

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

**BIOPROCESSING OF EDIBLE JELLY MUSHROOM TO
OBTAIN LONG-CHAIN GLYCOLIPIDS FUNCTIONAL AS
FOOD-GRADE ANTIMICROBIALS**

TUGBA KABASAKAL
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

GEBZE
2022

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

**BIOPROCESSING OF EDIBLE JELLY
MUSHROOM TO OBTAIN LONG-CHAIN
GLYCOLIPIDS FUNCTIONAL AS FOOD-
GRADE ANTIMICROBIALS**

TUGBA KABASAKAL
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

THESIS SUPERVISOR
PROF. DR. MELEK OZKAN

GEBZE

2022

**T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ**

**GIDA SINIFI ANTİMİKROBİYAL
ÖZELLİKLİ UZUN ZİNCİRLİ GLİKOLİPİD
ELDESİ İÇİN YENİLENEBİLİR JÖLE
MANTARIN BİYOPROSESİ**

**TUĞBA KABASAKAL
YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ANABİLİM DALI**

**DANIŞMAN
PROF. DR. MELEK OZKAN**

**GEBZE
2022**



YÜKSEK LİSANS JÜRİ ONAY FORMU

GTÜ Biyoteknoloji Enstitüsü Yönetim Kurulu'nun 19/07/2022 tarih ve 2022/16 sayılı kararıyla oluşturulan jüri tarafından 12/08/2022 tarihinde tez savunma sınavı yapılan Tuğba Kabasakal'ın tez çalışması Biyoteknoloji Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

ÜYE
(TEZ DANIŞMANI) :Prof. Dr. Melek Özkan

ÜYE :Prof. Dr. Ayten Yazgan Karataş

ÜYE :Dr. Öğr. Üyesi Fatma İnci Özdemir

ONAY

Gebze Teknik Üniversitesi Biyoteknoloji Enstitüsü Yönetim Kurulu'nun
...../...../..... tarih ve/..... sayılı kararı.

İMZA/MÜH

SUMMARY

Long-chain glycolipids (LGLs) from *Dacryopinax spathularia* (*D. spathularia*) have recently been reported as a promising, natural, food-grade antimicrobial agent. *D. spathularia* is one of the species of jelly mushroom in the family of *Dacrymycetaceae*, known to produce LGLs as a secondary metabolite during aerobic fermentation. To date, the safety of LGLs has been proven with several studies, yet very scant attention given into its fermentative production.

In this study, the growth behavior of *D. spathularia* and the antimicrobial activity of its secondary metabolite LGLs were investigated against *E.coli* BL21, *B. subtilis*, *P. aureginosa*, *S. aureus*, *S. cerevisiae*. The highest growth inhibition due to LGLs addition was 93.02% and achieved with *S. cerevisiae*. It was realized that the flask geometry and aeration are important for glycolipid production. Baffled shake flasks provided improved aeration during viscous *D. spathularia* cultivation and resulted in higher glycolipid production in a shorter time as compared to unbaffled shake flasks during 192 h cultivation at 180 rpm in malt extract soy peptone (MESP) medium. Moreover, a series of laboratory-scale fermentations were performed to determine the optimum operational parameters, e.g. pH, temperature, and medium composition, for LGLs production.

With its relatively easy, non-hazardous, and inexpensive production process, jelly mushroom glycolipids hold great potential not only in the food preservative market but also in the cosmetics and pharmaceutical industries. The findings of this study will bring into light the antimicrobial properties and fermentation behavior of *D. spathularia* and also provide data on the optimum fermentative production system for LGLs production.

Key words: Natural Glycolipids, *Dacryopinax spathularia*, Long-chain glycolipids, Natural Food Preservative, Fungi Fermentation

ÖZET

Son zamanlarda, *Dacryopinax spathularia*'dan (*D. spathularia*) elde edilen uzun zincirli glikolipidlerin (LGLs) umut vadeden, doğal, gıda sınıfı bir antimikrobiyal ajan olduğu bildirilmiştir. *D. spathularia*, aerobik fermentasyon ile ikincil bir metabolit olan LGLs'leri ürettiği bilinen *Dacrymycetaceae* familyasındaki jöle mantar türlerinden biridir. Literatürde bugüne kadar, LGLs'lerin güvenliği birçok çalışma ile kanıtlanmıştır, ancak fermentatif üretimine çok az ilgi gösterilmiştir.

Bu çalışmada, *D. spathularia*'nın büyüme davranışı ve ikincil metabolit olarak ürettiği LGLs'lerin antimikrobiyal etkisi, *E.coli* BL21, *B. subtilis*, *P. aureginosa*, *S. aureus*, *S. cerevisiae*'ye karşı araştırılmıştır. LGLs ilavesine bağlı en yüksek büyüme inhibisyonu %93.02 olarak *S. cerevisiae* üzerinde elde edilmiştir. Erlen şekil geometrisinin ve havalandırmanın glikolipid üretimi için önemli olduğu anlaşılmıştır. Viskoz özellik gösteren *D. spathularia* ekimi sırasında girintili çalkalama erlenleri daha iyi havalandırma sağlamıştır. Bununla birlikte, malt ekstrakt soya pepton (MESP) ortamında 180 rpm'de 192 saat büyütme sırasında girintili olmayan çalkalama şişelerine kıyasla daha kısa sürede daha yüksek glikolipid üretimi elde edilmiştir. Ayrıca, uygun operasyonel parametreleri belirlemek için laboratuvar ölçekli bir dizi fermentasyon gerçekleştirilmiştir (örneğin; LGLs üretimi için pH, sıcaklık, ortam bileşimi).

Nispeten kolay, güvenli ve ucuz üretim süreci ile jöle mantar glikolipidleri, sadece gıda koruyucu pazarında değil, aynı zamanda kozmetik ve ilaç endüstrilerinde de büyük potansiyele sahiptir. Bu çalışmanın bulguları, *D. spathularia*'nın antimikrobiyal özelliklerini ve fermentasyon davranışını aydınlatacak ve ayrıca LGL üretimi için optimum fermentatif üretim sistemi hakkında veri sağlayacaktır.

Anahtar kelimeler: Doğal Glikolipidler, *Dacryopinax spathularia*, Uzun Zincirli Glikolipidler, Doğal Gıda Koruyucusu, Mantar Fermentasyonu

ACKNOWLEDGEMENTS

I would like to thank my supervisors Prof. Dr. Melek Özkan and Dr. Yudi Yang for their support and guidance during my MSc thesis project. Their critical view and questions were inspiring and made me think critically about the experiments and the obtained results.

Next, I would like to thank Dr. HE Aimin, CEO Handary SA for his brilliant concept, insights, participation and financial support in this research.

I would like to thank to Ruchi Shah, R&D Director, Handary SA and all the members of the Biotechnology Laboratory (Department of Environmental Engineering, Gebze Technical University) for providing valuable knowledge and assisting the research. With their comments and contributions, this study has been improved in many ways.

Next, I would like to thank my friend, Zeynep Büşra Çakır, for her company in the lab. Our discussions about the experiments upgraded this dissertation. Besides, the long days and early mornings were definitely more fun with her company!

Furthermore, I would like to thank my friends Büşra Şahin and Habibe Kurt for their help during my experiments.

Lastly, I would like to thank my sister Kübra Kabasakal and my parents İsmail Kabasakal and Sevil Kabasakal. Not only during my MSc studies, but also during my whole journey as a student, you always listened to my exciting stories and guided me through harder times.

This thesis was under a signed collaboration with industrial partner Handary



SA Natural Shelf Life Solutions (Belgium), who owns the project idea and supports the thesis studies financially, and Gebze Technical University (Turkey), who provides laboratories, infrastructures and financial support.

I would like to express my deep gratitude to Handary SA for their valuable support to my thesis study and my career.

TABLE of CONTENTS

SUMMARY	i
ÖZET	ii
ACKNOWLEDGEMENTS	iii
TABLE of CONTENTS	iv
LIST of ABBREVIATIONS and ACRONYMS	vii
LIST of FIGURES	ix
LIST of TABLES	xii
1.INTRODUCTION	1
1.1.Aim and Contribution of Thesis	3
1.2.Natural Antimicrobials Used in Food Preservation	3
1.2.1.Antimicrobials of Plant Origin	4
1.2.2.Antimicrobials of Animal Origin	5
1.2.3.Antimicrobials of Microbial Origin	7
1.3.Glycolipids: A Promising Natural Food Preservative Agent	9
1.3.1.Antimicrobial Activity of LGLs	11
1.3.2.Toxicity Analyses of LGLs	12
1.3.3.Molecular Characteristics of LGLs	13
1.4.Synthesis of LGLs via Fermentation	14
1.4.1.Producing Organism: <i>Dacryopinax spathularia</i>	14
1.4.2.Production of LGLs by <i>D. spathularia</i>	16
1.5.Downstream Process of LGLs	19
1.5.1.Separation of LGLs: Centrifugation, Decantation, Filtration and precipitation	20
1.5.2.Solvent Extraction	21
1.5.3.Calorimetric Assays for LGLs Yield Determination	22
1.5.4. Usage of Ion Exchange Resins for Purification of LGLs	23
1.5.5. Chromatographic Techniques for Characterization of LGLs	23
2.MATERIALS AND METHODS	25
2.1.MATERIALS	25
2.1.1.Microorganism	25
2.1.2.Chemicals	25

2.1.3.Laboratory Devices	26
2.2.METHODS	27
2.2.1.Preparation of <i>D. spathularia</i> Glycerol Stocks	27
2.2.2. <i>D. spathularia</i> Fermentations	27
2.2.2.1.Cultivation Media	27
2.2.2.2.Inoculation	28
2.2.3.Follow-up of the Fermentation	28
2.2.3.1. Measurement of Growth by Absorbance at OD ₆₀₀	28
2.2.3.2. Cell Dry Weight Measurement	28
2.2.3.3. HPLC Measurement	29
2.2.3.4. TLC Measurement	29
2.2.3.5.Microscopy	30
2.2.4.Antimicrobial Tests	30
2.2.4.1.Antimicrobial Efficacy Test in Broth Cultures	30
2.2.4.2.Kirby-Bauer Antimicrobial Disk Assay Method	31
2.2.5.Downstream Processing Applied to the Fermentation Broth for Extraction of Glycolipids	32
3.RESULTS AND DISCUSSION	34
3.1. Shake Flask Fermentations	34
3.1.1. <i>D. spathularia</i> Cell Morphology	34
3.1.2.Growth Curve of <i>D. spathularia</i>	35
3.1.3.The Growth of <i>D. spathularia</i> in Baffled and Unbaffled SFs	36
3.1.4.The Growth of <i>D. spathularia</i> in Different Growth Media	38
3.1.5.The Effect of pH during <i>D. spathularia</i> Fermentation	41
3.1.6.The Effect of Temperature during <i>D. spathularia</i> Fermentations	42
3.2. Antimicrobial Activity Tests	43
3.2.1.Kirby-Bauer Antimicrobial Disk Assay with LGLs Precipitate	43
3.2.1.1. Disk Diffusion Assay with LGLs Precipitates from 7 days <i>D. spathularia</i> Cultivation in Baffled and Unbaffled SFs	44
3.2.1.2.Antimicrobial Effect of LGLs Precipitates Obtained from 14 days, 21 days and 28 days <i>D. spathularia</i> Cultivation in Unbaffled SFs	45
3.2.2.Antimicrobial Efficacy Tests in Broth Cultures	48

3.2.2.1. Antimicrobial Efficacy Tests with Diluted LGLs Precipitate from 21 days old <i>D. spathularia</i> cultures in unbaffled SFs	48
3.2.2.2. Antimicrobial Efficacy Tests in Broth Cultures with Concentrated LGLs Precipitate from 21 days and 28 days Old <i>D. spathularia</i> Cultures in Unbaffled SFs	49
3.2.2.3. The Effect of the Concentration of LGLs Precipitate Antimicrobial Activity	51
3.3. Chromatographic Analysis	53
3.3.1. TLC Analysis	53
3.3.2. HPLC Analysis	54
4. CONCLUSION	57
REFERENCES	58
BIOGRAPHY	70

LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Celcius
cm	: Centimeter
d	: Day
g	: Gram
h	: Hour
L	: Liter
m	: Meter
M	: Molar
µg	: Microgram
µl	: Microliter
µm	: Micrometer
mg	: Milligram
mL	: Milliliter
mm	: Millimeter
nm	: Nanometer
N	: Normality
%	: Percentage
t	: Time
v	: Volume
w	: Weight
AM-1	: Long-chain glycolipids from <i>Dacryopinax spathularia</i>
EFSA	: European Food Safety Authority
EOs	: Essential Oils
FAF	: Food Additive and Flavouring
FDA	: Food and Drug Administration

GLs	:	Glycolipids
GM2	:	Glucose Yeast
GOD	:	Glucose oxidase
GRAS	:	Generally Regarded as Safe
HMG	:	3-Hydroxy-3-Methylglutarate
HPLC	:	High Pressure Liquid Chromatography
HPLC-MS	:	High Pressure Liquid Chromatography Mass Spectrometry
LGLs	:	Long-chain Glycolipids
MESP	:	Malt Extract Soy Peptone
SFs	:	Shake Flasks
SmF	:	Submerged Fermentation
TLC	:	Thin Layer Chromatography
YMG	:	Yeast Malt Glucose

LIST of FIGURES

<u>Figure No</u>	<u>Page</u>
1.1 :	The factors causing food spoilage. 1
1.2 :	Natural antimicrobials from different origins. 4
1.3 :	Illustration of antimicrobial activity of glycolipids on spoilage microorganism. (a) The fatty acid unit of glycolipid establishes a bond with the cell membrane of spoilage microorganism. (b) The cell membrane of spoilage microorganism is damaged and ruptured by glycolipids. (c) Without the membrane, spoilage microorganism collapse and die. 10
1.4 :	Chemical structures of the three main glycolipid components 13
1.5 :	<i>Dacryopinax spathularia</i> grows on (a) decaying, decorticated, wood substrates in clusters and (b) individually. 15
1.6 :	<i>D. spathularia</i> . (a) Hyphae without clamp connection (black arrow), scale bar = 10 μ m. (b) Sausage-shaped, smooth; single septum, measured 0.5-3.8 microns. 16
1.7 :	Downstream processes: long-chain glycolipid extraction and purification methods 19
2.1 :	The steps of antimicrobial test in broth cultures 31
2.2 :	Downstream processing of the fermentation broth 32
3.1 :	<i>D. spathularia</i> cells A) under 10X, B) under 40X C) under 100X magnification after 120 h cultivation at 30°C and 180 rpm in malt extract soy peptone broth. Red colored arrows indicate budding spores, blue colored arrows indicate basidiospores and black colored arrows indicate probasidium and basidium formation. 34
3.2 :	Growth of <i>D. spathularia</i> and pH change in unbaffled flask A) Change in absorbance at 600 nm and pH B) Change in CDW and pH. 35
3.3 :	The change of CDW and absorbance (OD600 nm) during <i>D. spathularia</i> cultivation in A) baffled and B) unbaffled shake flasks. 37

3.4 :	pH change during <i>D. spathularia</i> growth in baffled and unbaffled flasks.	38
3.5 .:	Monitoring <i>D. spathularia</i> growth with absorbance (OD600 nm) (A) and CDW (B) in MESP, YMG and GM2 media during 168 h cultivation.	39
3.6 :	Change in pH during growth of <i>D. spathularia</i> in MESP, YMG and GM2 media	40
3.7 :	Growth of <i>D. spathularia</i> at pH 4.6 and 5.6.	41
3.8 :	Growth of <i>D. spathularia</i> at 30°C and 33°C.	42
3.9 :	Inhibition zone for <i>E. coli</i> BL21 and <i>B. subtilis</i> with GLs precipitate obtained from 7 days old <i>D. spathularia</i> culture in baffled shake flask.	44
3.10 :	Inhibition of <i>E.coli</i> BL21, <i>B. subtilis</i> , <i>P. aureginosa</i> , <i>S. aureus</i> , <i>S. cerevisiae</i> with GLs precipitate obtained from 14 days <i>D. spathularia</i> cultivation in unbaffled shake flasks.	45
3.11 :	Inhibition of <i>B. subtilis</i> and <i>S. cerevisiae</i> with GLs precipitate from 21 days old <i>D. spathularia</i> culture.	46
3.12 :	Inhibition of <i>B. subtilis</i> and <i>S. cerevisiae</i> with GLs precipitate from 28 days old <i>D. spathularia</i> culture.	46
3.13 :	The inhibition percentage of <i>E. coli</i> BL21, <i>B. subtilis</i> , <i>S. aureus</i> and <i>P. aeruginosa</i> and <i>S. cerevisiae</i> growth with GLs precipitate. Inhibition of <i>S. cerevisiae</i> growth with A) GLs precipitate from 21 days old and B) GLs precipitate from 28 days old <i>D. spathularia</i> culture.	49
3.14 :	Inhibition of <i>S. cerevisiae</i> growth with A) GLs precipitate from 21 days old and B) GLs precipitate from 28 days old <i>D. spathularia</i> culture.	50
3.15 :	Inhibition of <i>B. subtilis</i> growth with GLs precipitate from 28 day old <i>D. spathularia</i> culture.	51

- 3.16 : Growth inhibition by GLs precipitate in *B. subtilis* and *S. cerevisiae* cultures. (A) Absorbance (OD600) versus time during *S. cerevisiae* cultivation. (B) (OD600) versus time during *B. subtilis* cultivation. (C) Growth inhibition of *S. cerevisiae* culture due to different concentration of GLs precipitate. (D) Growth inhibition of *B. subtilis* culture due to different concentration of GLs precipitate. 52
- 3.17 : TLC analysis of LGLs from different cultivation times. (A) TLC plate stained with ethanol (95%, v/v). (B) TLC plate stained with sulphuric acid (10%, v/v). 53
- 3.18 : HPLC analysis of GLs precipitates from 7 days and 28 days and negative control. Blue solid line represents 28 days and red solid line represents 7 days. 55

LIST of TABLES

<u>Table No:</u>	<u>Page:</u>
1.1 : Plant-based natural antimicrobials used in food industry.	5
1.2 : Animal-based natural antimicrobials used in food industry.	6
1.3 : Microbial-based natural antimicrobials used in food industry.	7
1.4 : Antimicrobial glycolipids in food industry.	9
1.5 : Commonly used techniques for the extraction and purification of glycolipids.	22
2.1 : Laboratory devices used throughout the experiments.	26
2.2 : The composition of the MESP liquid medium.	27
2.3 : The list of the microorganisms used in the antimicrobial tests.	31
3.1 : Growth mediums used in medium screening experiments.	38
3.2 : The diameter of inhibition zones (in mm) obtained with LGLs precipitate from 7, 14, 21 and 28 days supernatants.	47
3.3 : Amount of LGLs and control solutions used for inhibition experiments.	50

1. INTRODUCTION

The world population has grown exponentially over the past centuries [Cohen, 2020]. During the 20th century alone, the population in the world has grown from 1.65 billion to 6 billion [Max Roser et al., 2013]. With the tremendous growth in the world population, the need to produce and transport food and beverages to far distances while maintaining its texture, nutritional value, and flavor has arisen.

Depending on the type of the product, physical, chemical, and microbial factors can cause spoilage and affect safety of food products throughout transport and retail operations (Figure 1.1) [Amit et al., 2017].

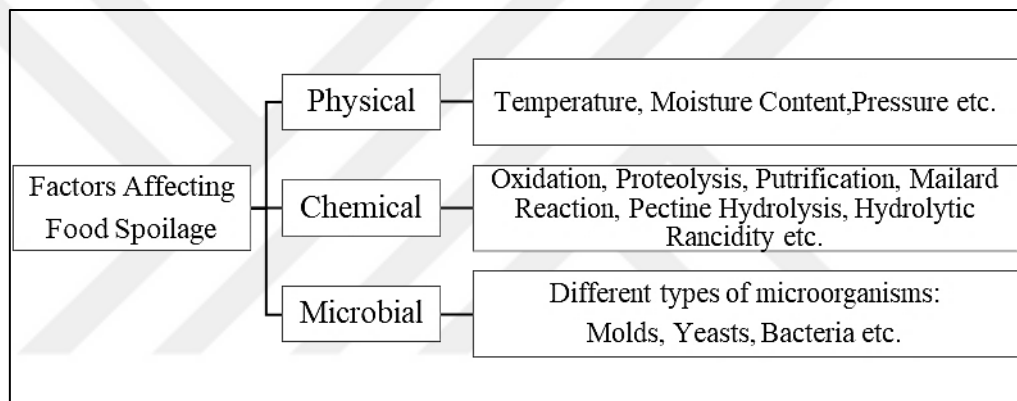


Figure 1.1: The factors causing food spoilage.

To minimize food loss and achieve a longer shelf-life, several conventional and modern preservation techniques like drying, freezing, chilling, pasteurization, irradiation, canning and addition of preservative agents have been utilized up to date [Amit et al., 2017]. Although these methods stop or greatly slow down spoilage, the problems like change of flavor, alteration in texture, and formation of undesirable substances have been faced throughout these applications. To cope with these problems, increasing research efforts have been put in developing methods that are more efficient, easy to apply, and natural in origin [Juneja et al., 2012][Kourkoutas and Proestos, 2020]. Until recently, the use of synthetic additives in food and beverages was considered as an efficient way for preventing the growth of microorganisms and making the food last longer [Abdulummeen et al., 2012][Carocho et al., 2014]. However, due to consumer concerns regarding potential adverse health effects of synthetic food additives such as allergenicity and

carcinogenicity, the use of natural compounds have become of great interest [Anand and Sati, 2013][Gultekin and Doguc, 2013][Mirza et al., 2017]. This has led natural food preservative agents to be favored for use in food and beverage industry.

In accord with this, numerous natural compounds derived from different sources like plants, animals and microorganisms have been discovered and employed to ensure safety and quality of products. Some examples of such compounds include peptides, enzymes, bacteriocins, bacteriophages, plant extracts (e.g., rosemary, basil, thyme, oregano, cinnamon, clove), fermented compounds (e.g., alcohol, propionic, citric acid) and glycolipids (e.g. rhamnolipids, sphorolipids) [Amiri et al., 2021] . The mechanism of preservation action for these compounds differs depending on their antimicrobial or antioxidant property. Considering the multiple factors involved in different preservation mechanisms, finding an efficient solution to provide sustainable and natural protection is not always easy. Yet, humans are lucky to have nature's helping hand; by inspiring the mechanisms already exist in nature, developing solutions to those challenges could be possible.

One example to such solutions is long-chain glycolipids of *Dacryopinax spathularia*. *D. spathularia* is a spatula-shaped edible jelly fungus which occurs on the wood substrates [Bensch, 1948; IMO Natural Solutions GmbH, 2017]. It produces glycolipid (GLs) molecules which exhibit antimicrobial property [Younes et al., 2021]. By producing glycolipids, *D. spathularia* keeps its food (e.g. wood substrate) spoilage free. In the same manner, those molecules have been shown to be effective for keeping food and beverage products spoilage free [Marquardt, 2019]. Their use as a preservative food additive in soft drinks has been permitted by Food and Drug Administration (FDA) in the US [IMO Natural Solutions GmbH, 2017].

With its natural origin and antimicrobial property, long-chain glycolipids hold great market potential not only for food and beverages but also cosmetics and pharmaceuticals [Faivre and Rosilio, 2010; Lourith and Kanlayavattanakul, 2009]. Further studies are needed to get deeper insights into properties of long-chain glycolipids and enlighten its production process. This thesis focuses on developing an optimized fermentative production and downstream purification system for long-chain jelly mushroom glycolipids.

1.1. Aim and Contribution of Thesis

D. spathularia was selected for fermented long-chain glycolipids (LGLs) production. LGLs from *D. spathularia* have been reported among the GRAS (Generally-Recognized-As-Safe) substances by FDA. The aim of this thesis was twofold consisting both upstream and downstream process development for fermentative production of LGLs. In this context, firstly fermentation behavior of *D. spathularia* strain, different culture mediums and substrate compositions were evaluated.

In the second stage, efforts were focused on optimization of the fermentation time to achieve highest antimicrobial activity, and also developing effective downstream strategies to recover the LGLs from fermentation end broth. Kirby-Bauer disk diffusion assay and cell density reading of actively growing liquid cultures at OD₆₀₀ were used to check the presence of growth inhibition due to antimicrobial activity of LGLs. The antimicrobial efficacy of LGLs against five different microorganisms, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Saccharomyces cerevisiae* were evaluated. To the best of our knowledge, such a comprehensive study to study both the characteristics of *D. spathularia* and antimicrobial activity of its secondary metabolite LGLs, has not been reported in the literature up to date.

1.2. Natural Antimicrobials Used in Food Preservation

Over the last few decades, a series of studies and scientific assessments have been conducted to discover novel natural antimicrobials for use in food industry [Barberis et al., 2018; Santos-Sánchez et al., 2017; Smid and Gorris, 2020]. In result, several naturally occurring antimicrobial compounds have been reported for their potential use in food and beverage protection. Depending on the origin of the compound, antimicrobials can be classified in three categories: antimicrobials of plant origin, antimicrobials of animal origin and antimicrobials of microbial origin (Figure 1.2.). Mostly those compounds are secondary metabolites of plants, animals or microorganisms [Rahman and Sarker, 2020][Bouarab Chibane et al., 2019].

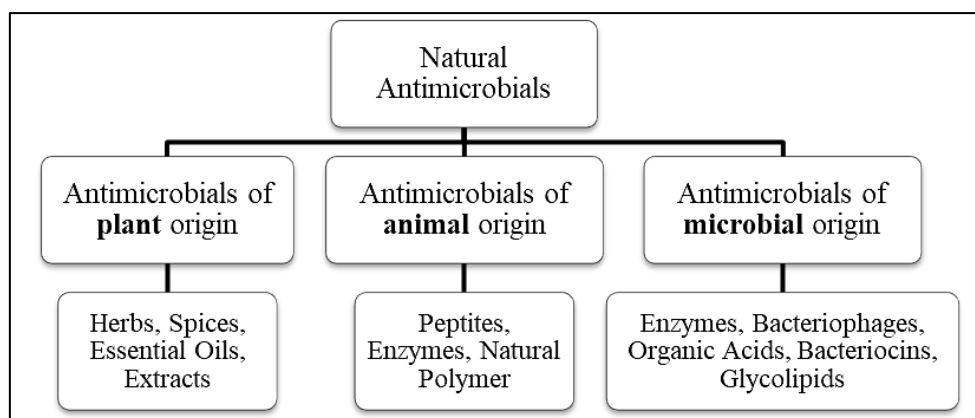


Figure 1.2: Natural antimicrobials from different origins.

Although, the secondary metabolites are not essential for the viability of producing organism, their antimicrobial property provide competitive advantages over food spoilage microorganisms and makes them valuable to utilize as food protective additives. This section provides an overview of natural antimicrobials from different sources, plants, animals and microorganisms, with a special focus for use in food industry.

1.2.1. Antimicrobials of Plant Origin

Plants are valuable sources of bioactive compounds that possess antimicrobial activity against food spoilage microorganisms. The four main categories of antimicrobial compounds from plant origin can be classified as spices, herbs, extracts and essential oils. Herbs and spices obtained from different plant parts have long been known for their antimicrobial activities in food and beverages [Martínez-Graciá et al., 2015; Tajkarimi et al., 2010]. Some of examples of antimicrobial herbs and spices have been known as peppers, chillies, coriander, clove, cumin, cinnamon, ginger, thyme, onion, oregano, rosemary and sage (See Table 1.1). Essential oils (Eos) extracted from different plants also contain ingredients functional as food grade antimicrobial. Plant parts, like leaves of basil, bulbs of garlic, clove buds and seeds of parsley have been known to serve as a source for Eos production. Saponins, flavonoids, carvacrol, thymol, citral, eugenol, and linalool are the compounds reported to provide antimicrobial function in Eos [Chen et al., 2020; Nguyen et al., 2020; Souza et al., 2022].

Table 1.1: Plant-based natural antimicrobials used in food industry.

Plant-based antimicrobials	Examples	References
Essential oils	Cinnamon	[Van Haute et al., 2016]
	Clove	[Sharma et al., 2020]
	Coriander	[Šojić et al., 2019]
	Lemongrass	[Hamad et al., 2019]
	Litsea Cubeba	[Dai et al., 2021]
Spices	Chilli pepper	[Clément et al., 2016]
	Garlic	[Mozaffari Nejad et al., 2014]
	Ginger	[Dixit and Luqman, 2015]
Herbs	Sage	[Moghimi et al., 2016]
	Rosemary, Thyme and Clove	[Saricaoglu and Turhan, 2018]
	Turmeric	[Gul and Bakht, 2015]
Extracts	Grape seed	[Memar et al., 2019]
	Pomegranate and Cranberry	[Daoutidou et al., 2021]

Most of the essential oils are classified as GRAS compounds, yet their use as a preservative agent faces challenges like changes in sensory properties of foods, the need for high concentrations to achieve effective microbial control, alterations in flavor and poor miscibility of Eos in aqueous environments [Mariod, 2016]. Moreover, when antimicrobials from essential oils used, the required dose to achieve effective antimicrobial function often outpaces the organoleptic acceptability level. To cope with these problems, recently different techniques like encapsulation and biodegradable edible coatings have been used. Further research is needed to develop an efficient way to utilize EOs in food systems.

1.2.2. Antimicrobials of Animal Origin

There are numerous antimicrobial compounds of animal origin, and they have often evolved as host defense mechanisms. Lysozyme, lactoferrin and transferrin are naturally occurring antimicrobial proteins found in the defense system of living organisms [Aminlari et al., 2014; Duran and Kahve, 2017]. The mechanism of antimicrobial activity of enzymes from animal origin often depends on their

membrane disruption and gap-formation ability. Lysozyme is classified as GRAS compound and its various forms either alone or in combination with other antimicrobials are commercialized for use as antimicrobial food additive [U.S. Food and Drug Administration, 2019]. Lactoferrin is another antimicrobial protein found in milk. Due to its iron-binding capacity and polycationic nature, lactoferrin shows antimicrobial function against foodborne pathogens like *Carnobacterium*, *Listeria monocytogenes* and *E. coli*. Its use on beef has been permitted in the United States [FDA, 2016]. Peptides like pleurocidin and protamine exhibit nonspecific mechanisms of antimicrobial action, by providing selectivity between different microorganisms (e.g. Gram-negative bacteria over Gram-positive bacteria) (See Table 1.2).

Table 1.2: Animal-based natural antimicrobials used in food industry.

Animal-based antimicrobials	Examples	References
Peptides	Pleurocidin	[Burrowes et al., 2004]
	Protamine	[Potter et al., 2005]
	Lactoferricin	[Niaz et al., 2019]
Enzymes	Lysozyme	[Moshtaghi et al., 2018]
	Lactoperoxidase	[Montiel et al., 2015]
Natural polymers	Chitosan	[Wang, 1992]
	Poly-L-Lysine	[Chheda and Vernekar, 2015]

Natural polymers like chitosan and poly-l-lysine also display antimicrobial function against food pathogens and spoilage microorganisms. Chitosan is a naturally occurring, polycationic biopolymer found in the shells of crustaceans, squid pen, and crawfish. Due to its antimicrobial activity against food spoilage organisms like fungi, and Gram-positive and Gram-negative bacteria, it has been extensively utilized as antimicrobial packaging film and coating agent. However, its use as a food preservative is often faces insolubility problems at neutral and higher pH values.

1.2.3. Antimicrobials of Microbial Origin

Microorganisms produce a broad range of components that can exhibit antimicrobial property against other microorganisms. Lactic acid bacteria (LAB) are the most important group of microorganisms, which are a source for natural antimicrobials. Bacteriocins of LAB contain ribosomally synthesized antimicrobial peptides used in food industry to prevent undesirable microbial growth [Cleveland et al., 2001]. Some examples of bacteriocins such as nisin, diplococcin, plantaricin, bulgarican, helveticin, and pediocin have been reported as food preservatives due to their antagonistic effect on important food pathogens [Deegan et al., 2006; Settanni and Corsetti, 2008; Yang et al., 2014] (See Table 1.3).

Table 1.3: Microbial-based natural antimicrobials used in the food industry.

Microbial-based antimicrobials	Examples	References
Enzymes	Glucose oxidase	[Li et al., 2019]
Bacteriophages	Ecoshield™	[Carter et al., 2012]
	Salmofresh™	[Sukumaran et al., 2016]
Organic acids	Propionic acid and Lactic acid	[Fernandez et al., 2017]
	Sorbic acid	[Salleh et al., 2021]
Bacteriocins	Nisin	[Bahrami et al., 2019]
	Natamycin	[Saito et al., 2022]
	Pediocin	[Ray, 2019]
	Enterocin	[Furlaneto-Maia et al., 2020]

Among all bacteriocins, nisin, pediocin and reuterin have been extensively studied to be employed by food processors as an additional barrier against undesirable bacterial growth. Nisin has been approved for use as food additive by FDA [Ebenezer et al., 1992]. It is produced by *Lactococcus lactis* and highly effective against Gram-positive foodborne pathogens or spore forming bacteria such as *S. aureus*, *M. luteus*, and *B. cereus* [Darbandi et al., 2022]. The antimicrobial activity of nisin is achieved by increasing permeability of the cytoplasmic membrane

and causing leakage of intracellular metabolites [Moshtaghi et al., 2018]. Reuterin (β -hydroxypropionaldehyde) is another antimicrobial compound effective against many pathogenic and food spoilage microorganisms. It is a water-soluble non-proteinaceous metabolite of glycerol, produced by some strains of *Lactobacillus reuteri* [Montiel et al., 2015]. It shows antimicrobial activity against both Gram-negative and Gram-positive bacteria, yeasts, and filamentous fungi.

Besides bacteriocins, enzymes such as glucose oxidase (GOD) also classified as antimicrobial food additive and received GRAS status by FDA [Candice, 2017]. GOD is mainly produced by *Aspergillus niger* and *Penicillium* species, and used as additional protection against spoilage organisms in baking products, dry egg powder and beverages [Anjum Zia et al., 2013; Li et al., 2019; Wong et al., 2008].

Bacteriophages as natural preservatives provide another favorable alternative to ensure food safety and quality. Bacteriophages are viruses that only targets and disrupts bacterial cells. By providing high selectivity against a specific type of spoilage bacteria, they extend the shelf life of food products. Products like EcoshieldTM and SalmofreshTM are examples of commercially available bacteriophages effective for reducing *E. coli* in beef, and *Salmonella* in chicken breast fillets [Carter et al., 2012; Sukumaran et al., 2016]. Upon use of bacteriophages in wide range of applications, problems like slow or incomplete fermentation have been reported. For instance, phage infection of starter cultures causes product downgrading and huge financial losses in dairy industry, particularly when LAB used as a starter culture [Marcó et al., 2012; Mills et al., 2019].

Apart from abovementioned antimicrobial agents, microbial glycolipids (GLs) have been also utilized to combat food spoilage organisms due to their excellent surface assembly and antimicrobial property [Mnif and Ghribi, 2016]. They are produced via various microorganisms and purified for further use in food industry [Abdel-Mawgoud and Stephanopoulos, 2018]. Depending on their molecular weights and sub-units, glycolipids are classified in different groups. The detailed information on molecular characteristics, fermentative production and downstream purification of GLs are given throughout Section 1.3.

1.3. Glycolipids: A Promising Natural Food Preservative Agent

Glycolipids are carbohydrate-attached lipids with important biological functions. As the name refers, they consist of two parts: carbohydrate group and fatty acid chains attached by a glycoside bond. The differences in carbohydrate and fatty acid group results occurrence of various type of glycolipids. Rhamnolipids, sophorolipids, mannosylerythritol lipids, cellobiose lipids, and xylolipids are some of the well-known components that provide diversity in glycolipids.

Owing to their superior structural properties such as amphipathicity, self-assembly and low molecular weight, GLs have gained particular interest to be utilized in various industrial applications. GLs of microbial origin especially have been widely studied to be used in food industry as natural, biodegradable antimicrobial biosurfactant.

GLs are produced by microorganisms either naturally or in specific culture conditions. Some of the glycolipid-producing microorganisms have been reported as *Pseudomona aeruginosa*, *Micrococcus luteus*, *Wickerhamiella domericqiae*, *Lactococcus lactis*, *Dacryopinax spathularia*, *Cryptococcus humicola* and *Candida bombicola* [Bitzer et al., 2018; Morita et al., 2011].

To locate the antimicrobial activity of glycolipids against wide range of food spoilage microorganisms, massive literature has been produced. Some examples of the recent studies on glycolipids are listed in Table 1.4 with the target and producing microorganism of GLs.

Table 1.4: Antimicrobial glycolipids used in food industry.

Type of Glycolipids	Producing Organism	Target Organism	References
Saphorolipids	<i>Starmerella bombicola</i> ATCC 22214 TM	<i>Botrytis cinerea</i> ,	[de O Caretta et al., 2022]
		<i>Sclerotium rolfsii</i>	
		<i>Rhizoctonia solani</i> and	
Rhamnolipids Table 1.4 continued	<i>Pseudomonas aeruginosa</i>	<i>Listeria monocytogenes</i>	[Magalhães and Nitschke, 2013]
Mannosylerythritol	<i>Pseudozyma aphidis</i>	<i>Listeria monocytogenes</i>	[Liu et al., 2020]

The efficacy of glycolipid's antimicrobial activity differs depending on the type of food pathogen and physico-chemical nature of the food product. Although the exact mechanism has not been fully described yet, the underlying mode of antimicrobial action mainly lies on disruption of the cell membrane and leakage of cellular components (Figure 1.3) [Shu et al., 2019, 2020]. The damage on the cell membrane results in inactivation of spoilage microorganism, hence the safety of food is ensured.

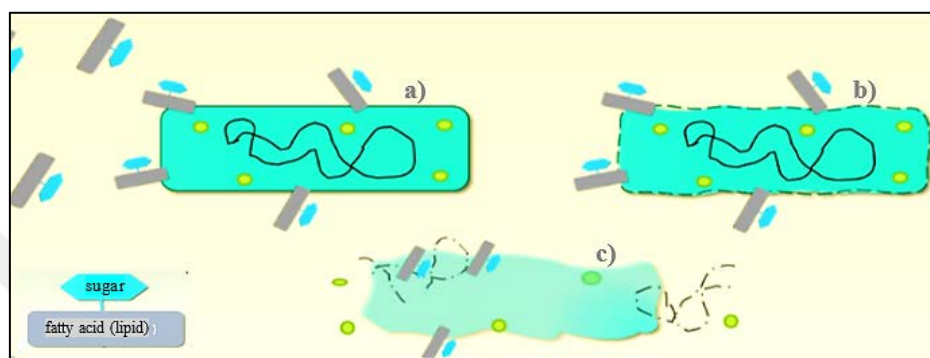


Figure 1.3: Illustration of antimicrobial activity of glycolipids on spoilage microorganism. (a) The fatty acid unit of glycolipid establishes a bond with the cell membrane of spoilage microorganism. (b) The cell membrane of spoilage microorganism is damaged and ruptured by glycolipids. (c) Without the membrane, spoilage microorganism collapse and die.

With careful formulations, the efficacy of antimicrobial activity in glycolipids can be improved to some extent; however the use of additional chemicals is often inevitable to increase the stability. In this context, there are not many products available in the market. The long-chain glycolipids (LGLs) from *D. spathularia*, however, are one of the excellent exemptions. These glycolipids, also called as AM-1, are obtained as a mixture through aerobic fermentation of non-genetically modified fungus, *D. spathularia* [Younes et al., 2021]. They can be utilized as food-grade antimicrobial in non-alcoholic beverages without the use of any additional chemicals or solvents. In the United States, AM-1 glycolipids are classified as GRAS substance due to their antimicrobial function in a variety of beverages, including fruit drinks, ready to drink teas, energy drinks, enhanced waters and juices [IMO Natural Solutions GmbH, 2017].

1.3.1. Antimicrobial Activity of LGLs

The antimicrobial activity of LGLs on food pathogens and spoilage organisms has been reported with several studies. In the GRAS notice of LGLs, the antimicrobial efficacy of LGLs was compared with two commonly known food preservative agents which are benzoic acid and sorbic acid [Gaynor, 2017]. The activity against five food spoilage organisms, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Zygosaccharomyces*, *Dekkera bruxeliensis* and *Byssoschlamys fulva* was determined by Minimum Inhibitory Concentrations (MICs) assay for LGLs, benzoic acid and sorbic acid. In result, MIC values obtained with LGLs were lower than sorbic acid and benzoic acid for all of the tested organisms. While the MIC value did not exceed 7.8 mg/L for LGLs, no readout was recorded below 250 mg/L for benzoic acid and sorbic acid.

Following this study, the minimum effective concentration of LGLs were determined for carbonated soft drinks, fruit drinks, teas, enhanced waters, energy drinks, sport drinks and juices. For this purpose, 10 mg/L LGLs were added to each beverage. Without LGLs, rapid spoilage was observed within a few days, while the LGLs added beverages remained without microbial growth during the test period. 1000 ppm sorbate and benzoate addition was needed to pass the test and 500 ppm was not sufficient in any of the tested beverages. Moreover with the results of this study, LGLs have been reported to show very potent activity against yeast and mold; good activity against Gram-positive bacteria; and weak or no activity against Gram-negative bacteria [Gaynor, 2017].

Apart from abovementioned studies, the antimicrobial potential of LGLs in dairy industry has been also investigated by a few researchers. In a very recent study, the antimicrobial activity of LGLs to control the growth of *Listeria monocytogenes* was investigated. LGLs were added in skim milk and also applied over polystyrene and stainless steel food processing surfaces to investigate its effectivity to prevent biofilm formation [Sun et al., 2021a]. In result, *L. monocytogenes* in skim milk was lowered below its enumeration level, and the antimicrobial effect was reported as highly depended on concentration of LGLs and fat content of milk. Moreover, significant reduction in biofilm formation was observed and initial growth of *L. monocytogenes* inhibited on Queso Fresco cheese. Similarly, another study was

carried out to determine the growth inhibition of LGLs on three predominant spore-forming spoilage bacteria in UHT whole and skim milk. Addition of 50 mg/L of LGLs was reported as bactericidal against *Paenibacillus odorifer*, *Bacillus weihenstephanensis* while same effect was achieved with 100 mg/L for *Viridibacillus arenosi* [Sun et al., 2021b]. Although these findings have paved the way LGLs to be utilized as shelf-life extender in dairy industry, further studies are needed to identify potential effects on the organoleptic properties of dairy products.

1.3.2. Toxicity Analyses of LGLs

Several toxicity experiments have been performed on rats and dogs in order to assess safety of LGLs for food uses. In a study carried out by Bitzer et al., pharmacokinetics, excretion balance, and tissue distribution of LGLs were examined on Sprague Dawley rats. Oral bioavailability was reported as very low at around 11% upon administration of 100 mg/kg of LGLs. Approximately 89% of the administered LGLs was recovered in expired air, urine, feces, and carcass [Bitzer et al., 2017a]. Another study performed by the same research group, focused on toxicity evaluation of LGLs in Beagle Dogs. For a period of 90 days, LGLs were introduced at doses of 150, 500 or 1000 mg/kg/day. The results were shown that LGLs up to 1000 mg/kg/day was well tolerated [Bitzer et al., 2017b]. Similarly, the subchronic toxicity of LGLs was studied in CrI:CD(SD) rats for 90 days. LGLs were administered via the drinking water at concentrations of 1.5, 5.0 or 15 mg/mL for 90 days. No changes in behavior, organs or in external appearance were observed on study animals during 90 days period [Bitzer et al., 2017c].

Further research was focused on investigation of potential reproductive and developmental toxicity of LGLs. For this purpose, three different dosages 150, 500, and 1000 mg/kg/day were administered prior to and during mating, throughout gestation, lactation, and postnatally. No side effects on intrauterine growth and survival, or fetal morphology were reported [Bitzer et al., 2018]. Moreover, the genetic toxicity potential of LGLs was evaluated in three in vitro test systems; bacterial reverse mutation assay (Ames test), the micronucleus test, and the mouse lymphoma thymidine kinase assay. Negative results in all three studies showed that LGLs does not cause any genotoxicity under the conditions of the experiments

[Bitzer et al., 2019].

With the aforementioned reproductive and prenatal developmental toxicity studies, the EFSA (European Food Safety Authority) Panel on Food Additives and Flavourings (FAF) set 10 mg/kg bw per day as an acceptable daily intake (ADI) for the use of LGLs from *Dacryopinax spathularia* [Younes et al., 2021]. Overall, the existent studies in literature have confirmed that LGLs have insignificantly low potential to cause any developmental or reproductive toxicity on humans.

1.3.3. Molecular Characteristic of LGLs

The compositions of long-chain glycolipids and specifications regarding its structure have been reported with several studies. In its GRAS statement approved by FDA, three structurally-related glycolipid components have been shared (Figure 1.4) [IMO Natural Solutions GmbH, 2017].

The core structure of LGLs is a hydroxylated long-chain fatty acid (C-26) with a glucopyranosyl-(1→2)-xylopyranosyl-(1→2)-xylopyranosyl trisaccharide group which is linked by glycosidic bond to ω-hydroxy position. Depending on the number of hydroxyl groups of the fatty acid moiety and acylation motifs of the sugar part, the LGLs differ in structure. Depending on acylation motifs, sugar part can contain acetyl, isovaleryl or 3-hydroxy-3-methylglutarate (HMG) unit.

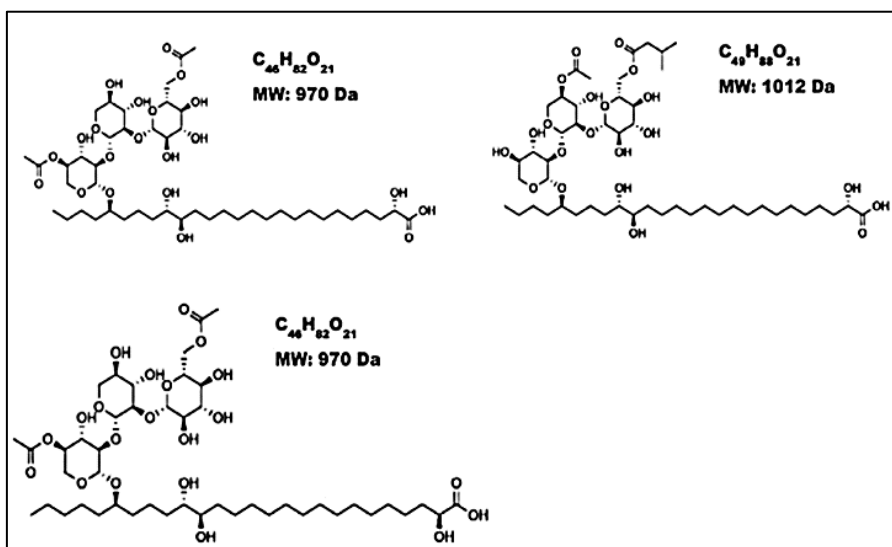


Figure 1.4: Chemical structures of the three main glycolipid components.

LGLs from *D. spathularia* are classified under eight groups based on their molecular weights, MW=886, 928, 954, 970, 1,012, 1,054, 1,072, 1,114 Da. Moreover it has been mentioned that LGLs within MW group 886 do not contain any acyl groups in its structural formula. However LGLs with 928, 954, 970, 1,012, 1,054 Da contain one acetyl group thus classified as mono-acetylated glycolipids. The highest MW groups, MW 1,072 and 1,114 Da, have 3-Hydroxy-3-Methylglutarate (HMG) as acyl groups. HPLC-MS analysis has been used to confirm the presence those MW groups in LGLs [Gaynor, 2017].

1.4. Synthesis of LGLs via Fermentation

Synthesis of LGLs is possible via various chemical and biological routes [Wakao and Suda, 2008]. Chemical synthesis offer good yields yet brings several economic and environmental considerations together. For instance, when produced via chemical routes, formation of toxic compounds might occur due to the use of non-sustainable sources such as petrochemical substrates [Abdel-Mawgoud and Stephanopoulos, 2018; Fleurackers, 2014].

In order to avoid such problems, biological routes such as enzymatic reactions and fermentative productions can be utilized [Grüninger et al., 2019; Liepins et al., 2021; Pöhnlein et al., 2015]. With biological pathways, producing biodegradable and ecofriendly GLs from renewable resources is possible. Biological routes are favored when the glycolipids are produced for use in food or pharmaceutical industry. In particular, fermentative productions have resulted in formation of highly active food-grade glycolipid products over the decade. During, the information regarding the fermentative production of glycolipids with a special focus on long-chain glycolipids from *D. spathularia* will be studied.

1.4.1. Producing Organism: *Dacryopinax spathularia*

D. spathularia is a species of fungi in the family of *Dacrymycetaceae* [Lopez et al., 2014]. It is also known as sweet osmanthus ear or under the scientific synonyms *Guepinia spathularia* and *Cantharellus spathularius*. It belongs to the phylum *Basidiomycota* and forms edible, fan-shaped, yellowish fruiting bodies

(Figure 1.5) [Bensch, 1948; Emberger and Gary, 2008; Martin, 1948].

It has a long history of food use in Chinese cuisine, e.g. a vegetarian dish called Buddha's delight [Meuninck, 2017]. It is also consumed in Malaysia, Japan, India and Cameroon [IMO Natural Solutions GmbH, 2017]. Moreover, recently its use for dietary and nutraceutical purposes has been recorded as safe [Gaynor, 2017; Kumar et al., 2021].



Figure 1.5: *Dacryopinax spathularia* grows on decaying, decorticated, wood substrates in clusters (a) and individually (b).

The family of Dacrymycetaceae does not exhibit any toxic or poisonous effect; however their basidiocarps are often tough and prevent their culinary use. The cultures of some *Dacrymyces* species, which are closely related to *D. spathularia*, have been reported for their use in production of carotene pigments [Farrow et al., 1958]. Besides long-chain glycolipids, carotenoids are the only known secondary metabolites which have been reported from a species of *Dacryopinax*. It has been also used for the treatment of several diseases and disorders such as anti-viral, anti-tumour, anti-bacterial, anti-immunomodulating, anti-inflammatory, anti-diabetic, nephroprotective, hepatoprotective [Kumar et al., 2018].

Bodman et al. studied nuclear patterns in basidium development in Dacrymycetaceae and provided a detailed analysis for *D. spathularia* [Bodman, 2018]. As it was reported, nuclear fusion at prophase I occur before epibasidia origin in *D. spathularia*. In another study cultural and cytological characterization of new Dacrymycetaceae species, *D. primogenitus* and *D. spathularia* was done. In result, the timing of movement of nuclei into the basidiospores was reported as varying in both species [McLaughlin et al., 2016]. Moreover basidiospores were reported as uninucleate at maturity in both *D. primogenitus* and *D. spathularia*.

D. spathularia was characterised by morphological methodology via phase contrast microscopy (Figure 1.6) [Alvarenga and Xavier-Santos, 2017; Solem, 2016]. As it was reported by Foster et al., basidiocarps caespitose of *Dacryopinax spathularia* grows in lines; stipitate and pileate; to 20 mm high. It has uniseptate spores, thick-walls, cylindrical abhymenial hairs. Its colour is yellow to orange when it is fresh, when dried first the colour turns from orange-brown to reddish brown, and then, dull white [Solem, 2016]. Internal hyphae of *D. spathularia* is branched, thin-walled, septate, pale yellow, 1–2.5 μm diameter, without clamp connections. [Alvarenga and Xavier-Santos, 2017; Martin, 1948].



Figure 1.6: *D. spathularia*. (a) Hyphae without clamp connection (black arrow), scale bar = 10 μm . (b) Sausage-shaped, smooth; single septum, measured 0.5-3.8 microns.

Morphological characterization and DNA sequencing of the 5.8S/ITS and 28S nuclear ribosomal DNA have been used for the taxonomic identification of *D. spathularia*. In a recent report provided by EFSA, *D. spathularia* was proven to be free of metabolites which demonstrate the structural properties of any mycotoxins or virulence factors previously described in the literature [Younes et al., 2021]. Additionally, no reports on poisonous or negative health impact of *D. spathularia* have been recorded in the literature.

1.4.2. Production of LGLs by *D. spathularia*

Production of LGLs was optimized with several bench-scale and pilot scale trials. In one of the first studies on *D. spathularia*, thirty-two strains representing sixteen species in the Dacrymycetales were tested their ability to decay wood using the soil block test [Seifert, 1983]. In this study, *D. spathularia* UBC 6101 strain was grown on malt yeast peptone agar for 3-6 weeks at 20° C, then was used to inoculate

50 ml balanced medium (D-Glucose, 10 g; 1 M NH_4Cl , 10 mL; 0.1 M KH_2PO_4 , 10 mL; 0.1 M K_2SO_4 , 2.5 mL; $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; thiamine, 100 µg; trace element solution) [Reid and Seifert, 1982]. In another study, the dacrymycetous fungal flora of Japan was investigated and *D. spathularia* cells were grown in slants of 0.1 % (w/v) cornmeal agar + 1.25 % (w/v) malt agar medium [Shirouzu et al., 2009]. In addition to those early studies, recently several nutrients such as glucose, yeast, malt, dextrose, cornmeal, trypticase soy, peptic digest of animal tissue, pancreatic digest of casein were screened in shake flasks for the optimization of culture medium [Justia Patents, 2012]. 500 ml Shake Flasks (SFs) were used with 200 ml working volume for medium screening experiments. During SFs experiments, pH, mycelial dry weight and amount of extractable material were measured to determine best culture medium. In addition, inhibition activities against two spoilage organisms *Bacillus subtilis* and *Zygosaccharomyces bailii* were evaluated in the agar diffusion assay. The results of experiments have shown that although the LGLs were produced in all of the tested mediums, the highest inhibition activity with respect to mycelial dry weight and the fermentation time was observed in glucose and yeast containing culture medium. The composition of glucose and yeast medium was specified as 2.0% for glucose, 0.1% for yeast extract. 3.3 g/L mycelial dry weight and 1.26 g/L extractable material were obtained with glucose and yeast medium and the final pH was recorded as 4.1 after 192 h. Moreover, the inhibition zone against *B. subtilis* and *S. cerevisiae*, were measured as 20 mm and 13 mm, respectively. With the microscopic control during production phase, it was seen that when glucose and yeast medium was used, the amount of LGLs stuck to the mycelial hyphae was less than all the other tested mediums. This has led glucose and yeast medium to be considered as advantageous in precipitation experiments and also for industrial scale fermentations since the majority of the LGLs will be located in the culture medium. Furthermore, the highest productivity was achieved with glucose and yeast medium much earlier than other fermentation mediums [Stadler et al., 2017].

Apart from culture medium, operational parameters such as pH and temperature were also studied to optimize LGLs fermentation. In accord with this, 200 ml SFs with 25% working volume were used for primary seed culture and then transferred into batches of 500 ml SFs with 40% working volume. Yeast (0.4%), malt (1%), glucose (0.4%) medium was utilized in seed SFs experiments at pH 6.3

[Stadler et al., 2017]. 240 rpm shaking speed and 23° C operational temperature were used in the first seed while these settings were changed 140 rpm and 28° C upon inoculation of second seed SFs. Thereafter, pilot-scale fermentation was performed in 40 L fermenter (Biostat LP42 fermentor (Bioengineering, Wald, Switzerland) with 30 L of glucose (2%) and yeast (0.1%) containing medium. 400 mL of culture broth (1.33% of total volume) from second seed SFs was utilized to inoculate the 40 L fermenter whilst the inoculation volume was increased to 1500 mL (5% of total volume) in the subsequent trials [Stadler et al., 2017, 2020]. In pilot scale fermenter, the culture was grown under 240 rpm stirring and 0.2 vvm (volumes of air per minute per volume of batch) aeration at 30 °C for 200 h. At the end of this process, 87 g of LGLs and 93 g of glycolipid sodium salts were obtained.

In the demonstrative scale production, 300 L (Bioengineering Type P, equipped with four Ekato Intermig® impellers) reactor was utilized with 200 L culture medium containing glucose (2%) and yeast extract (0.1%). For inoculation 20 L seed culture (10% of total working volume) was used. To prevent foaming during production, 0.03 ml/l of Clarol FBA 3003K (Cognis, Monheim, Germany) was added at the beginning of the fermentation. The fermentation was carried out at cascade; 33° C temperature, under 75 rpm agitation and 0.2 vvm aeration. The production was stopped after 300 h when there was no free glucose available and the oxygen partial pressure dropped until 20%. This process yielded 380 g LGLs [Stadler et al., 2020].

Besides these experiments, two fermentations were performed in 1 L SFs with 400 mL culture medium containing glucose (2%) and yeast extract (0.1%). Inoculation was done with 2 ml of *D. spathularia* to each SFs from seed cultures. The production was done at 200 rpm, at 32 °C for 360 h. At the end of these SFs batches, a total of 5.6 g/L and 6.2 g/L glycolipids were characterized by HPLC-MS [Stadler et al., 2017].

More details on the production process of long-chain glycolipids was shared in European and US Patent application of IMD Natural Solutions GmH [Stadler et al., 2017, 2020]. Submerged fermentation (SmF) was utilized for industrial scale production of long-chain glycolipids as it provides advantages like reduced time for biomass production and control of operational parameters (e.g. temperature, aeration and pH).

Overall, four different temperatures, 23°C, 28°C, 30°C and 33°C, were used at different scale LGLs fermentations and 30°C was recorded as the optimum

temperature. pH 6.0 was found as optimal among other tested pH values, i.e. 5.6, 6.0 and 6.3. Furthermore, the suitable fermentation medium for LGLs production was reported as glucose (2%) and yeast medium (0.1%).

Although all these trials provide information on LGLs, very scant attention has been given into fermentative production of LGLs in the literature. To the best of our knowledge, the data on the production of LGLs from *D. spathularia* is limited only with the patent application of IMD Natural Solutions GmH [Stadler et al., 2017, 2020]. Thus further research is needed to improve LGLs production.

1.5. Downstream Process of LGLs

Downstream processing (DSP) is very important to develop glycolipid production process which is energy-efficient, economic and environmentally friendly. When the fermentation stops, multiple DSP operations come into play for the recovery and purification of glycolipids. From commercial point of view, maximizing the recovery of glycolipids while reducing its production cost as much as possible is of great importance at any industrial-scale productions.

Up to date, several techniques such as precipitation, centrifugation, filtration and solvent extraction, have been developed to extract and purify LGLs (Figure 1.7) [Hubert et al., 2012]. In addition, broad range of systems differing in cost, size and complexity have been employed throughout DSP operations.

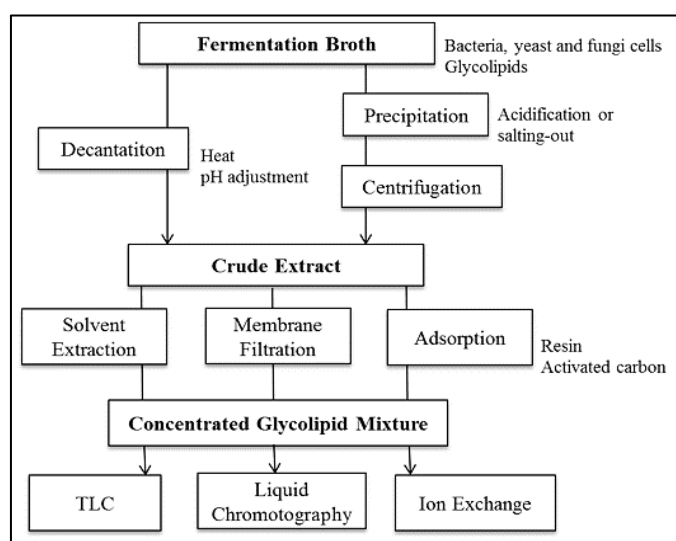


Figure 1.7: Downstream processes: long-chain glycolipid extraction and purification methods.

Among several technologies, making convenient selections and employing proper combinations of DSP equipment, significantly affect economic feasibility of the whole process. Thus, recently considerable effort has been put into downstream process development to achieve an efficient and profitable process.

1.5.1. Separation of LGLs: Centrifugation, Decantation, Filtration and Precipitation

As in many other fermentative productions, downstream processing of LGLs starts with the separation of the microorganisms from the fermentation broth. For this purpose, centrifugation has been mostly utilized as it facilitates the recovery of crude glycolipids from cell supernatant. However, when produced by fungus, glycolipids stick to the mycelial hyphae of the cells during production phase. To separate the glycolipids from mycelial hyphae, alkali pretreatment can be applied. For instance, Stadler et al. applied alkali treatment with 1 N sodium hydroxide solution for 1 h after 300 h fermentation of LGLs [Stadler et al., 2020]. In their study, the pH of fermentation broth was changed from 4.6 to 8 and this has led LGLs to largely release from the mycelia of *D. spathularia* cells. In another study, ethanol was used to dissolve the glycolipids before centrifugation [Hubert et al., 2012].

Once glycolipids are released from mycelia, the fermentation broth is centrifuged to remove mycelia from the culture medium. Afterwards, further elimination of the mycelia can be done by filtration. For example, a Pall (Dreieich Germany) 0.1 μm polysulfone membrane filter cassettes (diameter of fibers 1.4 mm, total area 24 m^2) microfiltration system was utilized to remove *D. spathularia* cells from glucose and yeast containing medium after alkali treatment and centrifugation [Stadler et al., 2020].

With the application of centrifugation and filtration, fermentation broth becomes totally cell-free, thereafter acidic precipitation or salting out procedures comes into play to obtain sediment of product. Depending on the producer organism, composition of the medium or produced metabolites; acidification or alkalization treatment can be utilized to make glycolipids less soluble in the aqueous phases. With the acidic or alkali treatments, glycolipids become easily available. Once the precipitation is formed, the supernatant is discarded via decantation or centrifugation and eventually the product is obtained. For instance, rhamnolipids were

precipitated either by acidifying the culture media or adding salts such as aluminum sulfate [Déziel et al., 2014; Schenk et al., 1995]. Similarly, long-chain glycolipids were precipitated by acidifying the filtered culture broth to pH 3 and 380 g dry glycolipids were obtained from 200 L fermentation broth at the end of the DSP process [Stadler et al., 2017]. Moreover, acidic precipitation method was employed for the extraction of succinoyl trehalose lipids [Franzetti et al., 2010].

1.5.2. Solvent Extraction

Despite centrifugation and precipitation are convenient methods, they cannot often remove all of the residuals from lipids or carbohydrate compounds. As an alternative to these methods, solvent extraction can be utilized to achieve more cleanly isolated glycolipid products. For this purpose, three polar organic solvents, chloroform, methanol and ethyl acetate have been mostly used up to date [Hubert et al., 2012]. For instance the extraction of sophorolipids was performed by using ethyl acetate [Elshafie et al., 2015].

Different solvents such as n-hexane, pentane or methyl tert-butyl ether were also reported to be useful for removing the residual substrates [Ashby and Solaiman, 2010; Qazi et al., 2022]. To extract trehalose lipids, either the combination of chloroform/methanol (2:1, v/v) or a mixture of ethyl acetate/methanol (8:1, v/v) were employed. In addition, rhamnolipid extraction was performed by using ethyl acetate or a mixture of chloroform/methanol (2:1, v/v) [Çakmak et al., 2017].

Similarly, long-chain glycolipids from *D. spathularia* were also extracted by using ethyl acetate [Stadler et al., 2020]. Apart from these studies, Smyth et al., provided very useful and explanatory research on commonly used glycolipids extraction methods [Smyth et al., 2010]. Table 1.5 demonstrates commonly utilized DSP methods with their advantages and disadvantages.

Table 1.5: Commonly used techniques for the extraction and purification of glycolipids.

Extraction / Purification Method	Pros	Cons	References
Acidic precipitation	Low cost, efficient in crude GLs recovery	Low recovery, non-specificity, solubility problems	[Venkataraman et al., 2021]
Solvent Extraction	Effective for the recovery of crude GLs, suitable for scale up, reuse of the solvents is possible	Expensive, large volume of solvent needed, mostly solvents are flammable or toxic	[Santos et al., 2016]
Adsorption on polystyrene resin	Effective to obtain highly pure GLs, fast process, reusability, recovery from continuous culture is possible	High costs of reduction phase, pollution of the resins in the presence of other organic materials	[Mnif and Ghribi, 2016]
HPLC, HPLC-MS, HPLC-ELSD	Speedy, reproducible, accurate, suitable for scale up	Elution of undesired compounds, low sensitivity for low molecular weight glycolipids	[Dardouri et al., 2021; Sarbu and Zamfir, 2018]
TLC	Simple, inexpensive, provides information on structural types of glycolipids	Not suitable for quantification, cannot identify complex mixtures, pollution by other impurities	[Smyth et al., 2014]

Although solvent extraction is a practical method, extracting a specific type of compound among other molecules is not always easy due to physical similarities among glycolipids. Thus further research is needed to maximize the selectivity of glycolipid of interest.

1.5.3. Calorimetric Assays for LGLs Yield Determination

Simplistic methods such as calorimetric assays also provide rough indication on glycolipid production yields. For instance anthrone or orcinol assay can be used to determine the presence of LGLs in the culture medium or the extract [JE and BT, 1962; Smyth et al., 2014]. These assays work by detecting the sugar moieties of the

glycolipids. Although these methods are inexpensive and allow direct application on the cell-free fermentation broth without the need for extraction, the impurities from other carbon sources can result in incorrect results. Thus these assays can be considered as a rough indicator of glycolipid production.

1.5.4. Usage of Ion Exchange Resins for Purification of LGLs

Ion exchange is another alternative method which can be employed for downstream processing purification of long-chain glycolipids. The most important advantage of this method is high selectivity and therefore providing products with high purity. Polymeric resins such as Amberlite XAD-2, Amberlite XAD-4 and Amberlite XAD-16 are effective in adsorbing low molecular weight organic substances from aqueous solutions. These resins have been also used for LGLs recovery since LGLs are hydrophobic in nature and have low molecular weight (ranging between 886 and 1.114 Da). For instance, Amberlite XAD-16 was used to recover LGLs from 30 L culture filtrate [Stadler et al., 2020]. 1000 g of Amberlite XAD16 was added to cell-free broth and left for overnight incubation. Resins were filtrated and incubated with methanol in ultrasonic bath for the recovery of LGLs. Then, eluate was evaporated and the solution was extracted with ethyl acetate. At the end, the protocol yielded 25 g of long-chain glycolipids crude extract. In an another study, using Amberlite XAD resin in combination with solvent extraction and heating was reported as effective for the recovery of mannosylerythritol lipids with high purity [Rau et al., 2005].

1.5.5. Chromatographic Techniques for Characterization of LGLs

Despite numerous examinations, the separation of specific type of glycolipids still remains difficult due to various structural resemblances within glycolipid classes. Chromatographic techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and size exclusion chromatography have been employed for the identification of individual glycolipid. TLC was utilized to obtain information on possible structural types of glycolipids found in the fermentation broth. In a study carried out on silica-gel TLC plates with a

solvent mixture of chloroform/methanol (9:1 v/v) and chloroform/methanol/water/acetic acid (65:15:2:0.1 v/v), mono- groups of rhamnolipids were distinguished from the disaccharide polar head groups [Monteiro et al., 2007].

Similarly, HPLC provides valuable information required for the identification of long-chain glycolipids. In a study, the use of a reverse phase HPLC was reported as efficient for separation of glycolipids from animal origin [Demopoulos et al., 1996]. Moreover, recent researches have shown that coupling evaporative light scattering detector (ELSD) or mass spectrometry (MS) with HPLC provide very precise quantification of LGLs. For instance, HPLC-MS was utilized to identify long-chain glycolipids from *D. spathularia* following 200 L demonstrative-scale fermentation and with the results process optimization of LGLs was successfully completed for an industrial scale production. [Stadler et al., 2020]. Diode array detector (DAD) at 200 and 210 nm was used to monitor separations and assess the purity of LGLs in the study. Additionally, HPLC-MS was also used for the analysis of rhamnolipids produced by *Pseudomonas aeruginosa* [Déziel et al., 2014].

Although chromatographic methods allow purifying gram quantities of glycolipids, their purification is still laborious since they only occupy a small portion of crude fermentation broth extracts. As it has been mentioned throughout this section, a single downstream processing technique is not sufficient for recovery of LGLs. Thus, more effort should be directed on developing downstream purification steps to obtain the LGLs at required degree of purity.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Microorganism

D. spathularia strain was delivered in an agar slant tube by DSMZ Germany.

For application in the experiments, replicates of the strain were prepared at Gebze Technical University. In accord with this, 250 mL baffled shake flasks (SFs) were filled with 50 mL of malt extract (30%, w/v), soy peptone (3%, w/v) (MESP) broth. The SFs were incubated at 30 °C in a rotary shaker and agitated at 180 rpm and an orbit of 2.5 cm. Also *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Saccharomyces cerevisiae* were used for antimicrobial activity tests. Stock cultures were opened under aseptic conditions and grown in appropriate media and incubation conditions.

2.1.2. Chemicals

The analytical grade chemicals which were used in this study are Sulfuric Acid (H₂SO₄) (Sigma-Aldrich), Acetone (Isolab), Yeast Extract (Merck), Peptone Bacteriological (Biolife), D-(+) Glucosemonohydrate (Merck), D-(+) Maltosemonohydrate (Sigma-Aldrich), Yeast Extract (Biolife), Agar Technical (Sigma-Aldrich), Sodium Chloride (Sigma-Aldrich), Sodium Hydroxide (Merck), Hydrochloric Acid Fuming (Isolab), Ethanol (Isolab), Sulfuric Acid (H₂SO₄) (Acs Reagent, Sigma-Aldrich), Glycerol (Fisher Scientific), Tryptone (Merck), Peptone from Soybean Enzymatic Digest (Sigma-Aldrich), Anhydrous Na₂SO₄ (Sigma-Aldrich). LB Agar (Lennox), D-(+) Glucosemonohydrate (Isolab), Sodium Chloride (Isolab), Agar (Isolab), Methanol (Isolab), Acetonitrile (Isolab), Chloroform (Isolab), Isopropyl (Isolab), Isooctane (Isolab), 2-propanol (Isolab), Nutrient Broth (Merck), Sulfuric Acid (H₂SO₄) (Isolab), Acetone (Sigma-Aldrich), Yeast Extract (Isolab), Peptone Bacteriological (Merck), D-(+) Glucosemonohydrate (Isolab), Isooctane (Merck).

2.1.3. Laboratory Devices

All laboratory devices used throughout the experiments were shown in Table 2.1.

Table 2.1: Laboratory devices used throughout the experiments.

LABORATORY DEVICES	BRANDS
Micropipettes	Axygen-Isolab
Vortex Stirrer	N-Biotek
Autoclave	Nuve, Nc,40l
Magnetic Stirrer	Wisestir
Water-Bath	Stuart
pH Meter	Hanna
Shaking Incubator	Biotek
Biosafety Cabinet	Mn Series, Nuve
Spectrophotometer	Model 4001/4, Genesys 20 Thermospectronic, Thermo Electron Corp., Waltham, Ma, Usa
Ultrasonic-Bath	Isolab
Drying Oven	Binder, Bd 53, 04-63908, Germany
Incubator	Wisecube Fuzzy Control System
Microcentrifuge	Weightlab, Wn-Cm15
Analytical Balance	Kern Acj / Acs
Centrifuge	Sigma 3 18k
Laboratory Freeze Dryer	Mrclab, Fdl-10N-50

2.2. METHODS

2.2.1. Preparation of *D. spathularia* Glycerol Stocks

Glycerol stocks of *D. spathularia* cells were prepared for long-term storage. For this purpose, firstly *D. spathularia* cells were grown in malt extract soy peptone (MESP) liquid culture for 120 h at a temperature of 30°C, 200 rotations per minute (rpm) and an orbit of 2.5 cm. At the end of the incubation period, the optical density (OD₆₀₀) was measured and microscopic examination was done to check contamination. Finally, glycerol stocks were prepared by adding 750 µL of a 50% (v/v) glycerol solution and 750 µL fermentation broth to a sterile 2 mL micro-centrifuge tube. Forty tubes were prepared and stored at -32 °C until further use.

2.2.2. *D. spathularia* Fermentations

2.2.2.1. Cultivation Media

For the seeds of fermentation, malt extract (30%, w/v), soy peptone (3%, w/v) (MESP) medium was used (Table 2.2) The pH of the MESP medium was adjusted to a pH of 5.6 with 1 M NaOH or 1 M HCl in all fermentations and the broth was autoclaved.

Table 2.2: The composition of the MESP liquid medium.

Component	Amount [g/L]
Malt Extract	30
Peptone from Soybean Meal	3

For all preparations, as well as for other experiments, deionized water was used. In the MESP medium, malt extract acted as the carbon source, and peptone from soybean meal as the main nitrogen source. Initial pH of the fermentation broth was adjusted to 5.6 ± 0.2 . All fermentations were performed in replicates in 250 mL shake flasks with a 50 mL (20%, v/v) working volume. Incubation conditions were at 30 °C in a rotary shaker and agitated at 180 rpm.

2.2.2.2. Inoculation

Autoclaved SFs with 50 mL (40% v/v) MESP broth were placed in a laminar flow. Each SF was inoculated with 1.5 mL (1.33% v/v) from a glycerol stock and incubated for 7 days at 180 rpm (orbit diameter 2.5 cm) and 30 °C. After incubation, the OD₆₀₀ was measured, cell dry weight (CDW) was calculated and contamination check via microscopic examinations was done.

2.2.3. Follow-up of the Fermentation

To evaluate the progress of the fermentation, samples were taken on a regular basis and analyzed with high pressure liquid chromatography (HPLC), pH meter, OD₆₀₀ and CDW measurements.

2.2.3.1. Measurement of Growth by Absorbance at OD₆₀₀

The optical density can provide an insight in the growth of the *D. spathularia* culture. To measure the OD₆₀₀, the sample was diluted with physiological water containing 9 grams NaCl per liter (0.9 % w/v) distilled water. A sufficient dilution had to be prepared in the range of 0.5 to 0.9 when OD₆₀₀ reading was above 3.3 abs. The optical density was measured by Genesys 20 Thermospectronic spectrophotometer at 600 nm, after measuring a blank standard which only contained physiological water.

2.2.3.2 Cell Dry Weight Measurement

To determine the biomass concentration of every sample during the fermentation, the cell dry weight was measured. 2 mL micro-centrifuge tube was placed in a drying oven at 105 °C for one hour and transferred in to the desiccator. After 30 min. in the desiccator, the weight of empty micro-centrifuge tube was taken and recorded. 1 mL of the fermentation broth was centrifuged for 10 minutes at 15000 rpm in a micro-centrifuge tube. The pellet was resuspended in distilled water and vortexed until a homogeneous sample was achieved. Thereafter, the suspension

was once more centrifuged at 15000 rpm for 10 minutes. The resulting pellet was again resuspended in distilled water, vortexed and put in a drying oven (Binder, Bd 53, 04-63908, Germany) at 105°C for 24 h. To determine the amount of biomass, the weight of pellet was taken also after pellet was dried and placed in desiccator.

2.2.3.3 HPLC Measurements

To follow up maltose and glycolipids concentrations during the fermentation, samples were analyzed by HPLC. For HPLC sample preparation, firstly centrifugation at 15000 rpm for 5 minutes was done and the supernatant was filtered with a polyether sulfone (PES) filter of 0.2 µm. 20 µL of filtered sample was taken and injected by manually to HPLC sample port.

Column was operated at 65 °C. The mobile phase was isooctane/2-propanol aqueous solution with a flow rate of 0.5 mL per minute for GLs detection [Demopoulos et al., 2006a]. 0.9 M H₂SO₄ with a flow rate of 0.8 mL per minute was used as mobile phase for maltose detection. The sample elution detection lasted 30 minutes, using ultra violet detector (UV) at 206 nm for GLs. For maltose analysis, the sample elution lasted 16 minutes, using refractive index (RI) detector. The results were visualized with the HPLC software.

2.2.3.4. TLC Measurement

TLC is a convenient method that has been routinely used for the separation and identification of lipids for purification of glycolipids. It was used for the separation of glycolipid after acidic precipitation in this study.

For this purpose, 60 Å asilica TLC plates (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) were used as stationary phase. Mobile phase was isopropyl acetate/methanol/acetic acid (100:10:1, v/v/v) [Kügler et al., 2015a]. The plates were dipped into 10 % (v/v) H₂SO₄ and development under 180 ° C air streams for 4–5 minutes and GLs were detected.

2.2.3.5. Microscopy

Gram staining and subsequent examination under the microscope were done to assess if contamination had occurred during fermentation. A drop of the diluted culture was spread on a glass slide, and then the drop was dried and fixed by passing the slide through a flame. Thereafter, the gram staining procedure was followed. Firstly, a drop of a 1 % crystal violet solution (SIAL) was added to the glass slide for 1 minute and rinsed off with distilled water. Secondly, the glass slide was dried and a drop of lugol (Sigma-Aldrich) was added for 45 seconds. The slide was flushed for 1 minute with 70 % ethanol and again dried. Finally, a drop of 0.5 % carbol fuchsin (Sigma-Aldrich) was added to the glass slide for 1 minute, flushed with distilled water and dried. To observe the staining, a drop of immersion oil was applied to the glass slide. Eventually, the slide was observed under a microscope with a 1000X magnification. *D. spathularia* is expected to be visualized as plant-like branches since hyphae are stained by crystal violet.

2.2.4. Antimicrobial Tests

The selection of proper and correct antimicrobial tests is of great importance for the detection of antimicrobial activity. Conventional techniques such as disc diffusion, broth microdilution and automated instruments, have long been utilized in antimicrobial tests so far. Due to their advantages like simplicity, reproducibility, ease in modifying and low cost, agar disk diffusion assay and broth cultures were used for antimicrobial activity detection in this study.

2.2.4.1. Antimicrobial Efficacy Tests in Broth Cultures

OD₆₀₀ readouts of actively growing liquid cultures with selected microorganisms were used to check the presence of growth inhibition due to antimicrobial activity of LGLs. Five different microorganisms used in antimicrobial tests of LGLs have been shown in Table 2.3.

Table 2.3: The list of the microorganisms used in the antimicrobial tests.

Name of the microorganism	Type
<i>Staphylococcus aureus</i>	Gram-positive bacterium
<i>Bacillus subtilis</i>	Gram-positive bacterium
<i>Pseudomonas aeruginosa</i>	Gram-negative bacterium
<i>Escherichia coli</i>	Gram-negative bacterium
<i>Saccharomyces cerevisiae</i>	Yeast

Luria-Bertani (LB) culture broth was used for *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli* and yeast malt (YM) broth was used for *S. cerevisiae* at 30 °C and 180 rpm. The steps of antimicrobial test have been demonstrated in Figure 2.1.

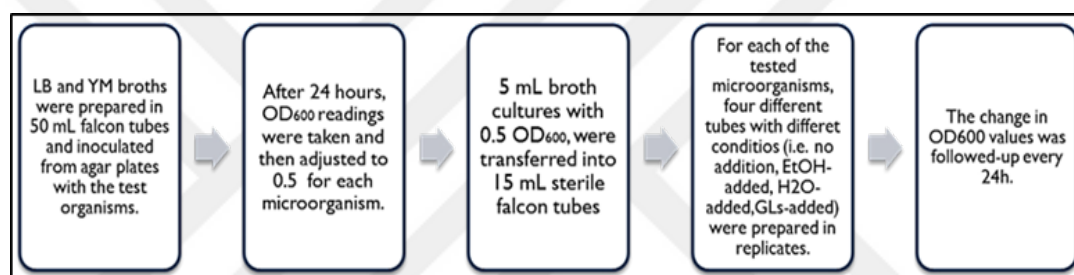


Figure 2.1: The steps of antimicrobial test in broth cultures.

Upon analyzing the OD₆₀₀ readouts, calculations were done to find inhibition ratios for each of the tested microorganisms and the inhibition percentages were demonstrated on bar graphs. The percentage of growth inhibition was calculated by taking negative control (water-added) as a basis.

2.2.4.2. Kirby-Bauer Antimicrobial Disk Assay Method

Kirby-Bauer test also known as the disk diffusion test is used for antibiotic susceptibility testing. It is considered among the most flexible susceptibility testing methods in terms of antimicrobial agents that can be tested. The technique basically requires placing disks loaded with antimicrobial agents on a lawn of tested microorganism on the surface of an agar medium, incubating the plate overnight, and measuring the presence or absence of a zone of inhibition at the end [Boyle et al., 1973; Hudzicki, 2009].

To detect antimicrobial activity of LGLs against different types of microorganisms, disk diffusion method was used in this study. For this purpose, the precipitate obtained after *D. spathularia* cultivation was dissolved in 1 mL deionized water, thereafter disks were impregnated to this solution and placed on Luria-Bertani (LB) and yeast malt (YM) agar plates on which test microorganisms were spreaded. *E. coli*, *B. subtilis*, *P. aureginosa*, *S. aureus* were grown on LB agar and *S. cerevisiae* was grown on YM agar plates for 24 h at 37°C. The presence of zone formation around GLs impregnated disks for inhibition of growth of test microorganisms was interpreted.

2.2.5. Downstream Processes Applied to the Fermentation Broth for Extraction of Glycolipids

The ability to isolate, purify and characterize the structure of LGLs is extremely important as it provides insight into the different conditions which effect production of glycolipids. To be able to drive the production in a certain direction a series of experimental techniques used in this study.

The purpose of downstream processing was to isolate, purify and concentrate the fermentatively synthesized glycolipids in this study. In line with this objective, the fermentation was ended up and the broth was harvested. The volume and percentage of solids of the broth was determined. The steps of antimicrobial test have been shown in Figure 2.2. Firstly, the culture broth resulting from fermentation was alkalized to pH 8 with 1 N sodium hydroxide solution to allow the glycolipids that partially stick to the cells under acidic conditions to become largely released from the mycelia.

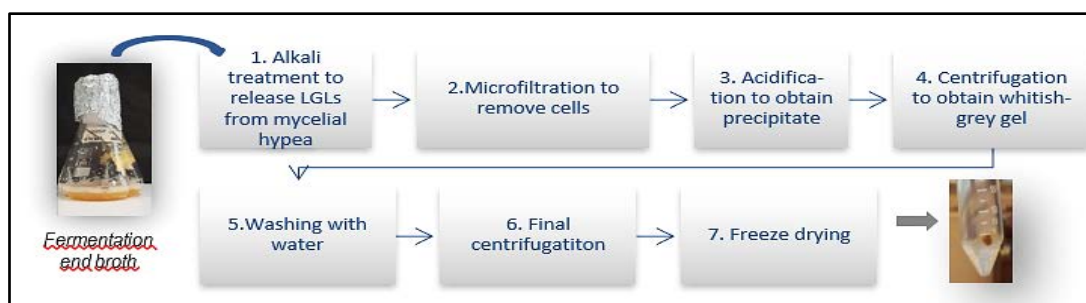


Figure 2.2: Downstream processing of the fermentation broth.

The culture broth was filtrated through a 0.22 μm membrane filter (diameter of fibers 1.4 mm) microfiltration system to completely retain the mycelia. The product was precipitated by acidifying the filtrate with 2 N hydrochloric acid to pH 3 and incubating for 16 h under cooling to 11° C. The fluid was removed by decantation via separating funnel and subsequent centrifugation (4500 rpm, 15 min), resulting in a whitish-grey gel. This crude product was washed immediately with water (pH 7), centrifuged again at (4500 rpm for 15 min) and freeze dried.



3. RESULTS AND DISCUSSION

3.1. Shake Flask Fermentations

3.1.1. *D. spathularia* Cell Morphology

D. spathularia is branched, pale yellow, has 1–2.5 μm diameter, without clamp connections. [Alvarenga and Xavier-Santos, 2017; Martin, 1948]. *D. spathularia* cells were observed under 10X, 40X and 100 X magnifications after 120 h cultivation at 30°C and 180 rpm in malt extract soy peptone broth (Figure. 3.1). In line with a recent study on taxonomical revision of the *Dacryopinax* species, aseptate to uniseptate basidiospores formation were also seen in the *D. spathularia* micrograph [Castro-Santiuste et al., 2020].

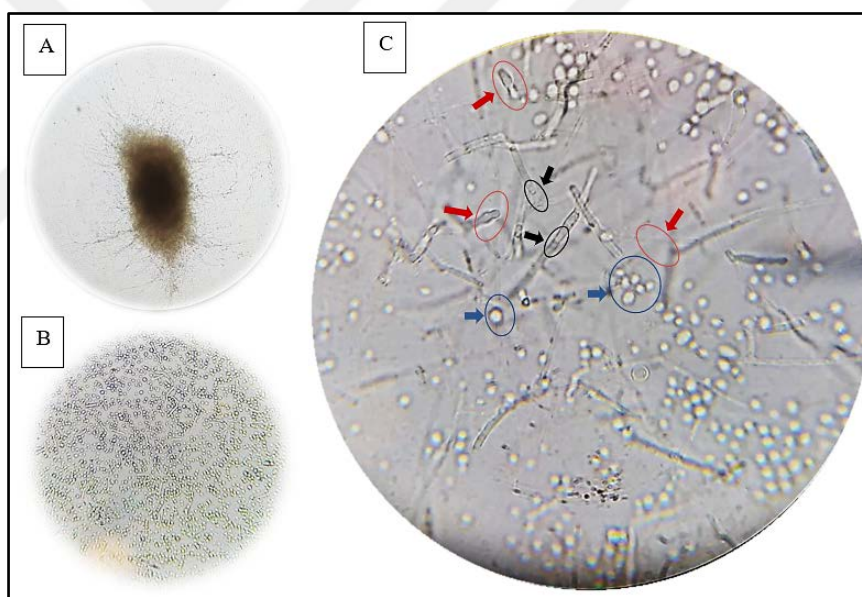


Figure 3.1: *D. spathularia* cells A) under 10X, B) under 40X C) under 100X magnification after 120 h cultivation at 30°C and 180 rpm in malt extract soy peptone broth. Red colored arrows indicate budding spores, blue colored arrows indicate basidiospores and black colored arrows indicate probasidium and basidium formation.

Moreover, probasidium and basidium of *D. spathularia*, which are represented with black colored arrows in Figure 3.1, were coincide with the representation done in a previous study [Shirouzu et al., 2009].

3.1.2. Growth Curve of *D. spathularia*

The growth curves are useful to gain insight into the growth characteristics of fungi species in different nutrient media and can help to optimize the conditions of fermentation parameters in favor of producing desired product. To better understand *D. spathularia* fermentations, growth curve of *D. spathularia* was demonstrated (Figure 3.2). For this purpose two shake flasks with 500 mL volume were filled with 150 mL malt extract soy peptone broth. Inoculation was done with 1.5% (v/v) *D. spathularia* cells with OD₆₀₀ of 13.01. Upon inoculation, the fermentation was followed-up during 28 days of cultivation.

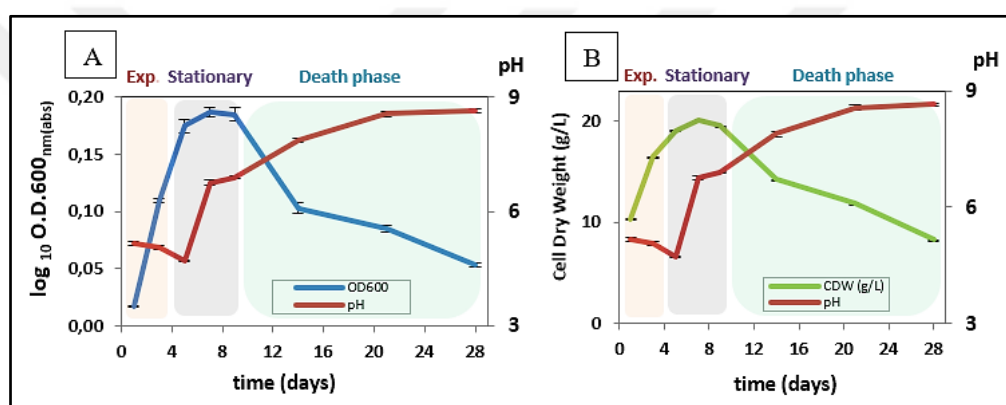


Figure 3.2: Growth of *D. spathularia* and pH change in unbaffled flask A) Change in absorbance at 600 nm and pH B) Change in CDW and pH.

There are four main phases of the growth curve: lag, log (exponential), stationary, and death [Bruslind, 2021]. The initial phase is the lag phase where cells are metabolically active but not dividing. During lag phase nutrients are harvested and adaptation to new environment is achieved. As it can be seen in Figure 3.2, no lag phase was seen during *D. spathularia* cultivation. This result can be attributed to the high inoculum concentration (OD₆₀₀=13.01) used at the beginning of the fermentation. To prevent long lag phase, the inoculum was taken from the end of the exponential growth phase (at 120th h) of seed culture. Besides inoculum concentration, it has been known from the literature that the size of the inoculum also influences the duration and efficiency of the lag phase [Robinson et al., 2001; Shida et al., 1975]. It is recommended to have at least 5% (v/v) of inoculum to decrease duration of the lag phase [Tsao, 1976]. In this study however, with 1.5% (v/v)

inoculum usage occurrence of long lag phase was prevented. Logarithmic phase started at around 24th h and continued until 72th hour.

Secondary metabolites are synthesized during or near the stationary phase of growth [Kügler et al., 2015b; Liepins et al., 2021]. Since the LGLs are secondary metabolites, the stationary phase is of great importance for the production of LGLs. According to the data obtained during the 28 days cultivation, the stationary phase started at 5th day of incubation and ended at 9th day. At day 10, cells entered death phase and this phase lasted till the end of cultivation.

There are not many publications on *D. spathularia* or its products and growth profile of this microorganism is not well defined in the literature, thus our findings cast a light on growth characteristics of *D. spathularia*.

3.1.3. The Growth of *D. spathularia* in Baffled and Unbaffled SFs

Shake flasks are commonly used for small scale cell cultivation as they provide shaking of the culture fluid and higher oxygen transfer rates. Oxygen is the most essential requirement for aerobic cultivations. The amount of biomass in a flask depends highly on the oxygen transfer rate. In order to ensure aeration and nutrient availability, different types of flasks including Fernbach flask, Sakaguchi flask, Erlenmeyer flask and baffled flask have been designed and utilized in different cultivations [Hartmann, 2020].

To determine the suitable flask type for *D. spathularia* cultivations, the use of baffled shake flask was compared with the use of unbaffled (Erlenmeyer) flask. Malt extract soy peptone (MESP) broth was used in both flasks. 250 mL baffled and unbaffled shake flasks filled with 50 mL (20%, v/v) working volume and inoculated with 750 μ L (1.5%, v/v) *D. spathularia* cells. The absorbance of inoculum was measured as 3.01 for the cultures grown in both flasks. pH, absorbance, cell dry weight (CDW) and maltose concentration were measured every 24 h upon inoculation.

After 192 h, cultivations were stopped and results were analyzed. Both CDW and OD₆₀₀ absorbance readouts taken from baffled SFs were higher than the unbaffled SFs. The end OD₆₀₀ and CDW of baffled SFs ($\log_{10}$ (8.74)= 0.94 and CDW=5.9 g/L) were higher than unbaffled SFs ($\log_{10}$ (0.21)=-0.67 and CDW=2.84 g/L) (Figure 3.3).

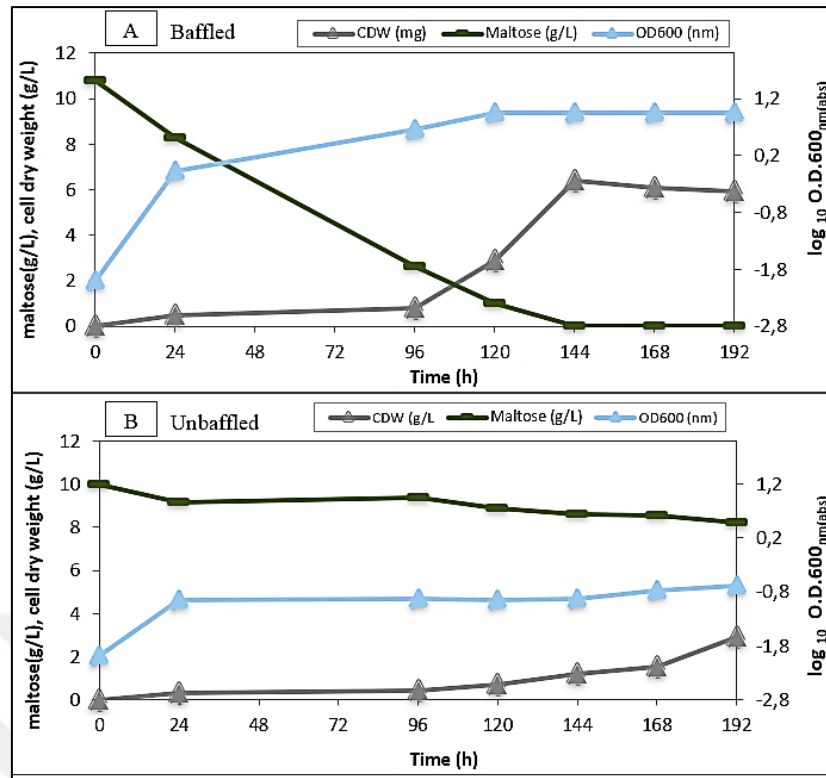


Figure 3.3: The change of CDW and absorbance (OD₆₀₀ nm) during *D. spathularia* cultivation in A) baffled and B) unbaffled shake flasks.

From these results, it was clear that higher amount of biomass were achieved with baffled SFs. These results tie well with a previous study on the effect of conventional flasks in shaking culture of *E. coli*. In the study, the maximum cell growth rates, k_G , and k_{La} in baffled flasks, were found 2 times and 5.9 times higher than those in the cylindrical flasks, respectively [Takahashi and Aoyagi, 2020].

Similarly, in the present study, due to insufficient oxygen supply poor *D. spathularia* growth was observed in unbaffled shake flasks. The start maltose concentration was 10 g/L in both unbaffled and baffled shake flasks. Only, 1.77 g maltose was consumed during 192 h cultivation in unbaffled shake flasks while all the maltose was consumed within 144 h in baffled shake flasks. The maltose consumption rates in unbaffled and baffled shake flasks were 1 mg/L.h and 70 mg/L.h respectively. The substrate consumption in baffled shake flasks was 70 times higher than the one in unbaffled shake flasks.

The start pH value in both baffled and unbaffled SFs was 5.6. The change in pH during the cultivation period was slightly different than each other in baffled and unbaffled shake flasks, yet the difference was so small (Figure 3.4).

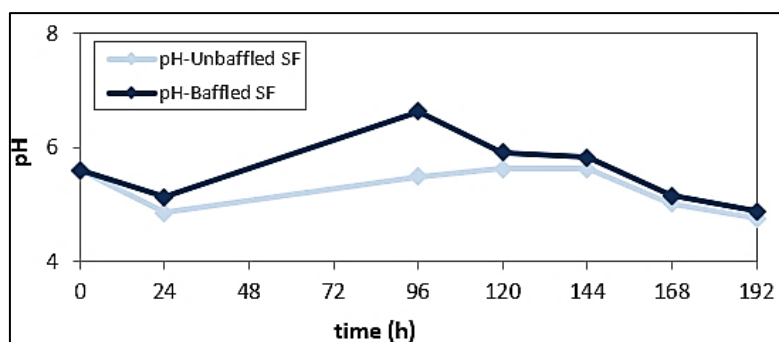


Figure 3.4: pH change during *D. spathularia* growth in baffled and unbaffled flasks.

As it has been shown in Figure 3.4, pH value dropped from 5.6 to 4.8 in unbaffled shake flasks. Similarly, in baffled shake flasks, first pH rised from 5.6 to 6.6 untill 96th h and then dropped to 4.9 at 192th h. No acid or base addition was needed over the period of cultivation.

Baffled shake flasks provided improved aeration during viscous *D. spathularia* cultivation. Besides, with baffles the liquid swirling motion was disrupted and culture pelleting was prevented. No spore aggregation was observed in baffled shake flasks during the 192 h of cultivation. Higher CDW and OD₆₀₀ values were obtained in baffled flasks.

Overall these findings have shown that the use of baffled shake flasks for *D. spathularia* cultivation is advantages as compared to use of unbaffled shake flasks in terms of the amount of biomass produced throughout cultivation.

3.1.4. The Growth of *D. spathularia* in Different Growth Media

Growth medium is of fundamental importance for all cultivations. Without appropriate media, the possibility of achieving sufficient amount of biomass and product is reduced. To determine suitable cultivation medium for *D. spathularia* fermentations, three different mediums were screened (Table 3.1).

Table 3.1: Growth mediums used in medium screening experiments.

Mediums	Composition
Malt extract soy peptone (MESP)	2% Glu., 3% Malt, 0.3% Soy peptone
Yeast malt glucose (YMG)	2% Glu., 0.4% Yeast Ext., 1% Malt Ext.
Glucose and yeast extract (GM2)	2% Glu., 0.1 % Yeast Ext.

250 mL SFs filled with 50 mL culture broths and inoculated with 750 μ L (1.5%, v/v) *D. spathularia* cells. The OD₆₀₀ of inoculum was 13 for each medium. pH, OD₆₀₀ and cell dry weight (CDW) were measured at time 0h, 24h, 48h, upon inoculation.

The results demonstrated that even though the *D. spathularia* cells were successfully grown in all of the tree mediums, the highest CDW and OD₆₀₀ values were achieved with YMG medium (27.7 g/L and log₁₀ (14.44)= 1.15 respectively) (Figure 3.5).

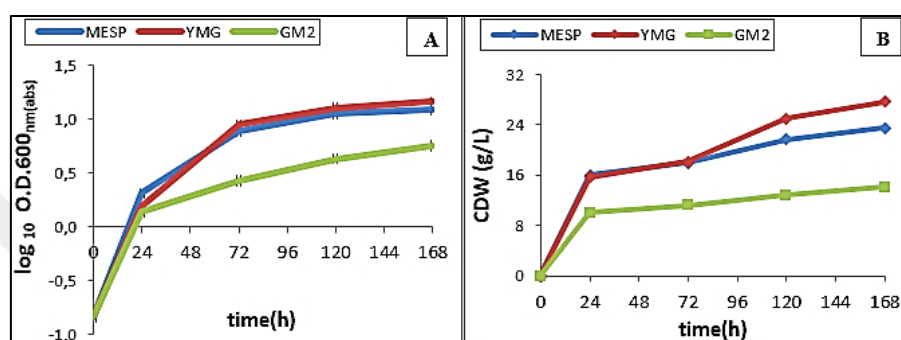


Figure 3.5: Monitoring *D. spathularia* growth with absorbance (OD₆₀₀ nm) (A) and CDW (B) in MESP, YMG and GM2 media during 168 h cultivation.

Stadler et al., previously recorded that the use of a simple medium containing dextrose or glucose as carbon source and a small amount of yeast extract could be superior against conventional complex mediums during *D. spathularia* cultivation [Stadler et al., 2020]. In line with the findings of Stadler et al., the highest cell turbidity and CDW readouts were obtained in glucose and yeast containing mediums at the end of our medium screening experiment. However, different than the findings of Stadler et al., the use of GM2 medium resulted in lowest amount of biomass at the end of the 168 h cultivation.

Through the cultivation period, the pH can control the accessibility of macro and micro nutrients, vital biological functions, microbial activity, and the effect of chemicals to the cells and desired product [Atasoy et al., 2019; Baccile et al., 2016]. Thus, monitoring and controlling the pH is of great importance.

To be able to reach high productivity and product recovery rate, it is very important to run the fermentation with correct pH value. At the end of the medium screening for *D. spathularia* cultivation, similar pH patterns in YMG and MESP mediums were observed (Figure 3.6).

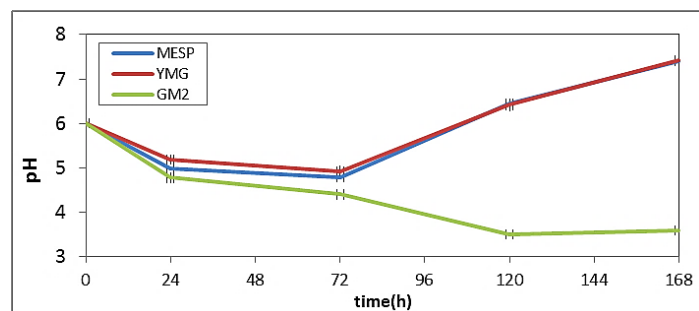


Figure 3.6: Change in pH during growth of *D. spathularia* in MESP, YMG and GM2 media.

pH was dropping between time 0 h and 72 h in both MESP and YMG medium, afterwards it raised till 7.3 in both medium. This change in the pH of MESP and YMG media could be relevant with the metabolism switch in *D. spathularia* cells. The type and concentration of secondary metabolites can be determined by physiology, developmental stage and environmental factors during growth [Isah, 2019]. When the primary substrates are nearly depleted, cells get stressed due to nutrient depletion and the production of secondary metabolites may increase [Ramakrishna and Ravishankar, 2011]. For example, deficiencies in nitrogen and phosphate were shown to lead to the accumulation of phenyl propanoids [Bryant et al., 1983]. Similarly, the use of different primary substrate sources may result alterations in pH values and the amount of LGLs produced during fermentation. The difference in the pH of GM2 and other two mediums, YMG and MESP, might be attributed to the use of different primary sources at start.

3.1.5. The Effect of pH on Growth of *D. spathularia*

Conventional process for recovery and purification of LGLs from fermentation broth generally involves one or several precipitation stages. During these stages, utilizing correct pH and pKa value makes great importance as it affects the efficiency of the recovery of LGLs from spent fermentation broth. To determine the optimum pH value for *D. spathularia* fermentation, two different pH values 4.6 and 5.6 were tested with shake flask experiment. As it is shown in Figure 3.7, the use of pH 5.6 for *D. spathularia* fermentation as compared to pH 4.6 resulted in higher OD₆₀₀ and CDW values.

The end OD₆₀₀ and CDW obtained with pH 4.6 ($\log_{10} (3.4) = 0.54$ and

CDW=14.2 g/L) were lower than pH 5.6 ($\log_{10}(6.0) = 0.78$ and CDW=20.0 g/L).

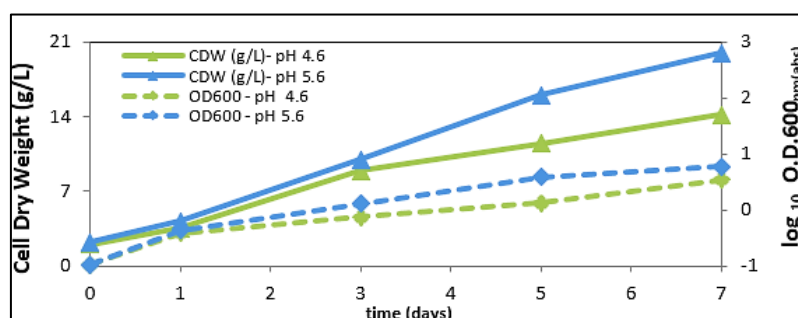


Figure 3.7: Growth of *D. spathularia* at pH 4.6 and 5.6.

Our findings showed that lowering the pH caused lower biomass production during *D. spathularia* cultivation. In accordance with the findings reported by Baccile et al., microbial glycolipids become water-soluble at basic pH and become insoluble upon lowering the pH [Baccile et al., 2016]. Also Stadler et al., mentioned using an appropriate pH, which is lower than the pK_a value of glycolipids and falls between the range of 4.0 to 5.0, could improve the efficiency of precipitation since the LGLs more dissociates in water [Stadler et al., 2017]. Although the exact value is highly compound-dependent, in particular the chain length and the number of double bond of the compound, the previous results revealed that LGLs are in dissociated form below pH 4 [Pashkovskaya et al., 2018].

Besides, it must be pointed out that no pH control was done in any of the shake flasks during our experiments. At this point it is important to highlight the fact that controlling the pH during cultivation period, could provide more stable system and higher amount of LGLs could be produced. For instance, Kuttiraja et al. were investigated the kinetics of lipid production by *Yarrowia lipolytica* SKY7 with and without the control of pH. The results revealed that by controlling pH at 6.5, 7.78 g/L lipid was produced while 6.58 g/L lipid was obtained when no pH control was done [Kuttiraja et al., 2018]. Overall, the study has shown that controlling the pH could enhance the lipid production by 15% as compared to pH-uncontrolled fermentation.

In light of these results, it is important to note that pH plays very critical role not only during cultivation but also in the following downstream purifications steps. Thus it must be kept below pK_a value to provide efficient recovery of the LGLs from fermentation medium during downstream processing applications. Furthermore, pH control during cultivation may improve the production of LGLs.

3.1.6. The Effect of Temperature on Growth of *D. spathularia*

Temperature is a vital parameter for fermentation processes. Each microorganism has optimum temperature and pH for its growth and activities. At temperature outside the optimum range the amount of biomass produced during cultivation is affected [Nascimento et al., 2022; Zhao et al., 2022]. This also has impact on the rate of substrate degradation and final product concentration. To determine the optimum temperature value for *D. spathularia* fermentation, two different temperature values 30°C and 33°C were evaluated in terms of their efficiency to give high biomass concentration (Figure 3.8). For this purpose two shake flasks with 500 mL volume were filled with 150 mL MESP broth and inoculated with 1.5% of total working volume. The fermentation lasted for 7 days at 180 rpm in a rotary shaker and at the end CDW and OD₆₀₀ values were interpreted.

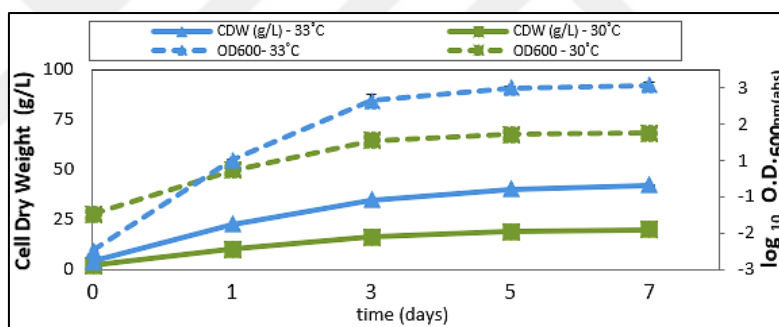


Figure 3.8: Growth of *D. spathularia* at 30°C and 33°C.

As it is shown in Figure 3.8, the use of 33°C for *D. spathularia* cultivation as compared to 30°C, resulted in slightly higher OD₆₀₀ and CDW values (the end OD₆₀₀ and CDW at 33°C and 30°C: log₁₀ (19.9) = 1.29, log₁₀ (18.4) = 1.26 and 22.2 g/L, 20.1 g/L respectively), yet no difference was observed on pH profiles of the two test temperatures.

We demonstrate that a 3-degrees °C increase in the fermentation temperature can affect the amount of biomass produced during fermentation. In conclusion, increasing the fermentation temperature resulted in an increase in cell densities which will most probably lead an increase in the concentration of LGLs produced during fermentation.

3.2. Antimicrobial Activity Tests

The GLs precipitate obtained at the end of the *D. spathularia* cultivation was tested for the presence of antimicrobial activity. For this purpose, firstly the fermentation was stopped, and the broth was alkalized with 1 M NaOH to pH 8. With this alkali treatment, GLs which were stuck to the mycelial hyphae of *D. spathularia* cells, released and directed in the fermentation broth [Stadler et al., 2017, 2020]. Then the cells were removed via microfiltration (0.22 μ m polysulfone membrane) and cell free broth was acidified to pH 3 with 1 N HCl solution. By lowering the pH, GLs were successfully precipitated and this precipitate was used in the antimicrobial tests.

3.2.1. Kirby-Bauer Antimicrobial Disk Assay with LGLs Precipitate

The disk diffusion method of Kirby-Bauer is a standardized, viable alternative to broth dilution method. In Kirby-Bauer method, test microorganism is placed on a plate of solid growth medium and white disks containing antimicrobial agent are added to the plate [Hudzicki, 2009]. After incubating the pathogenic microorganism to grow overnight, areas of clear media surrounding the disks show the inhibition of test organism by antimicrobial agent [Boyle et al., 1973]. In this study the Kirby-Bauer disk diffusion assay was employed to determine the resistance of the selected test microorganisms to glycolipids.

3.2.1.1. Disk Diffusion Assay with LGLs Precipitates from 7 Days *D. spathularia* Cultivation in Baffled and Unbaffled SFs

The first experiments to test antimicrobial effect of GL precipitate was performed with the precipitate obtained from 40 mL supernatant after 168 h cultivation in 250 mL baffled flask. The precipitate was dissolved in 1 mL sterile distilled water. Thereafter disks were immersed in LGLs solution for 1 min. and 3 min. durations and then placed on *E. coli* BL21 and *B. subtilis* cultures on Luria-Bertani (LB) agar plates (Figure 3.9). Cell-free fermentation which went through the same precipitation protocol was used as negative control.

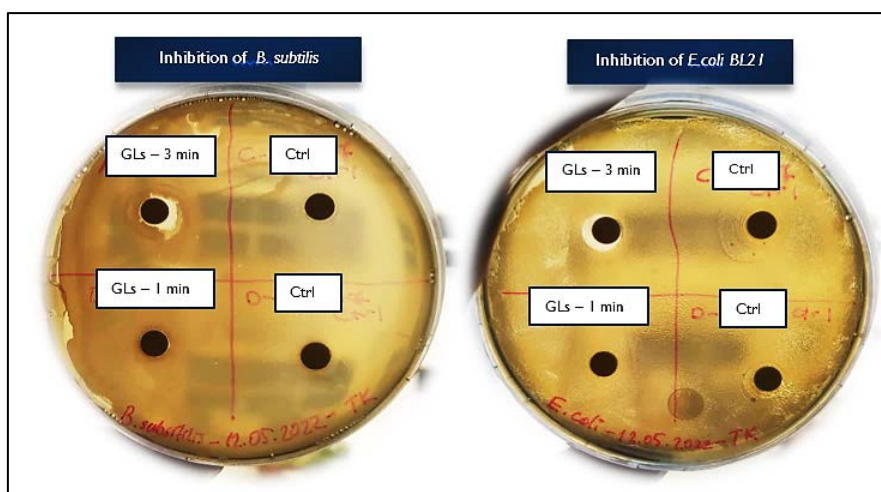


Figure 3.9: Inhibition zone for *E. coli* and *B. subtilis* with GLs precipitate obtained from 7 days old *D. spathularia* culture in baffled shake flask.

The results have shown the presence of antimicrobial activity against both *E. coli* and *B. subtilis*. The concentration of LGLs precipitate used in the experiment was 66 µg powder/mL. With this concentration, zone of inhibition around 3 min.-GLs impregnated disk on *B. subtilis* was measured as 0.7 mm while it was 0.6 mm on *E. coli* plate after 24 h incubation at 37°C. No zone formation was observed around 1 min.-GLs impregnated disks. Most probably, the use of longer impregnation duration (3-min.) allowed more LGLs to be absorbed by disks and also achieving sufficient concentration to inhibit the growth on both plates.

Moreover, it has been previously found that LGLs show antimicrobial activity mainly against Gram-positive bacteria, yeast and molds. Our findings were in accordance with these previously reported findings [Stadler et al., 2020]. Zone of inhibition on *B. subtilis* (Gram-positive) cells was higher than the zone formation on *E. coli* cells.

To investigate the effect of different flask type on antimicrobial activity of GLs precipitate, another shake flask experiment was performed in unbaffled shake flasks. In accord with this, 500 mL unbaffled shake flasks with 150 mL working volume were inoculated with 1.5% (v/v) *D. spathularia*, from seed culture (OD₆₀₀=13) and cultivated for 168 h. At the end of 168 h period, the fermentation was stopped and downstream processing steps were applied to supernatant (Figure 2.2). The precipitate obtained from 100 mL broth after 168 h cultivation, was 25.3 mg. The concentration used in the disk diffusion assay was 12.06 x 10⁴ µg powder/mL water.

The concentration used in disk diffusion assay from unbaffled shake flask was

higher than the concentration of precipitate from baffled shake flasks ($12.06 \times 10^4 \mu\text{g}$ powder/mL in unbaffled, $66 \mu\text{g}$ powder/mL in baffled), yet it did not result in larger formation of inhibition zone. Although the concentration obtained from unbaffled shake flasks was higher as compared to the one in baffled shake flasks, no inhibition zone was formed on the agar plates. Despite there were differences in the volume of the culture and inoculum size, the most effective parameter was thought to be the flask type based on these results.

3.2.1.2. Antimicrobial Effect of LGLs Precipitates Obtained from 14 Days, 21 Days and 28 Days *D. spathularia* Cultivation in Unbaffled SFs

To further investigate the antimicrobial activity of GLs precipitate in unbaffled shake flasks, 500 mL unbaffled flasks with 30% (v/v) working volume were inoculated with 1.5 % (v/v) *D. spathularia* ($\text{OD}_{600}=13$) and cultivated for 14, 21 and 28 days in MESP medium. The precipitates obtained from 100 mL broths at the end of 14, 21 and 28 days cultivation, were 26.1 mg, 27.7 mg and 29.0 mg respectively. The water volume to dissolve LGLs precipitate was lowered from 1 mL to 200 μL to achieve bigger inhibition zone formation.

LGLs impregnated disks were placed on *E.coli* BL21, *B. subtilis*, *P. aureginosa*, *S. aureus*, *S. cerevisiae* agar plates and the presence of zone of inhibitions were examined. The concentration of LGLs precipitate from 14 days cultivation was $13.05 \times 10^4 \mu\text{g}$ powder/mL. In result, no zone of inhibition was observed in any of the tested organisms after 24 h incubation at 37°C (Figure 3.10).

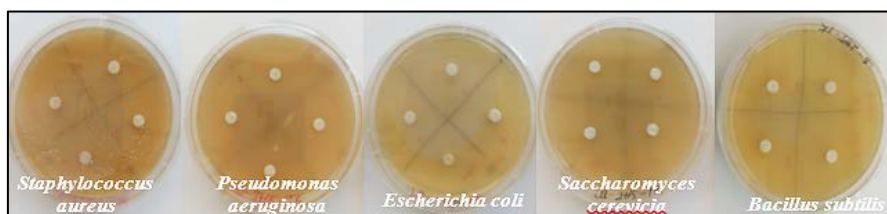


Figure 3.10: Inhibition of *E.coli* , *B. subtilis*, *P. aureginosa*, *S. aureus*, *S. cerevisiae* with LGLs precipitate obtained from 14 days *D. spathularia* cultivation in unbaffled shake flasks.

Contrary to our findings from disk diffusion assay with the LGLs precipitate from 7 days cultivation in baffled shake flask, we did not observe any zone of

inhibition on *E. coli* and *B. subtilis* plates in this experiment. These results have shown that the use of baffled shake flask does seem to shorten the time required to achieve sufficient amount of LGLs to inhibit the growth of test organisms. Moreover, knowing that *D. spathularia* produces LGLs during stationary phase as a secondary metabolite [Stadler et al., 2017, 2020], it could be also concluded that the time required to initiate the secondary metabolite production might be longer than 14 days in unbaffled flasks.

The concentration of the LGLs precipitate from 21 days cultivation was $13.85 \times 10^4 \mu\text{g powder/ mL}$ in the disk diffusion assay. With this concentration the growth of *S. cerevisiae* was inhibited 7 mm while no inhibition zone was observed on *B. subtilis* plate (Figure 3.11).

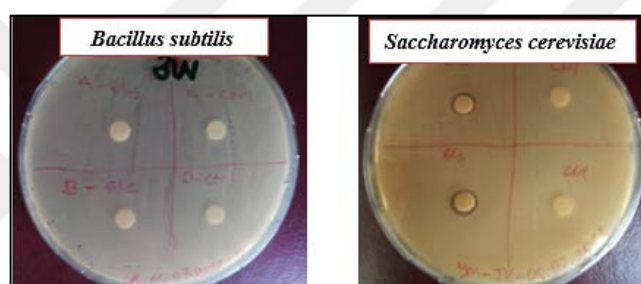


Figure 3.11: Inhibition of *B. subtilis* and *S. cerevisiae* with LGLs precipitate from 21 days old *D. spathularia* culture.

The concentration of the LGLs precipitate from 28 days cultivation was $14.5 \times 10^4 \mu\text{g/mL}$ in the disk diffusion assay. With this concentration the growth of *B. subtilis* and *S. cerevisiae* were inhibited 8 mm and 11 mm respectively (Figure 3.12).

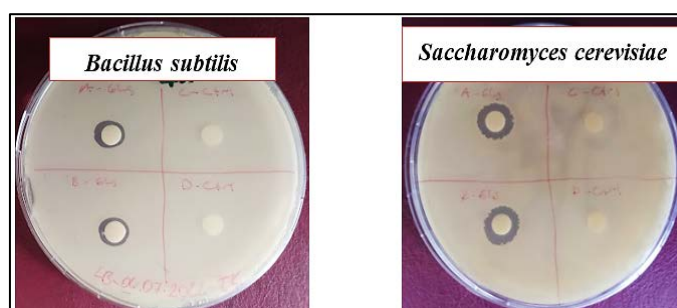


Figure 3.12: Inhibition of *B. subtilis* and *S. cerevisiae* with LGLs precipitate from 28 days old *D. spathularia* culture.

Table 3.2 shows the inhibition zones occurred in response to addition of LGLs from different cultivation times. As it can be seen in the Table 3.2 no inhibition zone was observed when LGLs precipitates from 7 days and 14 days cultivation were used in Kirby-Bauer disk diffusion assay. The growth of *S. cerevisiae* was inhibited more as compared to the growth of *B. subtilis* in both precipitates (from 21 days and 28 days supernatants). When comparing our results to those of previous studies, it must be pointed out that the our results confirm the antimicrobial activity of LGLs from *D. spathularia* against *B. subtilis*, *S. cerevisiae* and *E. coli* [Kumar et al., 2019; Stadler et al., 2017, 2020].

Table 3.2: The diameter of inhibition zones (in mm) obtained with LGLs precipitate from 7, 14, 21 and 28 days supernatants.

Organism	Cultivation period & Concentration of GLs precipitates			
	7 days	14 days	21 days	28 days
	Conc.: 12.6x10 ⁴ µg powder/mL	Conc.: 13.05 x 10 ⁴ µg powder /mL	Conc.: 13.85 x 10 ⁴ µg powder /mL	Conc.: 14.5 x 10 ⁴ µg powder /mL
<i>B. subtilis</i>	0 mm	0 mm	0 mm	8 mm
<i>S. cerevisiae</i>	0 mm	0 mm	7 mm	11 mm

From these findings, it can be concluded that the concentration of precipitate might have an impact on the antimicrobial effect of LGLs. However, this could be also relevant with the LGLs profile which might be different for different phases of growth. Therefore, not only the concentration but the type and ratio of LGLs produced might affect antimicrobial activity of the precipitates obtained. To reach sufficient amount of LGLs and perform successful disk diffusion assay, the precipitate must be recovered from at least 21 days cultivated culture when unbaffled shake flasks were used for cultivation.

3.2.2. Antimicrobial Efficacy Tests in Broth Cultures

To test antimicrobial activity of LGLs in broth cultures, the precipitates from 7, 14, 21 and 28 days cultivation in unbaffled shake flasks were utilized. OD₆₀₀ readouts of actively growing *E. coli*, *B. subtilis*, *P. aeruginosa*, *S. aureus*, *S. cerevisiae* were checked to see the growth inhibition due to antimicrobial activity of LGLs. The concentrations of LGLs from 7 days and 14 days were same with the concentrations used in disk diffusion assay (Table 3.3).

Similar to the results of our disk diffusion assays, no growth inhibition was seen with the precipitates from 7 days and 14 days of cultivation in unbaffled shake flasks. These results might be attributed to two things; first, the amount of LGLs precipitate produced within 14 days was not sufficient to inhibit the growth of the test organisms, and second, the LGLs grown in unbaffled shake flasks in 14 days do not function same as the LGLs produced in baffled shake flasks. More comprehensive experiments could be performed with LGLs precipitate from unbaffled and baffled shake flasks to better address the reason behind these results.

3.2.2.1. Antimicrobial Efficacy Tests with Diluted LGLs Precipitate from 21 Days old *D. spathularia* Cultures in Unbaffled SFs

Absorbance (OD₆₀₀ readouts) of actively growing *E. coli* BL21, *B. subtilis*, *P. aeruginosa*, *S. aureus*, *S. cerevisiae* were checked to see the growth inhibition due to antimicrobial activity of LGLs. The concentration of the LGLs precipitate used in this broth culture experiment was 6.93×10^4 µg powder/mL.

To perform the experiment, 25 mL LB and YM broths were prepared in 50 mL falcon tubes and inoculated from agar plates with the selected organisms. After 24 hours OD₆₀₀ values were adjusted to 0.5 for each microorganism. 5 mL broth cultures were transferred into 15 mL sterile falcon tubes. For each microorganism, four different parameters were prepared in replicates. The change in OD₆₀₀ was followed-up after 24 h.

The results showed that the growth in LGLs added cultures were slower than the cultures with no addition in *B. subtilis*, *S. aureus* and *P. aeruginosa*. These results provide evidence for antimicrobial activity of LGLs on *B. subtilis*, *S. aureus* and *P. aeruginosa* (Figure 3.13).

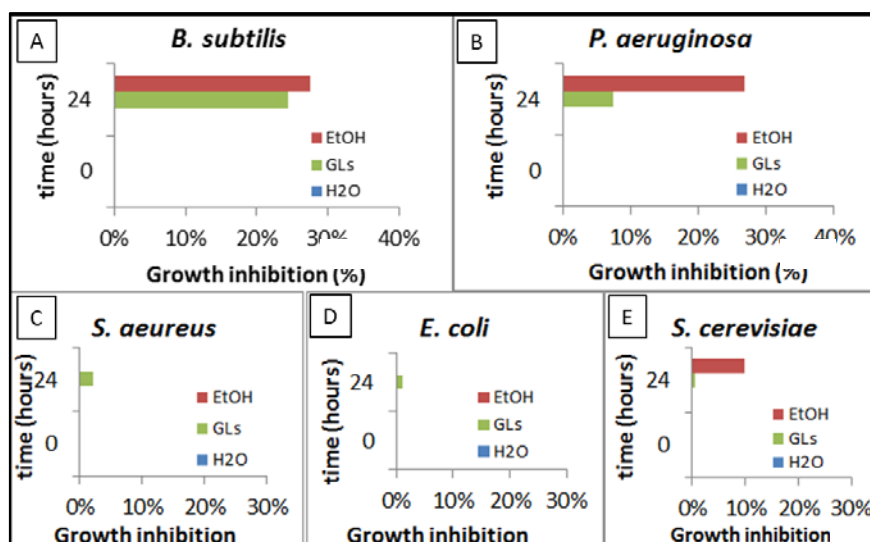


Figure 3.13: The inhibition percentage of *E. coli*, *B. subtilis*, *S. aureus* and *P. aeruginosa* and *S. cerevisiae* growth with GLs precipitate.

The inhibition due to LGLs addition was slightly lower than the inhibition due to EtOH addition in all of the tested organisms. Two highest inhibition were observed in *B. subtilis* (Gram-positive) and *S. aureus* (Gram-positive) broths during the cultivation period. These results in line with the previous studies mentioning that LGLs show antimicrobial activity mainly against Gram-positive bacteria, then yeast and mold [Stadler et al., 2017, 2020].

Overall, LGLs precipitate, 6.93×10^4 μg powder/mL, obtained from 21 days cultivated *D. spathularia* cells, inhibited the growth of *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *S. cerevisiae* by , 24%, 7%, 2%, 1%, 0%, respectively.

3.2.2.2. Antimicrobial Efficacy Tests in Broth Cultures with Concentrated LGLs precipitate from 21 Days and 28 Days Old *D. spathularia* Cultures in Unbaffled SFs

The concentration of the LGLs precipitates obtained from 21 days and 28 days *D. spathularia* cultures were calculated as 13.85×10^4 and 14.5×10^4 μg powder/mL, respectively. These precipitates were used for analyzing antimicrobial activity of the LGLs. OD₆₀₀ readouts of actively growing *B. subtilis*, and *S. cerevisiae* were checked to see the growth inhibition due to inclusion of LGLs in the cultures.

LB and YM broths were prepared and inoculated from agar plates with the *B. subtilis*, and *S. cerevisiae* organisms respectively. After 24 hours OD₆₀₀ values were

adjusted to 0.4 for each microorganism. 5 mL broth cultures were transferred into 10 mL sterile glass tubes. For each microorganism, different parameters were prepared in replicates (Table 3.3). The change in OD₆₀₀ values was followed-up every 24h.

Table 3.3: Amount of LGLs and control solutions used for inhibition experiments.

Microorganisms	Additions			
	Ctrl 1-No Addition	Ctrl 2-Deminarilized H2O	Ctrl 3-EtOH (70%)	LGLs precipitate
<i>B. subtilis</i>	0 µL	50 µL	50 µL	50 µL
<i>S. cerevisiae</i>	0 µL	50 µL	50 µL	50 µL

In line with the results of our disk diffusion assay, LGLs precipitate from 28 days resulted in higher growth inhibition than the precipitate from 21 days in *S. cerevisiae* cultures (Figure 3.14).

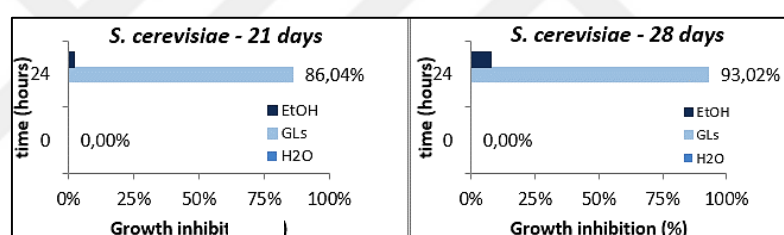


Figure 3.14: Inhibition of *S. cerevisiae* growth with A) GLs precipitate from 21 days old and B) GLs precipitate from 28 days old *D. spathularia* culture.

As it is shown in Figure 3.14, the inhibition occurred due to addition of precipitate from 28 days was higher than the precipitate from 21 days. The concentration of the LGLs precipitate from 28 days was 14.5×10^4 µg powder/mL, while it was 13.85×10^4 µg powder/mL, from 21 days. Accordingly, the inhibition was higher with LGLs from 28 days old culture, too.

In addition to *S. cerevisiae*, the growth of *B. subtilis* was also inhibited due to LGLs precipitate addition (Figure 3.15). Since the highest antimicrobial activity was achieved with the precipitate from 28 days, it was used in the subsequent trials.

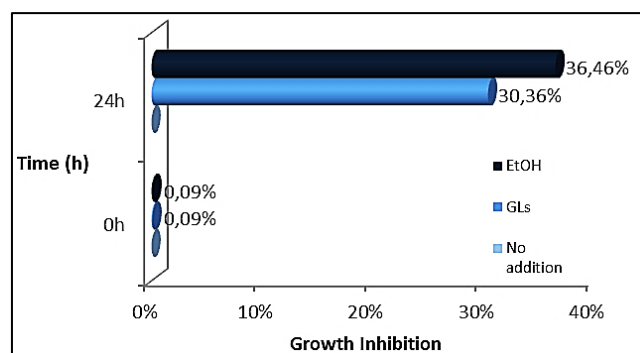


Figure 3.15: Inhibition of *B. subtilis* growth with GLs precipitate from 28 day old *D. spathularia* culture.

The inhibition 30.36% observed at time 24h. The inhibition caused by GLs precipitate was almost same as the inhibition occurred due to EtOH addition at time 24h. Although this result showed strong antimicrobial function of GLs, the inhibition effect may drop in the following days. In that case, it is important to note that optimization experiments would be required to preserve GLs with persistent growth inhibition activity.

Overall, these results further imply that the fermentation time significantly affect antimicrobial activity of GLs precipitate, hence it should be thoroughly studied to maximize the glycolipids antimicrobial activity.

3.2.2.3. The Effect of the Concentration of LGLs Precipitate Antimicrobial Activity

To investigate the effect of different LGLs concentrations on its antimicrobial activity, broth culture test was performed with *B. subtilis* and *S. cerevisiae*. Three different concentrations were applied as 725 µg powder/mL (C1), 362 µg powder/mL (C2) and 181 µg powder/mL (C3). Effect of GLs concentration on growth of microorganisms was tested for 72 hours of cultivation. Follow-up of the growth inhibition was done with OD₆₀₀ readings (Figure 3.16).

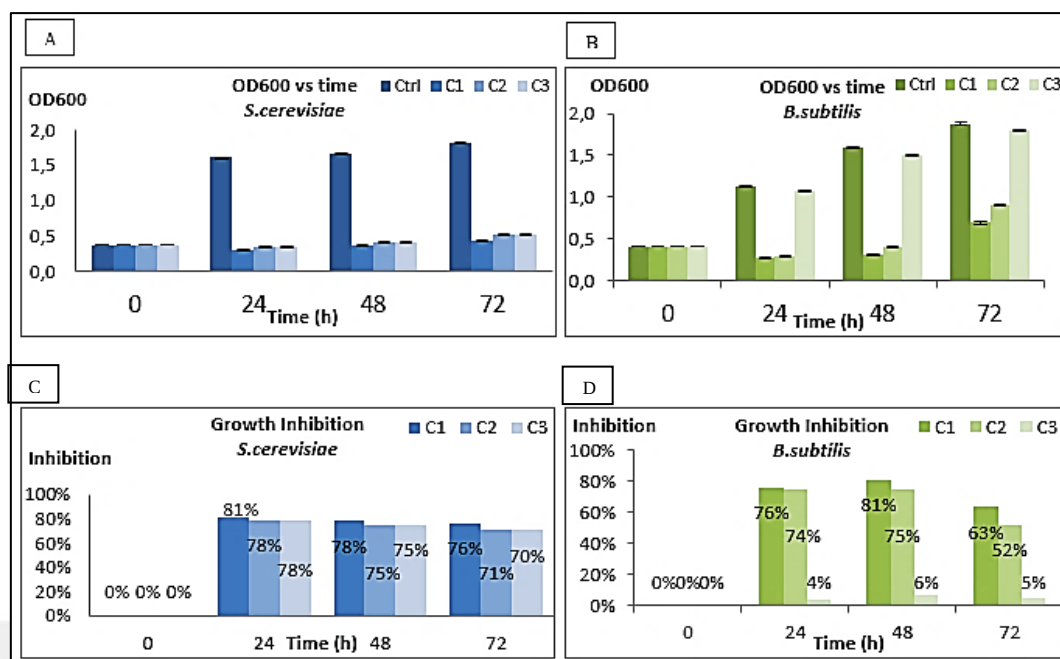


Figure 3.16: Growth inhibition by GLs precipitate in *B. subtilis* and *S. cerevisiae* cultures. (A) Absorbance (OD₆₀₀) versus time during *S. cerevisiae* cultivation. (B) (OD₆₀₀) versus time during *B. subtilis* cultivation. (C) Growth inhibition of *S. cerevisiae* culture due to different concentration of GLs precipitate. (D) Growth inhibition of *B. subtilis* culture due to different concentration of GLs precipitate.

The results showed that the inhibition effect was decreasing from higher concentration, C1 – 725 µg powder/mL, to lower concentration, C3 - 181 µg powder/mL in both cultures.

Moreover, the inhibition percentage was slightly dropping from time 24 h to time 72 h. The inhibition of effect GLs on growth of *S. cerevisiae* was higher than the inhibition on the growth of *B. subtilis* (*S. cerevisiae*: 81% at 24h, *B. subtilis*: 76% at 24h). Inhibition effect retained during 3 days of incubation (*S. cerevisiae* , from day 1 to day 3 : 81%, 78%, 76% and *B. subtilis*, from day 1 to day 3: 76%, 81%, 63%) . The highest inhibition was achieved with highest concentration (C1 – 725 µg powder/mL) applied.

Overall, these results suggest that not only the highest concentration (C1-725 µg powder/mL) but also the most diluted GL precipitate (C3- 181 µg powder/mL), was efficient to inhibit the growth of *S. cerevisiae*, however, for *B. subtilis* higher concentrations of GLs might be necessary to inhibit growth for longer periods of time; the resistance of this bacteria might be due to its sporulation property.

3.3. Chromatographic Analysis

Chromatographic analysis can be used very efficiently to study glycolipids. High-Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) techniques help in identifying different types of glycolipids based on their properties. In this study to identify glycolipids two conventional chromatographic methods were used.

3.3.1. TLC Analysis

The technique of Thin Layer Chromatography (TLC) has long been used to analyze glycolipid mixtures or to determine parameters for a preparative separation of glycolipid compounds using column chromatography [Fuchs, 2012]. In this study, LGLs obtained from supernatant was analyzed with TLC method. In line with this purpose, LGLs precipitates obtained after 7 days, 14 days, 21 days and 28 days of cultivation were loaded on TLC plates and stained with two different solvents (Figure 3.17). 60 Å silica TLC plates (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) as stationary phase and a mobile phase of isopropyl acetate/methanol/acetic acid (100:10:1 v/v/v) were used as running solvent.

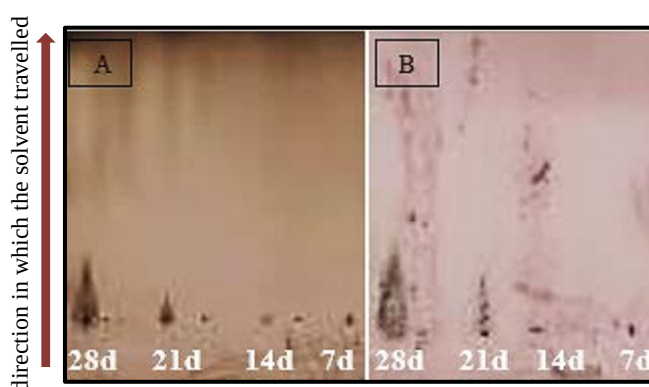


Figure 3.17: TLC analysis of LGLs from different cultivation times. (A) TLC plate stained with ethanol (95%, v/v). (B) TLC plate stained with sulphuric acid (10%, v/v).

Ethanol 95% (v/v) and sulphuric acid 10% (v/v) have previously been used for staining purposes after TLC chromatograph [Mani et al., 2016; Wang and Benning, 2011]. Thus in this study these two techniques were applied to observe LGLs. As it is

illustrated in Figure 3.17, the use of different solvents only changed the color of TLC plate but almost same lipid profile and concentrations were observed for two different solvent usage.

TLC plates are white in color. Changes in color were expected to be seen at the end of staining protocol and are accepted as a first indicator for successful completion of the protocol. In the study carried out by K  gler et. al., the TLC chromatogram of glycolipids produced by *Rouxiella* sp. DSM 100043 was obtained [K  gler et al., 2015b]. Similar to their results, we observed purple spots on the first two columns of the TLC plate where the precipitates from 28 days and 21 days were loaded (Figure 3.17-B).

According to the spots on TLC plates, the precipitates from 21 days and 28 days cultures contain GLs; however, no spots were seen on the columns 14 days and 7 days precipitates were loaded. The presence of larger spots observed with 28 days precipitate on both Figure 3.17-A and Figure 3.17-B, were attributed to the concentration difference between two different cultivation times.

Overall, these results further confirmed our previous findings regarding the absence or insufficient production of LGLs after 7 days and 14 days of incubation in unbaffled flasks. Moreover, it must be pointed out that for better separation of LGLs spots, further optimization in TLC protocol is necessary.

3.3.2. HPLC Analysis

HPLC-UV has long been used to separate compounds dissolved in a liquid sample and for quantitative analysis of the compound of interest. To compare glycolipid profile of precipitates obtained from 7 days and 28 days of cultivation, HPLC-UV analysis was performed. The separation of main glycolipid classes or subclasses by reverse phase and normal phase high performance liquid chromatography and UV detection, has been done previously [Demopoulos et al., 1996, 2006b]. Similarly, HPLC with UV detector was used in the present study. Isocratic elution was done with isooctane/2-propanol (1/1, v/v) mobile phase at 206 nm in 45 min. The column was kept at 65  C and the flow rate of mobile phase 0.5 ml/min. The results showing the difference between chromatograms of LGLs precipitate from 7 days and 28 days were demonstrated in Figure 3.18. Possible

LGLs peak in the sample from 28 days culture is seen at 11th minute of flow. In 7 days culture, it seems there is no LGLs production.

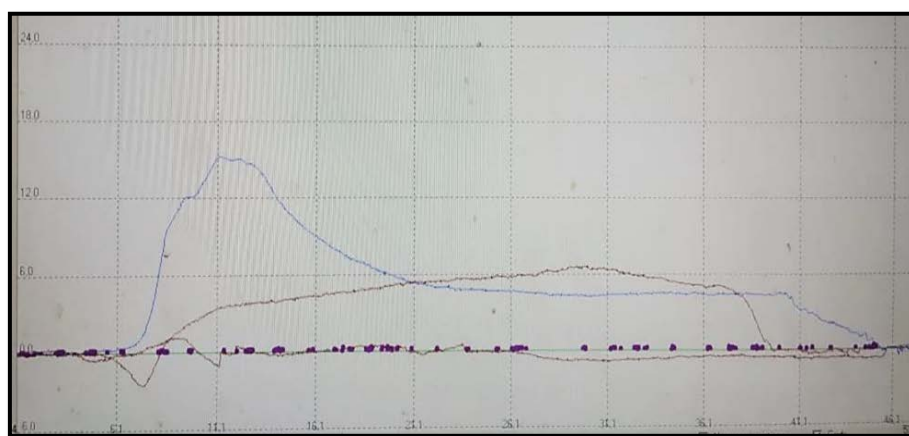


Figure 3.18: HPLC analysis of GLs precipitates from 7 days and 28 days and negative control. Blue solid line represents 28 days and red solid line represents 7 days.

In case of HPLC analysis, the purity of peak originated from the injected samples is very important, based on the peak area and height the concentration of the LGLs can be estimated. To be able to interpret the results properly, it is important to have standard solution with known concentration. Concentration of LGLs can be calculated by using LGLs standard in future studies.

As it can be seen in Figure 3.18, peaks which were not present in the chromatogram obtained from 7 days culture become prominent in chromatogram obtained from 28 days culture. Considering our previous antimicrobial test results which proved that precipitates obtained from 7 days and 14 days cultures did not show any antimicrobial activity, it can be concluded that the production of glycolipids was not started within first 14 days of *D. spathularia* cultivation in unbaffled shake flasks. Figure 3.18 shows increasing amount of LGLs in 28 days sample. Nonetheless, the peaks for LGLs are not well separated with the sugar column used during our HPLC analysis. More suitable HPLC column usage may provide better detection of different LGLs produced by *D. spathularia*.

Although making the exact quantification was not possible due to lack of LGLs standard, our results were comparable with the HPLC results of glycolipids standard used in a previous study [Demopoulos et al., 2006a]. Moreover, with the HPLC

analysis done in this study, the difference between lowest and highest LGLs containing sample was clearly revealed.



4. CONCLUSION

D. spathularia holds great potential to be utilized in food industry as it produces LGLs functional as food-grade antimicrobials. In this study, the growth behavior of *D. spathularia* and its antimicrobial activity against five test organisms were investigated with Kirby-Bauer disk diffusion assay and broth culture antimicrobial tests. The results have shown that the use of baffled shake flask during *D. spathularia* cultivation resulted in high biomass formation and antimicrobial activity; most probably due to better oxygen and substrate uptake.

The highest growth inhibition was recorded as 93.02% and seen on *S. cerevisiae* cells due to addition of LGLs (conc.: 14.5×10^4 μg powder/mL), from 28 days cultivation of *D. spathularia* cells. In addition, LGLs obtained from 21 days cultivation (conc.: 6.93×10^4 μg powder/mL), inhibited the growth of *B. subtilis*, *S. auerus*, *P. auereginosa*, *E. coli*, *S. cerevisiae* by , 24%, 7%, 2%, 1%, 0%, respectively within 24 hours. Longer cultivation period resulted increased growth inhibition in unbaffled shake flasks. In unbaffled shake flasks, 21 days cultivation was needed to obtain LGLs with antimicrobial activity, while 7 days was sufficient in baffled shake flasks.

Moreover, a series of laboratory-scale fermentations were performed to determine the optimum operational parameters, e.g. pH, temperature, medium composition, for LGLs production. The morphology of *D. spathularia* was demonstrated with microscopic examinations. The medium giving the highest amount of biomass was recorded as yeast malt glucose medium for *D. spathularia* cultivations. pH 5.6 resulted in higher biomass formation as compared to pH 4.5 and 33°C was more effective than 30°C to achieve higher amount of biomass.

Overall, the results of this study demonstrated the potential of LGLs to be utilized as a natural antimicrobial preservative agent. For future studies, conducting more comprehensive antimicrobial activity test with different food pathogens would be useful to further develop and confirm these initial findings.

REFERENCES

- Abdel-Mawgoud A. M., Stephanopoulos G. (2018), "Simple glycolipids of microbes: Chemistry, biological activity and metabolic engineering", *Synthetic and Systems Biotechnology* 3(1), 3–19.
- Abdulumumeen H. A., Risikat A. N., Sururah A. R. (2012), "Food: Its preservatives, additives and applications", *International Journal of Chemical and Biochemical Sciences* 1(10), 36–47.
- Alvarenga R. L. M., Xavier-Santos S. (2017), "New records of Dacrymycetetes (Fungi: Basidiomycota) from the Cerrado Biome (Brazilian Savanna) and Midwest Region, Brazil", *Check List* 13(4), 335–342.
- Aminlari L., Mohammadi Hashemi M., Aminlari M. (2014), "Modified Lysozymes as Novel Broad Spectrum Natural Antimicrobial Agents in Foods", *Journal of Food Science* 79(6), 1077–1090.
- Amiri S., Moghanjoughi Z. M., Bari M. R., Khaneghah A. M. (2021), "Natural protective agents and their applications as bio-preservatives in the food industry: An overview of current and future applications", *Italian Journal of Food Science* 33(9), 55–68.
- Amit S. K., Uddin M. M., Rahman R., Islam S. M. R., Khan M. S. (2017), "A review on mechanisms and commercial aspects of food preservation and processing", *Agriculture and Food Security* 6(1), 1–22.
- Anand S. P., Sati N. (2013), "Artificial Preservatives and Their Harmful Effects: Looking Toward Nature for Safer Alternatives", *International Journal of Pharmaceutical Sciences and Research* 4(7), 2496–2501.
- Anjum Zia M., Riaz A., Rasul S., Abbas R. Z. (2013), "Evaluation of Antimicrobial Activity of Glucose Oxidase from *Aspergillus niger* EBL-A and *Penicillium notatum*", *Brazilian Archives of Biology and Technology* 56(6), 956–961.
- Ashby R. D., Solaiman D. K. Y. (2010), "The influence of increasing media methanol concentration on sophorolipid biosynthesis from glycerol-based feedstocks", *Biotechnology Letters* 32(10), 1429–1437.
- Atasoy M., Eyice O., Schnürer A., Cetecioglu Z. (2019), "Volatile fatty acids production via mixed culture fermentation: Revealing the link between pH, inoculum type and bacterial composition", *Bioresource Technology* 292(10), 121889.
- Baccile N., Selmane M., Le Griel P., Prévost S., Perez J., Stevens C. V., Delbeke E., Zibek S., Guenther M., Soetaert W., et al. (2016), "PH-Driven Self-Assembly of Acidic Microbial Glycolipids", *Langmuir* 32(25), 6343–6359.

Bahrami A., Delshadi R., Jafari S. M., Williams L. (2019), "Nanoencapsulated nisin: An engineered natural antimicrobial system for the food industry", *Trends in Food Science & Technology* 9420–31.

Barberis S., Quiroga H. G., Barcia C., Talia J. M., Debattista N. (2018), "Natural Food Preservatives Against Microorganisms", *Food Safety and Preservation* 1(8), 621–658.

Bensch K. (1948), "*Dacryopinax spathularia*", *Lloydia* 11(2), 111–122.

Bitzer J., Henkel T., Nikiforov A. I., Rihner M. O., Thomas J. A. (2017a), "Pharmacokinetics, excretion balance, and tissue distribution of [14C]-labeled glycolipids and long chain fatty acids from *Dacryopinax spathularia* in rats", *Food and Chemical Toxicology* 109552–568.

Bitzer J., Henkel T., Nikiforov A. I., Rihner M. O., Henderson K. A. (2017b), "A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in Beagle dogs", *Food and Chemical Toxicology* 109544–551.

Bitzer J., Henkel T., Nikiforov A. I., Rihner M. O., Leuschner J. (2017c), "A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in CD® rats", *Food and Chemical Toxicology* 109569–584.

Bitzer J., Henkel T., Nikiforov A. I., Rihner M. O., Herberth M. T. (2018), "Developmental and reproduction toxicity studies of glycolipids from *Dacryopinax spathularia*", *Food and Chemical Toxicology* 120430–438.

Bitzer J., Henkel T., Nikiforov A. I., Rihner M. O., Verspeek-Rip C. M., Usta B., van den Wijngaard M. (2019), "Genetic toxicity studies of glycolipids from *Dacryopinax spathularia*", *Food and Chemical Toxicology* 123162–168.

Bodman S. M. C. (2018), "Morphology and Cytology of *Guepinia Spathularia*", *Mycologia* 30(6), 635–652.

Bouarab Chibane L., Degraeve P., Ferhout H., Bouajila J., Oulahal N. (2019), "Plant antimicrobial polyphenols as potential natural food preservatives", *Journal of the Science of Food and Agriculture* 99(4), 1457–1474.

Boyle V. J., Fancher M. E., Ross R. W. (1973), "Rapid, Modified Kirby-Bauer Susceptibility Test with Single, High-Concentration Antimicrobial Disks", *Antimicrobial Agents and Chemotherapy* 3(3), 418–424.

Bryant J. P., Chapin F. S., Klein D. R. (1983), "Carbon/Nutrient Balance of Boreal Plants in Relation to Vertebrate Herbivory", *Oikos* 40(3), 357.

Burrowes O. J., Hadjicharalambous C., Diamond G., Lee T. C. (2004), "Evaluation of Antimicrobial Spectrum and Cytotoxic Activity of Pleurocidin for Food Applications", *Journal of Food Science* 69(3), FMS66–FMS71.

Çakmak H., Güngörmedi G., Dikmen G., Çelik P. A., Çabuk A. (2017), "The true methodology for rhamnolipid: Various solvents affect rhamnolipid characteristics", *European Journal of Lipid Science and Technology* 119(10), 1700002.

Candice C. (2017), "Gras Notification for Glucose Oxidase From a Genetically Modified Strain Of *Trichoderma Reesei*", Feldbergstrasse 78 , D-64293 Darmstadt.

Carocho M., Barreiro M. F., Morales P., Ferreira I. C. F. R. (2014), "Adding molecules to food, pros and cons: A review on synthetic and natural food additives", *Comprehensive Reviews in Food Science and Food Safety* 13(4), 377–399.

Carter C. D., Parks A., Abuladze T., Li M., Woolston J., Magnone J., Senecal A., Kropinski A. M., Sulakvelidze A. (2012), "Bacteriophage cocktail significantly reduces *Escherichia coli* O157: H7 contamination of lettuce and beef, but does not protect against recontamination", *Bacteriophage* 2(3), 178.

Castro-Santiuste S., Sierra S., Guzmán-Dávalos L., Cifuentes J., Evans T., Martínez-González C. R., Alvaradosizzo H., Luna-Vega I. (2020), "Dacryopinax (Fungi: Dacrymycetales) in Mexico", *Phytotaxa* 446(1), 6–22–26–22.

Chen J., Liao C., Ouyang X., Kahramanoğlu I., Gan Y., Li M. (2020), "Antimicrobial Activity of Pomegranate Peel and Its Applications on Food Preservation", *Journal of Food Quality* 2020.

Chheda A. H., Vernekar M. R. (2015), "A natural preservative ϵ -poly-L-lysine: fermentative production and applications in food industry", *International Food Research Journal* 22(1), 23–30.

Clément K. K., Bakary C., Ibourahema C., Carole K. A., Rose K.-N. (2016), "Antimicrobial Activity of the Varieties of Peppers (*Capsicum*) of Côte d'Ivoire on Multiresistant Strains", *Int.J.Curr.Microbiol.App.Sci* 5(10), 875–890.

Cleveland J., Montville T. J., Nes I. F., Chikindas M. L. (2001), "Bacteriocins: safe, natural antimicrobials for food preservation", *International Journal of Food Microbiology* 71(1), 1–20.

Cohen J. E. (2020), "Population, population, and population", *The Bulletin of the Ecological Society of America* 101(3), 1–17.

Dai J., Li C., Cui H., Lin L. (2021), "Unraveling the anti-bacterial mechanism of *Litsea cubeba* essential oil against *E. coli* O157:H7 and its application in vegetable juices", *International Journal of Food Microbiology* 338108989.

Daoutidou M., Plessas S., Alexopoulos A., Mantzourani I. (2021), "Assessment of Antimicrobial Activity of Pomegranate, Cranberry, and Black Chokeberry Extracts against Foodborne Pathogens", *Foods* 2021, Vol. 10, Page 486 10(3), 486.

Darbandi A., Asadi A., Mahdizade Ari M., Ohadi E., Talebi M., Halaj Zadeh M., Darb Emamie A., Ghanavati R., Kakanj M. (2022), "Bacteriocins: Properties and potential use as antimicrobials", *Journal of Clinical Laboratory Analysis* 36(1).

Dardouri M., Mendes R. M., Frenzel J., Judite Costa &, Ribeiro I. A. C. (2021), "Seeking faster, alternative methods for glycolipid biosurfactant characterization and purification", *Analytical and Bioanalytical Chemistry* 4134311–4320.

Deegan L. H., Cotter P. D., Hill C., Ross P. (2006), "Bacteriocins: Biological tools for bio-preservation and shelf-life extension", *International Dairy Journal* 16(9), 1058–1071.

Demopoulos C. A., Kyrili M., Antonopoulou S., Andrikopoulos N. K. (1996), "Separation of several main glycolipids into classes and partially into species by HPLC and UV-detection", *Journal of Liquid Chromatography and Related Technologies* 19(5), 771–781.

Demopoulos C. A., Kyrili M., Antonopoulou S., Andrikopoulos N. K. (2006a), "Separation of Several Main Glycolipids into Classes and Partially into Species by HPLC and UV-Detection", [Http://Dx.Doi.Org/10.1080/10826079608005536](http://Dx.Doi.Org/10.1080/10826079608005536) 19(5), 771–781.

Déziel E., Lépine F., Dennie D., Boismenu D., Mamer O. A., Villemur R. (2014), "Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene", *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1440(2–3), 244–252.

Dixit S. S., Luqman N. (2015), "Antimicrobial Activity of Onion and Ginger against two Food Borne Pathogens *Escherichia Coli* and *Staphylococcus Aureus*", *MOJ Food Processing & Technology* Volume 1(Issue 4) .

Duran A., Kahve H. I. (2017), "The Use of Lactoferrin in Food Industry", *Academic Journal of Science* 07(02), 89–94.

Ebenezer R. V., James H., John M., Pieter V. (1992), "US5173297A - Bacteriocin from *Lactococcus lactis* subspecies *lactis* - Google Patents".

Elshafie A. E., Joshi S. J., Al-Wahaibi Y. M., Al-Bemani A. S., Al-Bahry S. N., Al-Maqbali D., Banat I. M. (2015), "Sphingolipids production by *Candida bombicola* ATCC 22214 and its potential application in microbial enhanced oil recovery", *Frontiers in Microbiology* 6(NOV), 1324.

Emberger, Gary (2008), "*Dactylopinax spathularia*",.

Faivre V., Rosilio V. (2010), "Interest of glycolipids in drug delivery: from physicochemical properties to drug targeting", *Expert Opinion on Drug Delivery* 7(9), 1031–1048.

Farrow W. M., Taben B., Roche L. (1958), "United States Patent Office - Microbiological Production of Carotenoids", (773), 184.

FDA (2016), "Gras Notice Inventory", US Food and Drug Administration 26(477), 1–126.

Fernandez B., Vimont A., Desfossés-Foucault É., Daga M., Arora G., Fliss I. (2017), "Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese", *Food Control* 78350–356.

Fleurackers S. (2014), "Biosurfactants versus Chemically Synthesized Surface-Active Agents", *Biosurfactants* 48–59.

Franzetti A., Gandolfi I., Bestetti G., Smyth T. J. P., Banat I. M. (2010), "Production and applications of trehalose lipid biosurfactants", *European Journal of Lipid Science and Technology* 112(6), 617–627.

Fuchs B. (2012), "Analysis of phospholipids and glycolipids by thin-layer chromatography-matrix-assisted laser desorption and ionization mass spectrometry", *Journal of Chromatography. A* 125962–73.

Furlaneto-Maia L., Ramalho R., Rocha K. R., Furlaneto M. C. (2020), "Antimicrobial activity of enterocins against *Listeria* sp. and other food spoilage bacteria", *Biotechnology Letters* 42(5), 797–806.

Gaynor P. (2017), "GRAS Notice 740 for Glycolipids from *Dacryopinax spathularia*", *Toxicology Regulatory Services* 26(1), 2–147.

Grüninger J., Delavault A., Ochsenreither K. (2019), "Enzymatic glycolipid surfactant synthesis from renewables", *Process Biochemistry* 8745–54.

Gul P., Bakht J. (2015), "Antimicrobial activity of turmeric extract and its potential use in food industry", *Journal of Food Science and Technology* 52(4), 2272–2279.

Gultekin F., Doguc D. K. (2013), "Allergic and immunologic reactions to food additives", *Clinical Reviews in Allergy and Immunology* 45(1), 6–29.

Hamad A., Nurlaeli E., Pradani D. Y., Djalil A. D., Hartanti D. (2019), "Application of Lemongrass as Natural Preservatives for Tofu", *Jurnal Teknologi Dan Industri Pangan* 30(2), 100–109.

Van Haute S., Raes K., Van der Meeren P., Sampers I. (2016), "The effect of cinnamon, oregano and thyme essential oils in marinade on the microbial shelf life of fish and meat products", *Food Control* 6830–39.

Hubert J., Plé K., Hamzaoui M., Nuissier G., Hadeif I., Reynaud R., Guilleret A., Renault J. H. (2012), "New perspectives for microbial glycolipid fractionation and purification processes", *Comptes Rendus Chimie* 15(1), 18–28.

Hudzicki J. (2009), "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol", *American Society for Microbiology*.

IMO Natural Solutions GmbH (2017), "GRAS Notice 740 for Glycolipids from *Dacryopinax spathularia*", *Toxicology Regulatory Services* 26(1), 2–147.

Isah T. (2019), "Stress and defense responses in plant secondary metabolites production", *Biological Research* 2019 52:1 52(1), 1–25.

JE H., BT H. (1962), "Carbohydrates, Determination of reducing sugars", *Methods Carbohydr Chem* 1380–394.

Juneja V. K., Dwivedi H. P., Yan X. (2012), "Novel natural food antimicrobials*", *Annual Review of Food Science and Technology* 3(1), 381–403.

Justia Patents (2012), "US Patent Application - #20140178444 issued June 26, 2014".

Kourkoutas Y., Proestos C. (2020), "Food Preservation: Challenges and Efforts for", *Foods* 9(391), 1–2.

Kügler J. H., Muhle-Goll C., Hansen S. H., Völz A. R., Kirschhöfer F., Kühl B., Brenner-Weiss G., Luy B., Syldatk C., Hausmann R. (2015a), "Glycolipids produced by *Rouxiiella* sp. DSM 100043 and isolation of the biosurfactants via foam-fractionation", *AMB Express* 5(1), 1–11.

Kumar A., Ali S., Lal S. B., Sinha M. P. (2018), "Mycochemical Screening and Determination of Nutritive Potency and Antioxidant Activity of Edible Macrofungi *Dacryopinax Spathularia* (Schwein) and *Schizophyllum Commune* (Fries)", *World Journal of Pharmaceutical Research* 7(16), 1311–1322.

Kumar A., Kumar M., Ali S., Lal S. B., Sinha M. P. (2019), "Anti-pathogenic efficacy of Indian edible macrofungi *Dacryopinax spathularia*(Schwein) and *Schizophyllum commune* (Fries) against some humanpathogenic bacteriae", *Journal of Emerging Technologies and Innovative Research* 6(1), 695–704.

Kumar A., Kumar M., Ranjan R., Sinha M. P. (2021), "Pharmacological Impact of Edible Macrofungi *D. Spathularia* Hematological Investigation Profile of Albino Rat", *International Journal of Advanced Research* 9(03), 527–533.

Kuttiraja M., Dhouha A., Tyagi R. D. (2018), "Harnessing the Effect of pH on Lipid Production in Batch Cultures of *Yarrowia lipolytica* SKY7", *Applied Biochemistry and Biotechnology* 184(4), 1332–1346.

Li X., Xie X., Xing F., Xu L., Zhang J., Wang Z. (2019), "Glucose oxidase as a control agent against the fungal pathogen *Botrytis cinerea* in postharvest strawberry", *Food Control* 105277–284.

Liepins J., Balina K., Soloha R., Berzina I., Lukasa L. K., Dace E. (2021), "Glycolipid Biosurfactant Production from Waste Cooking Oils by Yeast: Review of Substrates, Producers and Products", *Fermentation* 2021, Vol. 7, Page 136 7(3), 136.

Liu X., Shu Q., Chen Q., Pang X., Wu Y., Zhou W., Wu Y., Niu J., Zhang X. (2020), "Antibacterial Efficacy and Mechanism of Mannosylerythritol Lipids-A on *Listeria monocytogenes*", *Molecules* 2020, Vol. 25, Page 4857 25(20), 4857.

Lopez A., Garcia J., Arboles P. (2014), "Funga Veracruzana *Pluteus cervinus*", (February 2011).

Lourith N., Kanlayavattanukul M. (2009), "Natural surfactants used in cosmetics: glycolipids", *International Journal of Cosmetic Science* 31(4), 255–261.

Magalhães L., Nitschke M. (2013), "Antimicrobial activity of rhamnolipids against *Listeria monocytogenes* and their synergistic interaction with nisin", *Food Control* 29(1), 138–142.

Mani P., Dineshkumar G., Jayaseelan T., Deepalakshmi K., Ganesh Kumar C., Senthil Balan S. (2016), "Antimicrobial activities of a promising glycolipid biosurfactant from a novel marine *Staphylococcus saprophyticus* SBPS 15", *3 Biotech* 6(2), 1–9.

Marcó M. B., Moineau S., Quiberoni A. (2012), "Bacteriophages and dairy fermentations", *Bacteriophage* 2(3), 149.

Mariod A. A. (2016), "Effect of Essential Oils on Organoleptic (Smell, Taste, and Texture) Properties of Food", *Essential Oils in Food Preservation, Flavor and Safety* 131–137.

Marquardt P. (2019), "Natural glycolipids help control microorganisms in soft drinks | 2019-11-21 | Beverage Industry".

Martin G. W. (1948), "New or noteworthy tropical fungi", IV. *Lloydia* 11(2), 111–122.

Martínez-Graciá C., González-Bermúdez C. A., Cabellero-Valcárcel A. M., Santaella-Pascual M., Frontela-Saseta C. (2015), "Use of herbs and spices for food preservation: advantages and limitations", *Current Opinion in Food Science* 638–43.

Max Roser, Hannah Ritchie, Esteban Ortiz-Ospina (2013), "World Population Growth", *OurWorldInData.Org*.

McLaughlin D. J., Healy R. A., Arun Kumar T. K., McLaughlin E. G., Shirouzu T., Binder M. (2016), "Cultural and cytological characterization of *Dacryopinax primogenitus*, a new species in the *Dacrymycetaceae* with a fully sequenced genome",

Memar M. Y., Adibkia K., Farajnia S., Kafil H. S., Yekani M., Alizadeh N., Ghotaslou R. (2019), "The grape seed extract: A natural antimicrobial agent against different pathogens", *Reviews in Medical Microbiology* 30(3), 173–182.

Meuninck J. (2017), "Foraging mushrooms, Oregon: finding, identifying, and preparing edible wild mushrooms", *Falcon Guides* 74.

Mills S., Ross R. P., Neve H., Coffey A. (2019), "Bacteriophage and anti-phage mechanisms in lactic acid bacteria", *Lactic Acid Bacteria: Microbiological and Functional Aspects*, Fourth Edition 165–186.

Mirza S. K., Asema U. K., Kasim S. (2017), "To Study The Harmful Effects Of Food Preservatives On Human Health", *Journal of Medicinal Chemistry and Drug Discovery* 2(2347–9027), 610–616.

Mnif I., Ghribi D. (2016), "Glycolipid biosurfactants: main properties and potential applications in agriculture and food industry", *Journal of the Science of Food and Agriculture* 96(13), 4310–4320.

Moghimi R., Aliahmadi A., McClements D. J., Rafati H. (2016), "Investigations of the effectiveness of nanoemulsions from sage oil as antibacterial agents on some food borne pathogens", *LWT - Food Science and Technology* 7169–76.

Monteiro S. A., Sasaki G. L., de Souza L. M., Meira J. A., de Araújo J. M., Mitchell D. A., Ramos L. P., Krieger N. (2007), "Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614", *Chemistry and Physics of Lipids* 147(1), 1–13.

Montiel R., Martín-Cabrejas I., Medina M. (2015), "Reuterin, lactoperoxidase, lactoferrin and high hydrostatic pressure on the inactivation of food-borne pathogens in cooked ham", *Food Control* 51122–128.

Morita T., Ishibashi Y., Fukuoka T., Imura T., Sakai H., Abe M., Kitamoto D. (2011), "Production of Glycolipid Biosurfactants, Cellobiose Lipids, by *Cryptococcus humicola* JCM 1461 and Their Interfacial Properties", *Bioscience, Biotechnology, and Biochemistry* 75(8), 1597–1599.

Moshtaghi H., Rashidimehr A., Shareghi B. (2018), "Antimicrobial Activity of Nisin and Lysozyme on Foodborne Pathogens *Listeria Monocytogenes*, *Staphylococcus Aureus*, *Salmonella Typhimurium*, and *Escherichia Coli* at Different pH", *Journal of Nutrition and Food Security* 3(4), 193–201.

Mozaffari Nejad A. S., Shabani S., Bayat M., Hosseini S. E. (2014), "Antibacterial Effect of Garlic Aqueous Extract on *Staphylococcus aureus* in Hamburger", *Jundishapur Journal of Microbiology* 7(11), 13134.

Nascimento J. C. dos S., Ribeiro A. G., Pessoa R. A. S., Rabello C. B. V., Venâncio A., Porto T. S., Teixeira J. A. C., Porto A. L. F. (2022), "Effect of pH and temperature on phytase and biomass production by submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924", *Research, Society and Development* 11(6), e41311628994–e41311628994.

Nguyen L. T., Fărcaș A. C., Socaci S. A., Tofană M., Diaconeasa Z. M., Pop O. L., Salanță L. C. (2020), "An Overview of Saponins-A Bioactive Group", *Bulletin UASVM Food Science and Technology* 77(1), .

- Niaz B., Saeed F., Ahmed A., Imran M., Maan A. A., Khan M. K. I., Tufail T., Anjum F. M., Hussain S., Suleria H. A. R. (2019), "Lactoferrin (LF): a natural antimicrobial protein", *International Journal of Food Properties* 22(1), 1626–1641.
- de O Caretta T., I Silveira V. A., Andrade G., Macedo F., P C Celligoi M. A. (2022), "Antimicrobial activity of sophorolipids produced by *Starmerella bombicola* against phytopathogens from cherry tomato", *Journal of the Science of Food and Agriculture* 102(3), 1245–1254.
- Pashkovskaya A. A., Vazdar M., Zimmermann L., Jovanovic O., Pohl P., Pohl E. E. (2018), "Mechanism of Long-Chain Free Fatty Acid Protonation at the Membrane-Water Interface", *Biophysj* 1142142–2151.
- Pöhnlein M., Hausmann R., Lang S., Sylatk C. (2015), "Enzymatic synthesis and modification of surface-active glycolipids", *European Journal of Lipid Science and Technology* 117(2), 145–155.
- Potter R., Hansen L. T., Gill T. A. (2005), "Inhibition of foodborne bacteria by native and modified protamine: Importance of electrostatic interactions", *International Journal of Food Microbiology* 103(1), 23–34.
- Qazi M. A., Wang Q., Dai Z. (2022), "Sophorolipids bioproduction in the yeast *Starmerella bombicola*: Current trends and perspectives", *Bioresource Technology* 346.
- Rahman M., Sarker S. D. (2020), "Antimicrobial natural products", *Annual Reports in Medicinal Chemistry* 5577–113.
- Ramakrishna A., Ravishankar G. A. (2011), "Influence of abiotic stress signals on secondary metabolites in plants", *Plant Signaling & Behavior* 6(11), 1720.
- Rau U., La A. N., Roeper H., Koch H., Lang S. (2005), "Downstream processing of mannosylerythritol lipids produced by *Pseudozyma aphidis*", *European Journal of Lipid Science and Technology* 107(6), 373–380.
- Ray B. (2019), "Pediocin(s) of *Pediococcus Acidilactici* as a Food Biopreservative", 265–345.
- Reid I. D., Seifert K. A. (1982), "Effect of an atmosphere of oxygen on growth, respiration, and lignin degradation by white-rot fungi", *Canadian Journal of Botany* 60(3), 252–260.
- Robinson T. P., Aboaba O. O., Kaloti A., Ocio M. J., Baranyi J., Mackey B. M. (2001), "The effect of inoculum size on the lag phase of *Listeria monocytogenes*", *International Journal of Food Microbiology* 70(1–2), 163–173.
- Saito S., Wang F., Xiao C. L. (2022), "Natamycin as a postharvest treatment to control gray mold on stored blueberry fruit caused by multi-fungicide resistant *Botrytis cinerea*", *Postharvest Biology and Technology* 187111862.

Salleh F., Lani M. N., Chilek T. Z. T., Kamaruding N. A., Ismail N. (2021), "Lactic Acid Bacteria Producing Sorbic Acid and Benzoic Acid Compounds from Fermented Durian Flesh (Tempoyak) and Their Antibacterial Activities Against Foodborne Pathogenic Bacteria", *Applied Food Biotechnology* 8(2), 121–132.

Santos-Sánchez N. F., Salas-Coronado R., Valadez-Blanco R., Hernández-Carlos B., Guadarrama-Mendoza P. C. (2017), "Natural Antioxidant Ectracts As Food Preservatives", *Acta Sci. Pol. Technol. Aliment* 16(4), 361–370.

Santos D. K. F., Rufino R. D., Luna J. M., Santos V. A., Sarubbo L. A. (2016), "Biosurfactants: Multifunctional biomolecules of the 21st century", *International Journal of Molecular Sciences* 17(3), .

Sarbu M., Zamfir A. D. (2018), "Modern separation techniques coupled to high performance mass spectrometry for glycolipid analysis", *Electrophoresis* 39(9–10), 1155–1170.

Saricaoglu F. T., Turhan S. (2018), "Antimicrobial Activity and Antioxidant Capacity of Thyme, Rosemary and Clove Essential Oils and Their Mixtures", *J Inno Sci Eng* 2(1), 25–33.

Schenk T., Schuphan I., Schmidt B. (1995), "High-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*", *Journal of Chromatography A* 693(1), 7–13.

Seifert K. A. (1983), "Decay of Wood by the Dacrymycetales", <https://doi.org/10.1080/00275514.1983.12023787> 75(6), 1011–1018.

Settanni L., Corsetti A. (2008), "Application of bacteriocins in vegetable food biopreservation", *International Journal of Food Microbiology* 121(2), 123–138.

Sharma S., Barkauskaite S., Duffy B., Jaiswal A. K., Swarna J. (2020), "Characterization and antimicrobial activity of biodegradable active packaging enriched with clove and thyme essential oil for food packaging application", *Foods* 9(8), .

Shida T., Kazuo K., Mitsugi K. (1975), "Reduction of lag time in bacterial growth ", *J. Gen. Appl. Microbiol.* 2175–86.

Shirouzu T., Hirose D., Tokumasu S. (2009), "Taxonomic study of the Japanese Dacrymycetes", *Persoonia* 2316.

Shu Q., Niu Y., Zhao W., Chen Q. (2019), "Antibacterial activity and mannosylerythritol lipids against vegetative cells and spores of *Bacillus cereus*", *Food Control* 106106711.

Shu Q., Wei T., Lu H., Niu Y., Chen Q. (2020), "Mannosylerythritol lipids: dual inhibitory modes against *Staphylococcus aureus* through membrane-mediated apoptosis and biofilm disruption", *Applied Microbiology and Biotechnology* 2020 104:11 104(11), 5053–5064.

Smid E. J., Gorris L. G. M. (2020), "Natural Antimicrobials for Food Preservation", Handbook of Food Preservation 283–298.

Smyth T. J. P., Perfumo A., Marchant R., Banat* I. M. (2010), "Isolation and Analysis of Low Molecular Weight Microbial Glycolipids", Handbook of Hydrocarbon and Lipid Microbiology 3705–3723.

Smyth T. J. P., Rudden M., Tsaousi K., Marchant R., Banat I. M. (2014), "Protocols for the Detection and Chemical Characterisation of Microbial Glycolipids", 29–60.
Šojić B., Pavlić B., Ikonić P., Tomović V., Ikonić B., Zeković Z., Kocić-Tanackov S., Jokanović M., Škaljac S., Ivić M. (2019), "Coriander essential oil as natural food additive improves quality and safety of cooked pork sausages with different nitrite levels", Meat Science 157107879.

Solem R. (2016), " Fan-shaped Jelly Fungus (*Dacryopinax spathularia*) (Species ID: 18377)",.

Souza V. V. M. A., Almeida J. M., Barbosa L. N., Silva N. C. C. (2022), "Citral, carvacrol, eugenol and thymol: antimicrobial activity and its application in food", <https://doi.org/10.1080/10412905.2022.2032422> 1–14.

Stadler M., Bitzer J., Köpcke B., Reinhardt K., Moldenhauer J. (2017), "EP2717691B1 - Long chain glycolipids useful to avoid perishing or microbial contamination of materials",.

Stadler M., Bitzer J., Köpcke B., Reinhardt K., Moldenhauer J. (2020), "US20200154708A1 - Long chain glycolipids useful to avoid perishing or microbial contamination of materials",.

Sukumaran A. T., Nannapaneni R., Kiess A., Sharma C. S. (2016), "Reduction of Salmonella on chicken breast fillets stored under aerobic or modified atmosphere packaging by the application of lytic bacteriophage preparation SalmoFresh™", Poultry Science 95(3), 668–675.

Sun L., Forauer E. C., Brown S. R. B., D'Amico D. J. (2021a), "Application of bioactive glycolipids to control *Listeria monocytogenes* biofilms and as post-lethality contaminants in milk and cheese", Food Microbiology 95103683.

Sun L., Atkinson K., Zhu M., D'Amico D. J. (2021b), "Antimicrobial effects of a bioactive glycolipid on spore-forming spoilage bacteria in milk", Journal of Dairy Science 104(4), 4002–4011.

Tajkarimi M. M., Ibrahim S. A., Cliver D. O. (2010), "Antimicrobial herb and spice compounds in food", Food Control 21(9), 1199–1218.

Takahashi M., Aoyagi H. (2020), "Analysis and effect of conventional flasks in shaking culture of *Escherichia coli*", AMB Express 10(77), 1–6.

Tsao G. T. (1976), "Principles of microbe and cell cultivation, S. John Pirt, Halsted Press, Division of John Wiley and Sons, New York, 274 pages, \$34.00", *AIChE Journal* 22(3), 621–621.

U.S. Food and Drug Administration (2019), "GRAS Notice 853", 3667(853), .

Venkataraman S., Rajendran D. S., Kumar P. S., Vo D. V. N., Vaidyanathan V. K. (2021), "Extraction, purification and applications of biosurfactants based on microbial-derived glycolipids and lipopeptides: a review", *Environmental Chemistry Letters* 20:1 20(1), 949–970.

Wang Z., Benning C. (2011), "Arabidopsis thaliana Polar Glycerolipid Profiling by Thin Layer Chromatography (TLC) Coupled with Gas-Liquid Chromatography (GLC)", *Journal of Visualized Experiments : JoVE* (49), 2518.

Wong C. M., Wong K. H., Chen X. D. (2008), "Glucose oxidase: Natural occurrence, function, properties and industrial applications", *Applied Microbiology and Biotechnology* 78(6), 927–938.

Yang S. C., Lin C. H., Sung C. T., Fang J. Y. (2014), "Antibacterial activities of bacteriocins: application in foods and pharmaceuticals", *Frontiers in Microbiology* 5(5), 241–251.

Younes M., Aquilina G., Engel K., Fowler P., Frutos Fernandez M. J., Fürst P., Gürtler R., Gundert-Remy U., Husøy T., Manco M., et al. (2021), "Safety evaluation of long-chain glycolipids from *Dacryopinax spathularia*", *EFSA Journal* 19(6), 1–28.

Zhao Y., Liu S., Han X., Zhou Z., Mao J. (2022), "Combined effects of fermentation temperature and *Saccharomyces cerevisiae* strains on free amino acids, flavor substances, and undesirable secondary metabolites in huangjiu fermentation", *Food Microbiology* 108(11), 104091.

BIOGRAPHY

Tuğba KABASAKAL has got her bachelor's degree from the Department of Bioengineering, Manisa Celal Bayar University in 2019 and pursued her studies as a master's student at the Institute of Biotechnology at Gebze Technical University. During her MSc studies, she performed three internships, at the Faculty of Bioscience Engineering at the University of Ghent, Bio Base Europe Pilot Plant, Ghent, Belgium and Handary SA, Brussels, Belgium. Within the framework of her master's research, she worked on a project which was carried out within the graduate industry thesis collaboration agreement between Gebze Technical University (Turkey) and Handary S.A (Belgium). She made a poster presentation at GTU 6th Graduate Studies Symposium, and an oral presentation at the ISNFF 2022 Conference and Exhibition, namely "Bioprocessing of edible jelly mushroom to obtain long-chain glycolipids functional as food grade antimicrobial".