1.4. Usage areas of spent coffee grounds (SCG)

Coffee consumption has been steadily growing. Spent coffee grounds (SCG), coffee husk (CH), coffee silver skin (CSS), and coffee pulp are solid byproducts generated after coffee processing (Hejna, 2021). All of them have narrow usage areas like compost, fertilizer, and so on. SCG valorization has used a variety of techniques to create biobased products, including anaerobic digestion of SCG, extraction of SCG, transesterification of SCG, and fermentation of SCG by microorganisms (Rajesh Banu et al., 2020). The

development of a circular bioeconomy can be aided by using biorefinery processes in conjunction with an integrated strategy.

Biorefinery is a long-term process that uses a range of conversion and treatment processes to transform biomass into value-added chemicals, electricity, and fuels. However, the accessibility and economic worth of the manufactured product, as well as the waste qualities, affect the installation and advancement of a biorefinery process (Mata et al., 2018). SCG has been considered as an important raw material for a variety of processes as well as for biorefinery processing into high-value compounds recently. Through several integrated bioconversion processes, biorefinery aims to optimize the ultimate product value created from biomass. Pyrolysis, transesterification, hydrolysis, extraction, and fermentation are some of the processes that transform SCG into useful biofuels like bio-oil, biogas, hydrocarbon, bioethanol, and pellets, as well as value-added products like bioactive agents, compost, biochar, polyhydroxyalkanoates, carotenoids, phenolic compounds from SCG, and caffeine. Moreover, SCG lipids could also be utilized to make cosmetics (Ribeiro et al., 2013; Atabani et al., 2022; Rajesh Banu et al., 2020).

The SCG structure contains high levels of lignocellulose and lipids. Because of its carbon to nitrogen ratio, and the presence of minerals, polypeptides, and polysaccharides, SCG is a rich feedstock for fermentation processes. As a result, it might be used as a feedstock for manufacturing of biofuels like bioethanol and biodiesel. Waste from biofuel manufacturing might be utilized as an organic source to make compost and to manufacture pellets (Atabani et al., 2022). Biochar is also made up of oil removed SCG by pyrolysis. In addition, bio-oil is produced when manufacturing biodiesel. Biochar is employed in the industrial sector as combustible because of its high calorific value. SCG is also co-processed within biomass, like macroalgal biomass, activated waste sludge, and food waste in order to generate more biogas (Kim et al., 2017).

Vítěz et al. (2016) has reported on the creation of biogas from SCG by anaerobic digestion. Under mesophilic conditions, they reported 271–325 L production of methane per kilogram of dried SCG. To increase the recovery of methane through anaerobic digestion of SCG, an alkaline pre-treatment was used (Giroto et al., 2018). Lee et al. (2019) investigated an integrated biological and physicochemical method for recovering

SCG to manufacture fatty acid methyl ester (FAME), biogas, and lignin. FAME and lignin were recovered at maximum levels of 62.4% and 55.5%, respectively. The greatest methane output was 36.0 mL-CH₄/g-VS_{added} from the solid residues following extraction.

Haile et al. (2013) also investigated an integrated method involving the production of oil and biodiesel, as well as the use of waste for multiple product recovery, resulting in zero waste. SCG's oil was taken and processed to produce biodiesel. The residue obtained as a result of extraction of SCG and biodiesel manufacturing from SCG was utilized as a raw material for the manufacture of fuel pellets. Kwon et al. (2013) investigated the generation of sequential biodiesel and bioethanol by using SCG as raw material. Biodiesel and bioethanol yields from SCG depending on the consumption of sugar and lipid extraction were 97.5±0.5% and 0.46 g/g, respectively. SCG was also co-processed within macroalgal biomass (*Ulva*) by Kim et al. (2017), which increased bio-methanation by methane production of 190 mL/g chemical oxygen demand. In another study, 19.73% w/w oil was recovered from dry SCG, and it was utilized to make 73.4% (w/w) biodiesel (Haile et al., 2013). The solid residue left behind at the end of the extraction was utilized for producing bioethanol by hydrolysis with acid (H₂SO₄), and fermentation by *Saccharomyces cerevisiae* yielding 8.3% (v/v). The waste after the production of bioethanol was composted and solid fuel pellets were made with a carbon-nitrogen ratio (C/N) of 21.9:1 and a heating value of 20.8 MJ/kg, respectively. In terms of both economics and sustainable waste management, SCG conversion into biofuels has received greater attention (Karmee, 2018; Atabani et al., 2022).

In recent years, food waste has been evaluated as a feedstock for the production of industrially important microorganisms. Therefore, in this thesis study, the aim is to produce an industrial enzyme, a lipase, from SCG by microbial fermentation.

1.2. Production of Enzymes from Waste

Around 4,000 enzymes are produced now, with 200 of them being used commercially. Microorganisms are the primary source for the production of industrial enzymes (Tüysüz et al., 2019). The employment of microorganisms in the manufacture of enzymes provides a variety of benefits (Sharma et al., 2001). Microbial enzymes, in comparison

to plant and animal enzymes, are more stable under harsh circumstances and may be manufactured in larger amounts. Furthermore, organic waste would be used to produce microbial enzymes at a minimal cost. Enzyme-producing bacteria, on the other hand, can be processed fast and efficiently, and the genetic alterations required for the elevation of the production level of secondary metabolites can also be done more effectively on microbial cells (Gurung et al., 2013).

Remarkably, different wastes may be used as raw materials to produce enzymes or renewable energies. The European Union's new rules highlight the notion of biorefinery, in which waste from one business can be used as raw material for another (Ravindran and Jaiswal, 2016a). Many agro-industry wastes are lignocellulosic in origin, in other words, which are rich in cellulosic and hemicellulosic polysaccharides, as well as lignin, and include additional nutrients including proteins, lipids, pectin, and polyphenols. Sustainable industries maintain a stable supply of low-cost raw materials to function properly. The effective use of geponic waste has provided an important precedent as carbon and nitrogen supply to enzyme production in the middle of the 20th century. By introducing waste streams of specific sectors to agriculture-based enterprises, the waste storage problem can be solved. This necessitates realistic valorisation research on the utilization of waste in order to manufacture value-added goods like enzymes (Hassan et al., 2019).

Several research have been done in recent decades to investigate the effectual usage of agro-based waste and byproducts as a possible raw material to produce value-added goods. The majority of their works has been undertaken in agriculturally-dependent nations. Brazil, for example, is the world's greatest grower and trader in sugar cane, as well as the world's second-largest bioethanol supplier (Solomon et al., 2007). Currently, bioethanol production and concerned liquid fuel-based processes are the sole mercantile operations that employ agricultural waste as a feedstock. Fermentation techniques such as waste hydrolysis accompanied by fermentation or concurrent saccharification, separating a monosaccharide from a complex carbohydrate into its monosaccharide, and fermentation are used to achieve this. (Guan et al., 2016). Nevertheless, researchers have started to investigate possibilities for converting agricultural lignocellulosic leftovers as a feedstock to synthesize different enzymes (Ravindran and Jaiswal, 2016b).

A natural polymer called cellulose is present in lignocellulose, composed of repeated glucose monomers. Depending on the type of the waste product, food waste from plants contain starch, xylan, pectin, inulin, mannan, glucan, and other nutrients in addition to cellulose. Lignocellulosic waste appears to be a feedstock for the synthesis of enzymes that degrade lignocellulose due to its high polysaccharide content. Microorganisms synthesize enzymes, which are unique for every polysaccharide, allowing sugars to be released and digested for growth energy and cell upkeep. Since lignocellulose offers a cheap source of carbohydrates, its efficacy as a feedstock in industrial microbial processes has been extensively investigated (Yasin et al., 2013). Lignocellulosic waste has indeed been investigated for the manufacture of several industrially significant enzymes as a possible medium ingredient, particularly enzymes which can degrade cellulose due to the amount of cellulose it contains. Therefore, a huge amount of research has been carried out to utilize lignocellulosic waste. Before they may be utilized as a carbon source for manufacturing processes, lignocellulosic substrates must undergo a variety of upstream procedures. The lignocellulose's intrinsic molecular limitations and structural fractions prevent the profusion of microorganisms required for fermentation to obtain the favored products (Ravindran and Jaiswal, 2016b).

For that reason, current findings addressing enzyme synthesis from agro-based waste have focused on the influence of pre-treatment methods on yield. Pre-treatments are procedures frequently employed in lignocellulose-based feedstock production methods in which the intricate plant framework is disturbed physically, chemically, or by a mixture of approaches to increase the digestion rate (Hassan et al., 2018). Since the production of fermentable sugars from microbial fermentation of lignocellulosic waste has been used to treat lignocellulosic mass, the manufacture of enzymes which can degrade lignocellulose such as cellulase, hemicellulase, and laccase, is one of the most important issues. Laccase enzyme, to degrade lignin, is widely utilized in the bioremediation process, as well as in textiles, pulp, paper, and petroleum sectors (Rodríguez Couto and Toca Herrera, 2006). Otherwise, hemicellulase and cellulase enzymes are specifically used to manufacture bioethanol. The development of cost-effective production processes requires the on-site manufacture of cellulase in conjunction by the hydrolysis of biomass. Thus, Akolkar et al. (2005) had research about the production of lactase from fermented ragi with the help of *Lactobacillus acidophilus*

by submerged fermentation. Ragi is a grain that is cultivated in India as a food crop and is mostly consumed by rural inhabitants and its fermentation presents a perfect condition for the growth of yeast and lactic acid bacteria. In static flask culture, maximal enzyme activity was reported as 819 U/mL after 12 hours, but in shaking flask culture, enzyme activity obtained was only 648 U/mL after 16 hours.

Various lignocellulosic substrates have been used in several investigations to produce amylases in addition to cellulases and laccases. For instance, Salim et al. (2017) used a novel species of *Bacillus* to evaluate the synthesis of cellulase, α -amylase, protease, and pectinase utilizing olive oil cake, sunflower, soybean, wheat bran, and maize bran as growth substrates. Fernández Núñez et al. (2016) carried out a thorough investigation to improve a fermentation in solid state process utilizing various agro-based waste by *Rhizopus microsporus*. This study looked for four lignocellulosic sources of carbohydrates for amylase synthesis: wheat bran, soybean meal, sugarcane bagasse, and wheat flour type II. Data obtained from this study revealed that wheat bran was the best individual source of substrate for amylase synthesis as 392.5 U/g. Rajagopalan and Krishnan (2008) have produced amylase from sugarcane bagasse hydrolysate (SBH) by submerged fermentation method using catabolite-repressed *B. subtilis* KCC103. The absence of arabinose, glucose, and xylose in the ratio of 0.16:0.9:1.0 (w/w/w) was shown by HPLC analysis of SBH. The addition of SBH containing 1% reducing sugar (w/v) to the feeding medium resulted in the maximal production of α -amylase as 67.4 U/mL.

Besides lignocellulosic waste, food waste as vegetable and fruit waste from households is used to produce enzymes. Irshad et al. (2012) have studied the production of β -glucanase from orange peel by *Trichoderma viridi* through solid-state fermentation. After the fourth incubation day at 30 °C and pH 5.5, the highest activity was 412 ± 12 U/mL. Dhillon et al. (2012) have found that apple pomace has the potential to be used as a feedstock to produce cellulase and hemicellulase by *Aspergillus niger* NRRL-567 via fermentation in solid-state. Apple pomace had been used to produce organic compounds, bioenergy, fragrance chemicals, natural antioxidants, protein-enriched meals, mushrooms, and enzymes including pectinases, cellulases, and lignocellulolytic enzymes. Therefore, they recycled apple pomace for the synthesis of cellulase enzymes such as FPase (filter paper cellulase), BGL (β -glucosidase), xylanase, and CMCase

(carboxymethyl cellulase) since it is abundant in Canada. They were reported with maximal activity occurring between 48 and 72 hours of fermentation. After 48 hours of incubation, the activities of BGL and FPase were 60.09 ± 3.43 U/gram dry substrate (gds) and 133.68 ± 5.44 U/gds, respectively. Additionally, Li et al. (2014) have worked on the optimization of pectinase production by *Penicillium oxalicum* PJ02 from orange peel. According to their research, orange harvest was about 0.05 billion metric tons globally, with around 0.02 billion metric tons manufactured to orange juice, flavorings, and other by-products. Orange peel is the most common organic source by-product of the citrus processing sector, accounting for almost half of the weight of fresh oranges. Orange peel waste includes a variety of high-value compounds, including fiber (hemicellulose, lignin, and pectin), flavonoid, protein, and sugar. They studied the production of not only exo-pectinase but also of endo-pectinase since orange peel is high in pectin and reported 36.88 U/mL of exo-pectinase and 0.62 U/mL of endo-pectinase.

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are the third biggest category of industrially significant enzymes in terms of total sales (Tüysüz et al., 2019). Since it is the core of this work, lipase production from organic waste was explained in detail in the following part.

1.2.1. Lipase production from organic waste

Lipase, catalysed the hydrolysis and synthesis of esters, has essential industrial applications due to features such as catalytic activity over a wide temperature and pH range, substrate specificity, and enantioselectivity (Priji et al., 2014). Other catalyzed reactions that deviate from their normal physiological response account for the wide range of uses. Because of its applicability, it is used in the food sector, detergent and pharmaceutical industry, textile, paper, and cosmetic sectors (Kapoor and Gupta, 2012).

Lipase production is mainly affected by the type and concentration of carbon and nitrogen sources, pH, temperature, and ions in the environment. Microorganisms that grow in an oily environment have an ability to utilize oil and oily compounds to produce lipase (Saxena et al., 1999). Many microorganisms, including bacteria, yeast, and filamentous fungus, can generate lipase in submerged and solid-state cultivation (de Almeida et al., 2016).

When the literature is examined, it has been seen that, olive, sunflower, soybean, sesame, cottonseed, corn, peanut, and palm oils are plant-based oils and mostly used as carbon sources during microbial production of lipase (Wang et al., 2022). The concentrations of the oils used vary between 0.5% and 3% (Lima et al., 2003). Besides vegetable oils, various studies have recently examined the synthesis of lipase by utilizing various organic waste as substrates or by adding organic waste as inducers. Mazhar et al. (2006) conducted a comprehensive inspection on the production of lipases through fermentation in solid-state by utilizing agro-based waste as a substrate. In their study, 14 bacterial strains have isolated, tested, and compared for lipase synthesis. *Bacillus subtilis* strain NL-39 has chosen as the best producer of lipase among the three evaluated strains. The maximum activity of the enzyme was 34.93 U/ml when solid-state fermentation with soya bean meal was used. Other low-cost agro-industrial waste, such as sunflower meal, sugar cane bagasse, wheat bran, oat bran, and rice bran, produced lipase at rates of 23.83, 16.23, 12.17, 10.40, and 10.00 U/ml, respectively.

Alkan et al. (2007) worked on extracellular lipase production from melon waste by using *Bacillus coagulans* under fermentation in solid-state. After 24 hours of growth with 1% olive oil supplementation, the maximum production of lipase was recorded as 78,069 U/g. Palma et al. (2000) investigated lipase production by utilizing solid waste from industrial babassu oil manufacturing as a feedstock by *Penicillium restrictum*. Olive oil-enriched media had the greatest lipase activity of 27.8 U/g.

According to the research of Imandi and Rao (2007), India produces a lot of sugarcane bagasse, rice bran, and wheat bran. Therefore, they conducted a comparative investigation on the synthesis of extracellular lipase in solid-state fermentation by utilizing *Yarrowia lipolytica* NCIM 3589 with diverse complex substrates. In seven days of fermentation, the fermented substrate was reported as a mixed substrate of wheat bran and sugarcane bagasse by them. As a result, mixed substrate provided a maximum activity of lipase as 9.3 U/g. Rodriguez et al. (2006) also worked on lipase production by utilizing sugarcane bagasse as a carbon source through fermentation in solid-state. *Rhizopus homothallicus* (IRD13a) was employed for lipase synthesis. In only 12 hours of incubation, a maximum of 826 U/g was achieved. Hosseinpour et al. (2011) also investigated the extracellular lipase synthesis from *Aspergillus niger* strain NCIM 584

by using soya flour in a submerged culture. According to their work, the highest enzyme activity and yield were 6366 U/L and 76.4 U enzymes per g of soya flour, respectively.

Current residential and industrial actions result in an accumulation of negative-cost and low-value by-products and/or raw waste, that might have a severe effect on human life and environment owing to the existence of undesirable compounds. Waste cooking oils, for example, are produced when vegetable oils are heated to high degrees in food processing. Waste cooking oil is frequently dumped into the system of public sewage, and in facilities of wastewater treatment, these pollutants are regarded as additional remnants that must be cleaned, raising treatment expenses of water. Because of the environmental issues related with waste cooking oil, it is critical to recycle and valorize this pollutant waste. Moftah et al. (2011) carried out a thorough investigation of lipase production from oil cake waste by *Candida utilis* through fermentation in solid-state. From 1 ton of fresh olives, approximately 0.2 tons of olive oil and 0.5 tons of crude olive cake were generated. Although olive oil cake includes a lot of nutritionally beneficial components including carbohydrates, lipids, and polypeptides, it has a low level of crude protein, a lot of fiber, and a lot of unsaturated fatty acids. In their conclusion, they demonstrated that alkaline treatment of olive cake may generate lipase activity of 25 U/g. Moreover, Lopes et al. (2018) attempted to demonstrate the feasibility of substituting a costly edible oil such as olive oil for lipase synthesis with a non-edible oil waste such as waste cooking oil. The influence of mass transfer of oxygen is explored in batch cultures in a stirred tank bioreactor, and 12000 U/L of the highest lipase activity is reached at k_{La} of 16 h^{-1} as a consequence of their work.

There has been a significant push for the development of *Jatropha* plants and the use of their seeds in the manufacturing of biodiesel. It is obvious that the extraction of *Jatropha* oil will result in a large amount of remaining deoiled seed cake. Mahanta et al. (2008) assessed that deoiled *Jatropha* seed as a carbon source for lipase production through fermentation in solid-state by *Pseudomonas aeruginosa* PseA. The seed cake has promoted bacterial growth and lipase synthesis at a rate of 625 U/g. Babassu cake is a by-product of oil extraction process from babassu palm. Cavalcanti et al. (2005) focused on scaling up lipase synthesis in solid-state fermentation by utilizing *Penicillium simplicissimum* in packed bed bioreactors. According to the result of their work,

decreased temperatures and increased airflow rates might result in higher lipase activity. At a temperature of 27 °C and an airflow rate of 0.8 L/min, the highest activity of lipase (26.4 U/g) was recorded.

de Azevedo et al. (2020) also studied lipase production by *Aspergillus terreus* NRRL-255 using cacay butter and wheat bran as substrates under solid-state fermentation. According to their research, cacay is a prominent Amazonian tree growing around the foot of the Andes and in the Amazonian flats. Its seeds are rich in fat, with 12.27% monounsaturated fats, 11.69% saturated fats, and 76.05% polyunsaturated fatty acids, the bulk of which are oleic and linoleic acids. As a result, they set out to find the best conditions for lipase synthesis using butter and cacay oil as inducers and agro-based waste as carbon sources. The greatest lipase activity measured under ideal circumstances was 2,867.18 U/g. In terms of lipase characterization, the highest relative activity was achieved at pH 7.0 and 35 °C.

Although a lot of studies have been conducted for lipase production from different organic waste, to make microbial lipase production sustainable, it is very important to develop sustainable biotechnological processes and replace the substrate source with products with no added value. Therefore, in the present work, SCG was utilized as a source for lipase production from both yeast and bacteria within a biorefinery concept.

1.2.2. The concept of integrated biorefinery for zero-waste production

Zero waste was first used by Chemist Paul Palmer in the name of the Zero Waste Systems Inc. (ZWS) business built in Oakland, California, in the mid-1970s. This company wanted to ensure the reuse of chemicals released as residues in electronic products. In the 1970s, it traded the chemicals they collected without any charge to laboratory staff, scientists, and chemical companies. The company has become the largest chemical stock area of that time (Er, 2012). Over time, many organizations around the world have adopted the concept of zero waste and set the zero-waste target for storage and disposal. Zero waste is a waste management system that aims to minimize the generated waste, and it is based on sustainability in the production world, while it pushes individuals to act responsibly in living and usage areas. While many people misunderstand that it is just conventional recycling, on the contrary, zero waste does not encourage recycling.

Instead, it draws attention to the high costs and uncertainties in recycling practices. Recycling is one of the waste management alternatives, and although it has a place in the zero-waste model, it is the last refuge before it is sent to landfills, just like waste is composted. Zero waste management hierarchy forms the basis of the zero-waste management system. This hierarchy consists of five steps: reject what you do not need, reduce your needs, reuse what you consume, recycle what you cannot refuse, reduce and reuse, and rot the rest in compost (Johnson, 2013).

A biorefinery is a system that includes processes of biomass conversion and equipment for producing fuel, power, heat, and biochemicals from biomass (IEA Bioenergy Task 42, 2007). The concept's major goal is to optimize biomass usage while minimizing waste material and emissions from bioenergy product conversion by optimizing energy and material recovery (Trivedi et al., 2015). In the scientific community, the term "biorefinery" is gaining attraction. Some biorefinery complexes and non-traditional biomass sectors are already competitive in the market, and several pilot and demonstration facilities are in operation across the world. Biorefineries are categorized into three generations based on the raw materials utilized to manufacture biofuels and bio-based goods (Fernando et al., 2006; Ogier et al., 1999). As a result, first-generation biorefineries focus on typical agro-based biomass, such as corn, sugarcane, soybean or maize. Second-generation varieties depend on feedstock, contain lignocellulose, which is mostly consist of hemicellulose, cellulose, and lignin. Finally, the third generation includes industrial facilities that utilize microalgae, petroleum, forestry, urban waste, or agricultural waste as biomass of feedstock (Azapagic, 2014; Parajuli et al., 2015).

In the light of these explanations, the goal of this work is to use SCG for the synthesis of lipase by *Cryptococcus diffluens* (D44) and newly isolated *B. subtilis* strain BT2. Lipase was selected as a model enzyme to test the usage of spent coffee as a potential carbon and nitrogen sources for bioprocesses. In addition, the effect of SCG amount, temperature and pH on lipase production from *C. diffluens* D44 was investigated by using Box-Behnken design via Design-Expert program. Analysis of variance was performed with the help of the Design-Expert program to test the accuracy of the model formed as a result of the experiments designed according to the Response Surface Methodology (RSM). When more than one component is simultaneously influencing the response, the

RSM may be utilized to optimize the process by using statistical concepts (Yolmeh and Jafari, 2017). RSM has been extensively used to examine the impacts of several factors, including biomass loading, temperature, pH, chemical concentration, and others (Pereira et al., 2021). RSM is also widely preferred to be used in conjunction with Central Composite Design and Box-Behnken Design, two statistical approaches of experimental design, to choose the values of the parameters to be evaluated (Timung et al., 2015).

Using spent coffee grounds as a raw material in lipase production would also help us to provide alternative products to contribute to the elimination of our external dependence. After the bioprocess, the spent coffee grounds used by the microorganism were subjected to the pyrolysis process to develop a biorefinery concept with zero-waste. The possible use of thermochemical processes as an alternative food disposal option has lately sparked interest. They provide a simple and convenient method for reducing waste volume (>80%), shortening response times, increasing energy (Lee et al., 2019), the effectiveness of recovery (Czajczynska et al., 2017), and expanding the sorts of waste which may be sent away (Jahirul et al., 2012). Therefore, thermochemical methods are significant instruments for converting organic waste to energy/heat while potentially reducing ecological concerns. The thermochemical transformation of carbonaceous materials into solid, liquid, and/or gas products is known as pyrolysis. Pyrolyzing food waste may be more advantageous than conventional disposal options since its simplicity, low cost, and capacity to treat a wide range of feedstocks to create gases, charcoal, bio-oil, and biochemicals (Kim et al., 2020).

In the present work, we also investigated the pyrolysis of fermented SCG after lipase production. In the light of the literature review, no investigation has been conducted on the evaluation of environmentally, sustainable, and economically promising coffee waste in both lipase production and pyrolysis of fermented SCG. According to previous studies, it was found that 15-21.5% oil content was obtained from used coffee grounds depending on the extraction method (Loyao et al., 2018). Since coffee grounds include cellulose and hemicellulose, it was hypothesized the usage of the coffee ground by different microorganisms. Therefore, our research was to evaluate whether the coffee grounds containing cellulose, hemicellulose, mannan, galactan, and arabinan and protein as a

nitrogen source can be used directly by microorganisms without any treatment steps to produce biomass and industry-important enzymes with biorefinery concept.

As a result, it was aimed to bring a value that can contribute not only to society but also to the environment at a national and international level, support the zero-waste policy, and to contribute the creation of a sustainable future by realizing many of the global goals.

2. MATERIALS AND METHODS

2.1. Materials and Equipments

2.1.1. Microbial strains and chemicals

Two types of microbial strains as yeast and bacteria were used in this study. *Cryptococcus diffluens* (D44) used as yeast, were kindly provided from Prof. Dr. Tansel Yalçın (Ege University, Faculty of Science, Department of Biology, Basic and Industrial Microbiology Section). *B. subtilis* strain BT2 used as bacteria was isolated from soil sample at Kadıköy region of Istanbul.

All chemicals given in Table 2.1 were analytical grade and purchased from mainly two companies (Merck (Darmstadt, Germany) or Sigma Aldrich (St Louis, MO)) unless otherwise stated.

Table 2.1. Chemicals used throughout the experiments.

Chemical	Supplier
Acetic Acid	Merck
Acetone	Merck
Anis-aldehyde	Sigma Aldrich
Agar	Sigma Aldrich
Ammonium sulfate	Merck
Beef extract	Merck
Chloroform	Merck
Citric acid monohydrate	Merck
Coomassie Brilliant Blue G-250	Sigma Aldrich
D- Glucose monohydrate	Merck
3,5-Dinitrosalicylic acid	Merck
Ethanol	Sigma Aldrich
Ethyl Acetate	Merck
D-Fructose	Sigma Aldrich
Glycerol	Merck

Table 2.1. *continued.*

Chemical	Supplier
Glycine	Merck
n-Hexane	Merck
Isopropanol	Merck
Kanamycin sulfate	Sigma Aldrich
Magnesium sulfate heptahydrate	Merck
Malt extract	Sigma Aldrich
Maltose	Merck
Methanol	Merck
Nutrient Broth	Merck
Olive Oil	Komili
p-Nitrophenyl Palmitate	Sigma Aldrich
p-Nitrophenol	Sigma Aldrich
Peptone from casein	Merck
Peptone from meat	Merck
Phosphoric acid	Merck
Potassium Dihydrogen Phosphate	Merck
Potassium Phosphate Dibasic	Merck
Potassium Sodium Tartrate Tetrahydrate	Merck
Sodium Acetate	Merck
Sodium Carbonate	OMNI
Sodium Chloride	Merck
Sodium Hydroxide	Merck
Sodium Nitrate	Carlo Erba
Sucrose	Merck
Trisodium citrate dihydrate	Sigma Aldrich
Triton X-100	Amresco
Trizma Base	Sigma Aldrich
Tween80	Merck
Yeast Extract	Merck

2.1.2. Equipments

Equipments used in the thesis were listed below.

Table 2.2. List of equipments used.

Equipment	Supplier
Amicon Stirred Ultrafiltration Cell 8050	Millipore
Analytical Balance	Denver Instrument
Autoclave	Nuve OT 032
Automatic pipettes	Thermo Scientific
Deep Freezer (-20°C)	Arçelik No Frost
Deep Freezer (-80°C)	Thermo Scientific 88400V
Fume Hood	Hedlab X-Pro, EN
Heating Magnetic Stirrer	Velp Scientifica
Ice Machine	Bar Line
Incubator	Nuve FN400
Microplate Reader	Biotek-ELx808
Microtube Shacking Incubator	Labnet Vortemp 56 EVC
Orbital Shaker	Zhicheng
Peristaltic Pump	Cole Parmer
pH Meter	Hanna Instruments
Refrigerator	UĞUR
Spectrophotometer	Bio Rad SmartSpec Plus
Ultrasonic Bath	Bandelin
Vortex	Capp CRV-45X
Water Bath	LabO SM3

2.1.3. Buffers and solutions

Table 2.3. Compositions of buffers.

Buffers/solutions	Composition
30% glycerol solution	30% (v/v) glycerol and 70% (v/v) distilled water
50% glycerol solution	50% (v/v) glycerol and 50% (v/v) distilled water
Citric acid- sodium citrate buffer pH 4.0	0.05 M, 8.0 mL sodium citrate was mixed with 0.05 M, 13 mL citric acid
Dipotassium phosphate- monopotassium phosphate buffer pH 6.0	0.05 M, 2.0 mL dipotassium phosphate is mixed with 0.05 M, 18 mL monopotassium phosphate
Dipotassium phosphate- monopotassium phosphate buffer pH 6,5	2.937-gram dipotassium phosphate and 4.51-gram monopotassium phosphate were added to 1 L of distilled water
Dipotassium phosphate- monopotassium phosphate buffer pH 7.0	0.05 M, 22 mL dipotassium phosphate was mixed with 0.05 M, 6 mL monopotassium phosphate
Dipotassium phosphate- monopotassium phosphate buffer pH 7,5	6.406-gram dipotassium phosphate and 1.799-gram monopotassium phosphate were added to 1 L of distilled water
Dipotassium phosphate- monopotassium phosphate buffer pH 8.0	0.05 M 47 mL dipotassium phosphate was mixed with 0.05 M, 3 mL monopotassium phosphate
Glycine- sodium hydroxide buffer pH 8.5	3.75-gram glycine and 0.16 g sodium hydroxide were added to 1 L of distilled water
Glycine- sodium hydroxide buffer pH 9.0	0.05 M, 17 mL glycine was mixed with 5.0 mL sodium hydroxide

Tablo 2.3. *continued.*

Buffers/solutions	Composition
Glycine- sodium hydroxide buffer pH 10	0.05 M, 12.5 mL glycine was mixed with 8.0 mL sodium hydroxide
Sodium acetate- acetic acid buffer pH 5.0	0.05 M, 8.0 mL sodium acetate was mixed with 0.05 M 32 mL acetic acid
Sodium acetate- acetic acid buffer pH 5.6	0.05 M, 3640 mL sodium acetate was mixed with 0.05 M, 360 mL acetic acid
Sodium Carbonate 1 M	10.59 gram of sodium carbonate was dissolved in 100 mL distilled water

2.2. Methods

2.2.1. Treatments of SCG

2.2.1.1. Drying of SCG

Spent coffee ground (SCG) samples were sourced from 3 different local coffee shops, denoted as X, Y, and Z at Kadikoy, Istanbul. The samples were a combination of Arabica beans (*Coffea arabica*) and Robusta beans (*Coffea canephora*), two of the world's most popular coffee species (International Coffee Organization, 2020).

The effect of the amount of sample, temperature, and brand on drying of spent coffee grounds was first examined. The decreasing moisture content over time was calculated according to 3 different variables as X, Y, and Z. Firstly, by keeping the temperature constant, the coffee waste bought from the same coffee shop was dried in different amounts. Then, the amount of coffee waste bought from the same coffee shop was kept constant and dried at different temperatures. Finally, the coffee waste from different coffee shops were dried by keeping the temperature and quantity constant. All drying processes were done using oven. The moisture content of SCG was calculated by using the following equation.

$$\text{Moisture Content (\%)} = \frac{(\text{Weight Before Drying} - \text{Weight After Drying})}{\text{Weight Before Drying}} * 100 \dots \dots \dots (2.1)$$

3.3.3.6. Oil extraction from SCG

Soxhlet extraction was performed to measure the total oil content of spent coffee grounds (REF). Oil was extracted from spent coffee grounds dried at 60 °C for 24 hours, and the effect of the type of the solvents as sole hexane and hexane-isopropanol mixture (1:1, v/v), extraction period between 2-7 hours were tested on the efficiency of oil extraction from 10 g of dried SCG. 200 mL of solution was used for 10 grams of SCG in each trial. After extractions were completed, the solvents were separated from the spent coffee grounds and then vaporized on a rotary vacuum evaporator. To remove solvents that may still be present in the extracted oil samples, they were kept at room temperature for 18 hours, and then the volume of oil released was measured.

3.3.3.6. Sugar and phenolic compounds extraction from SCG

By examining the sugar extraction method parts of articles in the literature (Mussatto et al., 2011; Ballesteros et al., 2015; Go et al., 2016; Ballesteros et al., 2017; Juarez et al., 2017; Baruah et al., 2018), the effect of the concentration of sulfuric acid (H₂SO₄) (1-5%, v/v), amount of solution per spent coffee grounds (2-20%, w/v), and incubation time (1-3 h) were investigated on the efficiency of sugar and phenolic compounds extraction from oven-dried SCG. The yield of reducing sugar released was measured according to the method mentioned in 2.2.7.3., and the total phenolic compounds released were measured according to the method mentioned in 2.2.7.4.

2.2.2. Production of lipase enzyme from SCG

In the present work, considering the oil content of SCG, lipase was selected as the model enzyme and its production from both bacteria and yeast were investigated.

3.3.3.6. Production of lipase from SCG by *C. diffluens* D44 as yeast

Lipases produced by yeasts isolated from soil, wastewater of refinery waste, active sludge, as well as petroleum-contaminated soil show activity that seems promising. *C. diffluens* D44, *Rhodotorula slooffiae* D38, *Wickerhamomyces anomalus* D46, *Candida davisiana* D40, and *Cryptococcus anomalus* D57, a collection of recently explored yeast isolates found in petroleum refinery environment, have all been described as lipase generating. Based on screening tests, *C. diffluens* D44 was revealed as the best producer

of lipase among them. (Yalçın et al., 2013) Therefore, *C. diffluens* D44 was chosen as the lipase producer yeast strain in this thesis. *C. diffluens* D44 was stored at -80 °C in 15% glycerol stocks. Glycerol stock samples were inoculated on agar medium consisting of 0.3% malt extract (w/v), 0.3% yeast extract (w/v), 0.5% peptone (w/v), 1% glucose (w/v), 1.5% agar (w/v) at 28 °C and pH: 6.5 for 2 days. Then, agar plates were kept at +4 °C for further experiments.

For preculture preparation, 100 mL Erlenmeyer flasks with a final working volume of 20 mL were used and the medium containing 0.3% malt extract (w/v), 0.3% yeast extract (w/v), and 0.5% peptone (w/v) with an initial pH of 6.5 was prepared as preculture medium. It was sterilized in an autoclave for 15 minutes at 1.06 bar and 121 °C and then three colonies of *C. diffluens* D44 from agar plates were inoculated into sterilized preculture media. Flasks were incubated in an orbital shaker at 28 °C and 180 rpm for 18 hours. After the incubation period, cells were centrifuged at 7000 rpm for 6 minutes, and wet D44 pellets obtained were used to inoculate the production medium.

For lipase production, basal medium including 0.3% malt extract (w/v), 0.3% yeast extract (w/v), and 0.5% peptone (w/v) was used as basal production medium (Yılmaz and Sayar, 2015). The basal medium supplemented with carbon sources as 2% olive oil (v/v), %1-2 dried SCG (w/v), oil extract from SCG 2% (v/v), sugar extract from SCG with a reducing sugar content of 1% (w/v). All media of production were sterilized by autoclave at 121 °C for 15 min except oils. Oil extract from SCG and olive oil supplied from the market were sterilized by dry heat at 180 °C for 60 min in oven.

The wet cell pellet was then resuspended in media and transferred to lipase production media, where the starting optical density (OD) of 1.0 at 600 nm was adjusted. The initial optical density (OD) was kept constant at 1.0 in all trials. For 6 days, cells were allowed to grow at 250 rpm. Then, the effect of concentration of the best carbon source (1-10% w/v), the incubation temperature (28-35 °C), and the pH (4.0-9.0) were investigated to determine optimum conditions. Additionally, utilizing Box-Behnken design with the Design-Expert tool, the effect of SCG amount, temperature, and pH on lipase production from *C. diffluens* D44 was examined. To check the accuracy of the model created as a consequence of the experiments created using the Response Surface Methodology, analysis of variance (ANOVA) was carried out with the aid of the Design-Expert

program. Cells were taken after the cultivation period and centrifuged at 12000 rpm for 15 minutes at 4 °C, with the supernatant serving as extracellular crude lipase. The lipase activity of each medium was determined according to the method specified in method 2.2.7.1., and the concentration of protein was measured with regard to the method specified in 2.2.7.2.

3.3.3.6.1. Depigmentation of lipase from *C. diffluens* D44

Since the usage of SCG caused pigmentation, the removal of pigment from crude enzyme was investigated by using different protein precipitation methods.

Firstly, ammonium sulfate was used to precipitate proteins from the fermentation broth. This procedure was carried out by gently adding ammonium sulfate at 90% saturation to a solution containing crude lipase. The solution was stirred continuously overnight at 4 °C and then the mixture was spun down at 13000 rpm for 1 hour. After that, the pellet obtained was resuspended in 50 mM phosphate buffer at pH 7.0 and dialyzed within the same buffer for 18 h at 4 °C according to Dialysis Methods for Protein Research by Thermo Scientific.

Secondly, the precipitation of proteins by acetone and ethanol was carried out. The methods described by Thermo Scientific's "Acetone and Ethanol Precipitation of Proteins". To begin, acetone as four times the volume of crude lipase solution was added and then the mixture was incubated for 1 hour at -20 °C. After that, the solution was centrifuged at 13000 rpm for 20 minutes and the pellets were kept at room temperature for 45 minutes to evaporate any remaining acetone. Finally, pellets were dissolved in 50 mM potassium phosphate at pH 7.0 and dialyzed against the same buffer for 18 h at 4 °C to eliminate any acetone residue. For ethanol precipitation, a similar procedure to that used for acetone precipitation was applied.

Lastly, activated carbon was also used to remove pigments from crude enzyme solution. This study was done according to the method of Abdul Rahman et al. (2007) with small modifications. The immobilization of lipase was carried out by continuous shaking 2 g of activated carbon with 15 mL of crude lipase for 1 hour at 25 °C at 100 rpm. Then, the immobilized lipase was separated by centrifugation at 13000 rpm for 20 minutes.

2.2.2.2. Production of lipase from SCG by isolated strain BT2

Bacillus species are the source of numerous enzymes that present industrial importance (Barros et al., 2013). For this reason, *Bacillus* species that have an ability to utilize SCG were isolated from soil samples in Kadıköy region of Istanbul and lipase production from isolates were evaluated. For isolation of *Bacillus* species, one gram of soil was dissolved in 10 mL of sterile saline solution containing 0.9% (w/v) NaCl and then kept at 80 °C for half an hour to eliminate non-endospore-forming bacteria since *Bacillus* species are spore-forming bacteria (Remize, 2017). Then, samples were cooled, and serial dilution procedure was applied with 0.9% (w/v) NaCl. Diluted samples were spread on Petri dishes including nutrient agar at pH 7.0. After spreading, plates were incubated at 37 °C for 16 hours and then considering the morphological characteristics of the colonies observed, the different ones were transferred into separate petri dishes to purify each of them. To select the lipase producers, a nutrient broth medium supplied with 1% olive oil (v/v) was used, and isolates were incubated at 37 °C, 180 rpm for 16 hours. Then, lipase producers were determined by determining lipase activity according to the method specified in 2.2.7.1. Lipase producers selected were stocked in 25% glycerol solution at -80 °C for long-term storage.

The isolate named strain BT2 which showed the best lipase activity was identified based on 16S rRNA gene (~1.5kb) analysis. The identification of BT2 was achieved based on 16S rRNA gene sequence. Genomic DNA was isolated using EurXGeneMATRIX Bacterial & Yeast DNA isolation kit by following the instructions of the manufacturer. Amplification of the 16S rRNA gene within PCR protocol was carried out with universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTA-CGACTT -3'). Purification of the amplicon was performed by using the MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) by following the instructions of manufacturer. Sequencing of DNA with primers 27F and 1492R, was completed by BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA) and ABI3730XL Sanger Sequencing Analyzer (Applied Biosystems, Foster City, CA) by following the instructions of the manufacturer. 16S rRNA gene sequences with published names validly that belong to the close relatives of strain BT2 were retrieved from the GenBank database using blastn (Altschul et al., 1997). Maintenance

of strain BT2 was achieved by storing *isolated* strain in 25% glycerol stocks at -80 °C. Glycerol stock samples were used to inoculate nutrient agar at pH 7.0 and incubated at 37 °C for 16 hours to obtain single colonies to inoculate the preculture. Then, one colony of strain BT2 was inoculated into sterilized 20 mL nutrient broth as preculture media in 100 mL Erlenmeyer flasks and flasks were incubated in an orbital shaker at 180 rpm and 37 °C for 16 hours.

For lipase production from BT2, the effect of different carbon and nitrogen sources on the growth of strain BT2 and lipase production was evaluated. Glucose, fructose, maltose, molasses, and sucrose were used as carbon source, while beef extract, malt extract, peptone from casein, peptone from meat, and yeast extract was used as nitrogen source. Based on the carbon and nitrogen ratio of Nutrient Broth, 0.4 grams of carbon source and 0.32 grams of nitrogen source were used for 40 mL of medium. 1% olive oil (v/v) was added to each medium and the nutrient broth was used as a control. In addition, 1% dried SCG supplied nutrient broth was prepared to investigate the effect of SCG on the growth of *B. subtilis* strain BT2 and lipase production. Growth in microtiter plates was achieved in 96-well U-bottom plates (**Figure 2.1**). The growth of strain BT2 in agar plates including 1% SCG was shown in **Figure 2.2**.

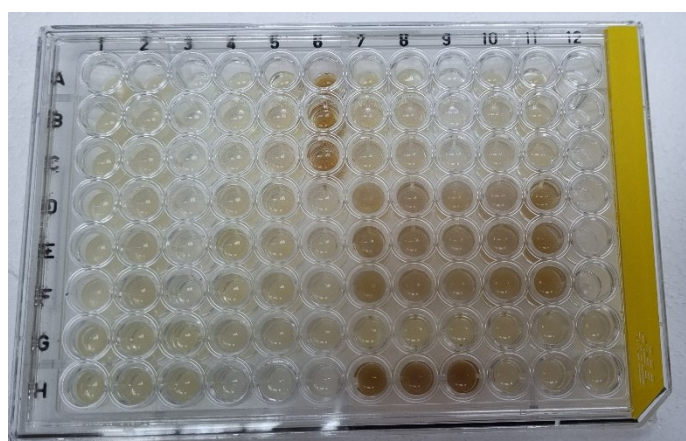


Figure 2.1. 96-well U-bottom plates for examination of the growth of *B. subtilis* BT2



Figure 2.2. Growth of *B. subtilis* BT2 on nutrient agar medium contain SCG (1% w/v)

To examine the effect of different oil sources on lipase production, 1% dried SCG, 1-2% olive oil, and 1% tributyrin were added to the nutrient broth. The surfactant effect was also investigated by adding 0.1% Tween80 to the same media. All trials were carried out at 37 °C and 180 rpm for 48 hours by using 50 mL growth medium in 250 mL flask. At the completion of the cultivation period, the fermentation broth was centrifuged at 12000 rpm for 15 minutes at 4 °C to remove cells, and supernatant was used as extracellular crude lipase. Finally, lipase activity and protein concentration of each medium were determined according to the method specified in section 2.2.7.1., and 2.2.7.2, respectively.

2.2.3. Characterization of lipase

2.2.3.1. Effect of temperature on lipase activity

2 mL substrate solution prepared by dissolving pNPP in 50 mM potassium phosphate buffer at pH 7 was mixed with 0.1 mL enzyme solution to investigate the influence of temperature on crude lipase and to identify the optimal temperature of crude enzyme. At temperatures ranging from 20 to 80 °C, the reaction mixture was incubated for 3 minutes. The optical density of the solution was measured at a wavelength of 410 nm. The highest activity value was accepted as 100%. Activities of others were calculated as relative activities.

2.2.3.2. Effect of pH on lipase activity

The buffers at pH ranges between 4.0 and 10.0 were used to find the optimal pH of the crude enzyme. For pH 4.0, pH 5.6, pH 7.0-8.0, and pH 8.5-10.0, 50 mM concentrations of citrate buffer, acetate buffer, phosphate buffer, and glycine-NaOH buffer were utilized, respectively. 2 mL substrate solution was mixed with 0.1 mL enzyme solution and incubated at 37 °C after dissolving pNPP in the above-mentioned buffers. The optical density of the solution was measured at a wavelength of 410 nm. The highest activity value was accepted as 100% and other activities were calculated as relative activities

3.3.3. Fatty acid sugar ester synthesis

According to Esra Büyük's thesis, 2.5 mL concentrated crude lipases from *C. diffluens* D44 using SCG were incubated with 1.66 g fructose and 200 µl vinyl decanoate in a 100 mL Erlenmeyer flask for 48 hours at 50 rpm and 50°C. In order to stop the reaction, 2 mL of hot water was put into the flask at the end of incubation. The organic phase was separated using 3.5 mL of ethyl acetate.

Since *C. diffluens* D44 crude lipase was used to synthesize sugar ester previously (Büyük, 2020), we investigated the lipase synthesized by *B. subtilis* strain BT2 from SCG for sugar ester synthesis. In this study, 45 mL spent coffee grounds sugar was used instead of fructose as a sugar source, and 500 µl spent coffee grounds oil was used instead of vinyl decanoate as a fat source. In addition, *B. subtilis* strain BT2 was used instead of *C. diffluens* D44 as a lipase producer. Incubation parameters and separation were kept the same, adhering to the previous work. After separating the organic phase, the organic phase was submitted to TLC and FTIR analysis to assess the synthesis of sugar esters.

2.2.5. Analytical methods

2.2.5.1. Lipase activity assay

The method described by Büyük's Master Thesis was modified and used to determine lipase activity (Büyük, 2020). 30 mg of pNPP was dissolved in 10 mL propan-2-ol and emulsified in 90 mL of 0.05 M potassium phosphate buffer pH 7.0, including 500 mg Triton X-100 for substrate preparation. 0.1 mL of enzyme solution was blended with 2

mL of pNPP-containing emulsion and incubated at 37 °C for 3 minutes. The absorbance was then spectrophotometrically measured at 410 nm against a substrate-free solution containing enzyme as a blank. The calibration curve was prepared by graphing various 4-nitrophenyl concentrations to calculate enzyme activity (**Appendix A**). Activity of samples was calculated by using the following equation.

$$Activity = \frac{\left(\frac{OD(410)}{\text{Slope of calibration curve}}\right) * \text{Dilution Factor} * \text{Reaction Volume (ml)}}{\text{Volume of Enzyme Solution (mL)} \times \text{Reaction Time (min)}} \dots\dots\dots (2.2)$$

“One unit of lipase activity (U) was defined as the amount of enzyme that liberates 1 μmol p-nitrophenyl for 1 minute under the assay conditions”. Activity assay was performed with duplicate measurement for each sample.

Additionally, enzyme activity was also measured using the oil emulsion technique according to the method of Soares et al. (1999) in order to ensure lipase production. 10 mL olive oil was mixed with 10 mL distilled water to make the substrate solution. The test mixture including 5 mL of the substrate, 2 mL of 50 mM sodium phosphate buffer (pH 7.0), and 100 μL of enzyme solution was incubated for 5 min at 37 °C. The reaction was stopped by adding 3 mL of acetone, ethanol, and water mixture (1:1:1) to roughly 0.3 g of the reaction solution, and the quantity of liberated fatty acids was determined by titrating the solution with 0.04 N KOH using phenolphthalein as an indicator. Under the assay conditions, one unit of enzyme activity was defined as the number of moles of free fatty acids released per mL per minute.

2.2.5.2. Measurement of protein concentration

The Bradford technique was used to determine the protein concentration (Bradford, 1976). For preparing the Bradford reagent, 50 mg of Coomassie Blue G250 was dissolved in 50 mL of methanol and 100 mL of 85% phosphoric acid (H₃PO₄) was added to the solution. Then, distilled water was used to adjust the total amount to 1 L and filtered to remove precipitates. For protein measurement, the enzyme-containing samples were mixed with Bradford reagent at a 1:1 (v/v) ratio and incubated for 15 minutes in the dark

at room temperature. The protein content was determined using bovine serum albumin as a reference and the absorbance of the samples was measured at 595 nm (Appendix A).

2.2.5.3. Determination of reducing sugar

The fermentable sugar content extracted from SCG was measured by using reducing sugar analysis. 3,5-dinitrosalicylic acid (DNS) technique was applied to detect the amount of total reducing sugar (Miller, 1959). For preparing DNS solution, 2.5 g of dinitro salicylic acid was dissolved in 50 mL of 2 M sodium hydroxide, then 75 g sodium potassium tartrate was added and agitated until completely dissolved to make DNS solution. Finally, distilled water is used to adjust the total amount to 250 mL. The standard curve of reducing sugar that is given in Appendix A. was prepared by using different concentrations of glucose solution (0.1-1.0 mg/mL) in distilled water.

To prepare the calibration curve, 1 mL from each concentration of glucose solution and 3 mL of DNS reagent were mixed and placed in a boiling water bath for 15 min. 1 mL of each glucose solution concentration was combined with 3 mL of DNS reagent and put in a boiling water bath for 15 minutes. It was promptly cooled after boiling, and 4 mL of distilled water was added. The absorbance of samples was measured using a spectrophotometer at 540 nm after it was vortexed. To create a standard curve, the glucose concentration was plotted against the absorbance. The curve's slope was computed and utilized in the calculations. According to the procedure described above, the amount of reducing sugar in a sample solution was also determined.

2.2.5.4. Determination of total phenolic compounds

The total phenolic content of extracts was determined in accordance with a protocol described by Yu et al. (2003). The total phenolic contents in the sugar extract solution of SCG were estimated using Folin–Ciocalteu reagent. In brief, the reaction mixture contained 50 µl sample, 250 µl of Folin–Ciocalteu reagent, 500 µl of 20% sodium carbonate, and 4.2 mL of pure water were kept in dark at ambient conditions for 30 minutes to complete the reaction. After 30 minutes, the absorbance at 765 nm was measured and used to calculate the phenolic contents. The calibration curve (Appendix A) prepared by using gallic acid as a standard was used to calculate phenolic content.

The unit of results were expressed as mg gallic acid (GAE)/g sugar extract of SCG.

2.2.5.5. Determination of fatty acid sugar esters by FTIR analysis

FTIR analysis was performed by commercial customer services of the Center for Nanotechnology and Biomaterials Applications and Research at Marmara University. FTIR analysis of the sample was carried out using a JASCO FT/IR-4700 (Japan) spectrophotometer in the range of 400–4000 cm^{-1} with sixteen scans per measurement.

2.2.5.6. Surface Morphology Analysis of SCG and SCG Variations by Scanning Electron Microscopy (SEM)

SEM analysis of SCG and SCG variations were performed in Assoc. Prof. Dr. Selim Ceylan's laboratory at Ondokuz Mayıs University in order to compare variations of SCG and to evaluate the utilization of SCG by microorganisms. These variations consisted of 5 samples: SCG remaining from oil extraction, SCG remaining from sugar extraction, SCG remaining from oil and sugar extraction, fermented SCG by bacteria, and fermented SCG by yeast.

3. RESULT AND DISCUSSION

3.1. Drying SCG, and Extraction of Oil and Reducing Sugar from dried SCG

3.1.1. Effect of SCG amount and temperature on drying of SCG

To determine the oil and reducing sugar content of SCG, all SCG used through this thesis was dried. For the determination of the best condition for the drying process, the effects of the amount of SCG, temperature, and brand type were evaluated.

First of all, the effect of the amount of SCG was evaluated (**Figure 3.1** and **Figure 3.2**) at constant temperature (50°C) by using SCG from same brand. As seen from **Figure 3.1** (Raw data is given in Appendix B, Table B.1.), the mass of SCG was decreased linearly during the first two hours at 10 and 15 gram of SCG. Then, the weight loss rate was reduced and reached the plateau after 5 hours of heat treatment. However, at 20 and 25 grams of SCG, linear loss in amount of the SCG was observed up to 5.5 hours of drying. According to these results, we assumed that the weight loss was associated with water loss and overall, 60% of water (**Figure 3.2**) was lost at all SCG weights studied.

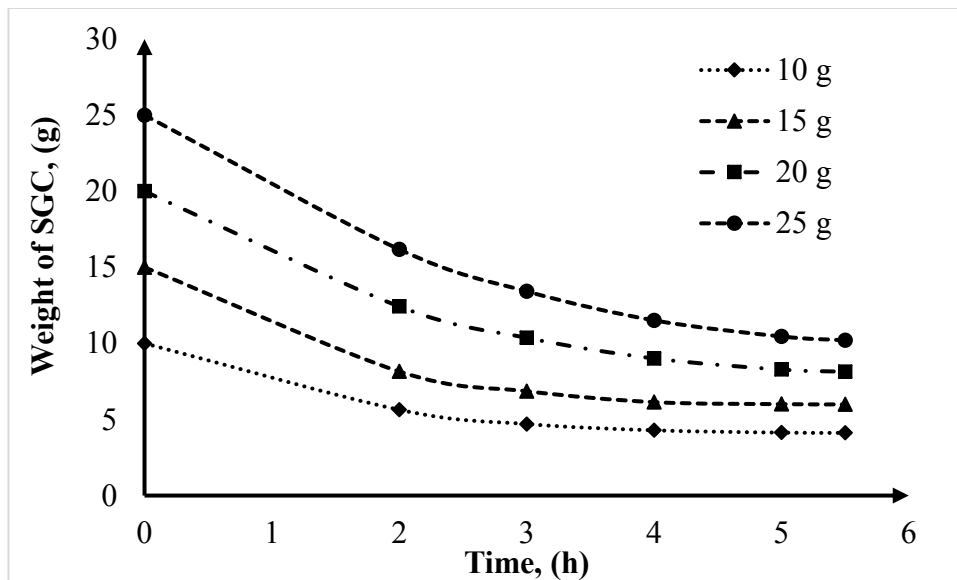


Figure 3.1. Weight loss profile of SCG (X brand) at 50 ° C and different SCG amounts

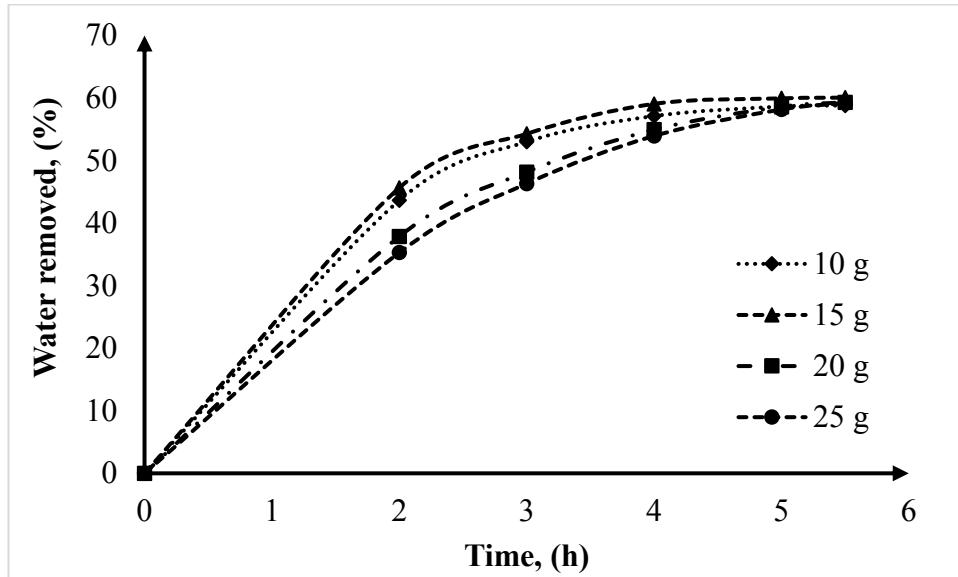


Figure 3.2. Water loss profile of SCG (X brand) at 50⁰ C and different SCG weight

Since the increase in the humidity ratio was parallel to the increase in SCG amounts, 25 grams of SCG were used for further experiments to evaluate the effect of temperature on drying processes (**Figure 3.3** and **Figure 3.4**). Raw data is given in Appendix B, Table B.2.

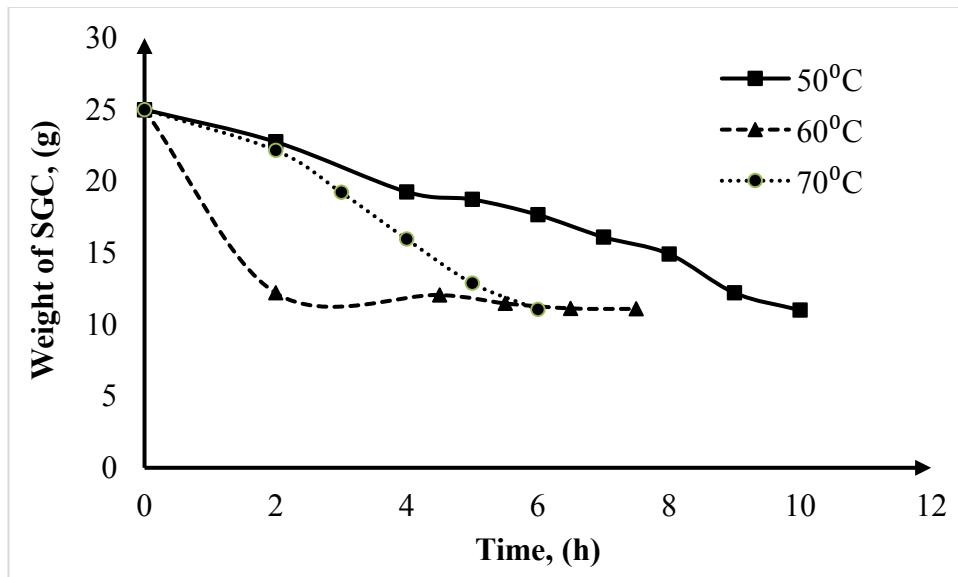


Figure 3.3. Effect of temperature on weight loss profile of SCG (Y brand)

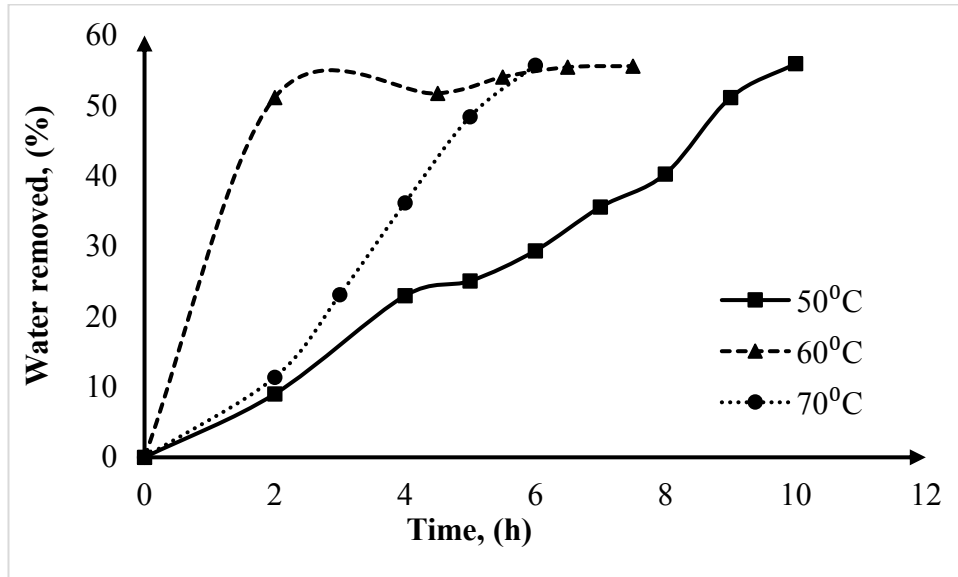


Figure 3.4. Effect of temperature on water loss profile of SCG (Y brand)

According to experimental results, as the drying temperature increased from 50^o C to 60^o C, the physically bound water evaporated faster (**Figure 3.3** and **Figure 3.4**), and 60% water loss was observed within 2 hours of drying. Prolonged drying at 60^oC did not increase water loss. However, at 50 and 70^oC, within the first two hours, similar water loss was observed. After two hours of drying, while the weight loss rate decreased at 50^oC, it continued to increase at 70^oC. These results made us think that 50^oC was not enough to remove water content, and weight loss at 70^oC could be associated with the deterioration in the structure of SCG. Since the effect of drying temperature affects the organic content of SCG (Rocha et al., 2021; Cervera-Mata et al., 2022), SCG was dried at 60^o C in each subsequent experiment in order to preserve the organic content of SCG. Furthermore, we investigated the effect of different brands on decreasing the humidity of SCG. Since the grinding coffee bean, the brewing conditions, and the storage conditions directly affect SCG, the effect of brands on the humidity removal rate was evaluated by using three different brands (**Figure 3.5** and **Figure 3.6**). Raw data is given in Appendix B, Table B.3.

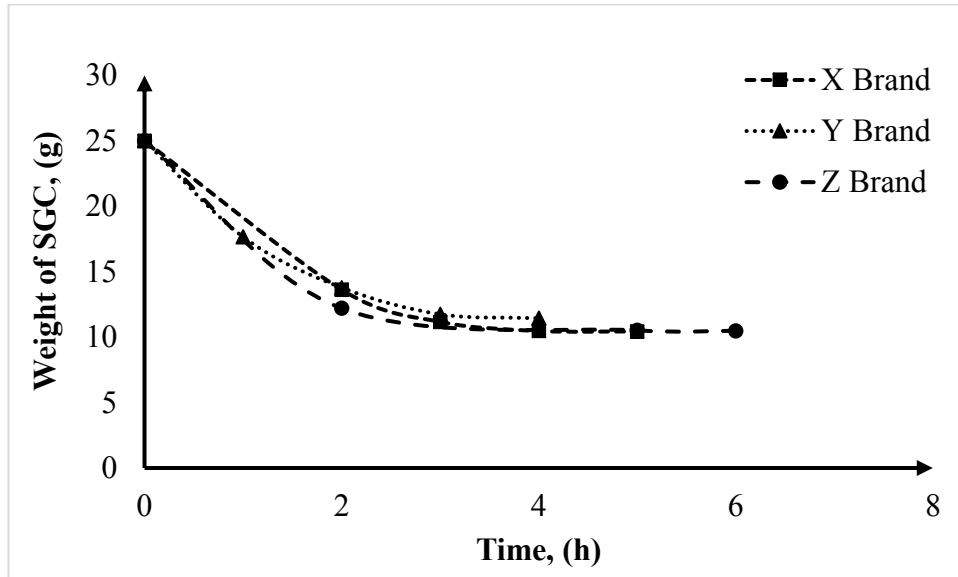


Figure 3.5. Effect of brand on weight loss profile of SCG (25 g SCG at 60⁰ C)

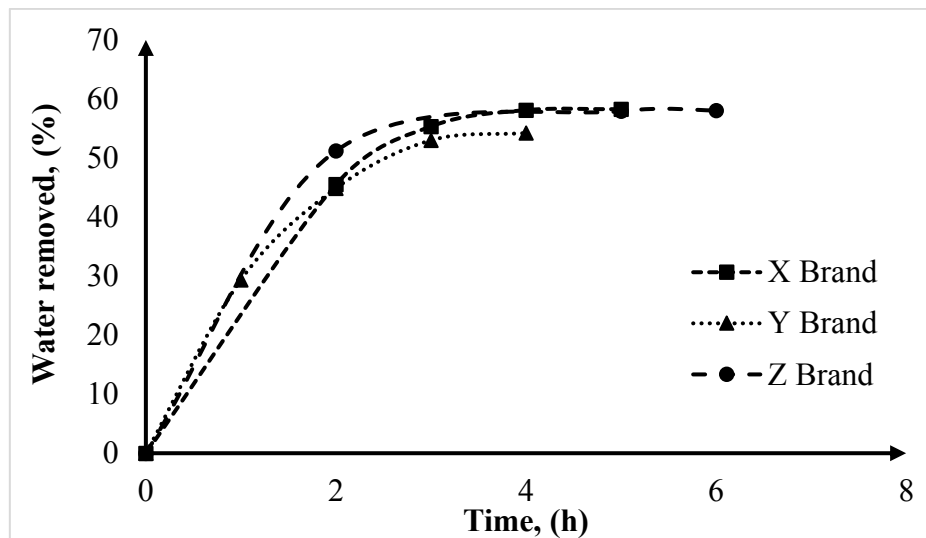


Figure 3.6. Effect of brand on water loss period of SCG (25 g SCG at 60⁰ C)

As seen from **Figure 3.5**, the similar weight loss profile was observed for SCG obtained from different brands. According to this profile, we assumed that the water evaporation rate was not affected by the brand.

In general, approximately 60% of the collected SCG was water. It is also consistent with the results obtained by previous researchers (Son et al., 2018; Tarigan et al., 2019). Because of the high-water content, dried SCG was used in further experiments.

3.1.2. Oil extraction from spent coffee grounds

To determine the oil content of SCG, oil extraction was carried out in Soxhlet apparatus using sole hexane and hexane: isopropanol mixture (1:1, v/v) (**Figure 3.7**).

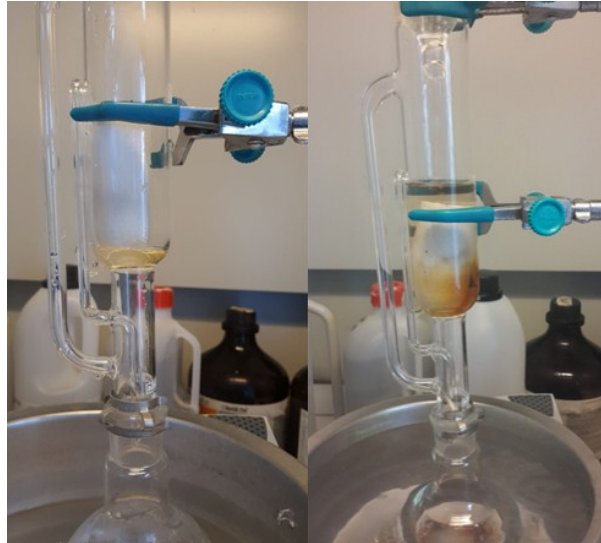


Figure 3.7. Extraction of oil from SCG by Soxhlet extraction apparatus

In the previous study by Caetano et al. (2012), hexane, isopropanol, and combinations of the two in different ratios (v/v; 50:50, 60:40, 70:30, and 80:20), as well as heptane, octane, and ethanol, were used to assess oil extraction rates. The best results were obtained from the hexane: isopropanol (1:1, v/v) mixture. Therefore, sole hexane and hexane: isopropanol mixture (1:1, v/v) were used. When sole hexane was used, 0.17 mL coffee oil/gram dried SCG was obtained, while hexane: isopropanol (1:1 v/v) yielded 0.2 mL coffee oil/gram dried SCG.

The antibacterial impact of the coffee oil extracted was also determined using the disk diffusion test (**Figure 3.8**) (Raba et al., 2015). As a microbial agent, *B. subtilis* BT2 was used. The positive control was 10 µg/mL kanamycin sulfate, whereas the negative control was sterile water.

According to the results of the experiment, the extracted coffee oil did not show an antibacterial effect on *B. subtilis* BT2 compared to the control as seen in the **Figure 3.8**.

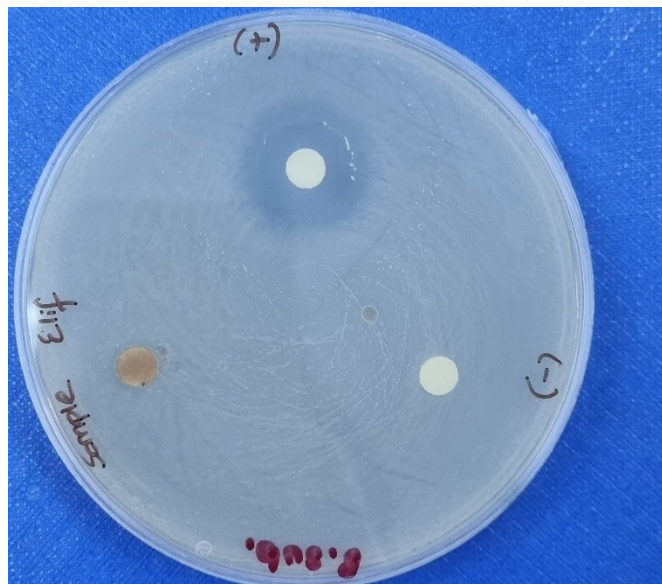


Figure 3.8. The anti-bacterial effect of spent coffee grounds oil

Raba et. al (2015) studied the antimicrobial effect of crude and high-temperature heated coffee oil. As a source of coffee oil, they used crude green coffee bean and roasted coffee bean and heated crude green bean, and heated roasted coffee. In the result of their study, after 6 hours of incubation, the quantifiable inhibition zone of *Enterococcus faecalis*, *Staphylococcus aureus* was observed. The inhibition zone of *Escherichia coli* was reported as smaller against all coffee oil samples compared to the control. They also reported that except *Salmonella*, *S. flexneri*, and *S. aureus*, no visible or quantifiable inhibitory zones were identified after 18 hours of incubation for the majority of examined bacterial species. Their findings indicated that neither Gram-positive nor Gram-negative bacteria are susceptible to the chemical components found in coffee oils. Similar to our results, the researched coffee oils had statistically negligible antibacterial action against all microbial strains tested as compared to the control (Raba et al., 2015).

3.1.3. Extraction of sugar and phenolic compounds from SCG

Cellulose, hemicellulose, and lignin are the primary components of plant cell walls in lignocellulosic biomass. Hemicellulose is the second most abundant natural carbohydrate biopolymer after cellulose, accounting for roughly 20–30 wt. % of plant material studied. Hemicellulose is a complex branching heteropolymer made of pentoses (xylose and arabinose), hexoses (mannose, glucose, and galactose) glucuronic acid, and a tiny

amount of rhamnose and fucose units (Ruiz et al., 2013). Since hemicellulose is tightly linked to cellulose and lignin in plant cell walls, pretreatment of lignocellulosic biomass is generally conducted before modification. To extract hemicellulose, chemical, physical, and biological pretreatments have been developed (Farhat et al., 2017). Since SCG contains 36.7% of hemicellulose, 21.2% of mannan, 13.8% of galactan, 8.6% of cellulose, and 1.7% of arabinan (Mussatto et al., 2011), in the present work, we examined the reducing sugar content of SCG that would be used for the growth of the microorganism and enzyme production.

As a result of all sugar extraction experiments performed according to the methods of previous studies, similar results, summarized in the **Table 3.1**, were obtained. According to our experimental results, the highest sugar yield as 38.16 ± 1.0 and 38.15 ± 1.7 g sugar/g SCG were obtained with 2.5 M HCl and 3% H₂SO₄. Go et al. (2016) investigated the hydrolysis of SCG by using sulfuric acid (3–5 %v/v) as hydrolyzing medium, over a period of 30–180 min at a solvent to solid ratio of 10 mL/g at 95 °C. They found that the optimum yield (26 and 31 g/g) of the available reducing sugar was achieved by using an acid concentration of 4% and a hydrolysis time of 120 min. According to the study by Ballesteros et al. (2015), 4 M 4L sodium hydroxide has mixed with 100 g of SCG and left overnight at room temperature, then centrifuged at 9700 g for 15 min at 4 °C. Three distinct polysaccharide extraction yields (Y1, Y2, and Y3) were identified, which might reflect crucial process economic characteristics.

Y1 denotes the total yield of the extraction, which is identified as g of lyophilized material per 100 g SCG; Y2 denotes the quantity of sugars extracted, which is identified as g of total sugars present in the lyophilized material per 100 g SCG; and finally, Y3 denotes the yield of the quantity of sugar extracted with regard to the total sugars present in the SCG, which is identified as g of total sugars in the lyophilized material per 100 of sugars from SCG. The overall sugar content recovered from SCG (lyophilized material) was 39%, while Y1, Y2, and Y3 were 6.05, 2.38, and 4.57 % (w/w), respectively (Ballesteros et al., 2015). They also used the autohydrolysis method for the extraction of polysaccharides from SCG in another study. Their ideal process conditions were 15 mL water/g SCG for 10 minutes at 160 °C. They found that a lyophilized material comprising 29.29 % (w/w) polysaccharides.

Table 3.1. Effect of extraction methods on sugar extraction from SCG

Type of Acid/Base	Dry SCG/acid or base (gr/mL)	Dry SCG/solution (gr/mL)	Temp. (°C)	Incubation time (min.)	Yield (% , gr reducing sugar/ gr SCG)	Yield (% , gr reducing sugar/ gr SCG)	Yield (% , gr reducing sugar/ gr SCG)	Yield (Avg.) (% , gr reducing sugar/ gr SCG)
2.5 M HCL	1/3.8	1/50	100	180	37.20	38.99	37.32	38.16 ± 1.0
%3 H ₂ SO ₄	1/0.3	1/10	121	60	55.06	39.82	36.47	38.15 ± 1.7
%2 H ₂ SO ₄	1/0.2	1/10	121	60	20.09	34.71	33.75	34.23 ± 0.5
%4 H ₂ SO ₄	1/0.4	1/10	121	180	60.25	36.64	29.78	33.21 ± 3.4
%4 H ₂ SO ₄	1/0.4	1/10	121	60	36.57	34.39	30.55	32.47 ± 1.9
%4 H ₂ SO ₄	1/0.4	1/10	121	120	49.85	32.75	31.84	32.29 ± 0.5
%3 H ₂ SO ₄	1/0.3	1/10	121	120	47.64	30.46	31.23	30.85 ± 0.3
%1 H ₂ SO ₄	1/0.1	1/10	121	60	29.05	30.71	30.87	30.79 ± 0.0
%2 H ₂ SO ₄	1/0.2	1/10	121	120	50.61	31.38	29.86	30.62 ± 0.8
%3 H ₂ SO ₄	1/0.3	1/10	121	180	68.15	31.33	29.78	30.56 ± 0.8
%1 H ₂ SO ₄	1/0.1	1/10	121	120	41.01	30.92	28.79	29.86 ± 1.1
%2 H ₂ SO ₄	1/0.2	1/10	121	180	66.47	30.69	28.74	29.71 ± 1.0
%1 H ₂ SO ₄	1/0.1	1/10	121	180	58.56	28.48	27.58	28.03 ± 0.5
%4 H ₂ SO ₄	1/0.4	1/10	95	120	39.11	21.23	19.52	20.37 ± 0.9
%5 H ₂ SO ₄	1/0.5	1/8	95	180	1.62	15.52	14.15	14.84 ± 0.7
%0.5 H ₂ SO ₄	1/0.05	1/8	163	45	9.95	9.29	8.18	8.73 ± 0.6
4 M NaOH	1/0.6038	1/8	25	Overnight	9.96	9.46	7.75	8.60 ± 0.9
Water	1/15	1/10	160	10	1.89	1.51	1.44	1.48 ± 0.0

Comparing our results of autohydrolysis with that obtained by Ballesteros et al., (2015), the result of our autohydrolysis study was approximately 20 times lower than their findings. The reason for this may be the difference in the equipment and the coffee beans used. They carried out their experiments in a 160-mL cylindrical stainless-steel reactor when autohydrolysis extraction from SCG was carried out by placing it in a 250 mL Schott bottle and then heating by the oven in my experiment.

In the study of Juarez et al. (2017), 29% (v/v) total reducing sugar per SCG was obtained at 5% v/v H₂SO₄ with a hydrolysis time of 3 h at 95 °C. According to the investigation by Mussatto et al. (2011), the best conditions for extracting hemicellulose sugars were 100 mg acid/g dry matter, 10 g liquid/g solid, and 163 °C for 45 minutes. Since the two highest results were obtained in 2.5 M HCL and 3% H₂SO₄, the second set of experiments was determined with two different acid solutions under different conditions as seen in the **Table 3.2**.

Evaluating the results of the previous experiments, the temperature was kept constant at 121 °C to find the approximate optimum values for sugar extraction from spent coffee grounds and all experiments were carried out in an autoclave device. Sugar extraction from SCG was performed with dry SCG/solution (gr/mL) ratio of 0.02, 0.025, 0.05, 0.1, 0.2, and incubation time (hours) of 1 and 3 hours, and the results are summarized in **Table 3.2**. At the same time, the ratio of total phenolic compounds of the extract obtained as a result of each experiment was analyzed as gallic acid equivalence and shown in **Table 3.3**.

As seen from **Table 3.2**, the highest sugar yield as 39.11 ± 1.8 and 38.43 ± 4.7 (% reducing sugar/gr SCG) were obtained when 3% H₂SO₄ solution was mixed at the rates of 0.02 dry SCG/solution (gr/mL) and 0.1 dry SCG/solution (gr/mL), respectively.

Acid hydrolysis is one of the often-employed chemical treatment methods. Various organic or inorganic acids, such as hydrochloric acid, sulfuric acid, nitric acid, oxalic acid, and formic acid, are utilized in the processing of lignocellulosic materials. When compared to these other acid pretreatments, dilute sulfuric acid hydrolysis is the most often employed (Baruah et al., 2018).

Table 3.2. Effect of process parameters on sugar extraction from SCG by using 2.5 M HCL and 3% H₂SO₄

Type of Acid/Base	Dry SCG/acid or base (gr/mL)	Dry SCG/solution (gr/mL)	Temp. (°C)	Incubation time (min.)	Yield (% , gr reducing sugar/ gr SCG)	Yield (% , gr reducing sugar/ gr SCG)	Yield (% , gr reducing sugar/ gr SCG)	Yield (Avg.) (% , gr reducing sugar/ gr SCG)
%3 H ₂ SO ₄	1/1.5	1/50	121	60	37.27	39.35	40.73	39.11 ± 1.8
%3 H ₂ SO ₄	1/0.3	1/10	121	60	33.31	42.57	39.41	38.43 ± 4.7
2.5 M HCl	1/3.8	1/50	121	60	37.15	35.73	36.37	36.42 ± 0.7
%3 H ₂ SO ₄	1/0.15	1/5	121	60	31.53	38.93	37.22	35.89 ± 3.9
2.5 M HCl	1/0.38	1/5	121	60	25.91	35.19	36.15	32.41 ± 5.7
2.5 M HCl	1/0.76	1/10	121	60	27.03	34.63	34.07	31.91 ± 4.2
%3 H ₂ SO ₄	1/0.6	1/20	121	60	21.86	37.19	35.72	31.59 ± 8.5
2.5 M HCl	1/1.52	1/20	121	60	21.71	35.19	33.47	30.12 ± 7.3
%3 H ₂ SO ₄	1/1.2	1/40	121	60	13.33	35.61	34.46	27.80 ± 12.5
2.5 M HCl	1/3.8	1/50	121	180	29.95	34.26	14.50	26.24 ± 10.3
2.5 M HCl	1/3.04	1/40	121	180	29.17	33.05	15.00	25.74 ± 9.5
2.5 M HCl	1/0.76	1/10	121	180	22.16	28.10	26.22	25.50 ± 3.0
2.5 M HCl	1/3.04	1/40	121	60	12.34	28.45	27.93	22.91 ± 9.2
2.5 M HCl	1/0.38	1/5	121	180	19.42	23.48	23.36	22.09 ± 2.3
2.5 M HCl	1/1.52	1/20	121	180	11.65	26.50	24.45	20.87 ± 8.0

Yet, the breakdown of lignin and hemicellulose may result in the production of several by-products by acid hydrolysis, including phenolic acids and furfurals (Brodeur et al., 2011). These compounds may be detrimental to subsequent microbial growth (Gao et al., 2017). Higher acid concentrations and/or operating temperature have been linked to increased formation of by-product. Moreover, high acid concentrations could be corrosive to equipments (Su et al., 2019). As a result, sugar extraction conditions from SCG must be optimized to limit by-product formation while maintaining high sugar yields.

As seen in **Table 3.3**, the highest yield of total phenolic compounds was obtained as 11.20 ± 0.6 (% mg of GAE/ g dry SCG) when 2.5M HCl solution was mixed at the rates of 0.02 dry SCG/solution (gr/mL). The second high yield of total phenolic compounds was obtained as 9.95 ± 0.3 (% mg of GAE/ g dry SCG) when 3% H₂SO₄ solution was mixed at the rates of 0.02 dry SCG/solution (gr/mL). Therefore, the second highest sugar extraction conditions, % 3 H₂SO₄ solution was mixed at the rates of 0.1 dry SCG/solution (gr/mL), were accepted as the optimum value in order to reduce the total phenolic compounds ratio.

3.2. Interference Between SCG and Lipase Activity Determination

In the present work, lipase activity was measured spectrophotometrically to demonstrate that the microorganism produces lipase to break down the oil of SCG according to method **2.2.11.1**. During the spectrophotometric measurements, to set the instrument to zero, a blank is required for any enzyme assay. Otherwise, the response can occur outside the system's field of observation. Normally, the assay combination without the initiating component is considered as the blank and it should be proven that the blank stays the same during the measurement time. However, lipase enzyme produced from SCG is brown in color due to SCG pigments (**Figure 3.9**). Plant extracts typically have intense colors because they include naturally occurring, highly conjugated pigments. In spectrophotometric experiments, the coloration of plant extracts might interfere with the determination of absorbance (Lankatillake et al., 2021) and there is a significant deviation in the blank, which might affect how the reaction proceeds and the assay's result, occasionally. These interactions will affect the assay solution, especially if it is held for a longer period over an extensive test series. Therefore, the reason for the deviation should be determined and, to the extent possible, removed (Bisswanger, 2014).

Table 3.3. Amount of phenolic compounds formed as a result of sugar extraction

Type of Acid/Base	Dry SCG/acid or base (gr/mL)	Dry SCG/solution (gr/mL)	Temp. (°C)	Incubation Time (min.)	GAE (mg of GAE/ g dry SCG)	GAE (mg of GAE/ g dry SCG)	GAE (mg of GAE/ g dry SCG)	GAE (Avg.) (mg of GAE/ g dry SCG)
2.5 M HCl	1/3.8	1/50	121	180	11.87	10.66	11.06	11.20 ± 0.6
%3 H ₂ SO ₄	1/1.5	1/50	121	60	10.30	9.80	9.74	9.95 ± 0.3
2.5 M HCl	1/3.04	1/40	121	180	9.66	9.57	10.24	9.82 ± 0.4
%3 H ₂ SO ₄	1/1.2	1/40	121	60	9.44	8.95	8.33	8.91 ± 0.6
2.5 M HCl	1/3.8	1/50	121	60	8.51	9.20	8.99	8.90 ± 0.4
2.5 M HCl	1/3.04	1/40	121	60	8.26	7.94	8.46	8.22 ± 0.3
2.5 M HCl	1/1.52	1/20	121	180	7.90	7.55	8.14	7.86 ± 0.3
%3 H ₂ SO ₄	1/0.6	1/20	121	60	8.13	7.51	7.63	7.76 ± 0.3
2.5 M HCl	1/1.52	1/20	121	60	6.67	6.70	6.83	6.74 ± 0.1
2.5 M HCl	1/0.76	1/10	121	180	5.32	5.27	5.69	5.42 ± 0.2
%3 H ₂ SO ₄	1/0.3	1/10	121	60	5.25	5.22	5.36	5.28 ± 0.1
2.5 M HCl	1/0.76	1/10	121	60	5.27	5.14	5.25	5.22 ± 0.1
2.5 M HCl	1/0.38	1/5	121	60	5.21	4.65	5.10	4.99 ± 0.3
%3 H ₂ SO ₄	1/0.15	1/5	121	60	4.02	3.89	4.00	3.97 ± 0.1
2.5 M HCl	1/0.38	1/5	121	180	3.94	3.43	3.92	3.76 ± 0.3

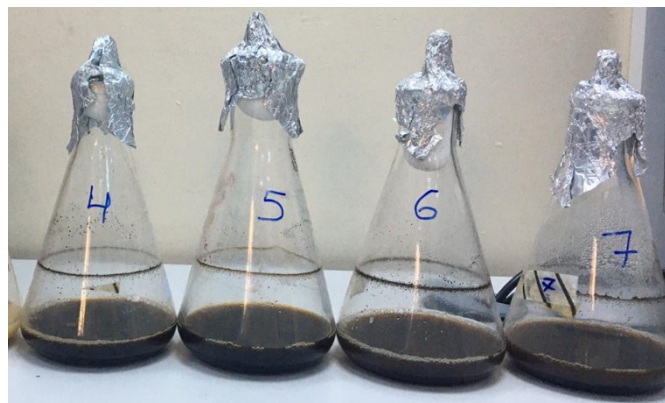


Figure 3.9. Lipase enzyme produced from SCG by *C. diffluens* D44

In the light of the above explanations, the most appropriate blank which yields reproducible results were determined by using different solutions as blank. For lipase activity as described in section 2.2.7.1., pNPP which is a chromogenic substrate was used. For substrate preparation, 3 mg of pNPP was dissolved in 1 mL propan-2-ol and emulsified in 9 mL of 0.05 M potassium phosphate buffer at pH 7.0, including 50 mg Triton X-100. For enzymatic reaction, 2.0 mL substrate solution was mixed with 0.1 mL crude lipase enzyme produced from SCG by bacteria or yeast. For preparing the blank, four different solutions were prepared.

One, denoted as buffer blank was prepared by mixing 2.0 mL phosphate buffer in the absence of pNPP with 0.1 mL lipase from SCG. When using the buffer blank, it showed more enzyme activity than it should, since the blank did not contain any pigment-containing solution and there was no chromogenic substrate in it. To be able to measure in this way, it would be necessary to calculate from the correlation between the absorbance differences using different blank types.

For the second blank, 2.0 mL substrate solution was mixed with the medium used for lipase production and denoted as sample blank. When using the sample blank, a false reading occurred due to the pigment difference between the assayed enzyme and the blank. This may be due to melanoidins. Melanoidins are generated when green coffee beans are roasted. These are the by-products of Maillard reaction which are high molecular weight nitrogenous molecules with a brown color (Moreira et al., 2017). It is known that the Maillard reaction occurs between the amino and carbonyl groups of organic materials non-enzymatically. According to literature, studies for melanoidin degradation are basically divided into three as physico-chemical, enzymatic and

biological (Santal and Singh, 2013) Since melanoidins are degraded during lipase production with the help of microorganism from SCG, it can be said that the color is bleached as seen in **Figure 3.10**. Therefore, the initial sample solution content contains a more intense color. Therefore, the absorbance value was measured as negative, although there was enzyme production when the sample buffer was used.

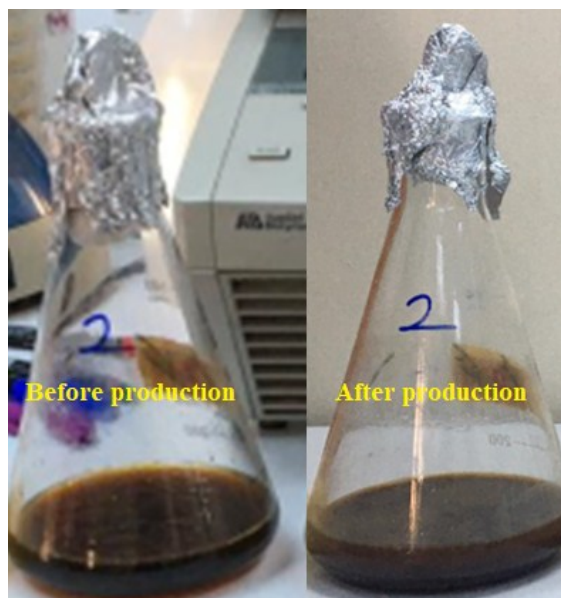


Figure 3.10. Before and after lipase production, production media views

The third blank was prepared by mixing 0.1 ml of inactivated enzyme solution and 2.0 mL substrate solution (denoted as boiled enzyme blank), and the fourth one includes 2.0 mL substrate-free phosphate buffer solution containing Triton X-100 and 0.1 mL lipase from SCG (denoted as enzyme blank). On the other hand, the desired result could not be obtained with the use of the boiled enzyme blank. The reason for this was that the denatured proteins formed turbidity when the enzyme was boiled. As described in section 2.2.7.1, the most successful results were obtained with the use of the enzyme blank. The reason for this is that with the use of enzyme blank, the color difference between the blank and the solution to be assayed has been eliminated. The only difference between the two was the color difference that would occur with the degradation of the chromogenic substrate.

Additionally, to terminate the enzymatic reaction, 1M sodium carbonate solution was used (Bhosale et al., 2021). However, in our work, the color change continues after this solution was added. This may be due to the low salting-out ability of the sodium carbonate

solution in the presence of SCG pigments as in the study of Song et al. (2022). Therefore, since the unreacted protein does not precipitate by the addition of sodium carbonate solution, it continued to actively degrade pNPP. For this reason, the step of adding stop solution was eliminated and the absorbance of the reaction mixture was measured immediately at the end of incubation period.

Although the pigments from the SCG were eliminated by the enzyme blank method, lipase activity was also determined using the oil emulsion technique described in method 2.2.7.1 to prove lipase production (Figure 3.11).



Figure 3.11. Determination of lipase activity by titration of free fatty acids

Finally, the supernatant separated from SCG and biomass by centrifugation at the end of lipase production, which is media containing extracellular lipase enzyme, was subjected to Gas Chromatography – Flame Ionization Detector (GC-FID) analysis to prove the production of lipase enzyme. The analysis was carried out at TÜBİTAK, Marmara Research Center. According to the results of the GC-FID analysis, the composition of fatty acids in the media containing the extracellular lipase enzyme was determined as 31.05% linoleic acid, 28.67% palmitic acid, 20.44% heptadecanoic acid, and 19.84% oleic acid. Although no fatty acid was added to the medium during the experiment, the fact that the microorganism breaks down coffee oil present in SCG and fatty acids released in the production medium. These results represent both the proof of active lipase production using SCG which is the most original aspect of this study. According to previous studies, linoleic (43%), palmitic (33.4%), and oleic (11%) acids have been found to be the main ingredients of SCG's oil (Vu et al., 2021). Of course, although the fatty acid types are basically the same, the difference in the composition may be due to the

type of coffee bean used, and the journey it has lived until it becomes spent coffee grounds, such as growing and roasting. In addition, the composition may have changed as the microorganism metabolizes fatty acids.

According to these results, it is possible to say that biological fatty acid extraction from SCG takes place during lipase enzyme production.

3.3. Production of Lipase from Spent Coffee Grounds by *C. diffluens* D44

To evaluate the utilization of SCG by *C. diffluens* D44, as described in section 2.2.2.1., basal medium including 0.3% malt extract (w/v), 0.3% yeast extract (w/v), and 0.5% peptone (w/v) (Yılmaz and Sayar, 2015) was supplemented with oil extract from SCG, sugar extract from SCG, and sole SCG, and used as lipase production medium (**Figure 3.12.**). The basal medium including (v/v) 2.0% olive oil was used as the control.

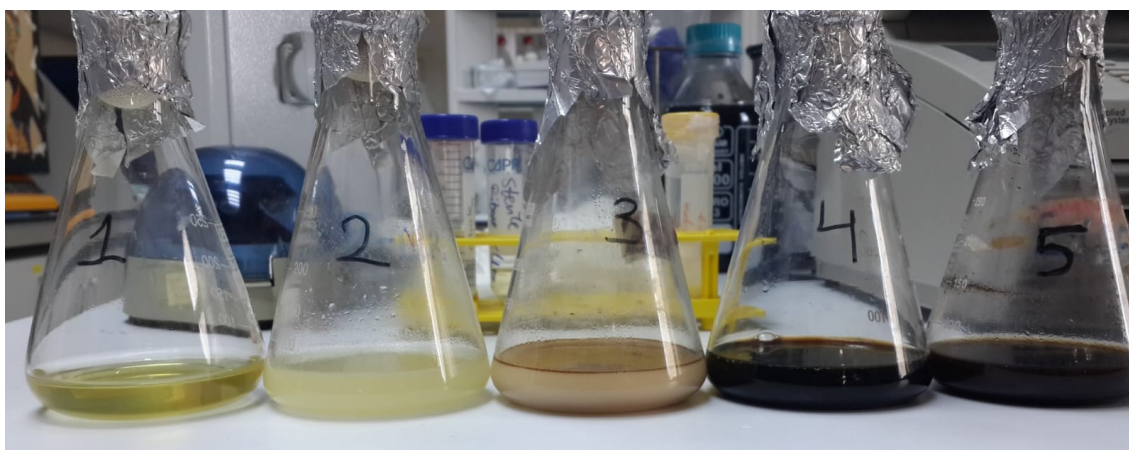


Figure 3.12. Lipase production media supplemented with olive oil, oil extract from SCG, sugar extract from SCG, and sole SCG

As seen in **Table 3.4.**, the lipase activity as 3.08 U/mL was obtained with media supplemented with dried SCG. Although it is almost half of that obtained by olive oil, this result shows that SCG can be used for the production of industrial lipase instead of being a food waste and will serve the national economy by reducing the resource with full recycling.

Table 3.4. Comparison of SCG-derived carbon sources with olive oil

Carbon sources, %	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)
Olive oil (2%, v/v)	6.903	0.057	121.778
Dried SCG (1%, w/v)	3.077	0.013	238.976
Oil extract from SCG (2%, v/v)	2.189	0.011	198.476
Sugar extract from SCG (1%, w/v)	1.169	0.018	63.456

For this reason, the effect of SCG amount, temperature and pH on lipase production from *C. diffluens* D44 was investigated by using Box-Behnken design via Design-Expert program (Table 3.5).

Table 3.5. The results of the experiments designed with the Design-Expert program

Parameters				OD₄₁₀				Activity (U/ml)
Run	Temp. (°C)	Waste (%)	pH	OD1	OD2	OD3	OD (mean)	
7	29.4	4.0	7.6	0.537	0.617	0.666	0.607	14.127
8	33.5	4.0	7.6	0.471	0.432	0.404	0.436	10.145
19	31.5	5.0	7.0	0.408	0.423	0.391	0.407	9.485
18	31.5	2.5	8.0	0.401	0.410	0.407	0.406	9.454
20	29.4	4.0	6.4	0.335	0.372	0.429	0.379	8.818
17	28.0	2.5	7.0	0.356	0.367	0.369	0.364	8.476
1	31.5	2.5	7.0	0.369	0.364	0.331	0.355	8.259
11	33.5	4.0	6.4	0.302	0.299	0.344	0.315	7.335
4	31.5	2.5	7.0	0.317	0.310	0.310	0.312	7.273
12	31.5	2.5	7.0	0.296	0.310	0.307	0.304	7.087
14	31.5	2.5	7.0	0.298	0.300	0.303	0.300	6.994
16	31.5	2.5	7.0	0.306	0.296	0.291	0.298	6.932
3	31.5	2.5	6.0	0.292	0.295	0.291	0.293	6.815
2	35.0	2.5	7.0	0.258	0.336	0.265	0.286	6.668
6	31.5	2.5	7.0	0.283	0.271	0.281	0.278	6.481
5	29.4	1.0	7.6	0.241	0.264	0.277	0.261	6.070
9	33.5	1.0	7.6	0.224	0.235	0.257	0.239	5.558
10	31.5	0.0	7.0	0.229	0.223	0.232	0.228	5.309
15	33.5	1.0	6.4	0.207	0.227	0.212	0.215	5.014
13	29.4	1.0	6.4	0.193	0.189	0.195	0.192	4.479

Analysis of variance (ANOVA) was performed with the help of the Design-Expert program to test the accuracy of the model formed as a result of the experiments designed according to the Response Surface Methodology.

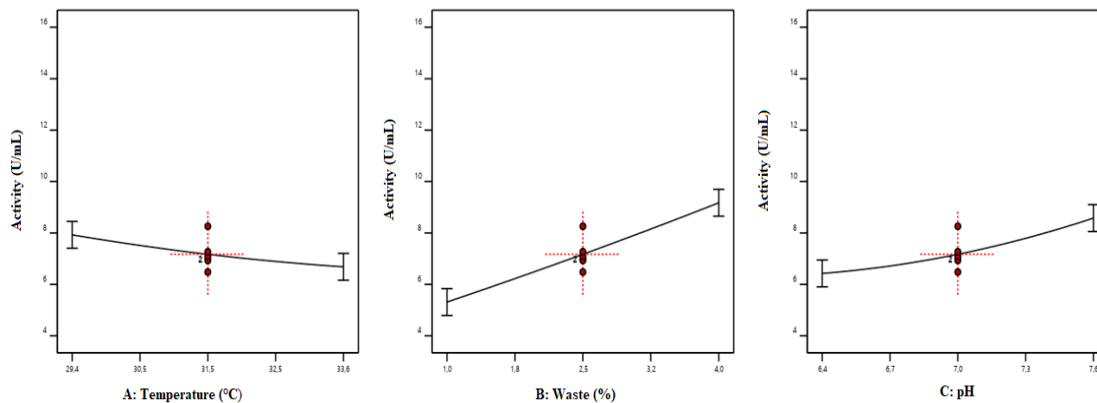


Figure 3.13. Observation of main effects of parameters according to ANOVA

Table 3.6. Result of ANOVA with respect to response surface methodology

Source	Sum of Squares	df	Mean Square	F-value	P-Value
Model	85.45	9	9.270	12.370	0.0003
A- Temperature	5.270	1	5.270	7.030	0.0240
B- Waste	50.840	1	50.840	67.850	< 0.0001
C- pH	15.790	1	15.790	21.070	0.0010
AB	3.750	1	3.750	5.010	0.0492
AC	1.570	1	1.570	2.090	0.1788
BC	4.470	1	4.470	5.970	0.0347
A2	0.261	1	0.261	0.349	0.5681
B2	0.076	1	0.076	0.102	0.7563
C2	1.590	1	1.590	2.130	0.1754
Residual	7.490	10	0.749		
Lack of fit	5.720	5	1.144	3.230	0.1119
Pure Error	1.770	5	0.354		
Cor Total	90.940	19			

When the tables and graphs according to ANOVA were examined, the temperature-waste and waste-pH interaction were low within the specified range. Although lack of fit value is expected, there are still linear effects with the chosen interval according to these results. This means that as the value of the factor increases or decreases, the activity changes

linearly. However, when doing an optimization, we expect it to fit a quadratic polynomial equation, not a linear one. The reason for this is to detect the minimum and maximum activity in the specified range.

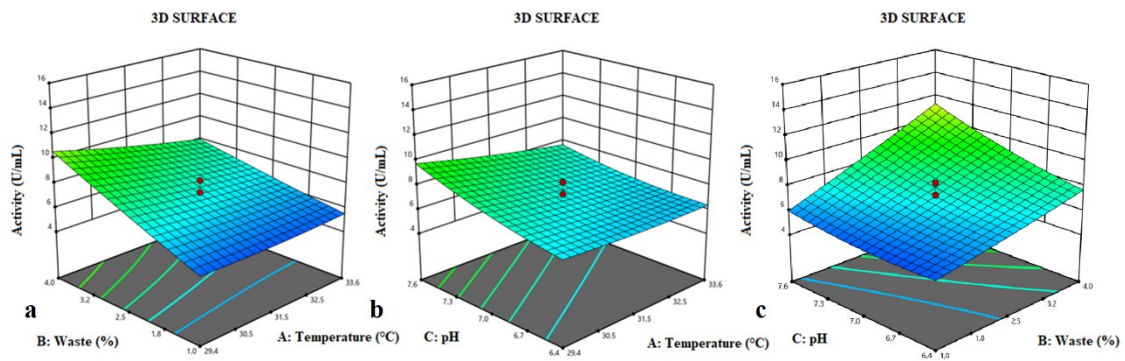


Figure 3.14. Three-dimensional (3D) response surface plots. (a) Activity (U/mL) as a function of waste (%) × temperature (°C); (b) Activity (U/mL) as a function of pH × temperature (°C); (c) Activity (U/mL) as a function of pH × waste (%).

Looking at the result of this design at the moment, the only parameter that approaches this situation seems to be pH. It was determined that the amount of activity decreased as the temperature increased. As the amount of waste increases, the activity continues to increase linearly.

In the situation when the temperature is minimum, the waste amount is maximum, the pH is within its range, and the activity is maximized, a new experimental design has been obtained where the temperature is 29.4 (°C), the waste rate is 4%, and the pH value is close to 7.6. According to the validation report of this design given in **Figure 3.15.** below, if the activity is obtained between 10.7 and 15.7 (U/mL) in the 95% confidence interval, we can say that the optimization process is successful in many perspectives.

Confirmation									
Two-sided Confidence = 95%									
Solution 1 of 49 Response	Predicted Mean	Predicted Median	Observed	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Activity	13,212	13,212		0,865637	1	1,11857	10,7197		15,7044

Figure 3.15. The result of the confirmation table given by the Design of Expert

The results of the experiments redesigned with the Design-Expert program are shown in **Table 3.7**. As seen in the table, the range of enzyme activity was between 10.7 and 15.7 (U/mL). Therefore, the optimization process can be considered successful. According to the results of the experiments carried out so far, the highest lipase enzyme activity produced from spent coffee grounds was found to be 14.6 U/mL with the use of 8.7% spent coffee grounds, while the pH of the production medium was at 8.3 at 29.4 °C. As seen in **Table 3.7.**, enzyme activity increased as the amount of waste increased unless the pH is too low.

Table 3.7. The results of the experiments redesigned with the Design-Expert program

Parameters				Absorbance at OD410				Activity (U/ml)
Run	Temp. (°C)	Waste (%)	pH	OD1	OD2	OD3	OD (mean)	
11	29.4	8.7	8.3	0.626	0.631	0.625	0.627	14.609
10	29.4	10.0	6.5	0.482	0.506	0.504	0.497	11.581
9	29.4	5.5	4.0	0.506	0.488	0.476	0.490	11.411
2	29.4	2.3	8.3	0.472	0.481	0.501	0.485	11.286
3	29.4	5.5	6.5	0.497	0.483	0.472	0.484	11.271
6	29.4	5.5	6.5	0.475	0.485	0.492	0.484	11.271
12	29.4	5.5	6.5	0.478	0.481	0.492	0.484	11.263
7	29.4	5.5	6.5	0.475	0.489	0.479	0.481	11.201
5	29.4	5.5	6.5	0.485	0.471	0.481	0.479	11.154
13	29.4	5.5	9.0	0.473	0.481	0.479	0.478	11.123
8	29.4	8.7	4.7	0.460	0.454	0.467	0.460	10.720
1	29.4	1.0	6.5	0.438	0.464	0.477	0.460	10.704
4	29.4	2.3	4.7	0.441	0.465	0.452	0.453	10.541

3.3.1. Depigmentation of crude enzyme

For pigment removal from crude enzyme solution, protein precipitation was performed using various agents described in **section 2.2.2.1.1**. As seen in **Figure 3.16.**, a lightening was observed in the color of the enzyme solution when ethanol was used as precipitating agent. However, when precipitated proteins were separated by centrifugation, the pigments of SCG remained in the precipitated proteins. The same observations were also

obtained with the precipitation using ammonium sulfate ($(\text{NH}_4)_2 \text{SO}_4$) and acetone. Therefore, the color in crude enzyme solution could not be removed using protein precipitation by ethanol, acetone, and ammonium sulfate.

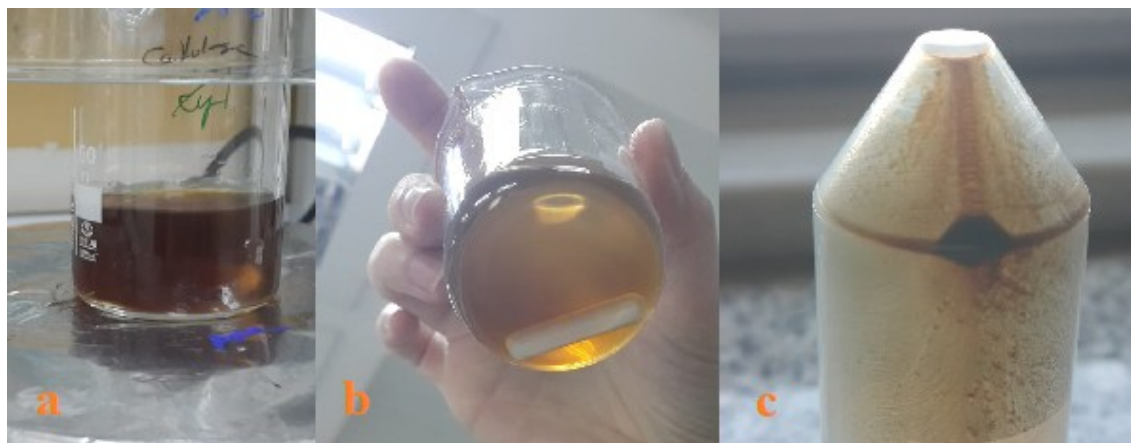


Figure 3.16. Precipitation of proteins by using ethanol

Since the activated carbon is generally used to remove pigments from liquid mixtures, we also evaluated the removal of pigment from crude lipase enzyme. As seen in **Figure 3.17.**, activated carbon was successful in removing color. However, since activated carbon is also used for the immobilization of enzyme (Pinto Brito et al., 2020), activated carbon was also adsorbed the crude lipase.



Figure 3.17. Precipitation of proteins by activated carbon

3.3.2. Characterization of *C. diffluens* D44 lipase from spent coffee grounds

3.3.2.1. Optimum temperature and pH of *C. diffluens* D44 lipase from SCG

During the cultivation of *C. diffluens* D44 with SCG, the organic compounds might be released from SCG to liquid medium. Therefore, we evaluated the effect of temperature and pH on D44 lipase obtained from SCG was investigated (**Figure 3.18** and **Figure 3.19**) and compared with D44 lipase produced from olive oil (Yılmaz and Sayar, 2015).

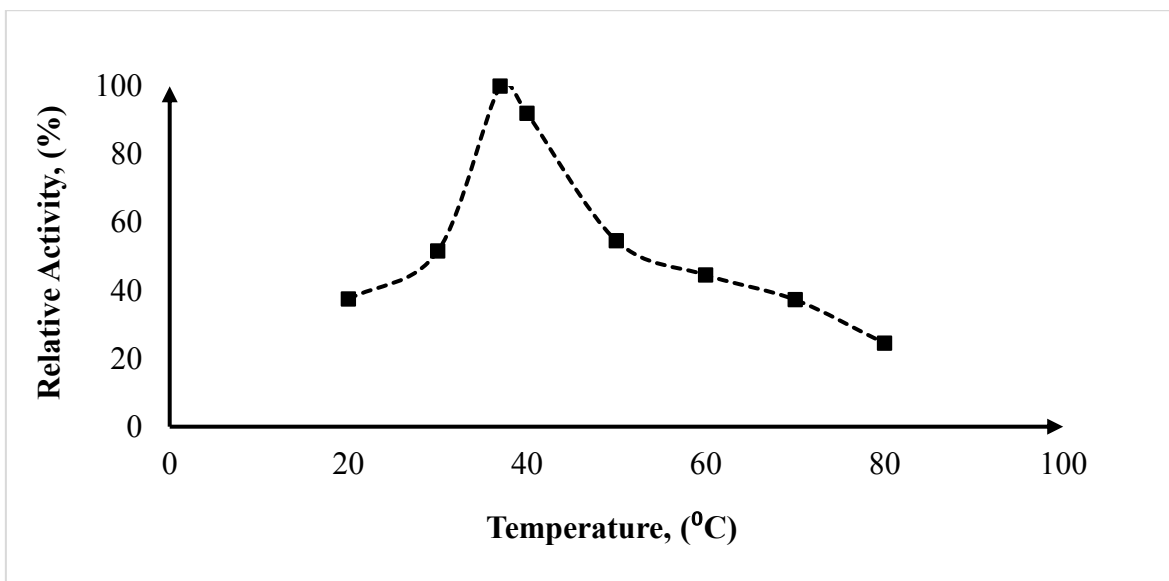


Figure 3.18. Optimum temperature of D44 lipase from SCG

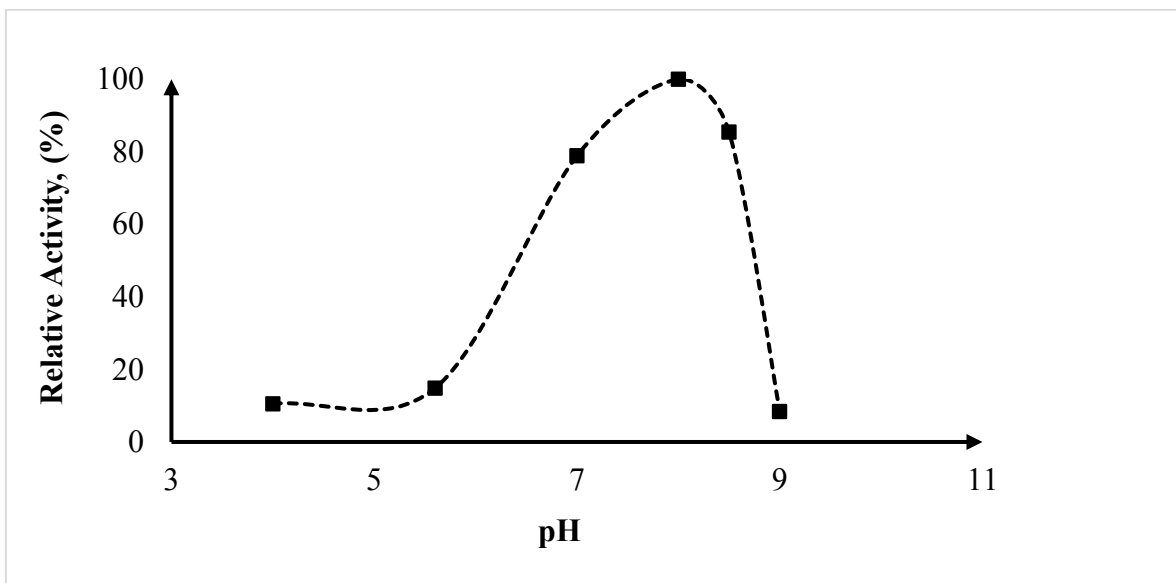


Figure 3.19. Optimum pH of D44 lipase from SCG

According to **Figure 3.18** and **Figure 3.19**, the optimum temperature and pH of D44 lipase produced from SCG were determined as 37 °C and 8.0, respectively (Raw data is given in Appendix B, Table B.4 and B.5). Yılmaz and Sayar (2015) studied the D44 lipase from olive oil, and optimum temperature and pH of D44 lipase were reported as 45°C and pH 9.0. Comparing our results with the results obtained by Sayar and Yılmaz (2015), using SCG as a substrate caused to reduce the optimum temperature and pH of D44 lipase.

According to the study of Kroh and Schumacher (1996), the covalent alteration of various amino acids as well as inter- and intramolecular crosslinking had an impact on enzyme activity. In addition, previous studies have shown that phenolic compounds have inhibitory effects on enzyme activity (Rohn et al., 2002; Martinez-Gonzalez et al., 2017; Yildirim-Elikoglu et al., 2021). Thus, the reduction in optimum temperature and pH of D44 lipase produced from SCG could be explained with the effect of organic compounds as phenolic compounds secreted from SCG to the fermentation medium on catalytic properties of the enzyme.

3.4. Production of Lipase from *B. subtilis* strain BT2

To evaluate the utilization of SCG by bacteria, strain BT2, which was isolated from soil samples from Kadıköy Region of Istanbul during screening for lipase producing bacteria, was used.

Based on 16S rRNA gene sequence analysis (**Table 3.8**), strain BT2 is 99.53% identical to *Bacillus subtilis*. In order to figure out the ability of *B. subtilis* BT2 to utilize SCG, different carbon, and nitrogen sources were investigated and compared with the utilization of SCG by BT2 strain.

Table 3.8. 16SrRNA sequence of strain BT2

27F-1492R:
GGAGGCAGCAGTAGGGAATTCTTCCGCAATGGACGAAAGTCTGACGGAG
CAACGCCGCCTGAGTGATGAAGGTTTTTGGATCGTAAAGTTCTGTTGTT
AGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTA
ACCAGAAAGCCACGGCTAATTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTTCATTG
GAAACTGGGGAACCTTGAGTGCAGAAGAGGAGAGTGGAATTCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACT
CTCTGGTCTGTAAGTACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
GATTAGATAACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT
AGGGGGTTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCT
TCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTG
CCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGACAGAACAAGGGCAGCGAAACCGCGAGGTTAA
GCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGA
CTGCGTGAAGCTGGAATCGGTAGTAATCGCGGATCAGCATGCCGCGGTG
AATACGTTCCCGGGCCTTGTACACACCGCC

3.4.1. Effect of carbon and nitrogen sources on lipase production from *B. subtilis* strain BT2

To investigate the impact of various carbon and nitrogen sources on the growth and lipase activity, glucose, fructose, maltose, molasses, and sucrose were used as carbon sources, while nitrogen sources included beef extract, malt extract, peptone from casein, peptone from meat, and yeast extract. The effect of different nitrogen sources on each carbon source was analyzed, and growth profiles are given between **Figure 3.20** and **Figure 3.21**. Lipase activity (U/mL), protein concentration (mg/mL) and specific activity (U/mg) obtained at each condition are tabulated in Table between **Table 3.9** and **Table 3.13**.

As seen in **Figure 3.20.**, the most effective nitrogen sources were malt extract and yeast extract when fructose was used as a carbon source, When using peptone from casein,

growth was insufficient and the cell entered the stationary phase in a relatively short period compared to other four nitrogen sources.

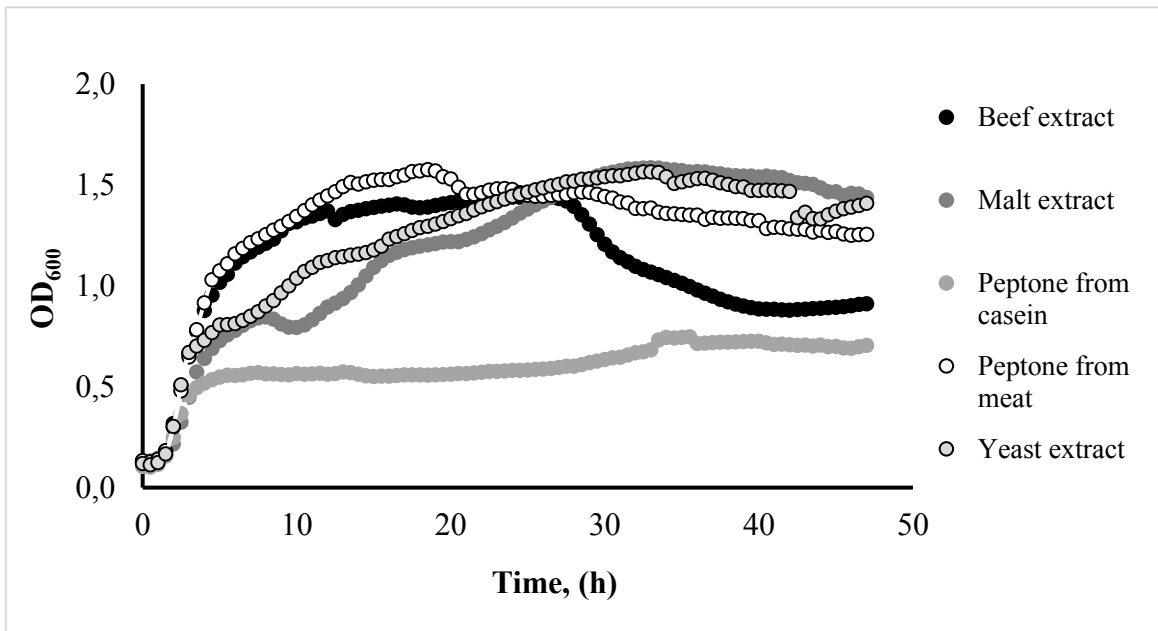


Figure 3.20. Effect of nitrogen sources on growth profile of *B. subtilis* BT2 using fructose as carbon sources

When the effect of nitrogen sources on lipase synthesis from fructose by BT2 strain was evaluated (**Table 3.9**), the highest lipase activity as 58.22 U/ml was found with yeast extract. Although strain BT2 was grown in malt extract, peptone from meat and malt extract, lipase activity was drastically decreased. For peptone from casein, the lowest lipase activity was observed since BT2 was not grown in the presence of peptone from casein as the nitrogen source and fructose as the carbon source. Therefore, if fructose is selected as the carbon source for lipase production from *B. subtilis*, it would be the right choice to use yeast extract as the nitrogen source.

Table 3.9. Effect of different nitrogen sources on lipase activity of *B. subtilis* using fructose as carbon source

Nitrogen Sources	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Act. (U/mg)
Yeast ext.	58.217	0.468	124.526
Peptone from meat	3.051	0.170	17.903
Malt ext.	2.468	0.142	17.354
Beef ext.	1.490	0.148	10.069
Peptone from casein	1.001	0.160	6.247

In the research of Haniya et al. (2017), fructose was determined as the best carbon source, while yeast extract was shown to be the best as a nitrogen source, with 55 U/mL lipase activity. The results obtained in their study are quite similar to our results.

When glucose was used as glucose, the best nitrogen source that yielded the highest lipase activity as 116.20 was malt extract (**Table 3.10**). Similar to fructose, peptone from casein negatively affected the growth of strain BT2 in the presence of glucose (**Figure 3.21**). However, the lipase activity produced in the presence of peptone from casein and glucose was 60 times higher compared with the activity obtained from fructose (**Table 3.10**). Contrary to the results of activity obtained with fructose, yeast extract negatively affect the lipase synthesis from strain BT2 in the presence of glucose.

Table 3.10. Effect of different nitrogen sources on lipase activity of *B. subtilis* using glucose as carbon source

Nitrogen source	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Act. (U/mg)
Malt ext.	116.201	0.269	432.049
Peptone from casein	62.176	0.327	190.306
Peptone from meat	45.875	0.468	98.116
Beef ext.	3.493	0.175	19.909
Yeast ext.	1.886	0.110	17.075

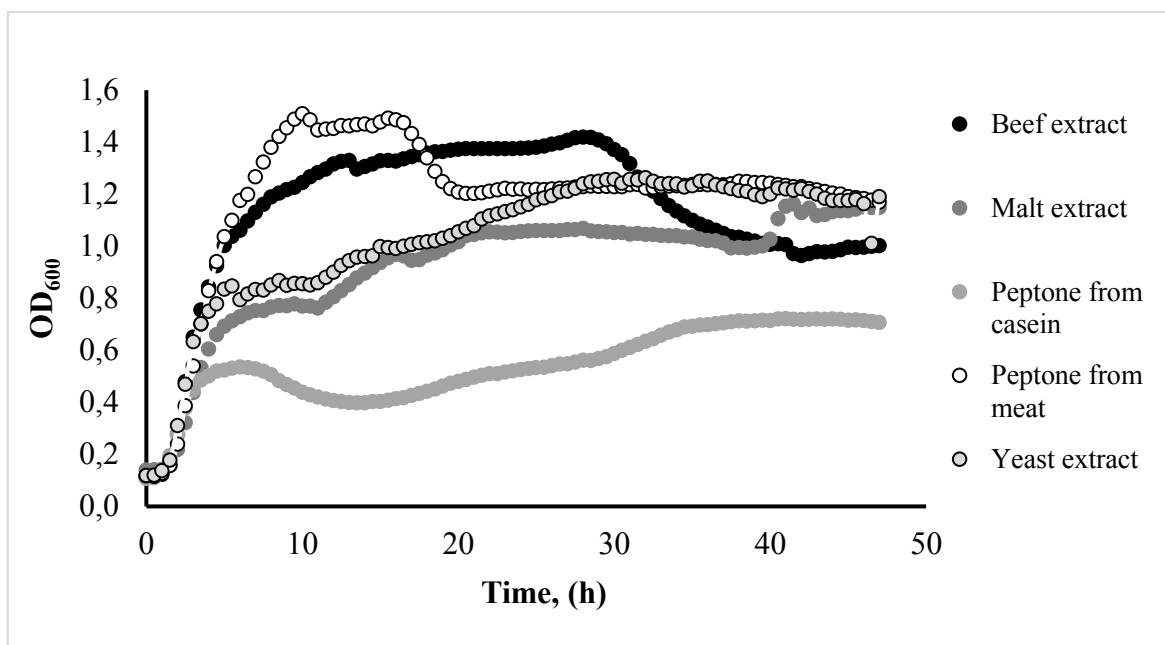


Figure 3.21. Effect of nitrogen sources on growth profile of *B. subtilis* BT2 using glucose as carbon sources

When we used maltose and sucrose as carbon sources, although *B. subtilis* BT2 was grown in the presence of all nitrogen sources studied (**Figure 3.22** and **Figure 3.23**), the lipase activity was very low compared to glucose and fructose at all nitrogen sources (**Table 3.11** and **Table 3.12**). The highest lipase activity was found to be 3.98 U/ml when using peptone from meat and maltose. With sucrose, the highest enzyme production as 5.01 U/mL was achieved by malt extract (**Table 3.12**).

Table 3.11. Effect of different nitrogen sources on lipase activity of *B. subtilis* using maltose as carbon source

Nitrogen sources	Activity (U/ml)	Protein Con. (mg/ml)	Specific Act. (U/mg)
Peptone from meat	3.982	0.217	18.323
Malt ext.	3.540	0.119	29.711
Beef ext.	2.841	0.230	12.373
Yeast ext.	1.514	0.149	10.177
Peptone from casein	1.234	0.219	5.641

Table 3.12. Effect of different nitrogen sources on lipase activity of *B. subtilis* using sucrose as carbon source

Nitrogen sources	Activity (U/ml)	Protein Con. (mg/ml)	Specific Act. (U/mg)
Malt ext.	5.007	0.168	29.761
Yeast ext.	2.701	0.173	15.654
Beef ext.	2.492	0.159	15.686
Peptone from meat	2.166	0.124	17.439
Peptone from casein	0.163	0.136	1.195

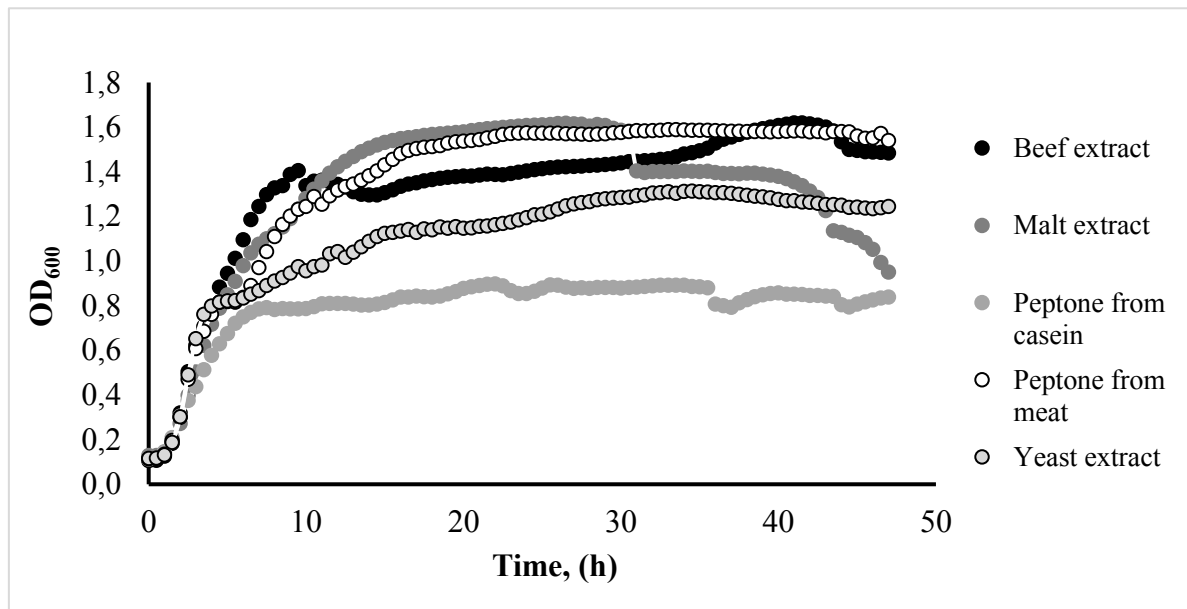


Figure 3.22. Effect of nitrogen sources on growth profile of *B. subtilis* BT2 using maltose as carbon sources

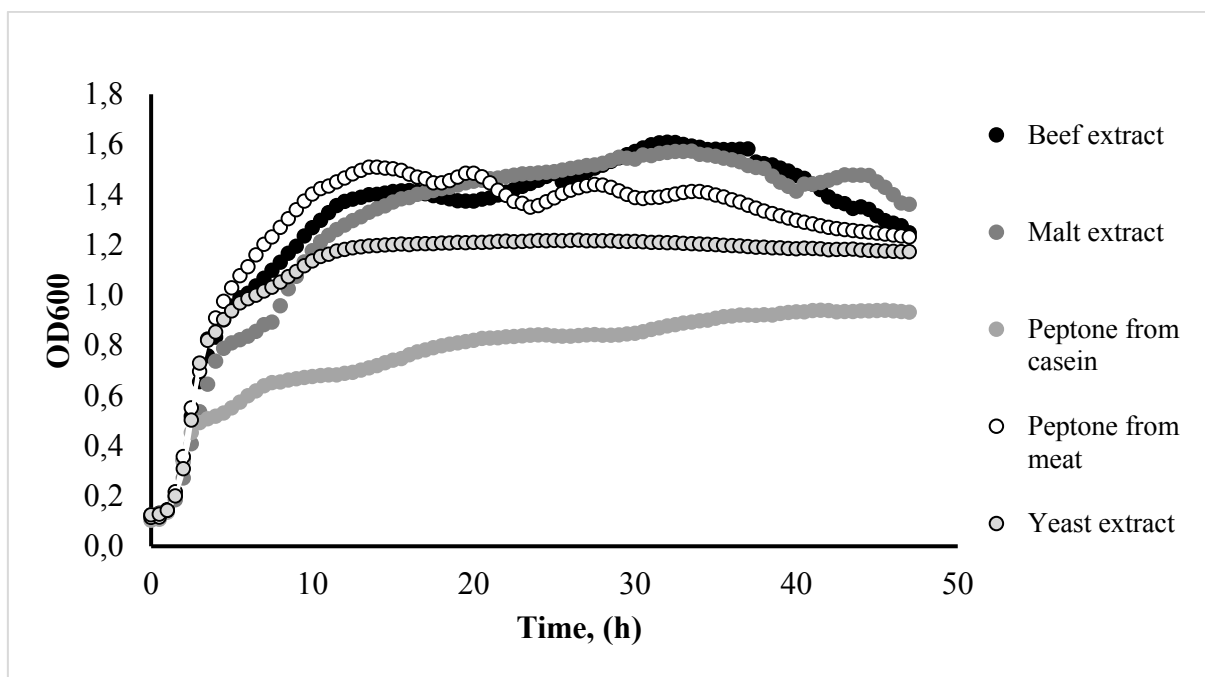


Figure 3.23. Effect of nitrogen sources on growth profile of *B. subtilis* BT2 using sucrose as carbon sources

Molasses is the main byproduct of the sugar industry and widely used in several fermentation processes. Therefore, we used molasses as a carbon source for lipase production from strain BT2 and the effect of different nitrogen sources was evaluated. As seen in **Table 3.13** and **Figure 3.24.**, the most effective nitrogen source for the growth of strain BT2 in the presence of molasses was malt extract. Although malt extract induces the growth of strain BT2, it yielded 10.60 U/mL of lipase activity which is six times lower than the highest activity (64.74 U/ml) obtained with peptone from casein. The lowest lipase activity (9.08 U/ml) was found by using yeast extract as a nitrogen source.

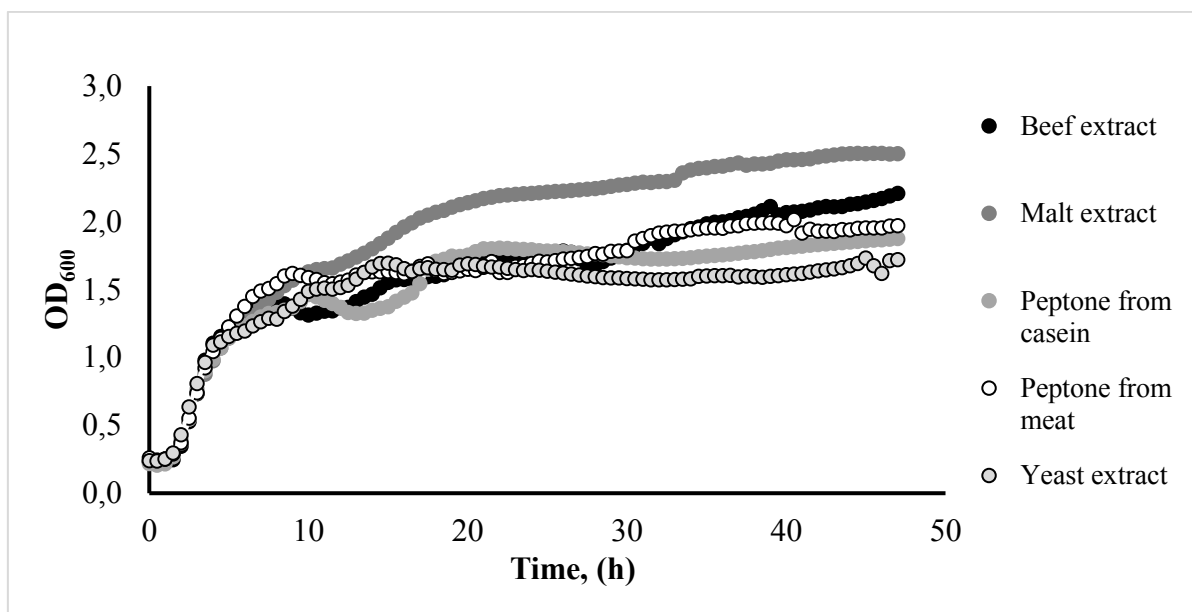


Figure 3.24. Effect of nitrogen sources on growth profile of *B. subtilis* BT2 using molasses as carbon sources

Table 3.13. Effect of different nitrogen sources on lipase activity of *B. subtilis* using molasses as carbon sources

Nitrogen sources	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Act. (U/mg)
Peptone from casein	64.737	0.514	125.840
Peptone from meat	39.122	0.399	98.070
Malt ext.	10.595	0.206	51.310
Beef ext.	9.222	0.213	43.294
Yeast ext.	9.082	0.243	37.324

As a result of research about the effect of carbon and nitrogen sources on lipase production from *B. subtilis* strain BT2, molasses and malt extract were the best carbon-nitrogen source pair for cell growth, while glucose and malt extract were for lipase activity.

When we examine effect of carbon and nitrogen sources on specific growth rate of strain BT2 in **Table 3.14**, The highest specific growth rate values were obtained when the

carbon source is selected as sucrose. Sucrose and peptone from meat were the best carbon-nitrogen source pair for specific growth rate.

Table 3.14. Effect of carbon and nitrogen sources on specific growth rate of strain BT2

Nitrogen Sources	Specific Growth Rate (h ⁻¹)				
	Carbon sources				
	Fructose	Glucose	Maltose	Molasses	Sucrose
Beef ext.	0.100	0.203	0.147	0.088	0.381
Malt ext.	0.063	0.214	0.139	0.110	0.642
Peptone from casein	0.078	0.068	0.105	0.134	0.815
Peptone from meat	0.311	0.296	0.111	0.148	0.910
Yeast ext.	0.045	0.111	0.041	0.055	0.844

Plants, animals, and microbes are just a few of the many species that may produce lipases (Szymczak et al., 2021). Bacterial lipases, in particular, play a significant role in commercial endeavors. Bacterial lipases are often produced in the presence of an organic nitrogen source on lipidic carbon, such as oils, fatty acids, glycerol, or tweens. (Gupta et al., 2004) However, the best sources of carbon or nitrogen for lipase production may vary by species of bacteria. According to the study of Sumarsih et al. (2019), glucose was found to be the best carbon source for lipase produced by *Micrococcus* sp. compared with sucrose and glycerol. This result is quite similar to the result obtained in this thesis. In this thesis, when malt extract was used as a nitrogen source, the use of glucose as a carbon source provided the highest lipase activity. In the investigation of Salihu et al. (2016), the optimum carbon source was discovered to be Tween80, which also has the capacity to stimulate the synthesis of lipase by *Aspergillus niger* AS-02. In another research, Bharathi et al. (2019) found that sucrose and yeast extract increased lipase production by various bacteria such as *Corynebacterium* sp., *Bacillus* sp., and *Pseudomonas* sp. when compared with other carbon and nitrogen sources.

3.4.2. Production of lipase from SCG by *B. subtilis* strain BT2

The main objective of this work is to investigate the utilization of SCG for the production of value-added product in order to develop environmentally friendly and sustainable processes. Therefore, we investigated the production of lipase from SCG by using *B. subtilis* BT2 (Table 3.15). Figure 3.25 shows that *B. subtilis* BT2 was grown in the presence of SCG and the specific growth rate in the absence and presence of SCG were calculated as 0.879 and 0.408 h⁻¹, respectively. When lipase expression by strain BT2 in the presence of SCG was evaluated, it was seen that *B. subtilis* BT2 produced lipase enzyme with SCG. Then the effect of different oil sources and surfactant on lipase production was examined and results are given in Table 3.16.

Table 3.15. Effect of SCG on lipase activity of *B. subtilis* BT2

SCG % (w/v) in NB	Activity (U/ml)	Protein Con. (mg/ml)	Specific Act. (U/mg)
1.0	13.250	0.267	49.599
0.0	4.518	0.142	31.761

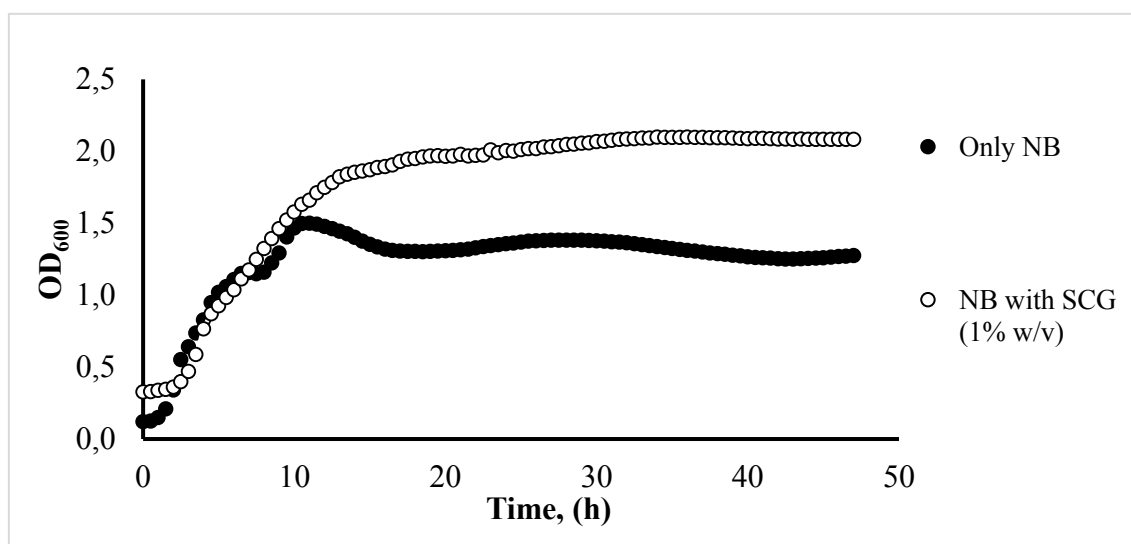


Figure 3.25. The effect of SCG on growth profile of *B. subtilis*

Table 3.16 presents that the highest activity was obtained as 58.87 U/ml from the medium containing 2% olive oil (v/v). However, approximately 70-75% of its results were obtained using 1% SCG (w/v) and 1% SCG (w/v) with 0.1% Tween80 mediums. Obtaining such a high result with the use of waste alone indicates that SCG can be used as an effective substrate source in lipase enzyme production. When the surfactant effect was examined, while the use of tween80 caused a decrease in enzyme activity when olive oil was used, it caused an increase in SCG and tributyrin.

Table 3.16. The effect of different oil sources and surfactant on lipase production from strain BT2

Lipase Production Media	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/μg)
NB + 2.0% Olive Oil	58.869	0.394	149.329
NB + 2.0% Olive Oil +0.1% Tween80	55.236	0.438	126.241
NB + 1.0% Olive Oil	50.579	0.355	142.381
NB + 1.0%SCG + %0.1 Tween80	44.711	0.656	68.198
NB + 1.0% SCG	40.426	0.601	67.295
NB + 1.0% Olive Oil + 0.1% Tween80	39.494	0.448	88.225
NB + 1.0% Tributyrin +0.1% Tween80	15.136	0.219	68.960
NB + 1.0% Tributyrin	7.452	0.081	92.149
NB	2.608	0.224	11.652
NB +0.1% Tween80	2.422	0.286	8.470

3.4.3. Characterization of *B. subtilis* strain BT2 lipase

3.4.3.1. Optimum temperature and pH of *B. subtilis* strain BT2 lipase

Optimum temperature and pH of the BT2 lipase produced from olive oil and SCG was determined as 37 °C (**Figure 3.26**) and 8.0 (**Figure 3.27**), respectively. The presence of SCG and organic compounds released from SCG during cultivation did not change the optimum temperature and pH of the enzyme. The raw data related to optimum temperature and pH of BT2 lipase are given in Appendix B, Table B.6, B.7, B.8, and B.9.

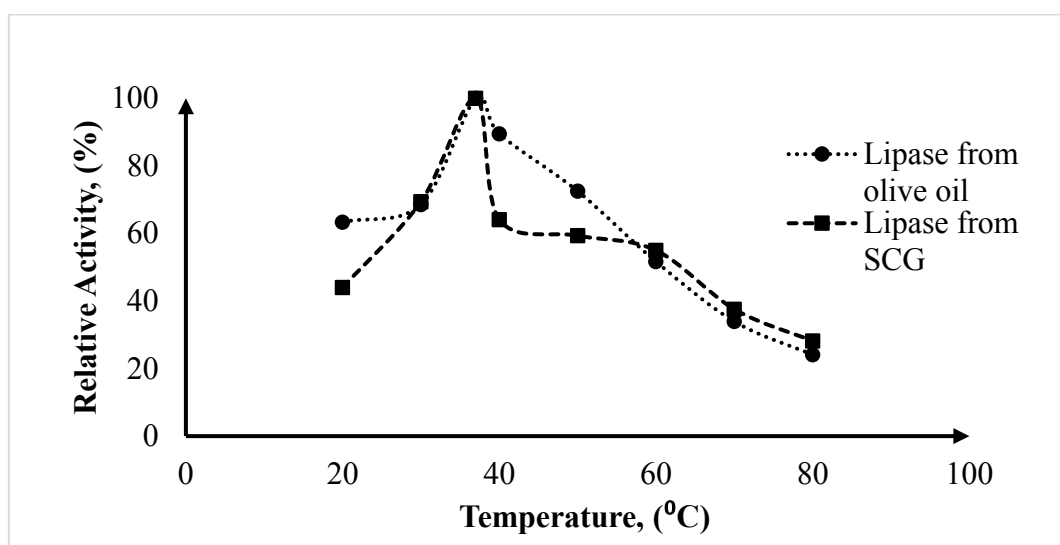


Figure 3.26. Optimum temperature of lipase enzyme from olive oil and from SCG by *B. subtilis* strain BT2

Comparing our results with the results reported by Haniya et al. (2015), optimum temperature and pH of *Bacillus cereus* strain PCSIR NL-37 lipase as 40 °C and pH 8.0 are very close to optimum temperature and pH of BT2 lipase. In another study, Iqbal and Rehman (2015) have characterized lipase from *B. subtilis* isolated from oil-polluted area in Pakistan, and they reported the optimum temperature and pH of enzyme as 50 °C and 7.0. Although the optimum pH of *B. subtilis* lipase was lower than BT2 lipase, optimum temperature of it is higher compared with the BT2 lipase. According to the results of studies of Ghori et al. (2011), the highest activity was obtained at 60 °C and pH 9.0 that are higher than that obtained for BT2 lipase.

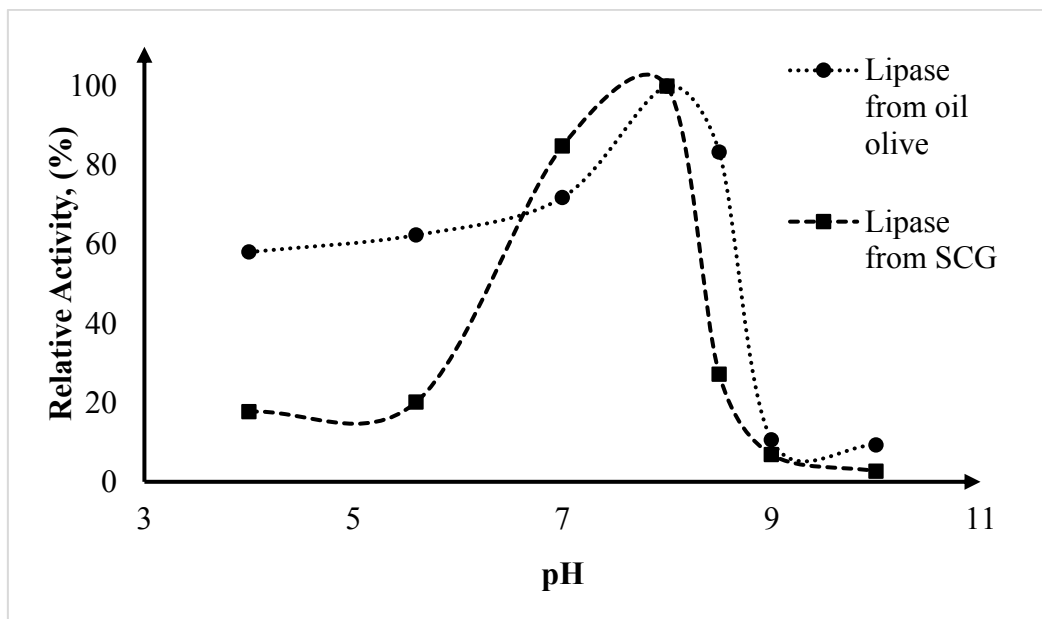


Figure 3.27. Optimum pH of lipase enzyme from olive oil and from SCG by *B. subtilis* strain BT2

3.4.4. Fatty acid sugar ester synthesis from *B. subtilis* strain BT2 lipase

Sugar esters are very important molecules used as non-ionic surfactants and antimicrobial agents in the food industry. Additionally, they are used in the pharmaceutical industry as anticancer agent (Marathe et al., 2022). Therefore, in the present thesis, the production of fatty acid sugar esters catalysed by BT2 lipase was investigated. The oil and sugars extracted from SCG were used instead of vinyl decanoate and fructose, and organic phase including sugar esters produced by catalysis of BT2 lipase were determined by Fourier Transform Infrared Spectroscopy (FTIR) (**Figure 3.28**).

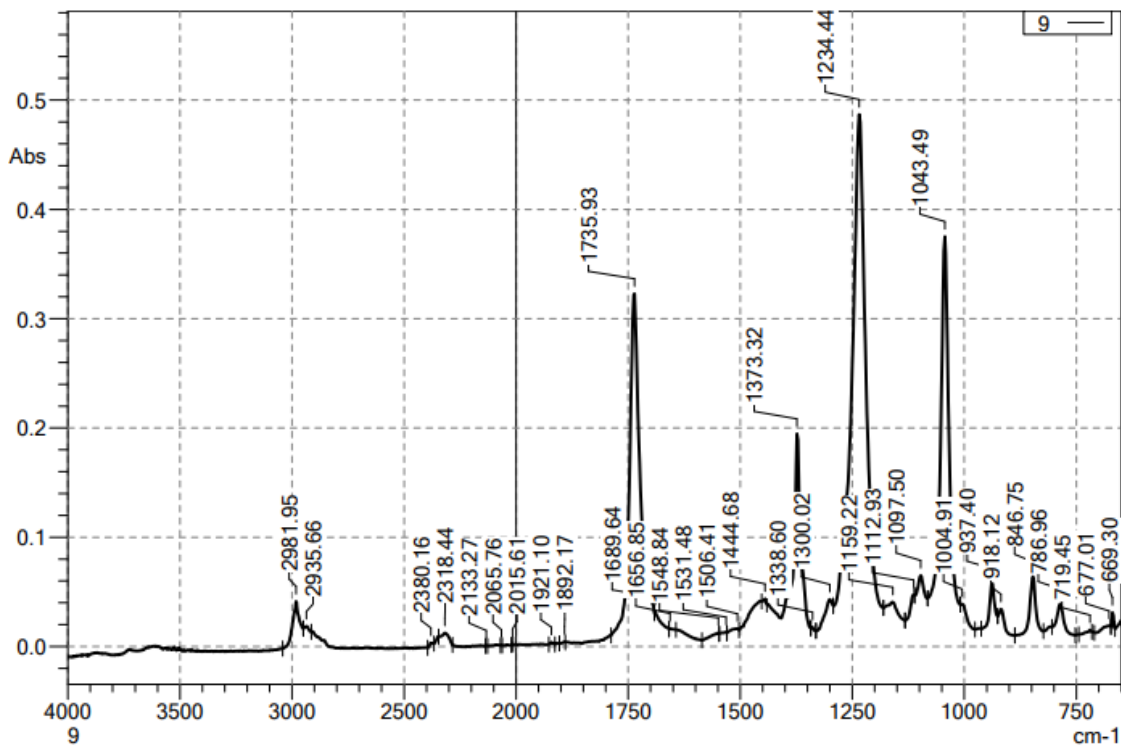


Figure 3.28. Sugar ester synthesis analysis with FTIR

Fourier Transform Infrared Spectroscopy (FTIR) is one of the frequently used methods to determine the functional groups from spectral bands of materials (Mouillet et al., 2011). Therefore, in the present work, the achievement of the synthesis of sugar esters from oil and sugar mixture extracted from SCG by BT2 lipase catalysis was determined by FTIR analysis.

Recently, Lee et al. (2022) reported that the sugars extracted from SCG included mainly mannose and galactose. When the FTIR spectrum of galactose was analyzed, the band having high intensity at 1043 cm^{-1} showed the stretching of C-O while bands at 763 , 835 , and 954 cm^{-1} indicated the deformation of C-H bond (Guyen et al., 2019). In addition, it is reported that stretching of O-H corresponded to low intensity peaks around 3125 , 3200 and 3382 cm^{-1} . Furthermore, the peaks of most sugars were obtained at 3315 , 2927 , and 2882 cm^{-1} (de Almeida et al., 2018). Moreover, Dandekar et al. (2009) studied the production of fructose esters, and they found the peaks at 2922.6 , 1736.8 , 1456.6 , 1088.2 , and 714.6 cm^{-1} indicated the C-H bond in $-\text{CH}_2$ or $-\text{CH}_3$, C=O ester bond, $-\text{CH}_2$, and $-\text{CH}_3$ groups, C-O ester bond and the (CH)₂ bond, respectively.

It can be seen in Figure 3.28. that FTIR spectrum of sugar esters synthesized BT2 lipase showed the similar pattern to that reported in the literature (de Almeida et al., 2018; Guven et al., 2019; Dandekar et al., 2009). The organic phase could include the SCGs sugar esters based on the stretching of C-O at 1043 cm^{-1} , C=O ester bond at 1735.83 cm^{-1} , C-H bond in $-\text{CH}_2$ at peaks between 2900 and 2982 cm^{-1} , and the $(\text{CH})_2$ bond at 719.45 cm^{-1} . The peaks between 2800 and 3000 cm^{-1} are also an indication of the presence of sugars in organic phase (Figure 3.28).

3.5. Surface Morphology Analysis of SCG and SCG Variations by Scanning Electron Microscopy (SEM)

The surface morphology of SCG and SCG variations was examined by SEM and presented in Figure 3.29. As can be seen in the figure below, the surface morphology of SCG was relatively smooth compared to variations of SCG. It is obvious that, the SCG variations generated by both the extraction and fermentation procedures have rougher and more porous surfaces. The use of both the oil and sugar of SCG resulted in a much more tubular appearance as seen in Figure 3.29 (D). Similar degradation patterns are seen in Figure 3.29 (E) and Figure 3.29 (F), providing further evidence that microorganisms use the organic content of SCG such as sugar and oil without any extraction process.

This result shows that SCG can be converted from waste to a value-added product by biotechnological procedures without any chemical extraction process, which is best novelty of this thesis.

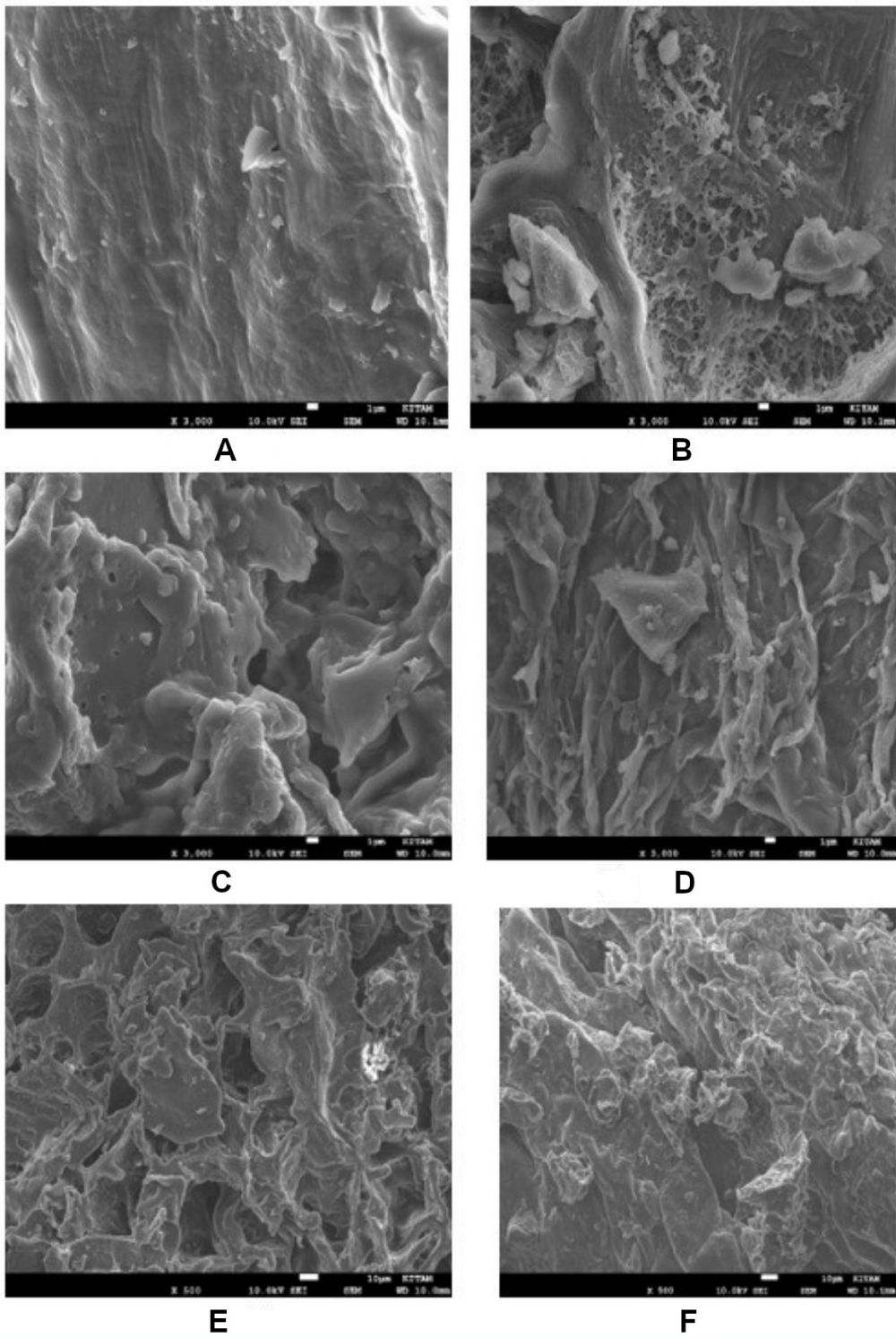


Figure 3.29. SEM Images of SCG and SCG Variations

(A) SCG; (B) SCG from oil extraction; (C) SCG from sugar extraction; (D) SCG from oil and sugar extraction; (E) SCG fermented by bacteria; (F) SCG fermented with yeast.

4. CONCLUSION

The carbon sources that are utilized are the most significant expense of bioprocesses. Hereby, processes that combine bioengineering concepts and green production technologies have become more significant in recent years. Hence, reduced operating expenses are essential for the creation of sustainable and efficient operations. The purpose of the proposed project was to promote the zero-waste initiative and to create a sustainable and cost-effective method by using SCG as a carbon source in bioprocesses.

Within the scope of this thesis, it was determined that the water content of the collected SCG was approximately 60%. To ensure that the SCG was stored for a long time without contamination, the drying process was applied to collect the amount of waste to be used within the specified period at once. The reason for this was to shorten the waste collection period in order to store waste with high water content without decomposition, and thus to prevent the carbon emissions that will be created by the vehicles while the waste is collected and transported. For the determination of the best condition for the drying process, the effects of the amount of SCG, temperature, and brand type were evaluated. According to the results of drying experiments, the water evaporation rate was not affected by the brand. Also, as the drying temperature increased from 50⁰ C to 60⁰ C, the physically bound water evaporated faster total water loss was observed within 2 hours of drying. When it was seen that the studies carried out above 60⁰ C did not affect the drying speed, no temperature increase was made to reduce the effect of drying temperature on the organic content of SCG and the drying temperature was kept constant at 60⁰ C.

While extracting the oil contained in SCG, the best results were obtained from the hexane: isopropanol (1:1, v/v) mixture, yielded 0.2 mL coffee oil/gram dried SCG. While extracting the reducing sugar contained in SCG, highest sugar yield as 39.11 ± 1.8 was obtained when 3% H₂SO₄ solution was mixed at the rates of 0.02 dry spent coffee/solution (gr/mL) with 9.95 ± 0.3 (%), mg of GAE/ g dry SCG).

Following the determination of the SCG's composition, the Box-Behnken design was used in the Design Expert software to optimize the parameters for lipase production from SCG by *C. diffluens* D44, a former lipase-producing yeast. The findings of the studies conducted thus far show that the maximum lipase enzyme activity produced from SCG was determined to be 14.6 U/mL with the use of 8.7% SCG, while the pH of the

production medium was at 8.3 at 29.4 °C. Also, the optimum temperature and pH of lipase produced from SCG by *C. diffluens* D44 were determined as 37 °C and 8.0, respectively. However, the lipase produced was dark in color due to the pigments from SCG. Hence, the depigmentation of the crude enzyme was attempted. Unfortunately, any agent other than activated carbon did not yield successful results and after activated carbon binds lipase, the activity was lost. No attempt was made to recover the lipase bound by the activated carbon. Maybe this can be studied, and color removal will be possible.

For evaluating the use of SCG by bacteria, *B. subtilis* BT2 that have an ability to utilize SCG was also isolated from the soil sample in Kadıköy, Istanbul. First of all, the effect of carbon and nitrogen sources on lipase production from *B. subtilis* strain BT2 were researched. According to the results of it, molasses and malt extract are the best carbon-nitrogen source pair for cell growth (2.5 OD at 600 nm), while glucose and malt extract are for lipase activity (116.201 U/ml). However, the highest specific growth rate values were obtained when the carbon source is selected as sucrose. Sucrose and peptone from meat are also the best carbon-nitrogen source pair resulting a specific growth rate of 0.910 h⁻¹. Then, the effect of SCG on growth, activity and specific growth rate was investigated. According to the findings, *B. subtilis* BT2 was grown in the presence of SCG and the specific growth rate in the absence and presence of SCG were calculated as 0.879 and 0.408 h⁻¹, respectively. When lipase expression by strain BT2 in the presence of SCG was evaluated, it was seen that *B. subtilis* BT2 produced lipase enzyme with SCG (13.250 U/ml). When the effect of surfactants on lipase activity was studied, it was shown that using Tween80 decreased enzyme activity when olive oil was utilized, on the other hand, it caused an increase in SCG and tributyrin. In addition, the presence of SCG and organic compounds released from SCG during cultivation did not change the optimum temperature (37 °C) and pH of the enzyme (8.0).

Moreover, one of the most important innovations in this thesis is the production method of sugar esters, since the oil and sugars extracted from SCG were used instead of vinyl decanoate and fructose. Finally, SEM images of the SCG and SCG variations used to investigate the reusability of them were taken and their structural changes were examined. It could be said that the SCG variants produced by the extraction and fermentation processes have surfaces that are rougher and more porous. The look became significantly more tubular when SCG's oil and sugar were extracted. Similar breakdown patterns are

seen in SCG that has been fermented by bacteria and yeast, supporting the idea that microorganisms utilize the organic components of SCG including sugar and oil without the need for extraction as well.

Large volumes of liquid and solid waste are produced by the food and agriculture industries, and this waste can indeed be harmful to the environment. Similarly, the coffee business can attest to this. One of the main wastes produced by the coffee industry is SCG. Additionally, the findings of this research demonstrate that SCG is a particularly abundant source of carbon for lipase production. According to the outcomes of the literature survey, the SCG has not yet been utilized in the production of lipolytic enzymes. Therefore, within the scope of this thesis, SCG was used for the first time in the production of a lipolytic enzyme without any chemical treatment. The best uniqueness and novelty of this thesis are demonstrated that SCG might well be converted to a value-added product such as lipase enzyme and sugar esters by biotechnological processes without the requirement for chemical extraction.

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APPENDICES

Appendix A

Table A.1. Raw data of the calibration curve using p-NPP as substrate

Buffer volume (ml)	Sample Volume (ml)	p-NP (μg)	p-NP (μmol)	OD410				p-NP ($\mu\text{mol/ml}$)
				OD1	OD2	OD3	OD (mean)	
0	0	0	0	0	0	0	0	0.00
2	0.1	83.466	0.6	0.176	0.177	0.175	0.176	0.27
2	0.1	166.932	1.2	0.351	0.352	0.349	0.352	0.53
2	0.1	250.398	1.8	0.546	0.548	0.544	0.546	0.80
2	0.1	333.864	2.4	0.720	0.721	0.723	0.722	1.07
2	0.1	417.33	3.0	0.912	0.907	0.908	0.909	1.33
2	0.1	500.796	3.6	1.11	1.08	1.08	1.09	1.60

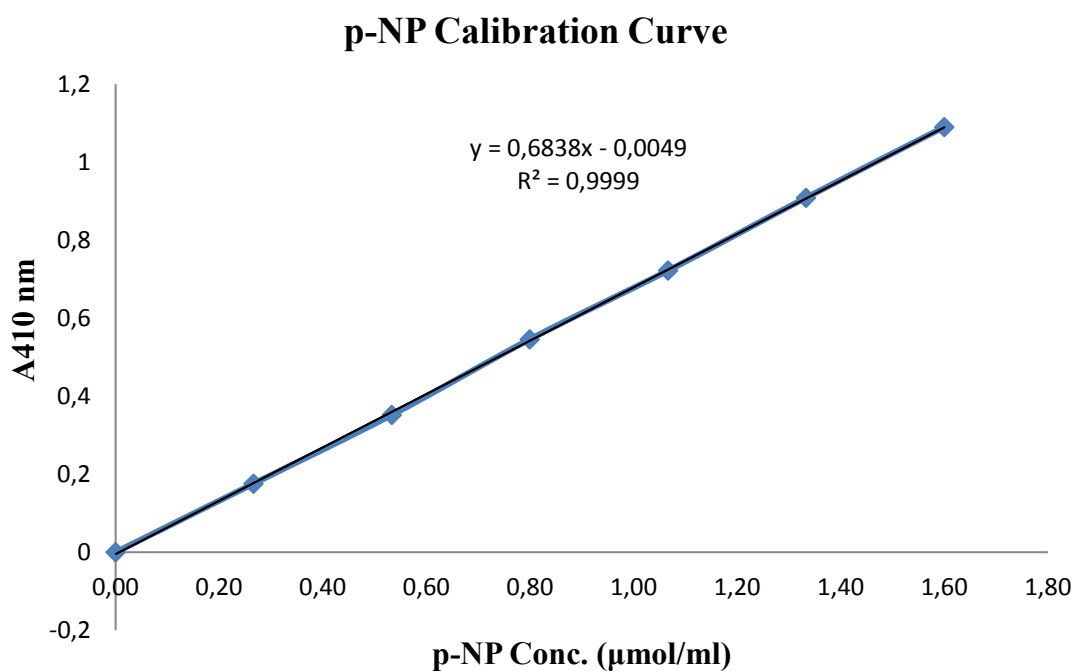


Figure A.1. Calibration curve used for enzyme activity calculation

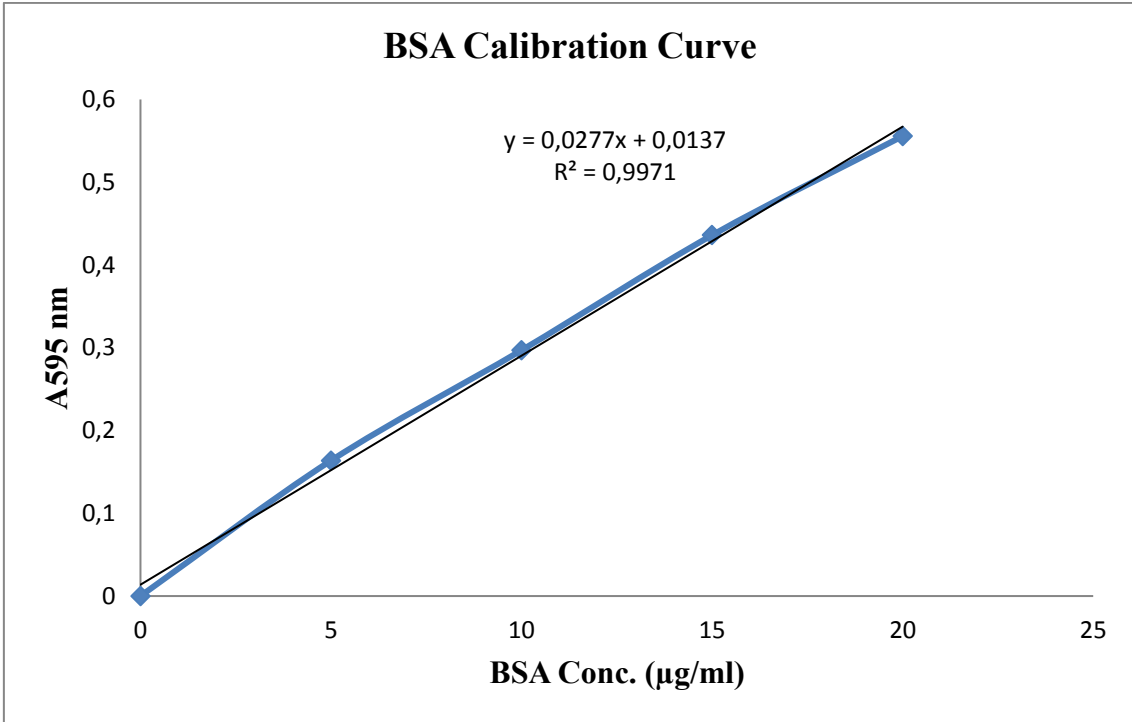


Figure A.2. BSA calibration curve used for Bradford protein assay

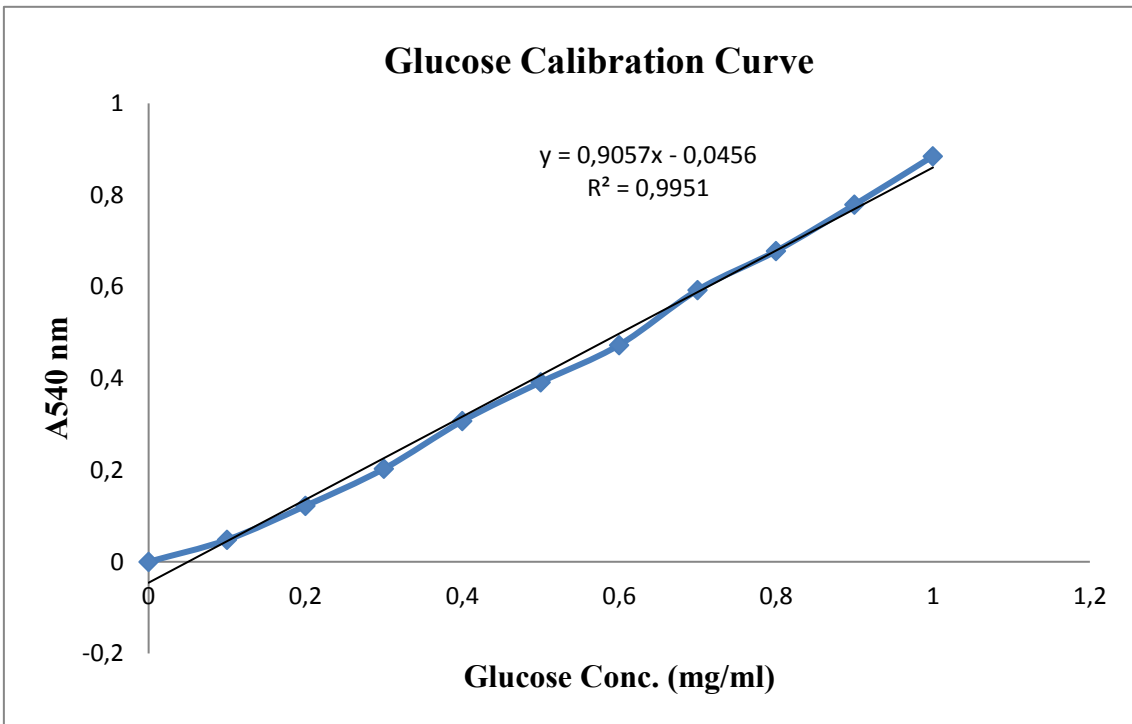


Figure A.3. Glucose calibration curve used for DNS assay

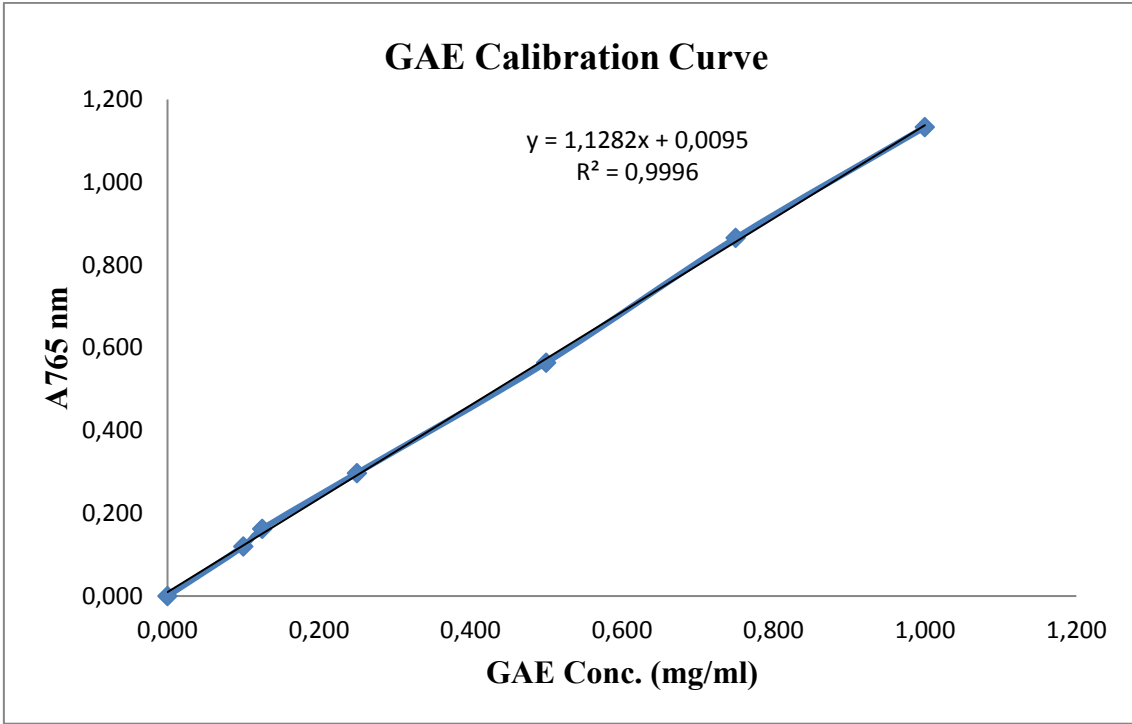


Figure A.4. GAE calibration curve used for Folin–Ciocalteu assay

Appendix B

Table B.1. Raw data for the effect of SCG amount on drying process

Time (h)	10 g	15 g	20 g	25 g
0.0	10.000	15.000	20.000	25.000
2.0	5.637	8.154	12.426	16.178
3.0	4.700	6.858	10.367	13.421
4.0	4.288	6.140	9.010	11.515
5.0	4.136	6.008	8.280	10.457
5.5	4.119	5.990	8.137	10.203

Table B.2. Raw data for the effect of temperature on drying process

Time (h)	50° C	Time (h)	60° C	Time (h)	70° C
0.0	25.000	0.0	25.000	0.0	25.000
2.0	22.746	2.0	12.203	2.0	22.155
4.0	19.255	4.5	12.060	3.0	19.219
5.0	18.731	5.5	11.475	4.0	15.955
6.0	17.657	6.5	11.126	5.0	12.885
7.0	16.103	7.5	11.087	6.0	11.056
8.0	14.923				
9.0	12.208				
10.0	11.002				

Table B.3. Raw data for the effect of brand name on drying process (25 g SCG at 60° C)

Time (h)	X Brand	Time (h)	Y Brand	Time (h)	Z Brand
0	25	0	25	0	25
2	13.622	1	17.659	2	12.203
3	11.164	2	13.782	5	10.515
4	10.472	3	11.75	6	10.484
5	10.434	4	11.441		

Table B.4. Optimum temperature of lipase enzyme from SCG by *C. diffluens* D44

Temperature (° C)	OD540			Activity (U/ml)	Relative Activity (%)
	OD1	OD2	OD (mean)		
20	0.74	0.74	0.74	17.26	37.45± 0.02
30	1.02	1.02	1.02	23.75	51.55± 0.09
37	1.98	1.98	1.98	46.07	100
40	1.82	1.83	1.82	42.38	91.99± 0.09
50	1.10	1.06	1.08	25.15	54.59± 1.11
60	0.87	0.90	0.88	20.53	44.55± 0.76
70	0.74	0.74	0.74	17.17	37.28± 0.19
80	0.44	0.53	0.49	11.29	24.51± 2.23

Table B.5. Optimum pH of lipase enzyme from SCG by *C. diffluens* D44

pH	OD540			Activity (U/ml)	Relative Activity (%)
	OD1	OD2	OD (mean)		
4.0	0.14	0.12	0.13	2.92	10.52± 0.87
5.6	0.18	0.17	0.18	4.12	14.83± 0.40
7.0	0.94	0.95	0.94	21.92	78.89± 0.64
8.0	1.20	1.19	1.19	27.79	100
8.5	1.03	1.01	1.02	23,75	85.46± 0.73
9.0	0.11	0.10	0.10	2.34	8.42± 0.37
10.0	0.00	0.00	0.00	0.00	0.00

Table B.6. Optimum temperature of lipase enzyme from olive oil by *B. subtilis*

Temperature (° C)	OD540			Activity (U/ml)	Relative Activity (%)
	OD1	OD2	OD (mean)		
20	0.89	0.89	0.89	20.66	63.36± 0.60
30	0.95	0.97	0.96	22.36	68.57± 0.22
37	1.39	1.41	1.40	32.60	100
40	1.25	1.25	1.25	29.17	89.46± 0.60
50	1.02	1.01	1.02	23.64	72.50± 0.59
60	0.72	0.72	0.72	16.84	51.64± 0.44
70	0.48	0.47	0.48	11.06	33.93± 0.31
80	0.34	0.34	0.34	7.85	24.07± 0.24

Table B.7. Optimum temperature of lipase enzyme from SCG by *B. subtilis*

Temperature (° C)	OD540			Activity (U/ml)	Relative Activity (%)
	OD1	OD2	OD (mean)		
20	0.97	0.99	0.98	22.76	43.97± 0.28
30	1.54	1.55	1.54	35.92	69.39± 0.02
37	2.22	2.23	2.22	51.77	100
40	1.42	1.43	1.42	33.16	64.06± 0.09
50	1.32	1.32	1.32	30.72	59.33± 0.05
60	1.22	1.22	1.22	28.47	54.99± 0.04
70	0.82	0.85	0.84	19.44	37.56± 0.62
80	0.63	0.62	0.63	14.57	28.14± 0.15

Table B.8. Optimum pH of lipase enzyme from SCG by *B. subtilis* strain BT2

pH	OD540			Activity (U/ml)	Relative Activity (%)
	OD1	OD2	OD (mean)		
4.0	0.53	0.52	0.53	12.23	17.71± 0.32
5.6	0.61	0.59	0.60	13.91	20.15± 0.39
7.0	2.54	2.50	2.52	58.60	84.88± 1.05
8.0	2.95	2.98	2.97	69.05	100
8.5	0.82	0.80	0.81	18.76	27.17± 0.46
9.0	0.21	0.20	0.20	4.76	6.90± 0.22
10.0	0.09	0.07	0.08	1.86	2.70± 0.28

Table B.9. Optimum pH of lipase enzyme from olive oil by *B. subtilis* strain BT2

pH	OD540			Activity (U/ml)	Relative Activity (%)
	OD1	OD2	OD (mean)		
4.0	2.24	2.22	2.23	51.70	58.08± 0.71
5.6	2.38	2.41	2.40	56.12	62.37± 0.10
7.0	2.75	2.77	2.76	64.50	71.88± 0.30
8.0	3.81	3.87	3.84	90.12	100
8.5	3.22	3.18	3.20	74.05	83.34± 1.16
9.0	0.415	0.398	0.41	9.27	10.59± 0.30
10.0	0.367	0.345	0.36	8.03	9.27± 0.36

