MEDIA OPTIMIZATION, SCALE-UP AND PILOT SCALE PRODUCTION OF *BACILLUS SPHAERICUS* MBI5 USED FOR MOSQUITO CONTROL

by
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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University
2016

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This work is dedicated to my family...

ACKNOWLEDGEMENTS

I like to start many thanks to my promotor Prof. Dr. Fikrettin Sahin for accepting me as a PhD candidate in his group, giving me a hand during the 43rd SIP meeting in Trabzon, 2010. Dear Sir, I always take energy from your fresh curiosity and endless inspiration. I would like to thank my supervisor Assist. Prof. Dr. Ali Özhan Aytekin for his precious encouraging support during the experimental and writing stages of the thesis. I have learned a lot of views from you during several lunch discussions we had. You always give encouraging support and help during fermenter study which was so new area for me. You make quite a nice example for a young researcher, especially your *on time* approach. And lastly, Dr. Emrah Nikerel provided a fruitful discussions we had at the final stage of my thesis. I enjoyed his smart and meaningful schemes to explain easily.

I also would like to thank to my thesis committee members, Prof. Dr. Meral Birbir, Assoc. Prof. Dr. İkbal Agah İnce, Asst. Prof. Dr. Emrah Nikerel for devoting their time to read my thesis, and attending the defense.

Since August 2010, I have spent and enjoyed most of my time in Genetic and Bioengineering Department. By the way, I would like to thank all my roommates, Mr.Selami Demirci, Miss. Ayşegül Doğan, Mrs. Neslihan Taşlı, Mr. Safa Aydın, Mrs. Esra Aydemir Çoban, Mrs. Gonca Altın Yılmazer, for their help and encouraging supports. I always remember our enjoyable roof chats (both scientific and social topics) in four seasons. And we have witnessed a lot of wonderful and special events such as good projects, journal club activities and three new family members have joined us (Selman Akif, Kerem, and Tarık Numan) for 5 consecutive years. Thank you guys!

I would like to thank to my colleague Mrs. Dilek Sevinç, Mr. Sadık Kalaycı, Mrs. Binnur Kıratlı, Mrs. Burçin Asutay, Mrs. Zişan Turan, Miss. Gökçe Kaya, Mr. Şahin Yılmaz and Mr. Şaban Kalay for their technical support. I want to say special thanks to İsmail Demir who is the oldest man in my Yeditepe University journey. Since 2008, we have met in a bus-ride and we have

been intimate. I always consulted to him before some critical decisions, İsmail Abi thank you for backing me up on free world. I would like to also thank all the friends and colleagues in the Department of Biotechnology for making my time in the department enjoyable. Thank you all.

Furthermore, I would like to thank our Bioprocess team members, Deniz Dilan Demirbağ, Deniz Dalca, Melis Kalkan and new member Kerem Kaya for collaborating on my research and laboratory contributions.

I would like to thank family members for their love and precious encouraging support during my education. Finally, I would like to thank my wife Serap KATI for her technical and emotional support during the PhD education.

ABSTRACT

MEDIA OPTIMIZATION, SCALE-UP AND PILOT SCALE PRODUCTION OF *BACILLUS SPHAERICUS* MBI5 USED FOR MOSQUITO CONTROL

Pest and disease vectors cause losses in the production of major crops and transmit mosquito-borne diseases worldwide. To reduce disease pathogen transmitting pathogen vector control should be properly designed with appropriate the control methods and long-term strategies. Chemical insecticides are applied in agricultural lands and on animals therefore they can cause easily spread different fauna via some physical effects such as wind, rain, motion of insects. Even if it is out of aim, these applications draw individually and collectively for the acute, subacute, and chronic poisoning with mutagenic, carcinogenic and teratogenic effects on humans, animals, bees, fish and some beneficial insects. In the world today, biopesticides are safe and environmental-friendly solution in order to control insect pest to prevent plant damage and infection diseases. *Bacillus sphaericus* (Bs) toxin has high specificity against insects of the dipteran family which are responsible vector-borne diseases. Bs produces large amount of toxin protein during sporulation phase and this protein gathers in the sporangial form as parasporal body. In the current study, a novel strain (Bs MBI5) has been produced based on power input scale up strategies. In this thesis, we have focused on fermentation development of novel Bs MBI5 strain producing highly effective biopesticide. The research achieved significant success in media and process optimization, process scale up and large scale production. This study is the first Bs cultivation research that applies using power input way to scale up of fermentation, and high yield fermentation using waste product.

ÖZET

SİVRİSİNEK MÜCADELESİNDE KULLANILAN *BACILLUS SPHAERICUS* MBI5 SUŞUNUN ÜRETİM ORTAMI OPTİMİZASYONU, ÖLÇEK BÜYÜTME VE PİLOT ÖLÇEKTE ÜRETİMİ

Zararlı ve hastalık taşıyıcı böcekler gıda mahsulllerinin üretiminde kayıplara ve sivrisinek kaynaklı hastalıkların dünya genelinde bulaşmasına neden olurlar. Bulaşıcı hastalıkları azaltmak amacıyla hastalık taşıyıcı böcekler ile mücadele uygun kontrol metodlar ve uzun süreli stratejiler ile dizayn edilmelidir. Kimyasal insektisitler tarımsal alanlarda ve hayvanlar üzerinde uygulanmaktadır ve bu nedenle bu kimyasallar kolayca farklı ekosistemlere böceklerin hareketi, rüzgar ve yağmur gibi fiziksel etkiler ile yayılmaktadır. Amacının dışında, bu kimyasal uygulamaları tek tek veya tümden insanlar, hayvanlar, arılar, balıklar ve bazı faydalı böcekler üzerinde akut, subakut, kronik zehirlenme, mutajenik, karsinojenik ve teratojenik etkiler meydana getirmektedir. Bugün dünya da, biyopestisitler salgın hastalıklara neden olan ve bitkilere zarar veren böcekler ile mücadele etmek amacıyla kullanılabilen güvenli ve çevre dostu çözümlerdir. *Bacillus sphaericus* (Bs) toksinleri, bulaşıcı hastalıklara neden olan dipteran ailesine karşı yüksek spesifik özelliğe sahiptir. Bs, sporlanma sırasında büyük miktarda toksin proteini üretir ve bu protein parasporal olarak sporangial form oluşturur. Bu çalışma da, yeni bir tür (Bs MBI5) sıvıya aktarılan güç olarak bilinen ölçek büyütme stratejisine göre üretildi. Bu tez de, biz oldukça etkili biyopestisit özelliği olan ve yeni bir tür olan Bs MBI5'in fermentasyonunun geliştirilmesine odaklandık. Bu araştırma, üretim ortamı ve proses optimizasyonu, prosesin ölçek büyütmesi ve büyük ölçekte büyütülmesi çalışmalarını önemli bir başarı ile tamamlamıştır. Bu çalışma, Bs üretiminde ölçek büyütme olarak sıvıya aktarılan güç metodu ve atık ürünlerden yüksek verimlilikte fermentasyonun gerçekleştirildiği ilk çalışmadır.

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LIST OF SYMBOLS/ABBREVIATIONS

o	Degree
ρ	Density of fluid
$	Dollars
P/V_L	Force per volume
µ	Micro
%	Percent
η	Viscosity
Abs	Absorbance
MAcid	Acid treatment molasses
ANN	Artifical neural network
ANOVA	Analysis of variance
Bin	Binary toxins
bp	Basepair
Bs	*Bacillus sphaericus*
Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis* subsp. *israelensis*
Ca	Calcium

CO_2	Carbon dioxide
CDW	Cell dry weight
C	Centigrade
CCD	Central composite design
Cfu	Colony forming unite
Cu	Copper
CSL	Corn steep liquor
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
d	Diameter
DDT	Dichloro diphenyl trichloroethane
DNS	Dinitrosalicylic acid
dH_2O	Distilled water
EPA	Environmental Pest Agency
EDTA	Ethylene diamine tetra acetic acid
EU	European Union
FAME	Fatty acid methyl ester
F primer	Forward primer
g	Gram
GP	Gram positive

h	Hour
HCl	Hydrochloric acid
IPM	Integrated Pest Management
L	Liter
LB	Lurian-bertani broth
Log	Logarithm
Mg	Magnesium
$MgCl_2$	Magnesium chloride
Max	Maximum
MRLs	Maximum residue levels
Tm	Melting temperature
MTBE	Methyltert butyl ether
mU	Microunit
Mn	Million
mL	Mililiter
mM	Milimolarity
Mtx	Mosquitocidal toxins
nm	Nanometer
Né	Newton number
N	Normality

NA	Nutrient agar
NB	Nutrient broth
NaOH	Sodium hydroxide
H_2SO_4	Sulphiric acid
OECD	Organisation for Economic Cooperation and Development
OD_{600}	Optic density at 600 nanometer wavelength
ppm	Parts per million
MPhys	Physical treatment molasses
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pH	Power of Hydrogen
KOH	Potassium hydroxide
RSM	Response surface methodology
R primer	Reverse primer
rpm	Revolution per minute
rps	Revolution per second
n	Revolution per minute
Re	Reynolds number
rRNA	Ribosomal ribonucleic acid

SDA	Sabroud dextrose agar
SDS	Sodium dodecyl sulphate
S	Substrate
TAE	Tris-acetate-EDTA
TSA	Tryptic soy agar
TSB	Tryptic soy broth
US	United States
USA	United States of America
USD	United States Dollars
UV	Ultra violet
V_L	Volume
y	Yield

1. INTRODUCTION

1.1 BIOPESTICIDES

Pest and disease vectors cause losses in the production of major crops and transmit mosquito-borne diseases worldwide. Vector borne infectious diseases risks nearly 18% of population [1]. All pesticides used in the worldwide target the insect pest; causes damage to human, forest and agriculture [2,3]. For instance; mosquitoes, blackflies, and other hematophagous insects, are real problem for humanity since ancient times. These insects are vectors, transporting many diseases from animal to man via transmission of pathogenic viruses, bacteria, protozoa and nematodes [4–6]. To reduce disease transmitting pathogen vectors control should be properly designed with appropriate the control methods and long-term strategies.

Chemical insecticides are applied in agricultural lands and on animals to the fight against pests and diseases. Over years, it has a great contribution to control them. Even if it is out of aim, these applications draw individually and collectively for the acute, subacute, and chronic poisoning with mutagenic, carcinogenic and teratogenic effects on humans, animals, bees, fish and some beneficial insects (e.g. silk worm) [7]. The chemical pesticides caused some irreversible problems due to the long-term uncontrolled usage. Problems can be summarized like human health effects (pesticide poisonings), animal poisonings and contaminated products, destruction of beneficial natural predators and parasites, pesticide resistance in pests, honeybee and pollination losses, crop losses, fishery losses, bird losses, groundwater and surface water contamination. It is estimated that the total indirect cost of the damage to environment and social economy is nearly $8.1 billion a year [8]. Due to the harmful effects of chemical pesticides, all around the world some programs worked to enhance protection of the human health, animals and environment. These program is sustainable pest management which based on integrated pest management (IPM). According to the OECD, the IPM should be promoted leading to a broader suite of control methods and lower risks to health and environment. OECD has defined

that IPM will become the accepted approach in the strategic objectives for 2024. This approach will be based on non-chemical and biological control solutions.

US Environmental Protection Agency (EPA) defined that “Biopesticides are certain types of pesticides derived from such natural materials as bacteria, plants, animals, and certain minerals” in 2008, Pesticides Unit [9]. Biological control agents, particularly entomopathogenic bacteria, can be used in mosquito control programs due to their specificity in their modes of action in the field [10]. Application of biopesticides have some advantages, unlike chemical pesticides, which have not adverse environmental effects such as residue accumulation, insect resistance, damage of non-target organisms and toxicity are environmentally benign [10–13]. Since biopesticides occur naturally in the environment and have specific target spectrum, they pose significantly lower toxicity risks than chemical insecticides.

In the world today, biopesticides are safe and environmental-friendly solution in order to control insect pest to prevent plant damage and infection diseases [14]. In the last five decades, the use of synthetic pesticides have shown adverse effects on the environment and human health [12,15,16]. To reduce such risks, consumers and governments are advocating for environmentally friendly methods and actions by regulating applications of synthetic pesticides. The EU legislations (EC No 396/2005) have defined the limit of chemical residues on the food and crops, known as maximum residue levels (MRLs). These limitations decreased the discovery of new synthetic pesticides due to the increasing of difficulty and cost. New launch of chemical pesticides from discovery to end product has higher cost than biopesticide (Table 1.1). Regulations, limitations on synthetic pesticide and organic agriculture demand have promoted increased interest in biopesticides, which will in the next decade, replace chemical biopesticides [9]. The increasing interest in biopesticides has also led to increased novel technological advances in terms of which high product performance, specificity, and enhanced pesticidal effects. More importantly, these biopesticides can be efficiently applied in the frame of integrated pest management (IPM) approaches [11,15,17,18].

Table 1.1. New Pesticides Launch Costs [18]

***Chemical Pesticides* (M)**	***Steps of New Products***	***Biopesticides* (M)**
$30-85	Discovery	$1
$110-146	Development	$2-4
$10-25	Regulatory	$1-3
-	Label	-
-	Launch	-
$150-256	***Total Cost***	$4-8

Market surveys demonstrated that market share for biopesticides is increasing with a rate of currently at more than 25% per year, and is bound to increase even more 5 years [11,19,20]. This is in contrast to the synthetic chemicals which has shown a gradual decline in recent 15 years. On the other hand, the biopesticide market size increased 26 fold from nearly $0.1 billion in 1993 to over $2.6 billion in 2014 (Figure 1.1) [21]. Notably, biopesticides account for 4% of the total pesticide market, which has been reported to be worth $55-60 billion US dollars [8,12]

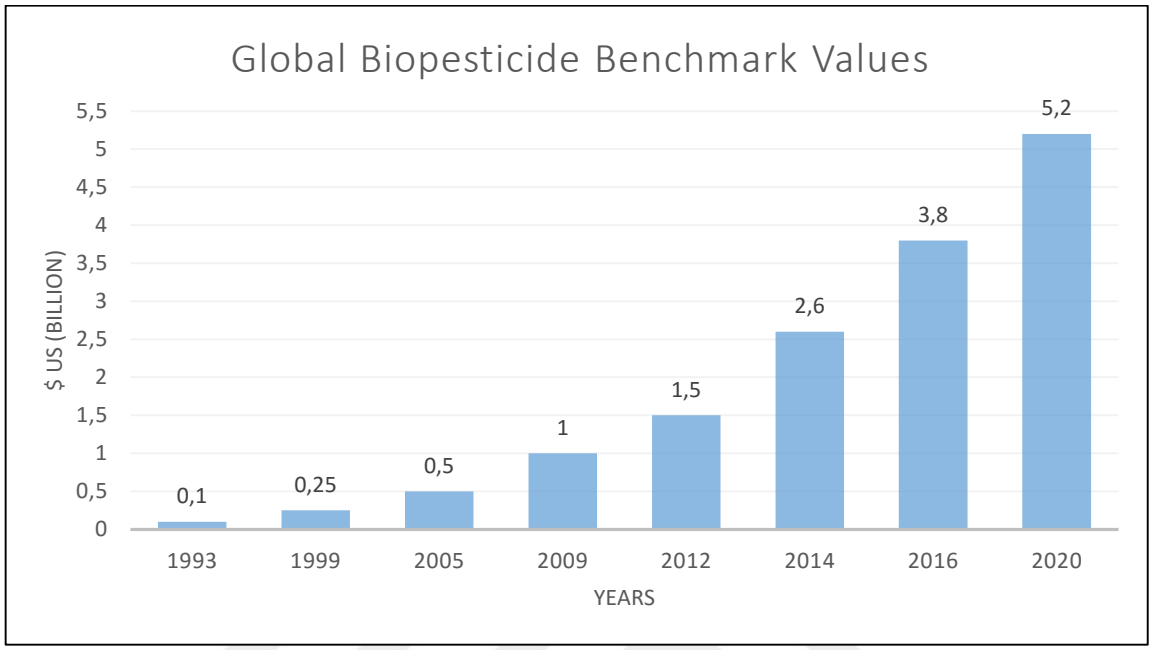


Figure 1.1. Total Global Market and Estimated Values in Next 5 Years for Biopesticides [21]

Globally, the trends of the application and development of biopesticide increased to nearly 1,400 biopesticide products being sold in the last decade [8]. The most common microbial biopesticides currently available in the market are listed in Table 1.2. These products have different formulations including dusts, granules, wettable powders, emulsions, and flowables. The type of formulation is critical in terms of production and application due to factors such as UV radiation, rain, pH, temperature. Therefore, considerations should be given to these factors so as to ensure the biopesticides have longer shelf life, are easy to use and have higher efficacy [19,22–24].

Table 1.2. Microbial Biopesticide Products in the global market [19]

Category of biopesticide	Products common name or trade name	Targets
The USA		
Bactericides		
A. radiobacter k84	Galltrol – A	Crown gall disease
P. agglomerans C9-1	BlightBan C9-1	Fire blight
P. agglomerans E325	Bloomtime	Fire blight
P. syringae pv. Tomato	AgriPhage	Bacterial speck
X. campestris pv.	AgriPhage	Bacterial spot
Fungicides		
B. licheniformis	EcoGuard	Fungal diseases
Bacillus mycoides	BacJ	Cercospora
B. pumilus GB 34	GB34	Seedling diseases – *Pythium* and Rhizoctonia
B. pumilus QST 2808	Sonata	Powdery mildew, downy mildew, and rusts
	Ballad Plus	
B. subtilis GB03	Companion	*Fusarium*, *Pythium*, *Rhizoctonia*
	Kodiak	
B. subtilis MBI 600	Histick N/T	Damping off
	Pro-Mix with Biofungicide	
B. subtilis subsp. amyloliquefaciens FZB24	Taegro	*Fusarium* and *Rhizoctonia* wilt diseases
P. aureofaciens Tx-1	Spot-Less	Turf fungal diseases
Pseudomonas	At-Eze	Soil and seed-borne fungi
P. syringae ESC 10	Bio-Save 10LP	Postharvest diseases
P. syringae ESC 11	Bio-Save 11LP	Postharvest diseases
Streptomyces griseoviridis K61	Mycostop	Fungi causing damping off, stem, and crown rots
	Biofungicide	
	Mycostop Mix	
S. lydicus WYEC108	Actinovate	Fungi causing damping off, stem and crown rots
	Actino-Iron	
Ampelomyces	PowderyGon	Powdery mildew
Aspergillus flavus AF36	*Aspergillus flavus* AF36	*Aspergillus flavus* producing aflatoxin

Table (continued)

C. minitans CON/M/91–08	Contans	*Sclerotinia minor, Sclerotinia sclerotiorum*
Gliocladium	Prestop	Seed-borne and soil-borne diseases
M. albus QST 20799	Arabesque	Postharvest diseases
Pseudozyma flocculosa	Sporodex	Powdery mildew
Trichoderma	Tenet	Soil-borne diseases
T. harzianum (*gamsii*) ATCC080	Bioten	
	Remedier	
T. harzianum ATCC	Binab	Wound healing
T. harzianum Rifai T-22	PlantShield	Seed and foliar diseases
	RootShield	
	T-22 Planter box	
T. harzianum T-39	Trichodex	Soil and foliar diseases
Trichoderma	Binab T	Soil and foliar diseases
Ulocladium	BOTRY-Zen	Botrytis and *Sclerotinia*
Verticillium albo-	DutchTrig	Dutch elm disease
P. syringae pv. tomato	AgriPhage	Tomato leaf spot
Candida oleophila	NEXY	Postharvest fruit molds
Fungicides/bactericides		
B. subtilis QST713	Serenade	Foliar fungal and bacterial diseases
Herbicides		
Bacillus cereus BP01	MepPlus	Plant growth regulator
Alternaria destruens	Smolder	Herbicide – dodder
Chondrostereum	Chontrol Paste	Herbicide – stump sprout inhibitor
Colletotrichum	LockDown	Herbicide – northern Jointvetch
Puccinia thlaspeos woad (dyer's woad	Woad Warrior	Herbicide – Dyer's woad
Insecticides		
B. popilliae	Milky Spore Powder	Japanese beetle grubs
Bacillus sphaericus Serotype H5a5b	VectoLex	Mosquito larvae
B. thuringiensis subsp.	Florbac	Moth larvae
B. thuringiensis subsp.	BMP	Mosquito and blackflies
B. thuringiensis subsp. israelensis EG2215	Gnatrol	Mosquito, flies
	Aquabac	

Table (continued)

B. thuringiensis subsp.	Agree WG	Plutella
B. thuringiensis subsp. kurstaki	Thuricide Forestry	Lepidopteran larvae
	Wilbur-Ellis BT 320	
	Dust	
	Dipel	
	Deliver	
	Biobit HP	
	Foray	
	Javelin WG	
	Green Light	
	Hi-Yield Worm Spray	
	Ferti-Lome	
	Bonide	
	Britz BT	
	Worm Whipper	
	Security Dipel Dust	
B. thuringiensis subsp. kurstaki BMP123	BMP123	Lepidopteran larvae
B. thuringiensis subsp. Kurstaki EG2348	Condor	Lepidopteran larvae
B. thuringiensis subsp.	Novodor	Colorado potato beetle
B. thuringiensis subsp. Kurstaki EG7826	Lepinox WDG	Lepidopteran larvae
B. bassiana 447	Baits Motel Stay-awhile	Ants
B. bassiana ATCC	Naturalis L	Various insects
B. bassiana GHA	Mycotrol ES	Various insects
	Mycotrol O	
	Botanigard 22WP	
	BotaniGard ES	
B. bassiana HF23	balEnce	Housefly
M. anisopliae F52	Tick-Ex	Ticks and grubs
Paecilomyces fumosoroseusApopka 97	PFR-97	Whitefly and thrips
Nosema locustae	Nolo-Bait	Grasshopper and crickets
	Semaspore Bait	
Anagrapha falcifera	CLV-LC	Lepidopteran larvae

Table (continued)

Gypsy moth NPV	Gypchek	Gypsy moth
H. zea NPV (previously *Heliothis zea)*	GemStar	Cotton bollworm, tobacco, budworm
Indian meal moth GV (*Plodia interpunctella* GV)	FruitGuard	Indian meal moth
Mamestra configurata	Virosoft	Bertha armyworm
Spodoptera exigua	Virus Spod-X	Beet armyworm
Saccharomyces	Bull Run	Fly attractant
Nematicides		
Bacillus firmus I-1582	BioNem	Nematodes
Pasteuria usgae	Econem	Nematodes
Myrothecium	DiTera	Nematodes
Paecilomyces lilacinus	MeloCon WG	Nematodes
Virucides		
Zucchini yellow mosaic virus – weak	AgroGuard-Z	Zucchini yellow mosaic
Europe		
Aureobasidium	Blossom Protect	Fire blight, postharvest diseases
Phlebiopsis gigantea	Rotstop	Conifer root rots
P. chlororaphis	Cedomon, Cerall	*Pyrenophora teres*, *P. graminea*, *Tilletia caries*, *Septoria nodorum*, *Fusarium* spp.
Pseudomonas sp.	Proradix	Root rots
S. griseoviridis K61	Mycostop	*Fusarium* wilt, *Botrytis* gray mold, root rot, stem rot, stem end rot, damping off, seed rot, soil-borne damping off, crown rot, *Rhizoctonia*, *Phytophthora*, wilt, seed damping off, early root rot
A. quisqualis AQ10	AQ10	*Leaf disease*
C. oleophila strain O		Postharvest disease
C. minitans CON/M-	Contans WG	*Sclerotinia sclerotiorum*, *S. minor*
G. catenulatum J1446	Prestop, Prestop mix	Damping off, gummy stem blight, gray mold, root rot, stem rot, wilt, storage diseases, foliar diseases, seed rot
P. flocculosa PF-A22 UL	Sporodex	Powdery mildew

Table (continued)

Trichoderma asperellum (ICC012) (T25) (TV1) (formerly T. harzianum)	Tenet	Fungal infections (*Pythium*,*Phytophthora, Botrytis, Rhizoctonia)*
Trichoderma atroviride IMI 206040 (formerly T. harzianum)	Binab T Pellets	*Botrytis cinerea*, pruning wound infection Chondrostereum purpureum
T. atroviride I-1237	Esquive	Fungal infections (*Pythium*,*Phytophthora, Botrytis, Rhizoctonia)*
Trichoderma gamsii (formerly *T.viride) (ICC080)*	Remedier	Fungal infections (*Pythium*,*Phytophthora, Botrytis, Rhizoctonia)*
T. harzianum Rifai T-22 ITEM 108 or KRL-AG2	Trianum P	Root diseases
T. harzianum Rifai T-39 (IMI 206039)	Trichodex	*Botrytis cinerea*, *Colletotrichum* spp.,
	Rootshield	*Fulvia fulva, Monilia laxa, Plasmopara viticola, Pseudoperonospora cubensis,Rhizopus stolonifer, Sclerotiniasclerotiorum*
T. polysporum and *T. harzianum*	Binab T Vector	Fungal pathogens, fairy ring, *Botrytis,Verticillium, Pythium, Fusarium,Phytophthora, Rhizoctonia, Didymella,Chondrostereum, Heterobasidion*
V. albo-atrum (WCS850) (formerly Verticillium dahliae)	Dutch Trig	Dutch elm disease
Fungicides/bactericides		
B. subtilis QST 713	Serenade	Fire blight, *Botrytis* spp.
Insecticides		
B. thuringiensis subsp.	Turex	Lepidoptera pests
B. thuringiensis subsp.	VectoBac	Sciarids
AM65		
B. thuringiensis subsp.	Dipel WP	Lepidoptera pests
B. thuringiensis subsp. kurstaki ABTS 351, PB 54, SA 11, SA12, and EG 2348	Batik	Lepidoptera pests
	Delfin	
B. thuringiensis subsp. *Kurstaki BMP 123*	BMP 123	Lepidoptera pests
	Prolong	

Table (continued)

B. thuringiensis subsp. *Tenebrionis NB 176*	Novodor	Coleoptera pests
B. bassiana ATCC	Naturalis L	Thrips, whitefly, mites
B. bassiana GHA	Botanigard	Whiteflies, aphids, thrip
Lecanicillium muscarium (Ve6)	Mycotal, Vertalec	Whiteflies, thrips, aphids
P. fumosoroseus Apopka 97	Preferal WG	Greenhouse whiteflies (*Trialeurodesvaporariorum)*
P. fumosoroseus Fe9901	Nofly	Whiteflies
Adoxophyes orana BV-0001 GV	Capex	Summer fruit tortrix (*Adoxophyes orana*)
Cydia pomonella GV	BioTepp	Codling moth (*Cydia pomonella*)
Spodoptera exigua	Spod-X GH	*Spodoptera exigua*
Bacillus polymyxa	Trade name not available	Crown gall
Bacillus sphaericus	Trade name not available	Crown gall
Fungicides		
B. cereus	Trade name not available	Bacterial wilt, sheath blight/rice false smut, bacterial wilt
B. licheniformis		Downy mildew, *Fusarium* wilt
B. subtilis	Trade name not available	Bacterial wilt, root rot, tobacco black shank, rice blast, rice false smut
Trichoderma spp.		Fungus downy mildew, *Rhizoctonia cerealis*, gray mold
Fungicides/bactericides		
P. fluorescens	Trade name not available	Bacterial wilt, root rot
Insecticides		
B. thuringiensis subsp.	Trade name not available	Lepidopteran pests
B. thuringiensis subsp.	Trade name not available	Lepidopteran pests
B. thuringiensis subsp.		Lepidopteran pests
Pseudomonas	Trade name not available	Locusts, grasshoppers
B. bassiana		*Monochamus alternatus*, *Dendrolimus punctatus*
M. anisopliae		Cockroaches, grasshoppers, locusts
P. lilacinus	Trade name not available	Nematodes
Pochonia		Nematodes
Dendrolimus cytoplasmic	Trade name not available	Virus Caterpillars

Table (continued)

NPV, *Ectropis obliqua hypulina* NPV, *Laphygma exigua* NPV, *Prodenialitura NPV, Buzura suppressaria NPV, Gynaephora ruoergensis NPV, Mythimna separata NPV*	Trade name not available	Virus Beet armyworm, lepidoptera, looper, *H. armigera*, *Laphygma exigua*
Periplaneta fuliginosa	Trade name not available	Cockroaches
Pieris rapae GV, *Mythimna separata GV, Plutella xylostella*	Trade name not available	*Pieris rapae*, *Plutella xylostella*
Japan		
B. thuringiensis kurstaki	Toarowaa Esmark Guardjet, Dipol, Tuneup Fivestar	Lepidopteran larvae
B. thuringiensis aizawai	Quark XenTari Florbac Sabrina	Lepidopteran larvae
B. thuringiensis aizawai +kurstaki	Bacilex	Lepidopteran larvae
B. thuringiensis japonensis	BuiHunter	Cockchafers and white grubs
B. bassiana	BotaniGard	Thrips, whiteflies, diamondback moth
P. fumosoroseus	Preferd	Whitefly, aphids
Lecanicillum longisporum	Vertalec	Aphids
Adoxophyes orana GV+Homona magnanima GV	Hamaki-Tenteki	*Adoxophyes honmai* and *Homona magnanima*
Steinernema carpocapsae	Bio Safe	Weevils, black cutworm, common cutworm, peach fruit moth
India		
Fungicide		
P. fluorescens	ABTEC Pseudo	Plant soil-borne diseases
	Biomonas	
	Esvin Pseudo	
	Sudo	
	Phalada 104PF	
	Sun Agro Monus	

Table (continued)

A. quisqualis	Bio-Dewcon	Powdery mildew
T. harzianum	Biozim	Soil-borne pathogens
	Phalada 105	
	Sun Agro Derma H	
	Eswin Tricho	
B. bassiana	Myco-Jaal	Coffee berry borer, diamondback moth, thrips, grasshoppers, whiteflies, aphids,codling moth
	Biosoft	
	ATEC Beauveria	
	Larvo-Guard	
	Biorin	
	Biolarvex	
	Biogrubex	
	Biowonder	
	Veera	
	Phalada 101B	
	Bioguard	
	Bio-power	
M. anisopliae	ABTEC	Coleoptera and lepidoptera, termites, mosquitoes, leafhoppers, beetles, grubs
	Verticillium	
	Meta-Guard	
	Biomet	
	Biomagic	
	Meta	
	Biomet	
	Sun Agro Meta	
	Bio-Magic	
P. fumosoroseus	Nemato-Guard	Whitefly
	Priority	
P. lilacinus	Yorker	Whitefly
	ABTEC	
	Paceilomyces	
	Paecil	
	Pacihit	
	ROM biomite	
	Bio-Nematon	

Table (continued)

Verticillium lecanii	Verisoft	Whitefly, coffee green bug, homopteran pests
	ABTEC	
	Verticillium	
	Vert-Guard	
	Bioline	
	Biosappex	
	Versitile	
	Ecocil	
	Phalada 107 V	
	Biovert Rich	
	ROM Verlac	
	ROM Gurbkill	
	Sun Agro Verti	
	Bio-Catch	
H. armigera NPV	Helicide	*H. armigera*
	Virin-H	
	Helocide	
	Biovirus-H	
	Helicop	
	Heligard	
Spodoptera litura NPV	Spodocide	*S. litura*
	Spodoterin	
	Spodi-cide	
	Biovirus-S	
Nematicides		
Verticillium		Nematodes
Australia		
Fungicide		
P. fluorescens	ABTEC Pseudo	Plant soil-borne diseases
	Biomonas	
	Esvin Pseudo	
	Sudo	
	Phalada 104PF	
	Sun Agro Monus	
	Bio-cure-B	
A. quisqualis	Bio-Dewcon	Powdery mildew

Table (continued)

T. harzianum	Biozim	Soil-borne pathogens
	Phalada 105	
	Sun Agro Derma H	
T. viride	Monitor, Trichoguard	Soil-borne pathogens
	NIPROT	
	Bioderma	
	Biovidi	
	Eswin Tricho	
	Biohit	
	Tricontrol	
	Ecoderm	
	Phalada 106TV	
	Sun Agro Derma	
	Defense SF	
Fungicides/bactericides		
B. subtilis		Soil-borne pathogens
Insecticides		
B. thuringiensis subsp.	Tacibio, Technar	Lepidopteran pests
B. thuringiensis subsp. Kurstaki	Bio-Dart	Lepidopteran pests
	Biolep	
	Halt	
	Taciobio-Btk	
B. bassiana	Myco-Jaal	Coffee berry borer, diamondback moth, thrips, grasshoppers, whiteflies, aphids, codling moth
	Biosoft	
	ATEC Beauveria	
	Larvo-Guard	
	Biorin	
	Biolarvex	
	Biogrubex	
	Biowonder	
	Veera	
	Phalada 101B	
	Bioguard	
	Bio-power	
M. anisopliae	ABTEC	Coleoptera and lepidoptera, termites, mosquitoes, leafhoppers, beetles, grubs
	Verticillium	
	Meta-Guard	

Table (continued)

	Biomet	
M. anisopliae	Biomagic	Coleoptera and lepidoptera, termites, mosquitoes, leafhoppers,
	Meta	
	Biomet	
	Sun Agro Meta	
	Bio-Magic	
P. fumosoroseus	Nemato-Guard	Whitefly
	Priority	
P. lilacinus	Yorker	Whitefly
	ABTEC	
	Paceilomyces	
	Paecil	
	Pacihit	
	ROM biomite	
	Bio-Nematon	
V. leccanii	Verisoft	Whitefly, coffee green bug, homopteran pests
	ABTEC	
	Verticillium	
	Vert-Guard	
	Bioline	
	Biosappex	
	Versitile	
	Ecocil	
	Phalada 107 V	
	Biovert Rich	
	ROM Verlac	
	ROM Gurbkill	
	Sun Agro Verti	
	Bio-Catch	
H. armigera NPV	Helicide	*H. armigera*
	Virin-H	
	Helocide	
	Biovirus-H	
	Helicop	
	Heligard	
S. litura NPV	Spodocide	*S. litura*
	Spodoterin	

Table (continued)

S. litura NPV	Spodi-cide	*S. litura*
	Biovirus-S	
Nematicides		
Verticillium		Nematodes
Bactericides		
A. radiobacter	NoGall	Crown gall disease
Fungicides		
T. harzianum	Trichodex	*Botrytis* spp.
Insecticides		
B. sphaericus	VectoLex	Mosquito larvae
B. thuringiensis subsp.	Agree, Bacchus, XenTari	Lepidoptera larvae
B. thuringiensis subsp.	Aquabac, BTI, Teknar,	Mosquito larvae
B. thuringiensis subsp. kurstaki	Biocrystal, Caterpillar, Killer, DiPel, Costar, Delfin	Lepidoptera larvae
M. anisopliae	BioCane, Granules	Gray-backed cane grub (scarabs)
M. anisopliae subsp.	Green Guard	Locusts and grasshoppers
M. flavoviride	Chafer Guard	Redheaded pasture cockchafer
H. armigera NPV	Heliocide	*Helicoverpa* spp.
	Vivus Gold	
	Vivus Max	
H. zea NPV	Gemstar	*Helicoverpa* spp.
	Vivus	
Africa		
Bactericides		
A. radiobacter	Crown Gall Inoculant	Crown gall
Fungicides		
B. subtilis 101	Shelter	Root and leaf diseases
B. subtilis 102	Artemis	Root and leaf diseases
B. subtilis 246	Avogreen	Root and leaf diseases
B. subtilis QST 713	Serenade	*Botrytis* spp.
A. quisqualis AQ10	Bio-Dewcon	Powdery mildew
T. harzianum	Eco-77	Root diseases
	Eco-T	
	Promot	
	Romulus	
	Rootgard	
	Trichoplus	
	Trykocide	

Table (continued)

T. harzianum 39	Trichodex	Root diseases
T. harzianum DB103	T-Gro	Root diseases
Fungicides/bactericides		
B. subtilis	Defender	Soil-borne fungi and bacteria
Insecticides		
B. thuringiensis subspp. aizawai and	Agree	Lepidoptera larvae
B. thuringiensis subsp.	VectoBac	Mosquito
B. thuringiensis subsp. kurstaki	DiPel	Lepidoptera larvae
	Rokur	
	Thuricide	
B. thuringiensis subsp.	Florbac WG	Lepidoptera larvae
B. bassiana	Bb Plus, Bb weevil,	Thrips, weevils, whiteflies
M. anisopliae subsp. acridum IMI 330189	Green Muscle	Locust
	Trade name not available	Lepidoptera larvae
Pseudomonas resinovorans bacteriophage	Agriphage	Insect pest control
Nematicides		
P. lilacinus	Bio-Nematon	Nematodes
P. lilacinus 251	PL Plus	Nematodes

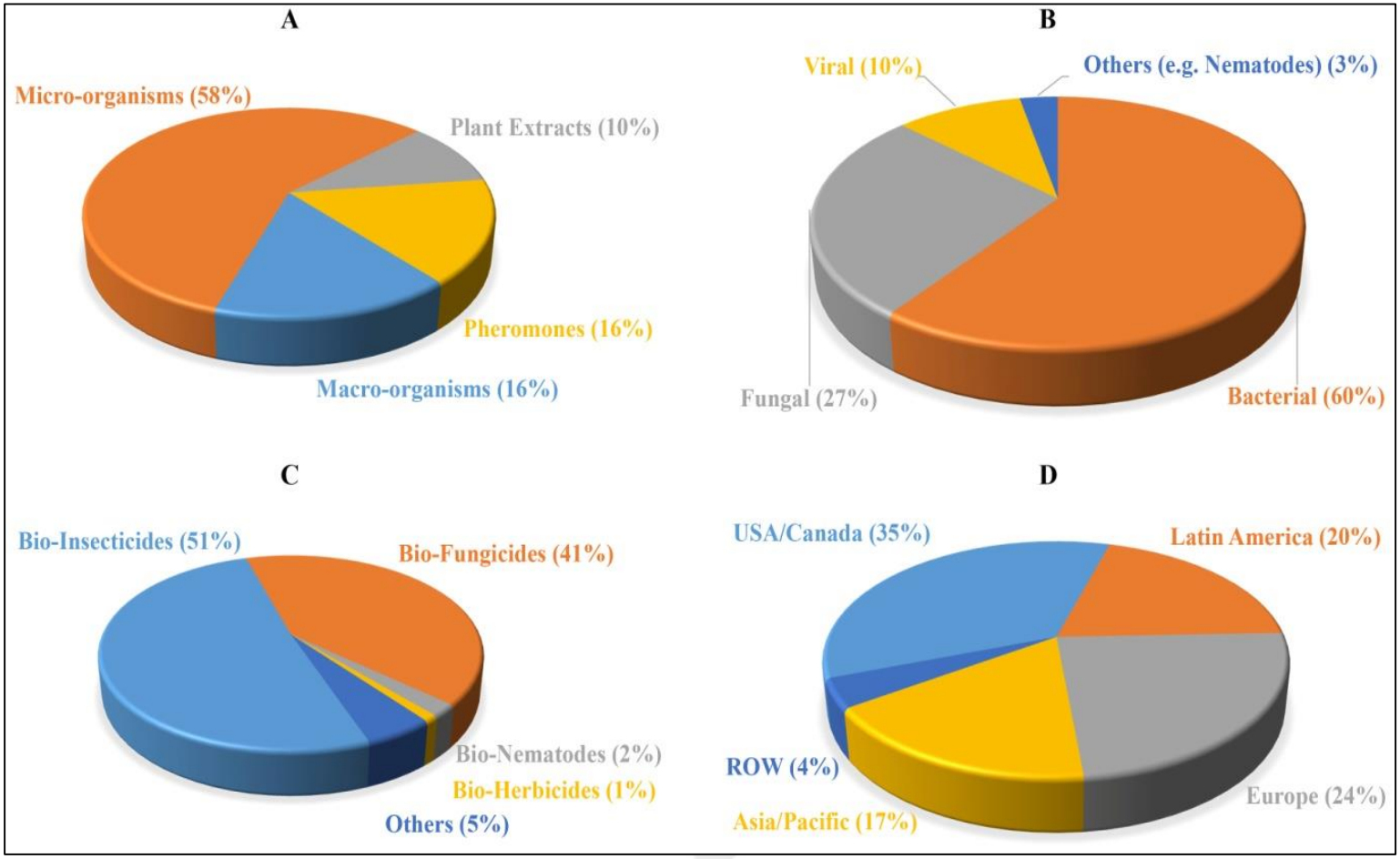


Figure 1.2. Various types of biocontrol products available in the world: A, B and C shows the categories of bio-control markets for biological control products, microbial control products and target-based bio-control agents, respectively. The usage of the biopesticide usage according to world market rates is shown in D. ROW means "rest of the world". These figures are based on data collected in the year 2015 from Dunham Trimmer LLC Biocontrol Database

The biological control market can be classified into three categories: (1), biochemical (pheromones and plant extracts); (2), microorganisms; and (3) macroorganisms (Figure 1.2). Microorganism have the largest share market (58%) in the biological control products (Figure 1.2, Panel A), while bacterial biopesticides have largest amount (60%) in the microbial control products category. Bacterial solutions are more widespread compared to other microbial solutions like viral, fungal (Figure 1.2, Panel B).

In agriculture, there are many types of insect-pest that cause damage to crops, causing approximately 40% decrease in crop harvests. The various types of biocontrol agents, which are specific different insect pests are shown in Figure 1.2, Panel C. Half of the microbial agents are used to address the problems associated with insect pests. In global scenario, biopesticides have been used largest share in USA and Canada with %35, followed by Europe with 24%, Latin America (20%), Asia/Pacific (17%), and lowest for Africa (4%) (Figure 1.2, Panel D). Whereas in USA, EPA encourages the development and use of biopesticides, Canadian universities provide a lot of research funds to improve of the applications of biopesticides. Europe has second biggest market share, the most effective markets being in Spain, Italy and France. The main means of livelihood in Asia is an agriculture and it has the largest biodiversity in worldwide for humanity and industry. Asia has the biggest size of food crops, and the continent has shown increasing levels of the usage of biopesticide. This has enhanced the yields of rice, maize, and vegetables. China is continuously extending biopesticide application area from 800,000 ha in 1972 to 27,000,000 ha in 2005. Nearly, 400 biopesticides had been registered in China from 2001 up to 2013 [25,26]. Various types of regulations have resulted to wide awareness among the public in Japan and India, thereby giving support in the production and application of biopesticides.

1.2 BIOLOGICAL CONTROL, CHEMICAL CONTROL AND SUSTAINABILITY

The pest or vectors must be controlled. If not, while pest can lead a catastrophe in agriculture, food crops and on the other side vectors can cause a great deal of infectious diseases in worldwide. Thus, different control methods have been used to struggle with them. There two main control methods: natural or chemical. Natural control methods contain biological materials. Biological control, biocontrol, or non-chemical control means use of the living organism (competitors, parasitoids, antagonists, pathogens, or predators) that is special naturally enemy, to reduce the damage caused by pest [11,20]. Chemical control agents are generally potent synthetic chemical pesticides. Both two methods carry some advantages and disadvantages.

Biological control methods provide a solution without harming humans, crops, and other non-target organisms. The biological control agents show highly specific activity to pest, for example insect family such as Lepidoptera-specific, Diptera-specific, or Coleptera-specific [3,27,28]. However chemical control agents have general mode of action. They do not select any insect or plant family. If it is a herbicide, it could be damage all of the plant types more or less [29]. Also, it can affect organisms other than plant such as bee, ant, and bird. Another biggest problem related to chemical agents are cross contamination. When a farmer apply the pesticide in field, pesticide can be easily carry to water resources by raining or irrigation. By the way, a pesticide can go from field to different habitats. Sometimes, when the farmers use biopesticides, they face some problems. The general one is that results are not homogenous, and consistent. So that, they sense confused in using and adopting these eco-friendly alternatives [13,19,30,31]. Another problem to reduce the farmer's faith and confidence is poor quality and short shelf-life of biological products. Poor quality comes from mainly low bacterial count. But formulation technologies will be achieved to produce viable and stable biological products.

It can be combine many different techniques and approaches to manage these pest. The main concept should be promote environmentally safe methods of pest and disease control. In this opinion, IPM program is the best way to choose the more suitable option in required situation.

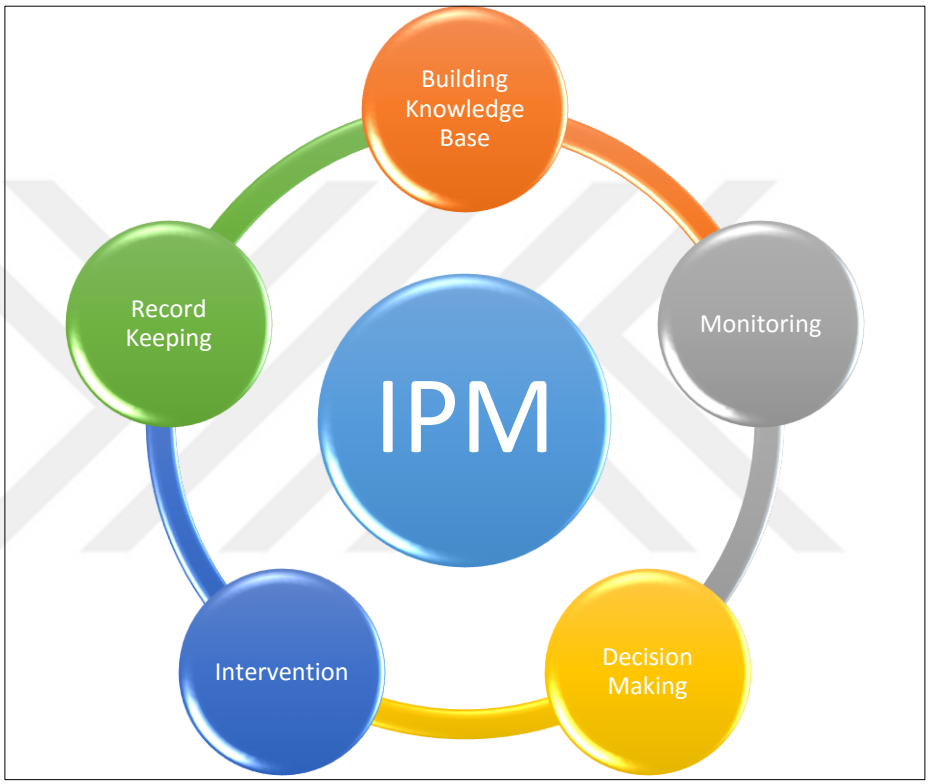


Figure 1.3. Steps of Integrated Pest Management (IPM).

IPM should base on less harmful solutions, environmentally responsible, and producing really healthy stages (Figure 1.3). IPM program can follow in five steps; building knowledge base, monitoring, decision making, intervention, and record keeping. Building knowledge part can be explain as learning about plant, learning about proper culture, rates of fertilization, learning about possible infection, how plant can be effected by pest, how you can identify its damage, and learning life stages of insect (egg, pupa, larvae, adult). Secondly, monitoring means regular inspection to catch a symptoms and signs of insect activity. Symptoms are things like the defoliation, leaf corruption, or sing of insect itself like eggs in back of leaf. Third step is decision after monitoring and detecting pest. You should decide and define the way what you can do.

This step is corner stone in IPM program. Fourth stage of building an IPM program is intervention. You can start by preventing the spread using some mechanical technics such as hand pick, row cover or kaolin clay spray. Later, you can use biological control technics which are natural enemies of pest. And finally you may choose to apply pesticide to reduce pest population. But the chemical compounds should be use safe, organic products approved government's regulations. And final step is recording which is used to keep the track up what you saw in your field and when you thought this will help you be prepared next year to figure out when you need to intervene [11,17,21,32]. In this way, you are going to be able to produce a beautiful, wonderful, and healthy solution.

1.3 *BACILLUS SPHAERICUS* IN BIOPESTICIDE

1.3.1 *Bacillus sphaericus* Characteristics and Classification

Bacillus sphaericus is a gram positive, obligate aerobe, mesophilic, spherical spore-forming bacterium that it is also naturally occurring and isolated from the soil. It has rod shaped cells, opaque, smooth and unpigmented. Bs grows on general nutrient agar from minimum temperature 10°C, to maximum 45°C. Its strains usually grow at pH 7.0-9.5; few strains grow at pH 6.0. Catalase, oxidase, urease, hydrolysis of casein, citrate utilization and hydrolysis of Tween 20 are positive. Bs deaminates phenylalanine and utilizes the citrate as a sole carbon source. It can grow up to 5% NaCl. Peptidoglycan layer consist of lysine and aspartate [33].

Bs has following taxonomy kingdom-Bacteria, phylum-Firmicutes, class-Bacilli, order-Bacillales, family-*Bacillaceae*, genus-*Lysinibacillus* [34]. *Bacillus* genus was reclassified as *Lysinibacillus* due to the fact that its cell wall contains L-lysine and D-aspartate distinguished from other members of this group. In Bergey's Manual of Systematic Bacteriology was emphasized that Bs has been divided into six DNA homology group and seven 16S rDNA sequence similarity groups according to Krych et al., 1980 and Nakamura et al., 2002 respectively [35,36]. The mosquitocidal activity exhibited strains are included in Group IIA which has lots of biotechnologically interesting strains [33,35,37].

1.3.2 *Bacillus sphaericus* Metabolism and Larvicidal Toxins

Bs do not utilize the general carbohydrate such as hexoses, pentoses and disaccharides. They prefer pyruvate, purine, pyrimidine and amino acids [38]. The inability of metabolize sugars is related to the absence of some key glycolytic pathway which are Embden-Meyerhof-Parnas (EMP) (glucokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase), hexose monophosphate pathway (phosphogluconate dehydratase), and Entner-Doudoroff (6-phospho-2-keto-3-deoxyglyconate aldolase) pathways [39]. In the last researches, these bacteria can use the carbohydrates without a good sugar transport system help of the glucokinase-encoding gene *glcK*, the phosphofructokinase-encoding gene *pfk*, and the phosphoglucose isomerase-encoding gene *pgi* [40–42]. In previous studies, Russel et al. did not neither control the sugar transport system nor detect *glcK* activity in NYSM medium [37,41].

Some strains of Bs can be used as biological control agent thanks to entomocidal toxin genes. The mosquito larvicidal Bs was firstly isolated by Kellen et al. from moribund larvae of *Culiseta incidensin* in California [43]. These toxins are produced a protein parasporal body that can be eaten by mosquito larvae. There are two types of toxin protein in Bs strains, the crystal protein or binary toxin (Btx) and mosquitocidal toxins (Mtx) [44–46]. Bs produces Btx during the sporulation and comprises 41.9- (Bin A) and 51.4-KDa (Bin B) [40,47,48]. Mtx toxins are produced during the vegetative growth and they are associated with the cell membrane of Bs [49]. Btx in Bs can synthesize in the early stages during sporulation and form a small crystal in the mother cell. Mtx toxins are found three types, Mtx1, Mtx2 and Mtx3, with molecular masses of 100-, 31.8- and 35.8-kDa, respectively [50–52]. Most of highly toxic strains synthesize Btx toxin and may contain one or more of Mtx toxins. However, only Mtx synthesized strains show low toxic effect against larvae [53]. Mtx toxin gene is irrelevant to Btx gene. When the cells pass the stationary phase, these Mtx toxins is degraded by the protease-positive strains. Thanabalu and Porter (1995) was showed that Mtx protein is synthesized in the vegetative phase in nine strains of Bs. The yield of Mtx among the nine strains were showed variation. The main reasons could be differences in *mtx* promoter strength or protease enzyme activity in vegetative cells in some strains. In previous studies, comparing the expression level of *mtx* gene between

13052.pC35 (protease negative) and 1693.pC35 (protease positive) strains, the amounts of Mtx decreased during sporulation of 13052.pC35 [51].

The toxicity high Bs strains which were isolated in last 30 years, they are generally associated with the DNA homology group IIA, flagellar serotype 5a5b. But there is no definite rule to common mosquitocidal strains limited to this serotype. Final researches show that serotype 1a, previously mentioned as low toxic strains, was detected Bin toxin genes. Also *mtx* gene is not present all of the serotypes. Due to these variation, the toxicity level is not predicted according to serotyping scheme. Six of the nine serotypes shows heterogeneity of the toxicity gene profile as listed Table 1 [54].

Table 1.3. Distribution of Mosquitocidal Toxin Genes in Some Strains of *Bacillus sphaericus* [54]

Strain	**Origin**	**Serotype**	***btx* gene**	***mtx* gene**
K	United States	1a	-	+
Q	United States	1a	-	+
9002	Indonesia	1a	+	+
9201	Indonesia	1a	+	+
9301	Indonesia	1a	+	+
BS-197	Indonesia	1a	+	+
SSII-1	India	2a2b	-	+
1889	Israel	2a2b	-	+
1883	Israel	2a2b	-	+
4b 1	Nicaragua	2a2b	-	-
LP24-4	Singapore	2a2b	-	-
LP35-6	Singapore	2a2b	-	-
17N	Caledonia	2a2b	-	ND
COK 1	United States	2a2b	-	-
K 8908	Indonesia	2a2b	-	-
BDG2	France	3	-	-
SL 42	United States	3	-	-
IAB 881	Ghana	3	+	-
LP1-G	Singapore	3	+	-
LP7-A	Singapore	3	+	-

Table (continued)

LP12-AS	Singapore	3	+	-
LP14-8	Singapore	3	+	-
LP20-E	Singapore	3	+	-
1593	India	5a5b	+	+
1691	El Salvador	5a5b	+	+
2013.6	Romania	5a5b	+	+
2362	Nigeria	5a5b	+	+
2317.3	Thailand	5a5b	+	+
2500	Thailand	5a5b	+	+
BSE18	Scotland	5a5b	+	+
BM1	United States	6	+	+
IAB 59	Ghana	6	+	+
S06 015	Iraq	6	-	-
IAB 481	Ghana	6	+	+
IAB 620.1	Ghana	6	+	+
IAB 460	Ghana	6	+	+
B55	Indonesia	6	-	-
COK 31	Turkey	9a9c	-	+
COK 34	Turkey	9a9c	-	+
2297	Sri Lanka	25	+	+
2627	Israel	25	+	-
IMR 6	Malaysia	25	+	+
1602	Canada	25	+	+
2173	India	26a26b	-	-
2315	Thailand	26a26b	-	-
2377	Indonesia	26a26b	-	-
LB29	Czech Republic	26a26b	-	-
BM2	United States	26a26b	-	-
S26 009	United States	26a26b	-	-
18W1.2	Iraq	26a26b	-	-
IMR 66.1	Malaysia	48	-	-
IAB 872	Ghana	48	+	+
Pr-1	Scotland	48	+	+

1.3.3 Toxin Specificity and Mode of Action of *Bacillus sphaericus*

The most important issue in the developing or applying pest control agents is the evaluation of the safety status about non-target organism which are cohabiting with target species. This issue is valid both chemical and biological control agents. Bs toxin has high specificity against insects of the dipteran family which are responsible vector-borne diseases. In the World Health Organization (WHO) records, Bs has not been noted any adverse effect on non-target and beneficial fauna coexisting with mosquito larvae [55]. In this application report, if Bs strains were used high rates of application, there is not adverse effect or acute mortality in honey-bees (*Apis mellifera*), on fish, predator invertebrate organisms, chironomids and other species of Nematocera. Also in mammalian safety tests including dermal toxicity, acute pulmonary infectivity, acute intraperitoneal infectivity Bs is not show any adverse effect [56–58]. This bacterial agent provides highly selective control opportunity.

Bs produces large amount of toxin protein during sporulation phase and this protein gathers in the sporangial form as parasporal body. The inclusion body contains protoxin (as a crystal protein) which are ready end of the sporulation. In the first studies related to toxicity effect on mosquito, the histopathological results showed that the midgut epithelium appears to be the primary site of action. At present, the mode of toxin action has been elucidated clearly. Bs' Btx crystal toxin contains two polypeptides of 42 kDa (BinA) and 51 kDa (BinB). Mosquito larvaes take into own metabolism these crystal protein during strained the water. After the solubilization and activation by the alkaline pH and intestinal proteinases, the 2 component proteins of the toxin, BinA (42 kDa) and BinB (51 kDa) bind to specific receptors on the brush border of epithelial cells of the gastric caecum and midgut. BinB protein associates with a single receptor, named as a GPI-anchored maltase [48,59–62]. When formed the toxin-receptor complex, a part of toxin get into the cell membrane lipid bilayer and cause pore formation (permeabilization) resulting in disruption of osmotic balance, lysis of the cells, and ultimately death of the insect [27,53].

1.4 GROWTH KINETICS

Microbial growth in broth culture medium is characterized by a successive phases: lag, log, stationary, and death phases. Characterizing and modeling the kinetics of sporulation and associated product formation of commercially important microbes at stationary phase are important parameters to describing substrate–product relationship of stationary phase products. Useful kinetic model for biopolymer synthesis could include balances on cell mass, product concentration, substrate utilization, and a single limiting substrate. One of the very important practical applications of this model is the evaluation of the product formation kinetics. Mathematical models of such kinetics facilitate data analysis and provide a strategy for solving problems encountered in fermentations. Kinetic data are needed to develop basic understanding of fermentation processes and to permit rational design of continuous fermentation processes. Final product yields and substrate conversions are the criteria with the main attention toward the productivity.

1.4.1 Growth Kinetics Models

1.4.1.1 Monod Model

The idea of microbial growth kinetics has been dominated by an empirical model (eq. 1) originally proposed by Monod (1942). The Monod model introduced the concept of a growth limiting substrate. The exponential growth phase can be characterized by the following first order equation which states that the rate of increase of cell mass is proportional to the quantity of viable cell mass at any instant time:

$$\frac{dX}{dt} = \mu X \tag{1.1}$$

where, dX/dt is the growth rate(g L h^{-1}); X is the concentration of biomass (g/L); μ is the specific growth rate (h^{-1}). The relationship between the specific growth rate and the concentration of the limiting substrate is described by the following Monod equation:

$$\mu = \frac{\mu_m S}{Ks+S} \tag{1.2}$$

where, μ max is the maximum specific growth rate (h^{-1}); S is the concentration of the limiting substrate (g/L); K_s is the substrate concentration at one-half the maximum growth rate.

Another form of the **Monod equation** (Simpkins & Alexander, 1984);

$$-\frac{dS}{dt} = \mu \frac{max\, S(So + X'o - S)}{Ks + S} \tag{1.3}$$

They took extreme ratios of X and S in above equation as special cases of Monod equation and divided these cases into **Non-growth** and **Growth** situations.

1.4.1.2 Logistic Model

The microbial growth is governed by a hyperbolic relationship and there is a limit to the maximum attainable cell mass concentration which is described by the logistic equation.

$$\frac{dX}{dt} = \mu \left[1 - \frac{X}{Xm}\right] X \tag{1.4}$$

where, μ describes the initial specific growth rate (h^{-1}) and X_m the maximum cell mass concentration (g/L).

1.5 BIOPROCESS OPTIMIZATION

Optimization plays a critical role to developing the performance of a system, a process, or a product. For example, given a chemical reaction, optimization can be used to determine conditions that result in highest product yield [63]. Optimization is commonly used to achieve high productivity of desired products [64–68]. Optimization of process and media can significantly decrease the cost of biotechnological production. In classical method, components were added or removed (chemical, physical or molecular; medium ingredients, pH, temperature, fermentation period, inoculum size, shaking rate, stop/start specific gene activity etc.) to find the critical factors and specific requirements e.g. growth and product formation. This basic method was called “one variable at a time”. Besides being laborious, this method typically misses the interactive effects among the variables. On the other side, statistical optimization yields better results accurate information on interaction in shorter time with smaller effort. In statistical optimization, two general methods are often employed to predict “right” combination of parameters, which are artificial neural network (ANN) and response surface methodology (RSM).

ANN based models can be implemented for monitoring, control, classification and simulation of different biological processes. This method uses mathematical nodes/neurons to build a network that can form complex pattern of relationships [69,70]. ANN can be combined evolutionary algorithms (e.g. genetic algorithm) to find the best fitting parameters [71]. RSM is another solution which based on mathematical and statistical techniques to propose the influences of individual factors, build models, evaluate the effects of several factors. This flexibility provides to select and achieve the optimum conditions for desirable responses.

1.5.1 Response Surface Methodology (RSM) And Central Composite Design (CCD)

Response surface methodology, developed by Box and Wilson, is an empirical statistical technique which is based on mathematical techniques employed the fitting of a polynomial model to the experimental data [72]. This polynomial data provides predictions on the behavior of the experimental system. The main purpose is to optimize the system, to achieve improved

performance and learn more the system itself (e.g. the interactions among the parameters). RSM is based on choosing an experimental design that will define the type of experimental matrices should be carried out. The effect of the variables on the response can be shown using a second-order polynomial. For this, alternative experimental design schemes exist like three level factorial, Box–Behnken, central composite, and Doehlert designs [73]. The RSM can be represented by mathematical model as surface graphical perspective.

In RSM, there are some key terms and application stages related to optimization.

Experimental design is a set of experiments constituted by different level combinations of variables. CCD can be used not only to study first order effects, but also second order interactions. It produces a set of combinations from variables that is carried out and get experimental results of response.

Factors/independent variables are the input parameters which can be changed freely of each other. Typically in bioprocess optimization are pH, temperature, concentration of components, incubation time, mixing rate, inoculum size etc.

Levels of a variable are obtained from the experimental design. For example, 4 levels of glucose concentration: 1, 5, 10 and 20 g/L.

Responses or dependent variables are the results of the experiments from experimental design sheet. Usually, values are absorbance, colony forming units, net emission intensity and others.

Residual is the difference between calculated and experimental results for each experiment. If the residual value is low, the mathematical model is adequate.

In RSM, to design experiments and building models, the below schemes should be followed:

1. Determination of factors which have major effects on process.
2. Selection of the design and executed the experimental set according to the matrix.
3. Obtained the fitting polynomial function from experimental results in light of mathematical and statistical treatment.
4. Evaluation of the goodness of fit.
5. Control of the optimal region according to necessity and possibility.

6. Validation of the optimum rates for each variables [63,74].

Central composite design (CCD) is a design strategy which allows the selection of the most important variables of process build up the estimation of curvature and interaction effects using second-order factorials [68,73]. Factors of CCDs' experiments are distributed at three levels that they are factorial points (coded value of +1 or -1), star (axial) points (coordination value of +α or – α), and center points (value of 0 point). Each level value uses different for estimation of main and two-factor interactions (factorial points), rotatability and orthogonality (axial points), estimation of pure experimental error [75,76].

1.5.2 Production Media Components

The cost-effective manner while designing media is important to compete with other chemical control agents. Economically attractive production media is a prerequisite to start the progress. Building a fermentation media some critical points should be evaluated [45,53]. These parameters are cheaper raw materials, locally available sources, and simplified pretreatment stages. Much effort in fermentation process optimization has been made to produce biopesticide economically from several inexpensive waste substrates [77].

Molasses is a cheap media source which can obtain easily from a sugar factory. In the world, sugar agriculture has high production amount (Figure 1.4). Also in Turkey, there are a lot of companies to buy cheap and locally available molasses. 25 sugar factories are managed by government and also there are 8 private sugar companies (http://www.turkseker.gov.tr/). Totally 33 sugar companies are located 30 different cities (Figure 1.5). The main reasons for generally using molasses as a substrate in fermentation are lower price carbon sources and its valuable content besides sucrose [78,79]. It can contain minerals, organic compounds and vitamins, which are well support in complex media.

Figure 1.4. World major sugar feedstock countries

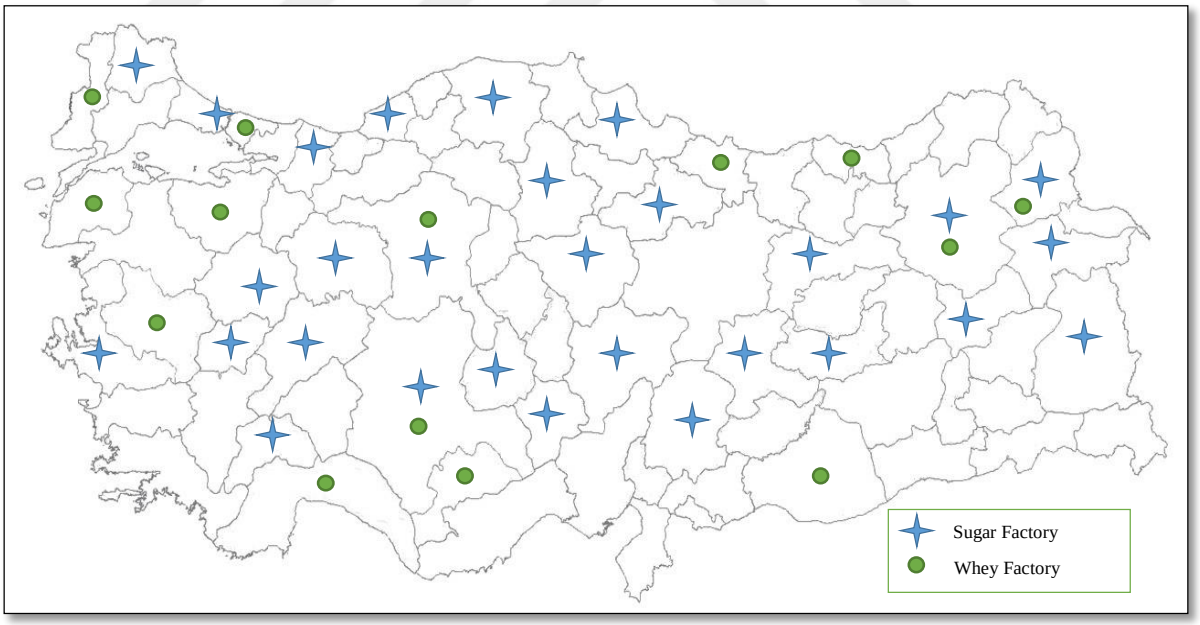


Figure 1.5. Feedstock holding places of molasses and whey in Turkey.

Whey protein can be recovered from cheese whey as by-product. In a year, in United States 1.2 billion tons of cheese whey are obtained from cheese manufacturing [80]. Cheese whey contains nearly 7% total solids, of which 70% is mainly lactose, 13% is proteins, 1-2% is lipids. In Turkey, annually 452.000 tone cheese are utilized and whey is produced nine times the rate of

cheese production [81–83]. Every one kilogram cheese production, 9 kg whey is produced. In lots of location, cheese manufacturers produce whey during cheese production. And in a widespread area, whey protein can be found easily in Turkey (Figure 1.8).

Corn steep liquor (CSL) is also another inexpensive energy sources for fermentation process. CSL is a major by-product of corn steeping process. It has a nutritional and functional supplement in the fermentation process because of containing important nutrients, vitamins, minerals, amino acids and growth stimulants like biotin [84,85]. CSL has been used as a nitrogen source for high level enzyme expression. It was utilized for production of exopolysaccharide and ethanol [84,86].

1.6 SCALE UP STRATEGIES

Shake flasks are the bioreactors most frequently used in biotechnology. The variety of tasks in which shake flasks are applied is very wide such as; establishment of basic process conditions, strain screening, medium development, etc. Furthermore, shake flasks are commonly used because since they are very easy to handle and many experiments can be conducted simultaneously with little supervision [87]. Commercialization of fermentation involves the scale-up of laboratory fermentation to pilot scale and industrial scale bioreactor [88] Unfortunately, the transfer process from shake flasks to bioreactor is difficult and poorly understood, mainly because of the lack of knowledge concerning the influence of the operation conditions on mass transfer, hydrodynamics and power input [87,89].

Usually, the productivity of the desired product is high in flask scale, and is drastically reduced as the scale is enlarged when one attempts to translate the conditions of the flasks to stirred bioreactors. Accordingly, results obtained in the shake flasks can be used only as preliminary indicators. Pre-designation of scaling-up studies must be proved in studies carried out in a bench scale bioreactor, to determine the conditions for successful industrial production [90]

The scale-up criteria are specific for each systems. For the performance of the bioprocess, the most critical point is selecting the scale-up principle depending upon the transport property [91]. The method for scaling up a fermentation system is commonly based on empirical criteria such

as pH, temperature, constant power input per unit volume (P/V), a constant mass transfer coefficient, constant mixing time and a constant impeller tip velocity [90].

P/V is especially important in complex two-phase liquid systems as drop diameter depends on the maximum energy dispensation rate or power consumption. Furthermore, the extraction efficiency in extractive processes should be influenced by power input as it determines the mean drop size and the dispersion of the immiscible extractive organic phase. In this respect, Cull et al. [92] showed that P/V was the best scale-up criteria to keep constant the interfacial area in a two liquid phase bioconversion process using geometrically similar stirred tank reactors. Therefore, given the two-liquid phase nature of extractive bioprocesses, it seems reasonable to use P/V as a scale-up criterion [91].

Although, agitation power per unit volume (P/V) is among the most often used parameters for scale-up, the scale-up method to maintain dissolved oxygen (DO) at a constant level became popular by the developing of sensor technologies for DO concentration. Because of applied shear stress, culturing filamentous microorganisms such as fungi or actinomycetes in a large bioreactor could expose cells to damage. In parallel with this situation, the product yield is reduced. Therefore, a settlement shall be tried to achieve a sufficient level of DO with minimum possible shear stress [90].

The industrial implementation of process relates to conjunction with the transfer of mass, heat and momentum. These parameters are scale-dependent and changes in small-scale to large-scale. The process control starts with following stress factors, understanding the physiological responses, and analyzing the interactions of the various physical and chemical parameters. In table 1.4, there are some fermentation parameters, coefficient, and terms implicated in mixing, aeration, oxygen and heat transfer, suitable as scale-up variables to be kept constant alone or combined with each other [93–95]

Table 1.4. Equations for scale-up fermentation parameters [93]

Parameter/coefficient		***Mathematical characterization***	***Symbol explanation***
1-	Power input (P); volumetric power input (P/V)	$P = 2\pi nM = N_{Po}\rho n^3 d_I^5 [kgm^2 s^{-2} = W]$	P=power input; n=stirrer speed; M=momentum; N_{Po}=dimensionless power number; ρ=density of the medium
2-	Dimensionless power number (N_{Po})	$N_{Po} = P/\rho n^3 d_I^5$	d_I =impeller diameter
3-	Impeller tip speed (v_{tip})	$v_{tip} = 2\,\pi\, n\, d_I [m/sec]$	n=stirrer speed; d_I =impeller diameter
4-	Reynolds number (Re)	$Re = n\, d_I^2 \rho/\eta$	Re= Reynolds number; n=stirrer speed; d_I =impeller diameter; ρ=density of the medium; η= dynamic viscosity
5-	Modified dimensionless power number	$N'_{Po} = N_{Po} Re^3 d_F/d_I = P d_F \rho^2/\eta^3$	N'_{Po}= Modified dimensionless power number; N_{Po}= dimensionless power number
6-	Aeration rate (volume per volume per minute, vvm)	$A_R = F_G/V_R [m^3/m^3\ min]$	A_R=Aeration rate; F_G = volumetric gas flow rate; V_R=fermenter reaction volume
7-	Oxygen transfer rate (OTR)	$OTR = k_L a(C_G - C_L) = k_L a\, L_{O2}(pO_{2G} - pO_{2L}) \left[kg \frac{O_2}{m^3} h\right] with\ C_G = \frac{0{,}526 p_i}{36} + T\ [mg/L]$	OTR=oxygen transfer rate from gas to liquid phase; k_L=mass transfer coefficient; a=specific interfacial surface area; C_G=oxygen saturation concentration in the gas phase; C_L=measured oxygen saturation concentration in the liquid phase; L_{O2}=oxygen solubility in the liquid phase; pO_{2G}=partial pressure of oxygen in the gas phase; pO_{2L}=partial pressure of oxygen in the liquid phase; p_i= vessel back pressure [bar]

1.7 CURRENT TECHNOLOGIES AND STRATEGIES IN PRODUCTION OF *BACILLUS SPHAERICUS*

The biological control agent Bs has been paid attention of production because of economic, health, social, and environmental importance. From the first discovery of Bs as insect pathogens [43], to date, numerous academic and commercial formulations have been done. The main studies can be categorized as finding insect specificity, determination toxin types, development of low-cost media, and media optimization.

1.7.1 Insecticidal activity

Kellen et al. [43] had first reported in 1965 as a pathogen for mosquito larvae. Bone and Tinelli was applied Bs spores against nematode (*Trichostrongylus colubriformis*) eggs and showed nematicidal effect [96]. Toxicity against *Culex quinquefasciatus* was studied using purified protoxin of Bs and was shown to be active [97]. Bs 1593 strain was test against *Anopheles gambiae* larvae in the field. The applied concentration to control the larvae was found at low rates [98]. Malarial vector *Anopheles stephensi* fourth instar larvae were controlled by Bs and some plant extract combination (*Leucas aspera*) [5].

Another recent study was performed about cell line of *Anopheles gambiae* larvae that it was followed the response for Bin toxin action. Bin toxin protein kills cells via toxin uptake, vacuole formation and autophagy [99].

1.8 THE AIM OF THE STUDY

First, a novel entomopathogenic microorganism could be discovered. It is characterized and identified according to biochemical, morphological and molecular properties. To enlighten of mosquitocidal toxin profiles.

Second, the focus of the study would be defined on selecting the appropriate growth media components for Bs MBI5 production. Various ingredients (carbon sources, nitrogen sources, and minerals) were investigated by comparing the cell density using one variable at a time approach.

Third, the performance and effect ratio of selected complex media for Bs MBI5 cultivation was optimized with respect to Response surface methodology and central composite design. Economic analysis was also evaluated to give information for selecting a cost-effective medium potentially for large-scale production of Bs MBI5.

Finally, followed by the medium investigation and optimization, a fermentation scale-up process from a 500 mL flask, 5 L and to 30 L reactor was performed using novel strain Bs MBI5. The performances of the different fermentation scales were evaluated by cell mass production, cell density, vegetative cell amount, spore amount, and protein amount. Last but not least stage, after lyophilization of the final products from different fermentation progress were evaluated in vivo larval toxicity on mosquito larvae.

2. MATERIALS

2.1 MEDIA AND CHEMICALS

Tryptic Soy Broth (TSB)	MERCK	GERMANY
Tryptic Soy Agar (TSA)	MERCK	GERMANY
Sabroud Dextrose Agar (SDA)	Acumedia	USA
Potato Dextrose Agar (PDA)	MERCK	GERMANY
Nutrient Agar (NA)	MERCK	GERMANY
Calcium Carbonate	SIGMA	USA
Yeast Extract	MERCK	GERMANY
Glucose mono hydrate	Riedel-de Haën	GERMANY
Lactose mono hydrate	MERCK	GERMANY
Mannose	Fluka	SWITZERLAND
Sucrose Carlo Erba	Carlo Erba	ITALY
96 % Ethanol	MERCK	GERMANY
Agarose	SIGMA	USA
2X master mix PCR solution	Fermentas	USA
Primestar Taq polymerase	Takara	JAPAN
Generuler1 kb DNA ladder	Fermentas	USA
PageRuler Plus Presatined Protein Marker	Thermo Scientific	USA

Imperial Protein Stain	Termo Scientific	USA

2.2 LABORATORY EQUIPMENTS AND DEVICES

Erlenmayer Flask	Isolab	GERMANY
Petri Dish	Isolab	GERMANY
Ependorf Tubes 2 ml, 1.5ml, 0.5 ml	Isolab	GERMANY
Serological pipettes 25, 10, 5, 2 ml	Grenier-Bio or Axygen	USA
Inoculation Loops	Isolab	GERMANY
Pasteur Pipette	Isolab	GERMANY
Cryotubes	TPP	SWITZERLAND
Sherlock Microbial Identification System	Newark	GERMANY
Incubator	Memmert	GERMANY
Incubator Shaker Certomat IS	Sartorius Stedim	GERMANY
Labculture Sterile Cabin Class II Type A2	ESCO	SINGAPORE
Centrifuge Allegra 64R	Beckman Coulter	USA
Benchtop centrifuge 1-14 SIGMA	SIGMA	USA
Magnetic stirrer	Heidolph	GERMANY
Fridge	Artico	DENMARK
Autoclave HICLAVE HV-85	HIRAYAMA	JAPAN
Water bath	Memmert	GERMANY

Vortex MX-S	DragonLAB	CHINA
Nanodrop 2000	Thermo Scientific	USA
-80 °C freezer	Sanyo	USA
Microwave	ARÇELİK	TURKEY
Spectrophotometer Ultrospec 3000	Pharmacia Biotech	SWEDEN
Plate reader Multiscan Spectrum	Thermo Labsystems	USA
Thermal cycler My Cycler	BIO-RAD	USA
Thermal Block Mixing block	BIOER	CHINA
3 L Fermentor Minifors	INFORS HT	SWITZERLAND
30 L Fermentor BIOSTAT C Plus	Sartorius Stedim Biotech	GERMANY
DV-III Ultra Programmable Rheameter	Brookfield	USA
Sonicator Digital Sonifier 250	Branson	USA

2.3 STRAIN

The entomopathogenic strain of *Bacillus sphaericus* MBI5 was isolated from larval habitat of Istanbul, Turkey in the spring and summer of 2008. Aerobic, rod shaped, endospore forming bacterium. It is widely distributed in soil and water habitats. This organism was characterized by 16S rRNA technique.

3. METHODS

3.1 CHARACTERIZATION OF BACTERIUM

Bacillus sphaericus MBI5 was identified according to biochemical, fatty acid profile, and genomic results. Biolog analysis was performed to determine the chemical and carbon source utilization. FAME analysis was used to define the cell wall fatty acid composition. Also some genomic studies were finished to understand the microorganism species.

3.1.1 Carbon Substrate Utilization (BIOLOG)

Bacterial strains were tested for carbon substrate utilization by using Biolog Microplate (GP) system. A pure culture was isolated from BUG + M agar. When the culture was pure, it was subcultured on BUG + M + T (0.25% maltose swabbed with thioglycolate). Then thioglycolate was added to the agar plate: before streaking the strain, precisely 8 drops from a thioglycolate dropper was added into 3 ml of sterile water. A sterile swab was dipped into the solution to moisten the cotton tip. A thin film of liquid was spreaded across the entire surface of the agar medium. For the thioglycolate to dry on the agar, it was allowed for approximately 5 minutes. Secondly Biolog's sterile stick was used to touch a colony and make a plus sign (+) on the center of the agar media going across each of the two lines and media were incubated at 27°C for 24h. Cells were harvested with sterilized plastic loops, and suspended in sterile saline (0.85 NaCl pH: 6). The Biolog GP Microplates were preconditioned at 27°C for 24h, then inoculated by adding 150 µl of each bacterial suspension into the reaction wells of microplate using a multichannel micropipetter. The plates were incubated at 27°C for 12-24h. The color reaction inditory positive utilization of each carbon substrate was read by Microplate reader and the results (metabolic fingerprint) for each bacterial strain were compared with the Biology GP database with Microlog Software (v 4.20.03).

3.1.2 FAME Analysis

Extraction and identification of whole-cell fatty acid methyl-esters (FAME's) by gas chromatography was performed. Bacteria were streaked onto TSA and incubated at 80°C for 24h. A loopfull of cells were harvested from TSA plate of each strain and added to 1.2 M NaOH in 50% aqueous methanol in a screw cap tube, then incubated at 100°C for 30 min in water bath. After, saponified samples were cooled at room temperature for 25 min. They were acidified and methylated by adding 2 ml 54% 6 N HCL in 46% aqueous methanol and incubated at 80°C for 10 min in water bath. After rapid cooling, methylated fatty acids were extracted with 1, 25 ml 50% methyltert butyl ether (MTBE) in hexane. Each sample was mixed for 10 min and bottom phase was removed with a Pasteur pipet. The top phase was washed with 3 ml 0.3 M NaOH. After mixing 5 min, the top phase removed for analysis. Fatty acid methyl esters were separated by gas chromatography FAME profiles of the strains were identified by the commercial TSB6 database with Microbial Identification System software (v 6:0). The cellular concentrations of the fatty acids for each strain were determined and strains were identified at species level.

3.1.3 Isolation of Genomic DNA

Bacterial genomic DNA was isolated using commercial kit and performed according to the manufacturer's instructions (Invitrogen Pure Link Genomic DNA Mini Kit).

3.1.4 16S rRNA Analysis

16S rRNA genes of the bacterial DNA isolates (MBI5) were amplified by the PCR using purified DNA and primers 27f and 1492r. PCR amplifications was caried out in total volume of 50 µl reaction mixture containing 0.2 mM of 27f and 1492r primers for total 16S, 0,2 µl of DNA polymerase, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 mM MgSO4, 10mM Tris and 50 ng template DNA. PCR conditions were as follows : preamplification 94°C for 5 min : denaturation at 94°C for 30s : annealing at 55°C for 40s : elongation at 72°C for 2 min repeated 35 cycles and then post amplification for final extension 10 min at 72°C.

3.1.5 Polymerase Chain Reaction (PCR) of Genes

To amplify the BinA and BinB genes from the genome and bacteria (colony PCR), Finnzymes High-Fidelity DNA Polymerase kit was used. The PCR amplification was carried out in eppendorf thermal cycler in 20 µl reaction volume. The reaction was subjected for amplification from genomic DNA to initial denaturation of 2 min at 95°C and subsequent 35 cycles each comprising denaturation of 92°C for 50 s, annealing at 55°C for 50 s and elongation at 72°C for 50 s. For colony PCR, the reaction was subjected to initial denaturation of 7 min at 95°C and subsequent 35 cycles each denaturation of 92°C for 50 s, annealing at 55°C for 50 s and elongation at 72°C for 50 s. The pipetting instruction listed below (Table 3.1).

Table 3.1. PCR Pipetting Instructions

Component	***Volume / 20 µl reaction***
H_2O	11.4 µl
5x Fhusion HF Buffer	4 µl
10 mM dNTPs	0.4 µl
10 mM Forward Primer	0.5 µl
10 mM Forward Primer	0.5 µl
DNA	3 µl
DNA Polymerase	0.2 µl

3.1.6 Agarose Gel Electrophoresis

1% agarose gel was prepared with the buffer, Tris Acetate- EDTA buffer (Sigma). The gel preparation protocol is given in detail below;

- A 1% agarose gel was made by mixing 0.5 g agarose with 50 ml TAE buffer.
- The mixture was heated in a microwave oven until all agarose had melted and the solution had started to boil.
- It was waited to get a cool solution approx. 60-65 °C.
- 2.5 µl ethidium bromide was added into solution and gently mixed.
- The gel was poured into gel tray and the comb was set. All bubbles were removed.
- After 20 min, when the gel had solidified, the tray was released from all components.
- The tray was set into tank containing TAE and samples were loaded with marker.
- The gel was run at 80 V for 40 min. Finally, gel was visualized under UV light.

3.2 ANALYTICAL PROCEDURES

3.2.1 Total Protein Amount Determination

The total soluble protein in the medium was estimated by Bradford method. The supernatant was used for total protein analysis in 96 well-plate. Each well plate was containing 150 uL supernatant and 150 uL 2N Bradford Assay Solution. The assay was read at 595nm and used bovine serum albumin (BSA) as a standard.

3.2.2 Biomass Determination

The bacterial growth was determined by measuring the optical density at a wavelength of 650 nm. The biomass concentration was calculated using a calibration curve established from the relationship between optical densities at 650 nm and the dry cell weight.

3.2.3 Vegetative Cell Amount Determination

The number of viable cells in the cultures were measured by the plate count method as shown Figure 3.1. 0.1 ml of the liquid culture was added into centrifuge tubes containing 0.9 ml of

sterile PBS. Each tubes were vortexed a short period. Tenfold serial dilutions of each sample were prepared and 0.01 ml of 10^{-5}, 10^{-6}, 10^{-7} dilutions was spread onto same nutrient agar plates. Each dilutions was done three replicate.

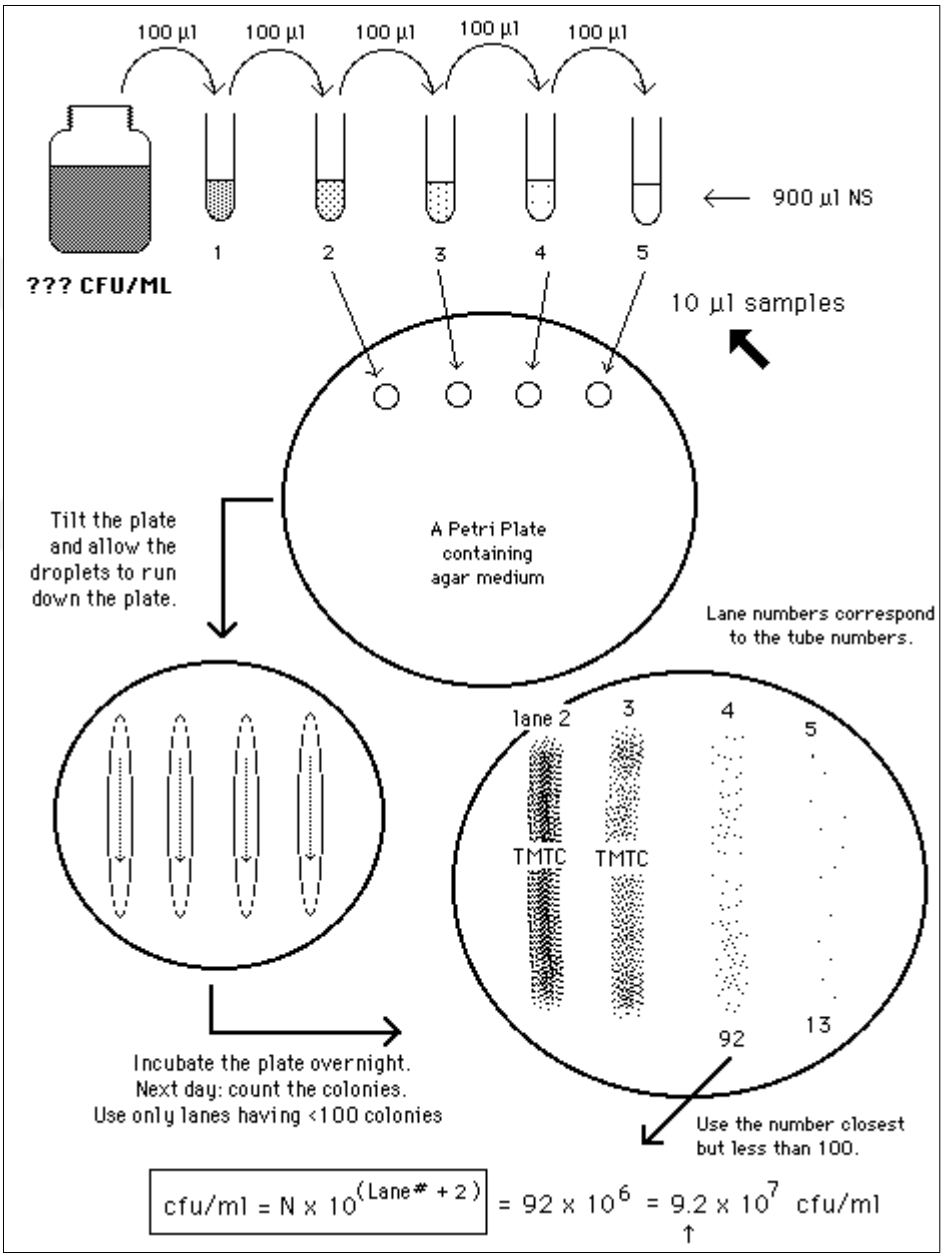


Figure 3.1. Calculation number of cell in the culture using plate count method

3.2.4 Spore Counting

To calculate the number of the spores in culture, it was heated at 80°C for 20 min, serially diluted and plated on nutrient agar plates as mentioned above. After incubation, the developing Bs colonies were counted and expressed in spores/mL.

3.2.5 Glucose Determination

The glucose concentration was monitored by dinitrosalicylic acid (DNS) assay in a 96-well plate. The reaction mixture containing 150 uL sample and 50 uL DNS solution (g $0,1L^{-1}$: 3,5-dinitrosalicylic acid 1g; 20g Potassium Sodium Tartarate-Rochella salt; 2M NaOH 20 ml), which was incubated at 37C for 10 min. After incubation, the reaction mixture was read at 540 nm using microplate reader. A standard glucose solution (5g L^{-1}) is prepared in a day advance for any structural deformation or changes. A Calibration curve is prepared. A straight line can easily be obtained in the range 200–1600mg L^{-1} glucose. All of the experiment was carried out triplicate.

3.2.6 In Vivo Larval Toxicity Assay

The toxicities of fermentation products to third instar larvae and early fourth instar of *Culex* spp. were verified by in vivo larval assay. Larvae were reared on an artificial diet (fish bait) in the presence or absence of different amounts of the toxin product. After fermentation, the spore and protein mixing was centrifuged to separate from supernatant. Then centrifugation, the pellet was lyophilized to storage. Spores with associated toxin were plated for viable-cell counts and were bioassayed against mosquito larvae. The bacteria were diluted into 100 ml of tap water containing 10 larvae and some feed in 250 ml flasks. Each flask was kept at nearly 25 ºC with a light-dark photoperiod of 16h: 8h. Each treatment was performed triplicate. Surviving larvae were counted at 24 h and calculated 95% lethal concentrations (LC_{95}) from average of mortality.

3.3 BIOPESTICIDE PRODUCTION MEDIA

To find the optimum media, flask trials were initially started with commercial media such as nutrient broth (NB), lurian-bertani broth (LB), tryptic soy broth (TSB). The commercial media trials were used as a standard to compare the yield with complex media trials. Each standard media was designed alone and combined with some minerals. Also to grab the cost effective media, some agricultural and food by-products were used as complex media composition. These by-products are molasses, whey and corn steep liquor (CSL). As done in commercial media, each complex media combination were observed with adding/deleting the minerals like magnesium sulphate, manganese sulphate, potassium salts, sodium salts.

3.3.1 Pretreatment Stages of Complex Media

3.3.1.1 Molasses Pretreatment

According to sugar process, two different types of molasses can be produced. The sugar beet molasses is a byproduct of progressing sucrose production from sugar beet. Another type is starch molasses which is byproduct of dextrose production from corn. In the scope of this study, sugar beet molasses was used to obtain fermentation media. This molasses was supplied by Adapazarı Sugar Factory (Adapazarı, Turkey). Raw molasses can be used as a crude after onefold or twofold dilution with distelled water. But in somecases molasses is treated with different pretreatment processes such as sulphuric acid treatment, cation exchange resin addition, tricalcium phosphate treatment, potassium ferrocyanide and EDTA treatment [100–102]. These processes are needed to removed the growth inhibiting contents which are heavy metals, volatile organic acid and some alcohol components [103,104]. Especially sulphuric acid treatment is used to hydrolysis some polysaccarides into monosaccarides. In the present study two different molasses types were employed. The crude molasses was diluted with dH_2O twofold (%, w/v) before direct usage (named as Molasses Physical, MPhys) and acidification (named as Molasses H_2SO_4, MAcid). To prepare the molasses physical that 100 g the raw molasses was diluted with 200 ml distilled water, after that the diluted molasses was centrifuged

at 6000*g*, for 20 min. The second type of molasses was molasses H_2SO_4. The pH of diluted molasses is adjusted to pH 3 with 6M H_2SO_4. It was mixed for 1 hour at 60 ºC. After that, the final pH was reached at 7 with 10M NaOH.

3.3.1.2 CSL and Whey Pretreatment

CSL was diluted 50 gr in 1L of distilled water. The pH of diluted CSL was adjusted to 7.0 with 1M KOH. After pH adjustment, CSL was centrifuged at 4,000 rpm for 15 min. And sterilized at 121C for 15 min. CSL was provided by Cargill Company, Bursa.

Whey powder was purchased from a Pınar A.Ş in Izmir. Whey concentration in the medium was about 50g/L.

3.4 BIOPROCESS OPTIMIZATION AND RESPONSE SURFACE METHODOLOGY

3.4.1 Investigation of Key Components

To investigate the key energy sources both carbon and nitrogen was performed using one-at-a-time approach which is the main concept of fermentation media was defined by shake flask trials. Each carbon and nitrogen sources was revised to understand the best suitable component for production. Before starting the media optimization via Design Expert software, limits of carbon sources, nitrogen sources and minerals were enlightened and tested.

3.4.2 Experimental Design for Biomass Production Using Central Composite Design

The levels of five significant factors and the interaction effects between various medium constituents which influence the biomass and protease production significantly were analyzed and optimized by the response surface methodology, using a CCD design. A small CCD with four variables was used to optimize the response. In the present work, the selected variables

were molasses, CSL, $MnSO_4$ and $MgSO_4$ concentration and each variable was analyzed at five levels coded as - α, -1, 0, +1 and + α (Table 3.2). The CCD of 30 runs include 16 runs for factorial design, 8 runs for axial points and 6 runs for replications of the central points. The levels of factors used for experimental design are given in Table 3.3.

Table 3.2. The variables and their levels for the central composite experimental design

Independent variables	**Symbols**	**Units**	**Levels**				
			-α	**-1**	**0**	**+1**	**+α**
Molasses	A	%, v/v	3.787	10	25	40	46.213
CSL	B	%, v/v	3.787	10	25	40	46.213
$MnSO_4$	C	%, w/v	-0.692	0.2	0.85	1.5	1.7692
$MgSO_4$	D	%, w/v	-0.692	0.2	0.85	1.5	1.7692

Table 3.3. Experimental design for central composite design of response surface methodology ([a] Center points)

Run	**Factor 1 A: Molasses (%, v/v)**	**Factor 2 B:CSL (%, v/v)**	**Factor 3 C: $MnSO_4$ (%, w/v)**	**Factor 4 D: $MgSO_4$ (%, w/v)**
1	40.00	40.00	1.50	1.50
2[a]	3.79	25.00	0.85	0.85
3	25.00	46.21	0.85	0.85
4	10.00	10.00	0.20	1.50
5	10.00	10.00	1.50	0.20
6	10.00	10.00	0.20	0.20
7	10.00	40.00	1.50	0.20
8[a]	25.00	25.00	0.85	0.85
9	40.00	40.00	0.20	0.20
10	25.00	25.00	0.85	-0.07
11	40.00	40.00	0.20	1.50
12	40.00	40.00	1.50	0.20
13	25.00	25.00	0.85	0.85
14	46.21	25.00	0.85	0.85
15	40.00	10.00	0.20	1.50
16	10.00	40.00	1.50	1.50
17	25.00	25.00	0.85	1.77
18[a]	10.00	40.00	0.20	0.20

Table (continued)

19[a]	10.00	10.00	1.50	1.50
20	40.00	10.00	1.50	1.50
21	25.00	25.00	0.85	0.85
22	40.00	10.00	1.50	0.20
23	10.00	40.00	0.20	1.50
24	25.00	25.00	1.77	0.85
25	25.00	25.00	0.85	0.85
26	25.00	25.00	0.85	0.85
27[a]	25.00	3.79	0.85	0.85
28	25.00	25.00	0.85	0.85
29	40.00	10.00	0.20	0.20
30[a]	25.00	25.00	-0.07	0.85

RSM was used to define the optimal levels of key factors after the optimal region of each significant variable was determined. Therefore, the predicted response can be calculated from the second-degree polynomial, Eq. 3.1, which includes all interaction terms.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j}^{n} \beta_{ij} x_i x_j + \sum_{j=1}^{n} \beta_{jj} x_j^2 \quad (3.1)$$

where *Y* is the predicted response. In this study, four variables are involved; hence, *n* takes the value of 4. Thus, by substituting the value of 4 for n, Eq. 3.2 becomes:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (3.2)$$

where Y is the predicted response variable; β_0 is the constant term, and $X_1, X_2, X_3,$ and X_4 represent the codded values of molasses, CSL, $MnSO_4$, and $MgSO_4$, respectively. $\beta_1, \beta_2, \beta_3, \beta_4, \beta_{11}, \beta_{12}, \beta_{13}, \beta_{14}, \beta_{22}, \beta_{23}, \beta_{24}, \beta_{34}$ are the model coefficients.

The statistical significance of the model equation and the model terms were evaluated in Design Expert software using Fisher's test. According to the correlation coefficients R and R^2, which explain the quality of fit of the regression model, the contour plots were determined as a two-dimensional graphical representation that generated response surface curves.

3.5 OPTIMIZED PRODUCTION MEDIUM AND FERMENTATION

The completion of media optimization, software has provided the extra optimization tools to find higher desirable options. These tools are factor goal and importance, response goal and importance. Factor/response goal is specified target for each factor/response. Possible options include maximize, minimize, move towards a target value [105]. Also setting a factor to be "exactly equal to" a specific value. Factor importance is the specified importance value for this factor in relation to the other factors and responses [67,106]. The default is +++ (three pluses). If it is more important to achieve one factor or response than another, give the more important factor or response a higher weight. The optimization module in Design-Expert searches for a combination of factor levels that simultaneously satisfy the requirements placed on each of the responses and factors. To use optimization, you must first analyze each response to establish the appropriate model [107,108]. Optimization of one response or the simultaneous optimization of multiple responses can be performed graphically or numerically.

3.5.1 Fermentation Procedure

3.5.1.1 Shake Flask Experiment

Cultures were carried out in 500 ml Erlenmeyer flasks during 48 h, containing 100 ml of culture medium at 150 rpm and 30 ºC in an orbital incubator shaker. A 2% (v/v) inoculum of the pre-culture was used to inoculate each culture media.

3.5.1.2 Bench and Large-scale Bioreactor

The optimized media and higher desirability solution were used as production medium in bioreactor trials. Fermentations were carried out in two stirred-tank bioreactors—bench- and large-scale. Details of bioreactor dimensions are summarized in Table 3.4. The final validation of the optimized medium was used in a 5L bioreactor (Infors HT, minifors) for lab-scale production during cultivation of Bs MBI5. The bioreactor was equipped with two Rushton type turbines and baffles. The optimized medium (pH 7) was sterilized in autoclave at 121 ºC for 15 min.

Figure 3.2. Lab-scale 5L batch type bioreactor (Minifors, Infors HT)

30L bioreactor (Sartorius Ag, Biostat C plus) was used for large-scale production. It has three Rushton type turbines and baffles. It was sterilized in situ at 121 ºC for 15 min.

Figure 3.3. Large-scale 30L bioreactor (Biostat C plus, Sartorius)

CSL was sterilized separately and was mixed aseptically via automatic feed pump in bioreactor before cultivation. The medium was inoculated with 2% of inoculum and fermentation was performed at 30 ºC for 48 h with uncontrolled pH. Samples were withdrawn periodically at an interval of 4 h and analyzed for protein amount, CDW, viable cell amount, and sporulation estimation. Other fermentation parameters, such as temperature, pH, dissolved oxygen and airflow were continuously monitored using microprocessor-controlled probes.

Table 3.4. Dimensions of bench- and pilot-scale bioreactors

Bioreactor	Bench scale	Large scale
Total volume (L)	5	42
Working volume (L)	3	30
Impeller (six-blade Rushton turbines)	2	3
Stirrer Diameter, D_i (m)	0.06	0.105
Vessel Diameter, D_v (m)	0.15	0.265
D_v / D_i	2.5	2.52
Liquid height, H_L (m)	0.3	0.545
H_L / D_v	2	2

3.6 SCALE UP STRATEGY: POWER INPUT

The fermentation of aerobic microorganisms in shaking flask has been affected by power consumption in fermentation broth. Power consumption is related to characterizing and upscaling of cultures [109]. Shaking flask experiments constitute the biotechnological development to determine optimal medium composition or to find suitable microbial strain. In this study, a new method, introduced by Büchs, was used to find specific power consumption in a shaking flask on rotary (orbital) shaker [110]. It has been demonstrated that the specific power consumption in shaking flask at low viscosity may generally be defined by the modified power number (or Newton number) which is a function of the flask Reynolds number, according to following equation:

$$Ne' = \frac{P}{\rho . N^3 . D^4 . V_L^{1/3}} = C . f(Re) \quad (3.3)$$

The operating conditions in shaking flask was found as “in-phase” which means the bulk of the liquid within the flask circulates same way with the shaking table. According to the Büch [110], the modified Newton number (*Ne’*) is plotted over the flask Reynolds number (*Re*) using 2143 measuring points of shaking flasks results in a closed form (Figure 3.4).

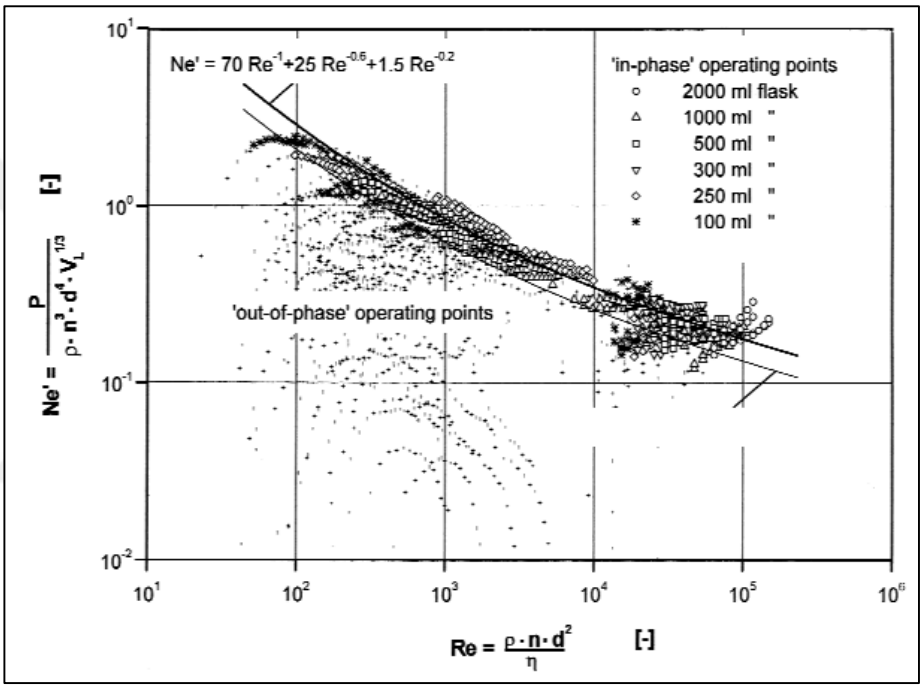


Figure 3.4. Closed presentation of all measuring points (2143) for unbaffled shaking flasks in form of the modified Newton number (Ne’) dependent on the flask Reynolds number (Re) with variation of flask size, viscosity, shaking diameter, and shaking frequency. The final differentiation of “in-phase” (large symbols) and “out-of-phase” (small cross symbols) operating conditions [110]

These “in-phase” points are fitted using a least-square-error method resulting in the following equation:

$$Ne' = 70\,.\,Re^{-1} + 25\,.\,Re^{-0.6} + 1.5\,.\,Re^{-0.2} \tag{3.4}$$

This correlation consists of a laminar (Re^{-1}), a transition ($Re^{-0.6}$), and a turbulent term ($Re^{-0.2}$) analogous to well-known hydrodynamic problems. Using the fitted curve Eq. (3.4) and the definition of the modified Newton number (*Ne'*) according to Eq. (3.3), the specific power consumption of favorable operating conditions (in-phase system) can now be determined in advance thanks to Büchs [109,110].

In aerated system the dimensionless aeration number (Na) can be associated to the ratio of the effective power applied in aerated and non-aerated systems. These correlations were extremely important to the energetic control and to the monitoring of the bioprocess performance, being specific to the geometry and diameter of the impeller [111]. The ratio of power requirements in aerated vs. nonaerated vessels, P_a/P vs. a dimensionless aeration rate N_a:

$$N_a = \frac{Q}{N_i.D_i^3} \tag{3.5}$$

where (Q is volumetric gas rate, N_i and D_i are velocity of impeller and diameter of impeller, respectively) has been correlated as shown in Figure 3.4.

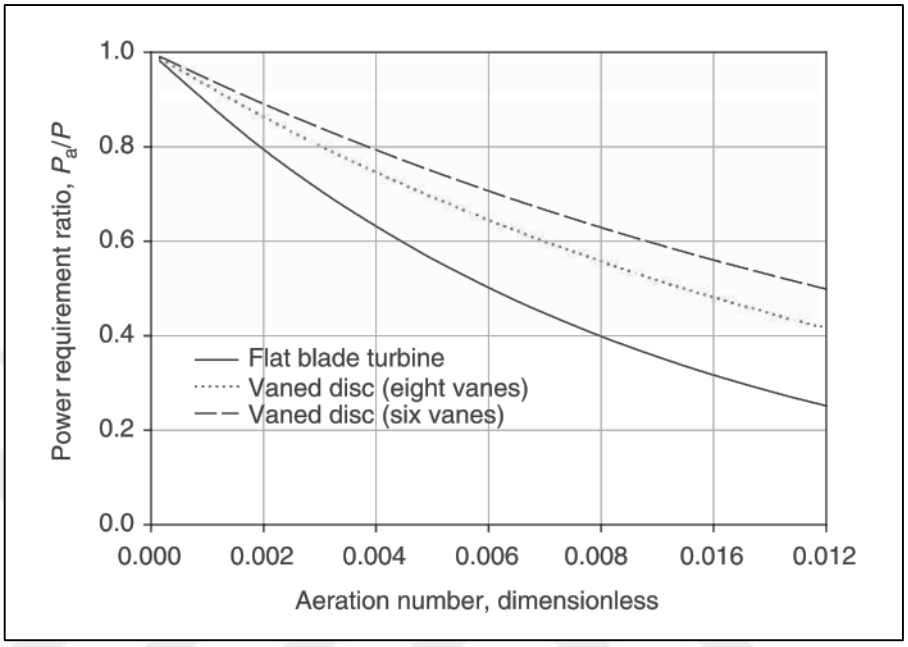


Figure 3.5. Ratio of power requirement for aerated versus non-aerated systems as s function of Na [112]

The combination of power and Reynolds number was the next step for correlating power and fluid-flow dimensionless number, which was to define power number as a function of the Reynolds number which was defined as:

$$Re = \frac{\rho N D_i^2}{\mu} \tag{3.6}$$

where (ρ is fermentation broth average density, N is velocity of impeller in a second; rps, μ is fermentation broth viscosity) plotted as dimensionless power input versus impeller Reynolds number; the plot is known as a power graph. The plot is presented in Figure 3.5.

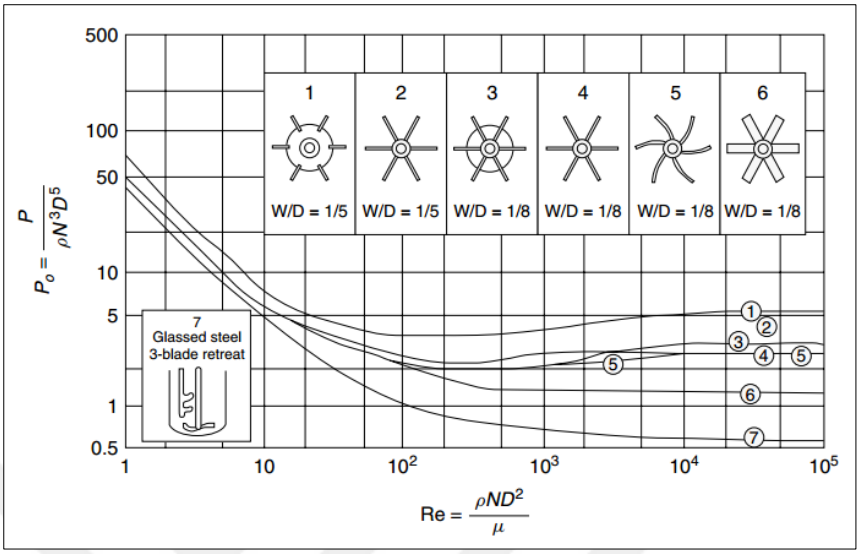


Figure 3.6. Power number vs. Reynolds number (impeller) for various impeller geometries [112,113]

After define the Reynolds number from Eq. 3.4, if it is greater than 10^4, the flow is turbulent [111,112,114]. Based on a Reynolds number, the power number is defined from Figure 3.5.

The power number is a dimensionless number that is the ratio of ungassed power to gassed power in a normal bioreactor.

$$P_0 = N_P\, \rho\, N_i^3 D_i^5 \tag{3.7}$$

where P_0 is ungassed power, in W or hp. Then the defined the P_0 from power number equation (Eq. 3.5), using the plot of P_g/P_0 versus Na (Figure 3.4), the ratio of gassed power to ungassed power is defined. Finally, for power input value as a gassed power per unit volume, the ratio of P_g(gassed power) to fermentation (V) is used as following:

$$\frac{P_g}{V_L} = Watt/m^3 \tag{3.8}$$

4. RESULTS

4.1 IDENTIFICATION OF STRAIN

To identify the strain, FAME Analysis (Table 4.1), BIOLOG (Figure 4.1) and 16S-rRNA sequencing analysis (Figure 4.2) were performed.

When compared fatty acid profiles of MBI5 and reference BS, The major cellular fatty acids in MBI5 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45,00%) and $C_{16:0}$ iso, 12,65% and minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.60%), $C_{16:0}$ (1.72%), $C_{17:1}$ iso ω10c (1,43%). In contrast, the reference *B. sphaericus* has different amounts of fatty acids. Besides, another fatty acid (14:0 iso 3OH) only appears at reference *B. sphaericus*. Consequently, significant similarities in fatty acids profiles were found between *B. sphaericus* YS73 and MBI5. Both MBI5 and *B. sphaericus* YS73 were identified with MIDI as *Bacillus-sphaericus*- GC subgroup E.

Table 4.1. Comparison of Bs MBI5 fatty acid profiles with reference *B. sphaericus* YS73

Numeric Names of Fatty Acids (Peak names)	**Percent % MBI 5**	**Percent % *B. sphaericus* YS73**
14:0 iso	2.02	1.26
14:0	0.60	0.85
15:0 iso	45	46.61
15:0 anteiso	10.87	7.89
14:0 iso 3OH	-	1.05
16:1 w7c alcohol	9.93	6.80
16:iso	12.65	5.48
16:1 w11c	3.31	5.62
16:0	1.72	1.64
17:1 iso w10c	1.43	4.92
Sum In Feature 4	1.65	2.58
17:0 iso	6.11	10.86
17:0 anteiso	4.70	4.45
18:1 w9c	-	-

BIOLOG assay results revealed that each bacteria use different metabolites which can be seen from the changes of microplates. With Biolog GP plates, positive identifications were obtained after 48 h incubation. MBI 5 was performed 0.470 similarities with *B. sphaericus* YS73. The results of FAME and BIOLOG analysis are not enough to have an accurate decision about bacterial species so that 16S-rRNA gene sequencing was performed for the identification of bacterial strains.

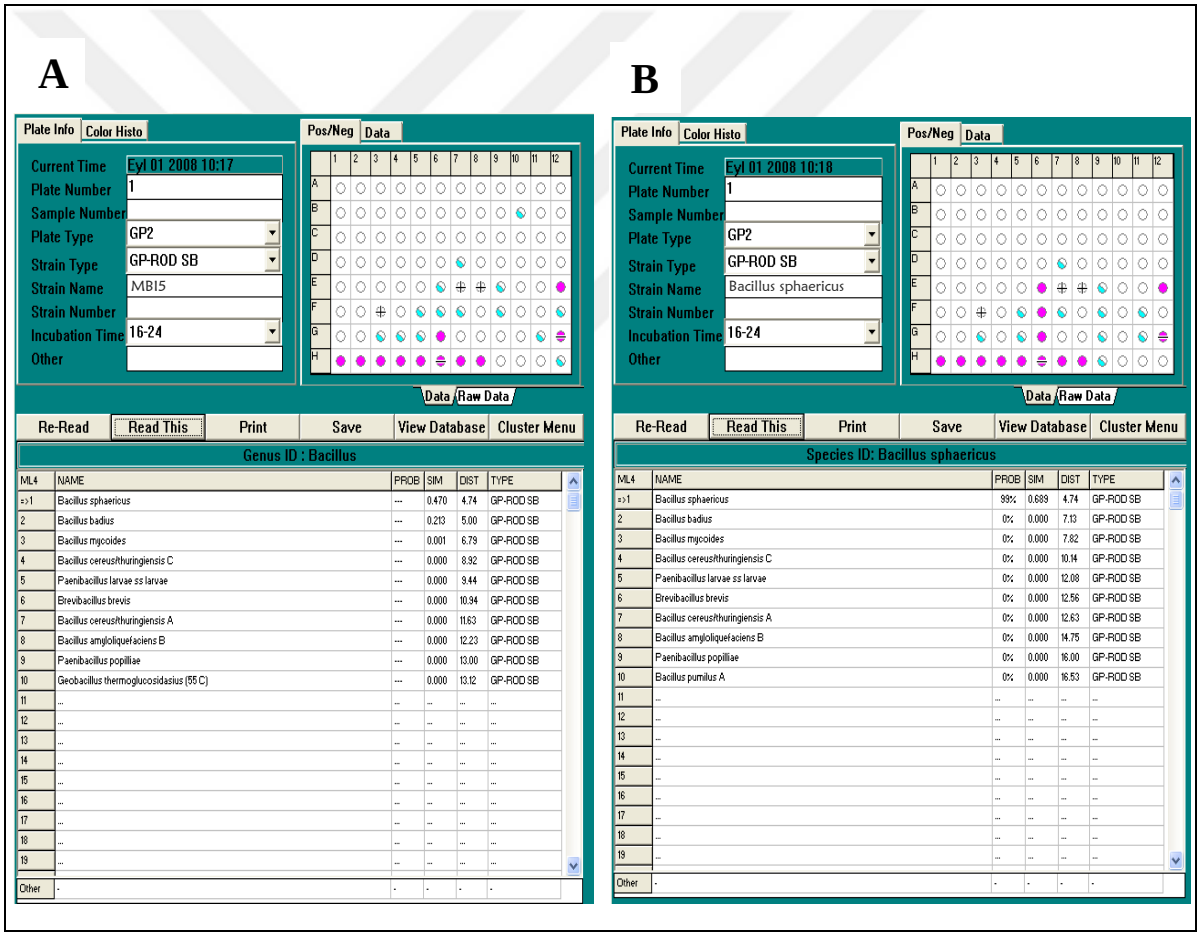


Figure 4.1. The results of BIOLOG micro assay of Bs MBI5 (A) and reference *B. sphaericus* YS73 (B)

According the sequencing results and phylogenetic tree, MBI5 differ from known *Bacillus* species. All these results show that MBI5 is a new strain that have remarkable mosquitocidal effects against larvae.

Moreover, molecular identification of MBI5 strain was performed. Firstly, genomic DNA isolated from MBI5 (Figure 4.3). All genomic DNA of strains isolated well.

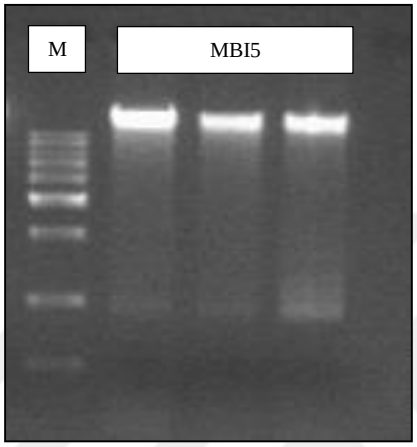


Figure 4.2. Agarose gel result of genomic DNA isolation from MBI5 and the marker (10 kb)

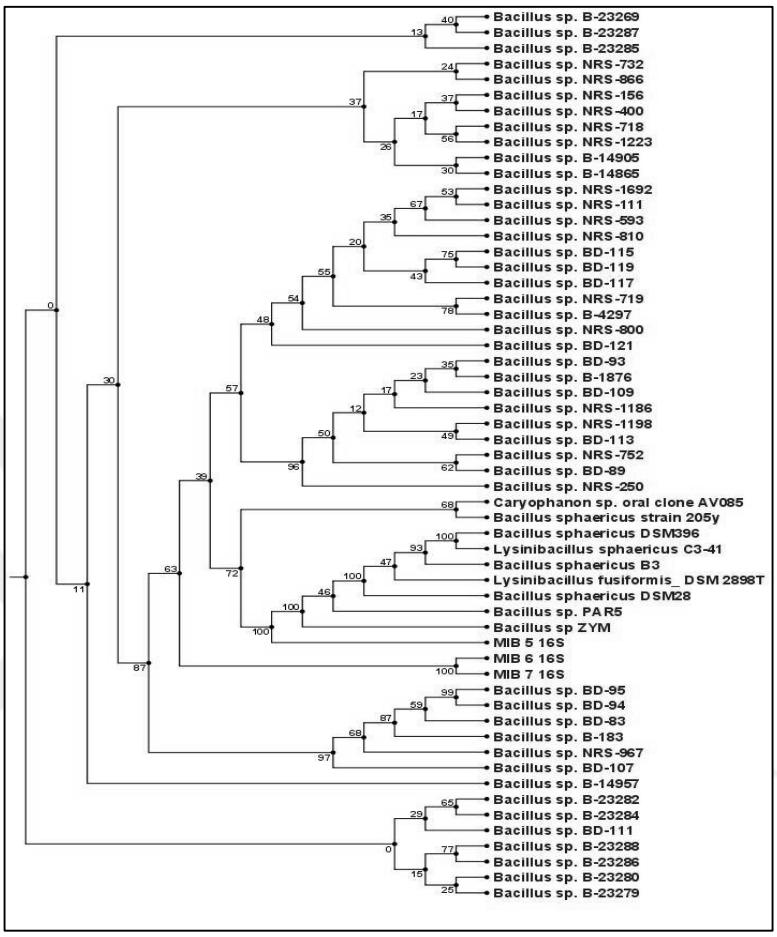


Figure 4.3. Phylogenetic tree of Bs MBI5 according to 16S-rRNA gene sequencing

The genes were cloned from the bacterium (MBI5) and considered the genome of MBI5 to perform further experiments such as molecular cloning and protein isolation. After isolation of genomic DNA from MBI5, gradient PCR was performed using new designed primers for both BinA and BinB genes to find out best Tm values (Figure 4.4). According to results, the 55 °C was found to be the optimum temperature degree for both genes.

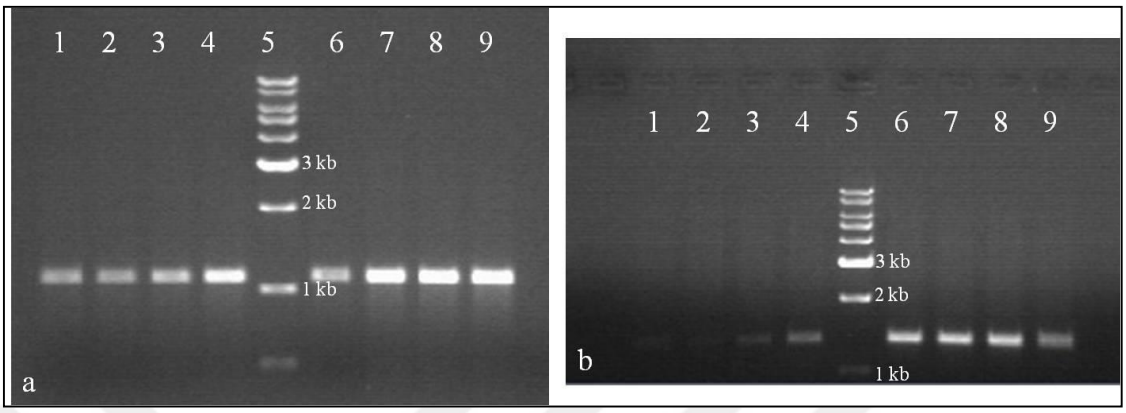


Figure 4.4. Agarose gel result of gradient PCR results. (a) BinA gene, (b) BinB gene. Line 1, 2, 3, 4, 6, 7, 8, 9 indicate the annealing temperature 48, 48.5, 49.5, 50.9, 52.8, 54.4, 55.4, 56 °C, respectively. Line 5 shows the marker (10 kb)

4.2 GROWTH KINETICS

On the basis of the experimental data, a batch fermentation kinetic model was tried to develop the production of *B. sphaericus* MBI5. The main media components were presented in Table 4.2. Related to each glucose concentration growth graphs were figured out at Figure 4.5. All of the batches exponential growth was observed for long period (4-12h), followed by a decelerating growth phase and afterwards, the cultures entered stationary phase. Following the initial 12h, protease production increased with increase in cell mass and seemed to be growth associated. Maximum cell growth was obtained at 2 h. However, maximum protein amount was secreted during the decelerating growth phase and subsequent stationary phase signifying non-growth associated production phase.

Table 4.2. Media contents for kinetic studies of *B. sphaericus* MBI5

Component(g/L)	**KNT 0.1**	**KNT 0.5**	**KNT 1**	**KNT 5**	**KNT 10**	**KNT 20**
Glucose	0.1	0.5	1	5	10	20
Yeast extract	0.2	0.2	0.2	0.2	0.2	0.2
$(NH_4)_2SO_4$	2	2	2	2	2	2
$MgSO_4.7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2
KH_2PO_4	2	2	2	2	2	2

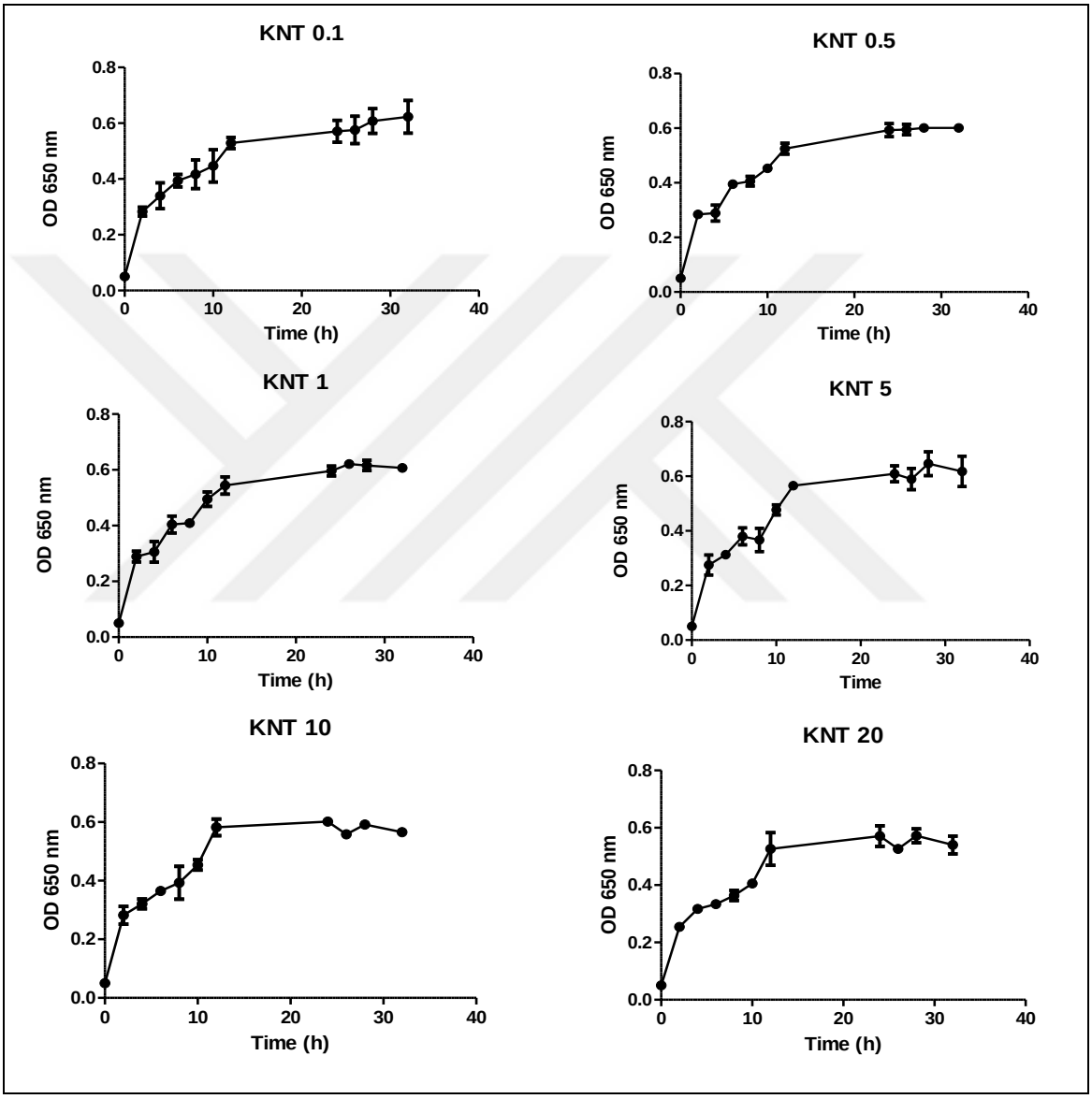


Figure 4.5. Growth scheme of Bs MBI5 on different glucose concentration

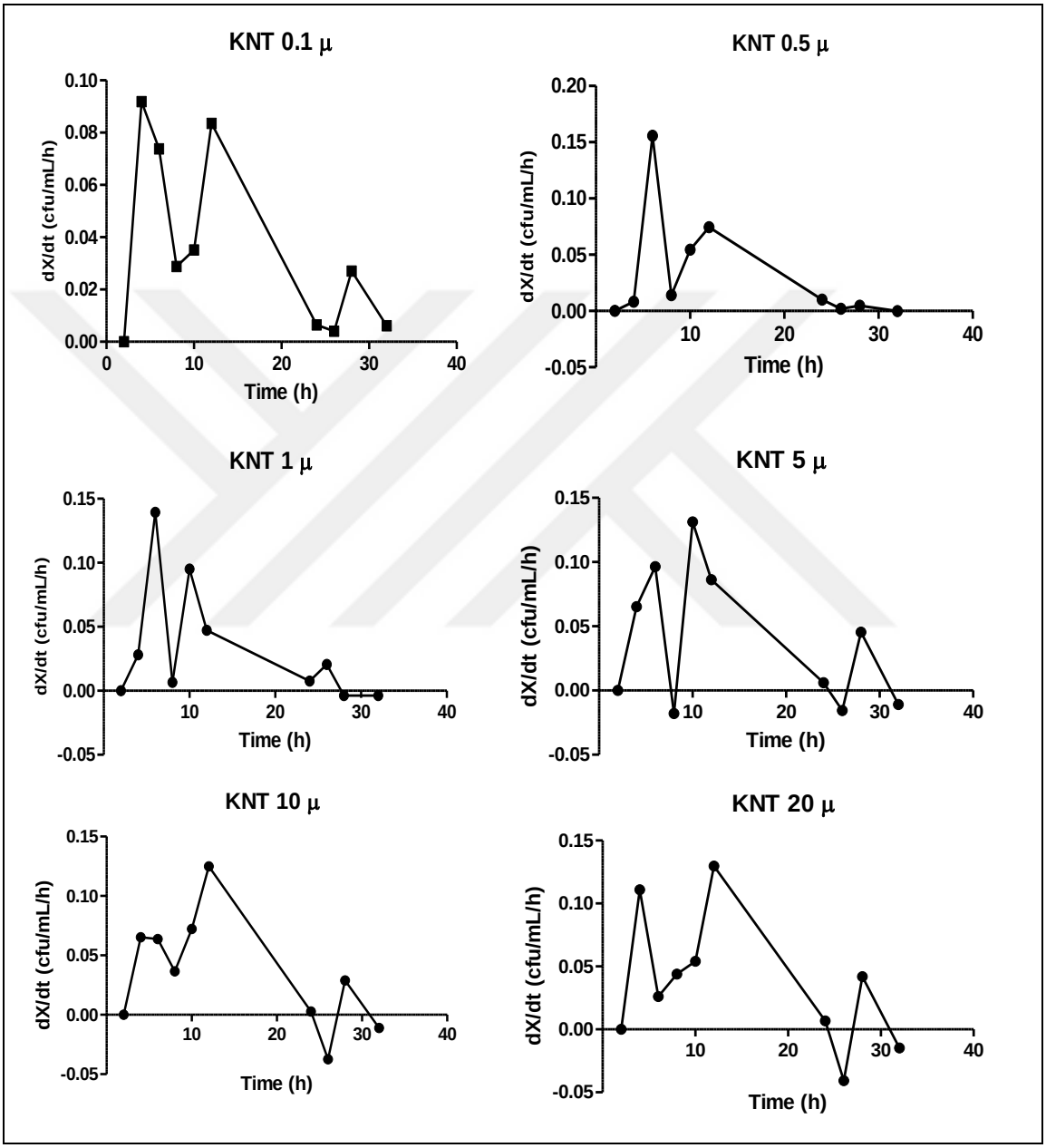
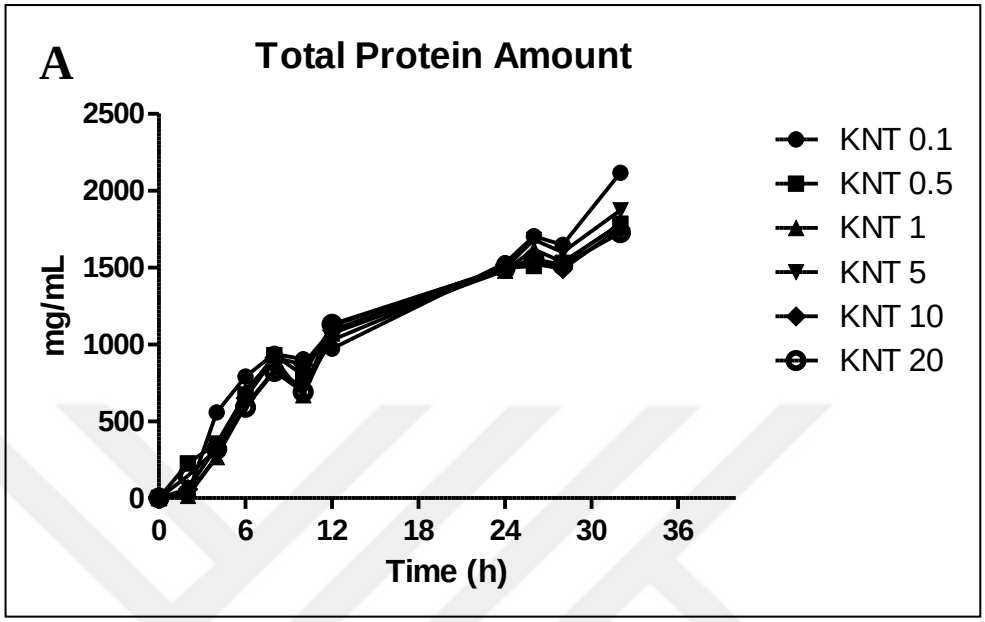


Figure 4.6. Rate profiles of growth (dX/dt) for different glucose concentration media



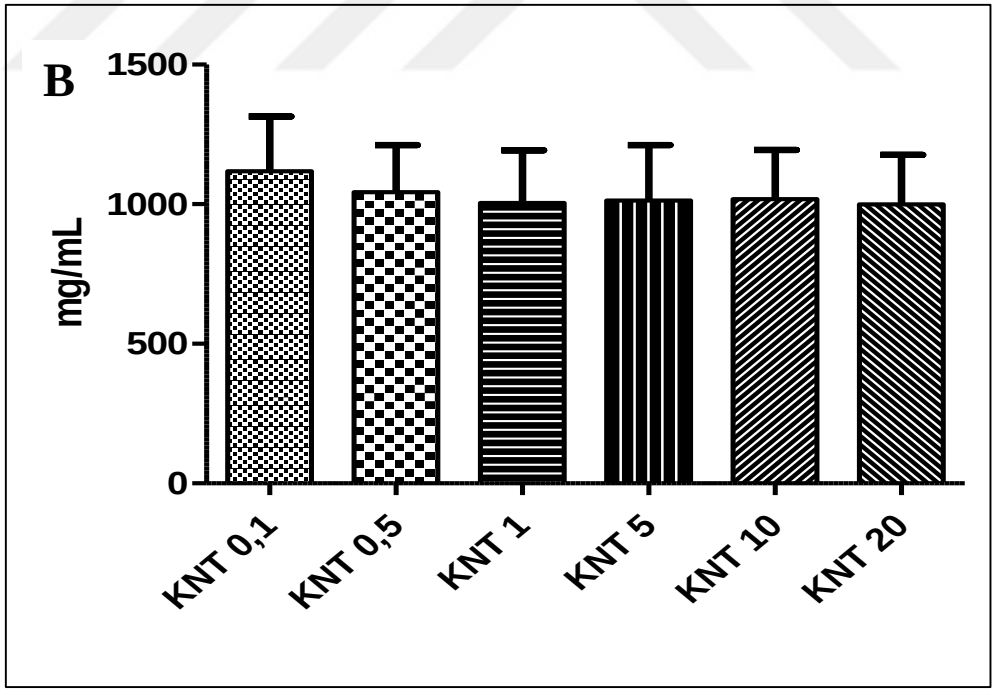


Figure 4.7. Total protein amount of Bs MBI5 during different glucose concentration A) Total protein production regime depends on time, B) Total protein amount end of the cultivation

Table 4.3. Effect of initial glucose concentration on biomass and protein production by Bs MBI5

Initial Glucose Concentration (mg mL^{-1})	**X_{max} (Log cfu mL^{-1})[1]**	**P_{max} (mg mL^{-1})[2]**	**$Y_{P1/S}$ (Log cfu mg^{-1})[3]**	**$Y_{P2/S}$ (mg mg^{-1})[4]**	**Productivity (Log cfu mL^{-1} h^{-1})[5]**
0.1	7.493 (at 32 h)	2115.7	74.93	2.12E+04	0.23
0.5	7.478 (at 28 h)	1783.4	14.96	3.57E+03	0.27
1	7.492 (at 26 h)	1777.9	7.49	1.78E+03	0.29
5	7.509 (at 28 h)	1872.3	1.50	3.74E+02	0.27
10	7.478 (at 24 h)	1752.3	0.75	1.75E+02	0.31

On plotting the rate of cell growth (dX/dt) as a function of fermentation time as presented in Figure 4.6, it was observed that the all of the glucose concentration growth rate was reached peak before fermentation of 12 h.

Although the highest yielding cell amount and protein amount were observed 74.93 Log cfu mg^{-1}, 2.13E+04 mg mg^{-1} at 0.5mg mL^{-1} glucose concentration, the 105mg mL^{-1} glucose concentration's fermentation time of 24 h a, the productivity of 0.31 Log cfu mL^{-1} h^{-1}was considered to be optimum (Table 4.3).

In batch fermentation of different glucose concentration media, cell growth and protein amount increased simultaneously. However, the growth rate was decreased at stationary phase, the protein amount was secreted during post stationary growth phase (Figure 4.7).

[1] X_{max}: Maximum cell growth, data were presented as Log_{10}.
[2] P_{max}: Maximum total protein production.
[3] $Y_{P1/S}$: Yield coefficient of cell growth based on glucose.
[4] $Y_{P2/S}$:Yield coefficient of total protein Formation based on glucose.
[5] Productivity was calculated at the time when cell (P1) reached X_{max}.

4.3 BIOPESTICIDE PRODUCTION MEDIUM

Biopesticide production by Bs MBI5 was studied through submerged fermentation of different substrates. Each of substrates were taken in 500 mL shake flask and 100 mL of selective production medium was mixed. Firstly, to find growth profile and optimized various parameters required for maximum biopesticide production were used commercial media. Also the commercial media were combined some minerals to increase the biomass production and induction of spore production. The selection of a substrate for large-scale enzyme production by fermentation depends on its availability and cost. In this regard, secondly several low cost agro residues were used for production of biopesticide.

4.3.1 Commercial Media Trials

The effect of some commercial media on cell density was studied. The production rate was measured by optical density at 650 nm. According to the optical density, the maximum biomass was observed in TSB medium at 48h (Figure 4.8).

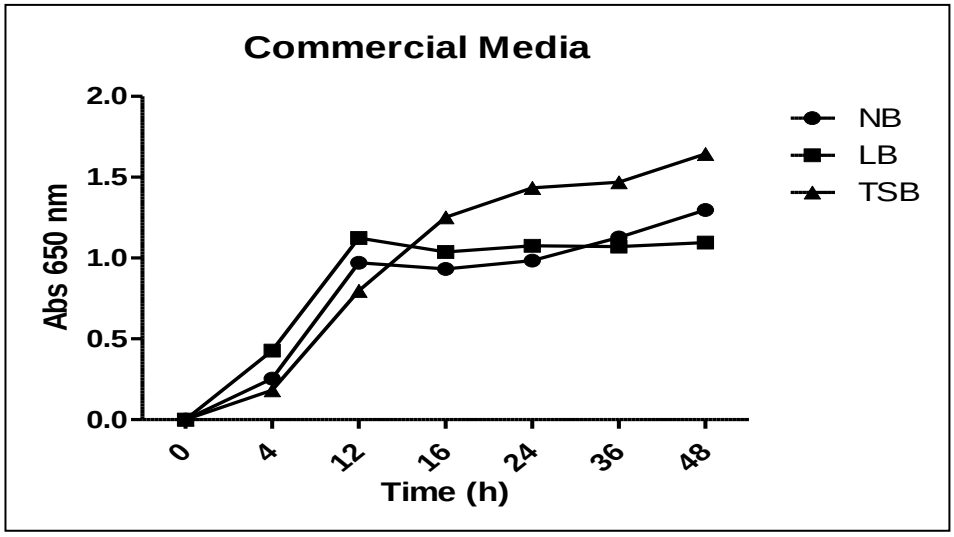


Figure 4.8. Growth profile of Bs MBI5 on standard media

4.3.2 Effect of Mineral Supplementation for Commercial Media

Evaluation of mineral effects on biopesticide production, magnesium sulphate (0.5 g/L), manganese sulphate (0.03 g/L) and their combination was performed to add to commercial media. The addition of minerals was increased the cell density according to mineral free media which means high biopesticide production.

After mineral addition into NB, the $MgSO_4$ and its combination with $MnSO_4$ were showed the high cell density (Figure 4.9).

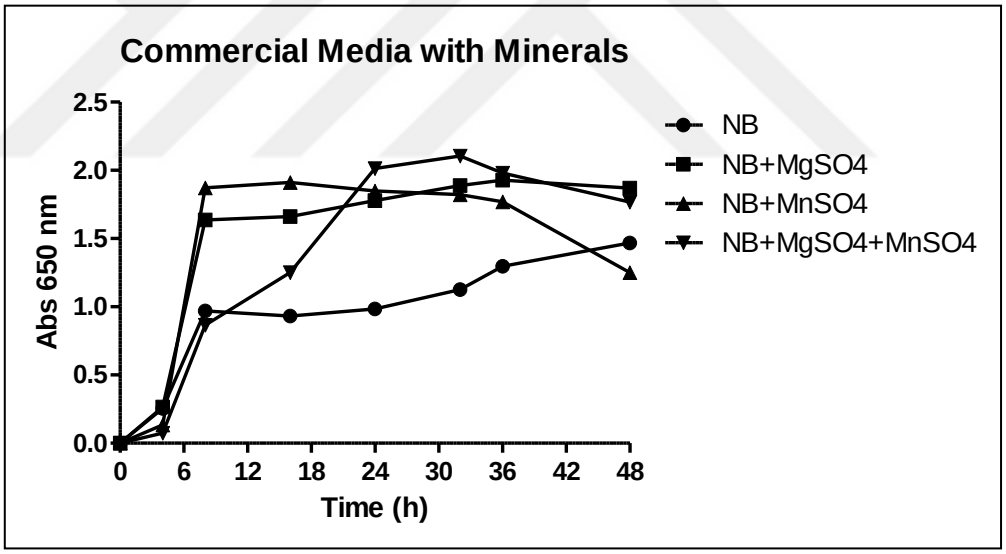


Figure 4.9. Growth profile of Bs MBI5 on NB and NB+minerals additive

The three minerals combination in TSB was increased the cell density according to mineral free TSB (Figure 4.10). Similar results were obtained in LB medium (Figure 4.11).

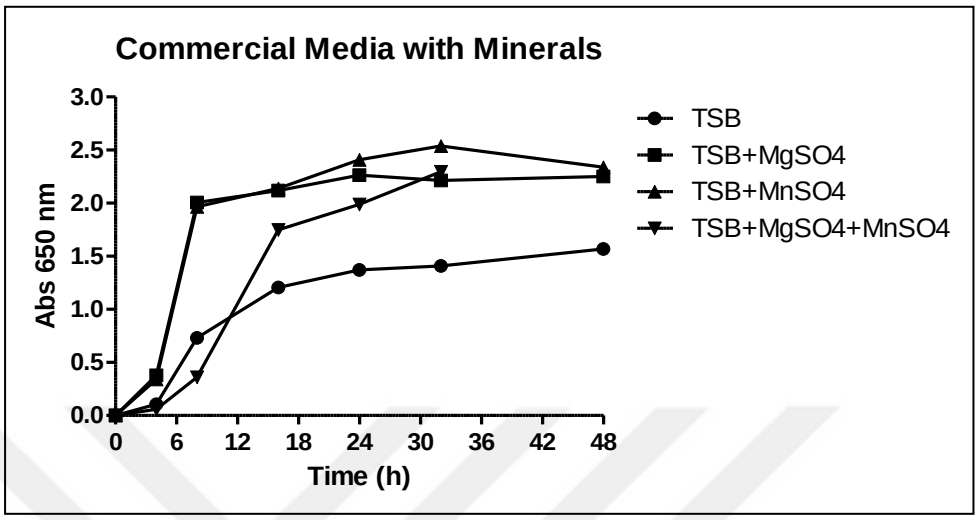


Figure 4.10. Growth profile of Bs MBI5 on TSB and TSB+minerals additive

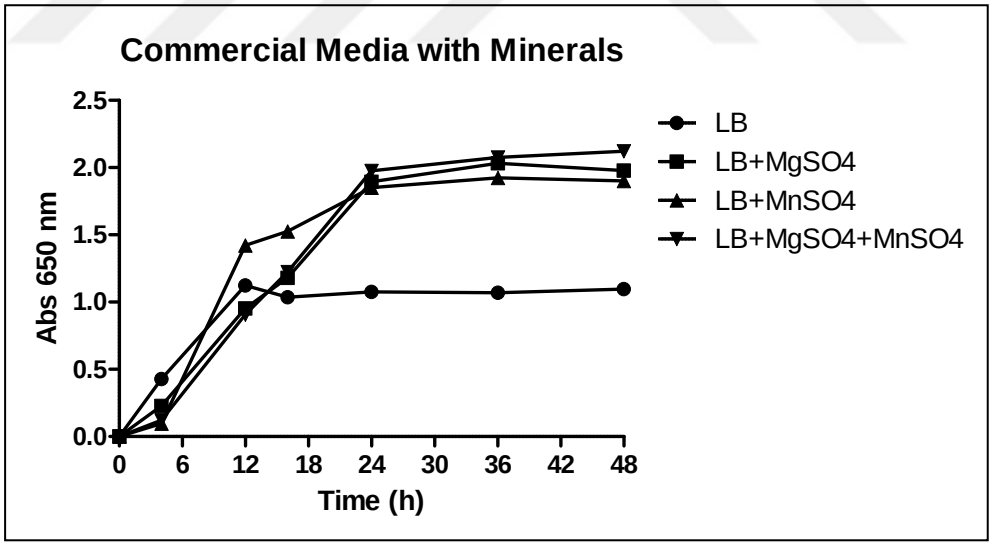


Figure 4.11. Growth profile of Bs MBI5 on LB and LB+minerals additive

4.3.3 Complex Media Trials

The complex media trials were started with determination of molasses types' effect on production. MAcid and MPhys media were used 5% and 10% ratio on production of biopesticide. The MPhys had greater influence on cell density as compared the molasses type tested. 10% MPhys medium showed highest effect on biopesticide production (Figure 4.13). When MAcid media were used, they had so negative effect on production and the cell density was nearly zero point at 650 nm, as shown figure 4.12.

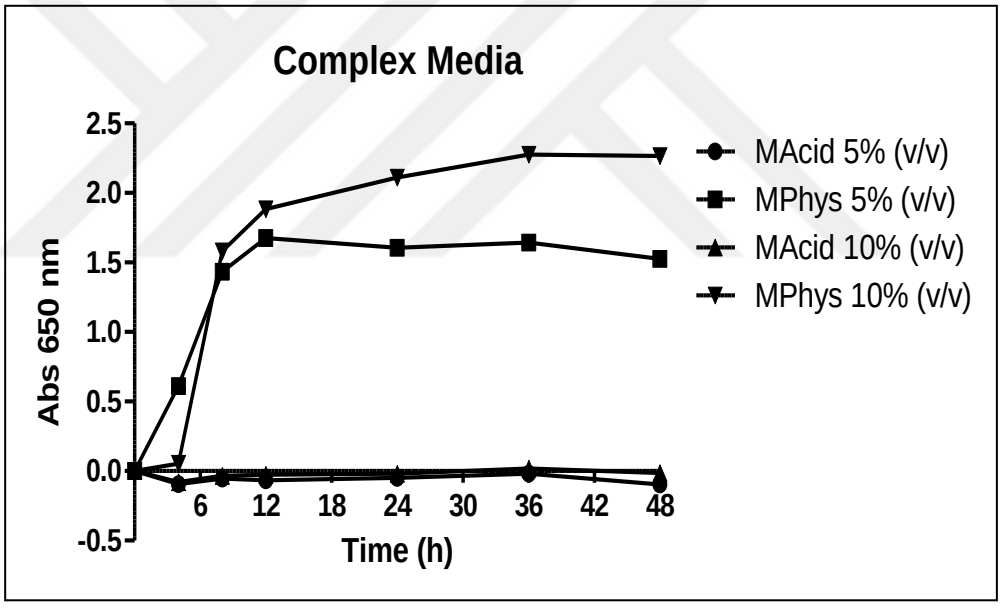


Figure 4.12. Growth profile of Bs MBI5 on molasses and some other by-product combinations

CSL (20%, v/v) and whey (20%, v/v) were added into the different molasses type media as nitrogen sources. CSL addition into both molasses type showed a good effect on cell density. Whey addition showed a meaningful activity with MPhys medium on growth (Figure 4.13).

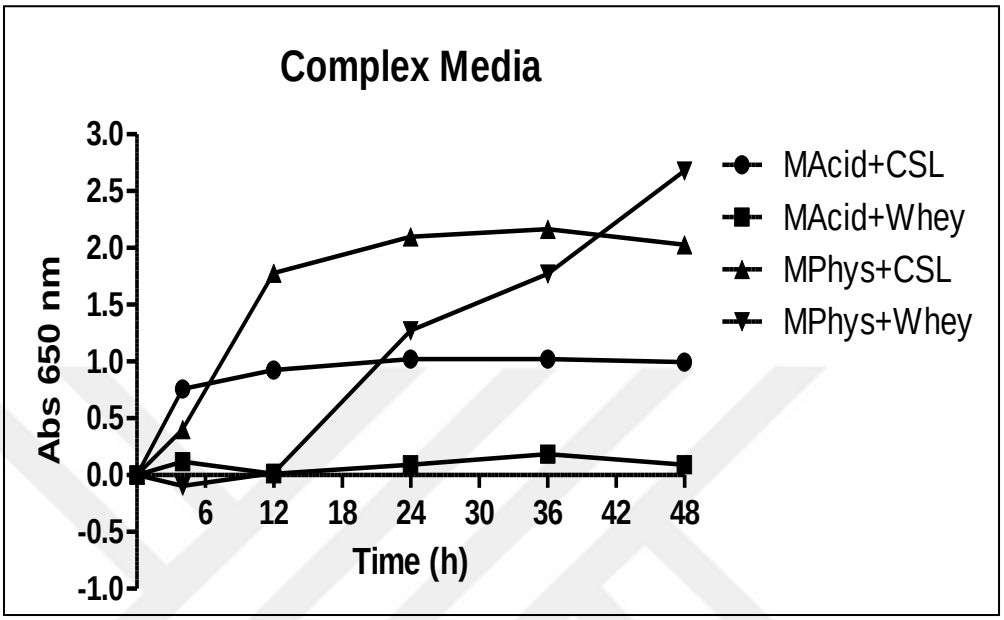


Figure 4.13. Growth profile of Bs MBI5 on molasses and some other by-product combinations

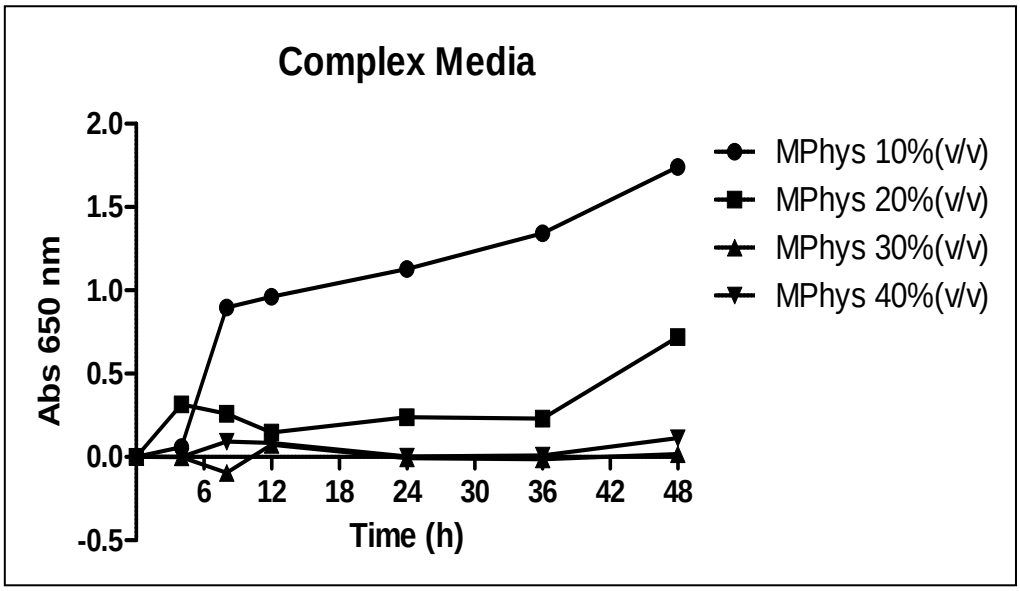


Figure 4.14. Growth profile of Bs MBI5 on different amount of molasses physical

The amount of MPhys media showed a critical role on production as a carbon source. The analysis showed that there was an inverse relationship between the production of biopesticide and amount of molasses. Cell density reached the highest amount at 10% MPhys concentration (Figure 4.15).

The effect of nitrogen source amount was studied using from 10% to 50% (v/v) concentration CSL media. The higher cell density was seen up to 40% (v/v) CSL amount (Figure 4.15).

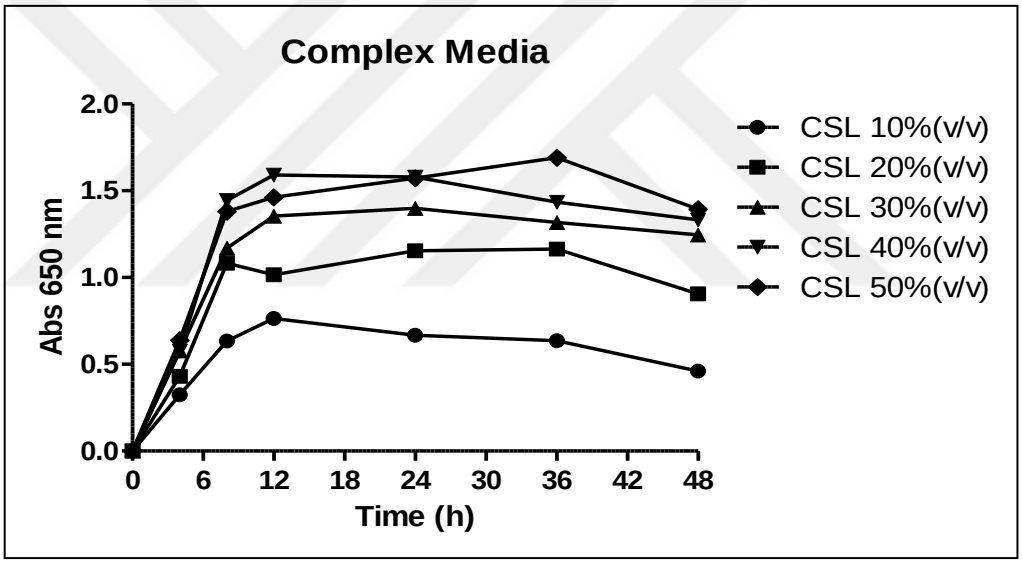


Figure 4.15. Growth profile of Bs MBI5 on different amount of CSL

4.3.4 Effect of Mineral Supplementation for Complex Media

Magnesium sulphate (0.5%, g/L) was added into MPhys (10%, v/v) plus CSL (40%, v/v) and MPhys plus whey (20%, v/v). The $MgSO_4$ had significant effect with MPhys plus CSL medium on cell density at 48h. Whey usage as a nitrogen source did not give good results on growth (Figure 4.16).

When assessed supplementation of potassium salts (KH_2PO_4, 0.25 g/L; K_2HPO_4, 0.25 g/L) and sodium salts ($NH_4H_2PO_4$, 0.25 g/L; NH_4HPO_4, 0.25 g/L), potassium salts increased the cell density (Figure 4.17). Both salts were added into MPhys (10%, v/v), CSL (40%, v/v), and $MgSO_4$ (0.5%, v/v) medium combination.

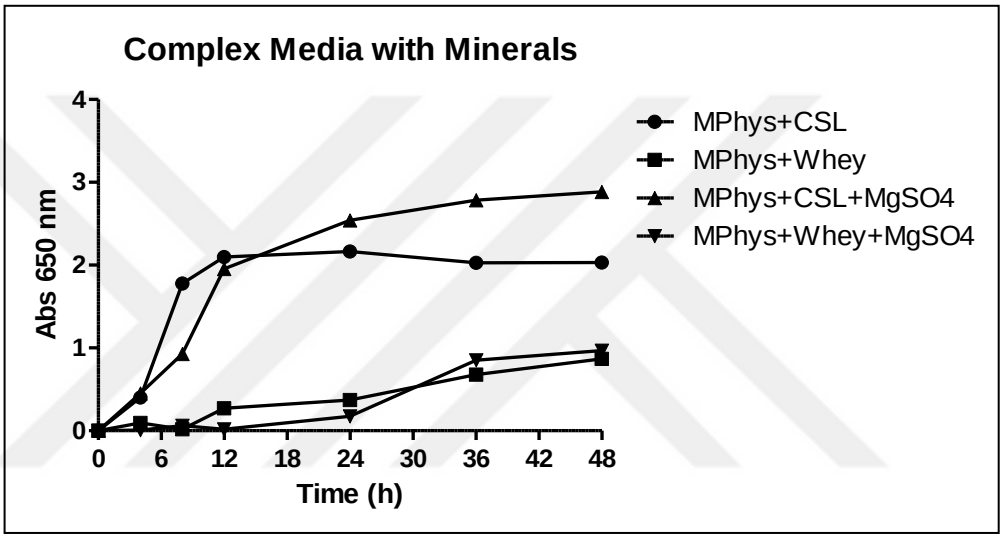


Figure 4.16. Growth profile of Bs MBI5 on different complex media with mineral additive

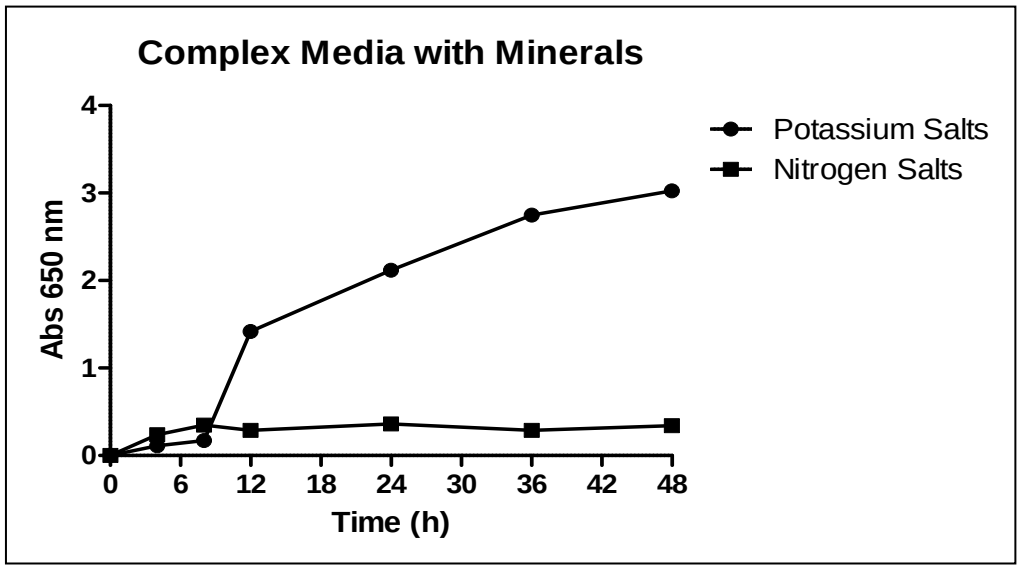


Figure 4.17. Growth profile of Bs MBI5 under effect of potassium and nitrogen salts

4.3.5 Effect of Some Different Sugars For Growth

Influence of different sugars were evaluated using glucose (8 g/L), fructose (8 g/L), sucrose (8 g/L and 16 g/L). The less growth profile was seen on glucose supplemented medium. Molasses physical and sucrose (16 g/L) increased the cell density at 48 h (Figure 4.18).

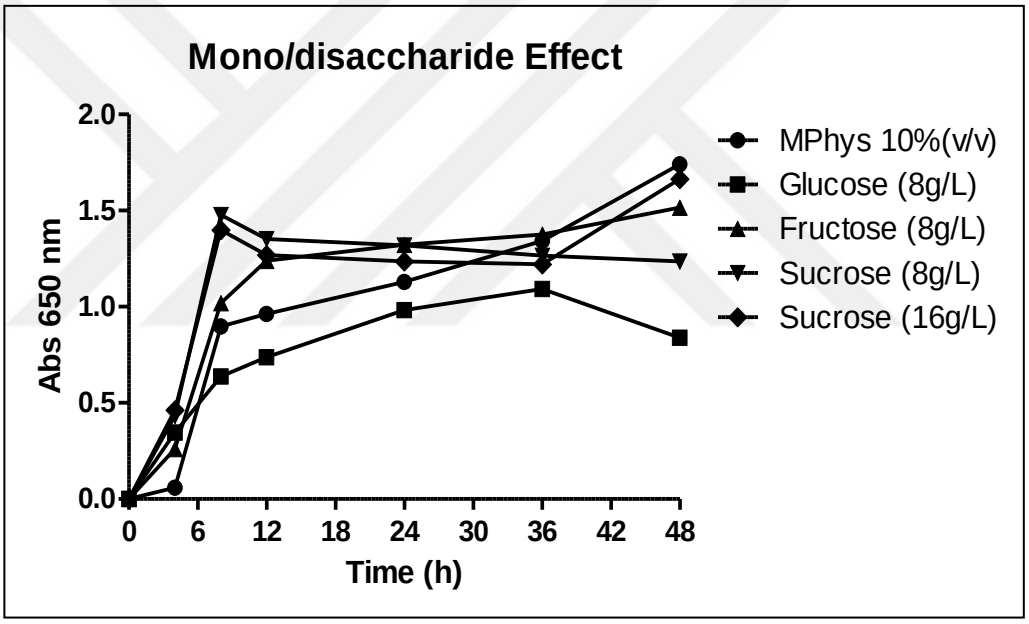


Figure 4.18. Growth profile of Bs MBI5 under effect of different sugars

4.3.6 Effect of Inoculum Size and Age

The effect of inoculum size and age on biopesticide production is shown in Figure 4.19 and 4.20, respectively. . To study the effect of inoculum size, 100 ml of production media were inoculated at a level of 2.0, 5.0, and 10% (v/v) from an 18h old bacterial culture broth. The effect of age on inoculum was studied by inoculating 100 ml of the production medium with 2%

inoculum of 12 to 36h old culture of Bs MBI5. It was depicted that increase in inoculum age and size had not much influence on biopesticide accumulation.

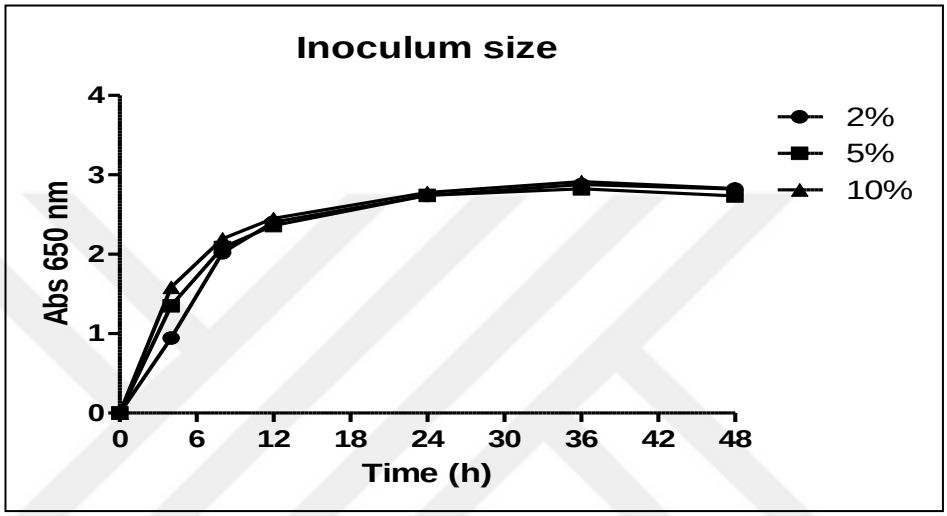


Figure 4.19. Growth profile of Bs MBI5 under effect of different inoculum size

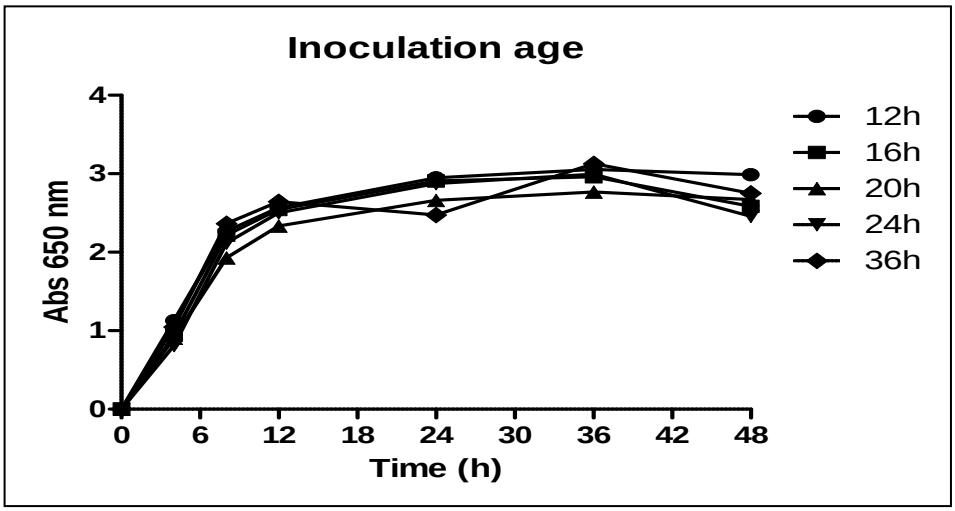


Figure 4.20. Growth profile of Bs MBI5 under effect of different inoculant age

4.4 BIOPROCESS OPTIMIZATION

4.4.1 Statistical Optimization and Validation of The Model Equation

The process was tried to develop, improve and optimize by using RSM which is a combination of mathematical and statistical techniques. It is used to evaluate the relationship between the four variables (MPhys, CSL, $MnSO_4$, and $MgSO_4$) and the three important response (cell dry weight, protease activity, and specific protease activity). The CDW response and the specific protease activity were transformed using power and the inverse square root to obtain a significant model at –1.74 and –0.5 of lambda, respectively. The protease activity response was obtained as a linear model equation using none-transformation (lambda=1.0). The values of CDW were best-fit using a second-order polynomial equation, and the other responses were calculated by a first-degree polynomial equation. The equations of the models based on the coded values are as follows:

$$((\text{CDW (g/L)})^{-1.74} = 0.32+0.049*A-0.011*B+0.00376*C-0.00366*D-0.059*A*B-0.050*A*C-0.0056*A*D+0.049*B*C+0.017*B*D+0.00741*C*D-0.043*A^2-0.050*B^2+0.045*D^2 \quad (4.1)$$

$$\text{Protease activity (mU/mL)} = 497.08 + 83.66*A+80.04*B+55.29*C-3.69*D \quad (4.2)$$

$$1.0/\text{Sqrt}((\text{Specific Protease Activity (mU/mg)}) = 0.027+0.00431*A-0.000612*B-0.00637*C+0.00073*D \quad (4.3)$$

where A is molasses, B is CSL, C is $MnSO_4$, and D is $MgSO_4$.

After the experimental results were finished, the responses value were listed in table 4.4.

Table 4.4. Composition of various experiments of the central composite design and corresponding results on Bs MBI5 growth and protease production

Runs	Factors				Responses		
	A: MPhys (%, v/v)	**B:CSL (%, v/v)**	**C:$MnSO_4$ (%, w/v)**	**D:$MgSO_4$ (%, w/v)**	**Y1: CDW (g/L)**	**Y2: Protease activity (mU/mL)**	**Y3: Specific Protease activity (mU/mg)**
1	40.00	40.00	1.50	1.50	4.293	622.002	1216.960
2	3.79	25.00	0.85	0.85	6.693	391.567	9036.151
3	25.00	46.21	0.85	0.85	3.978	620.269	1772.197
4	10.00	10.00	0.20	1.50	4.713	210.882	540.722
5	10.00	10.00	1.50	0.20	5.582	336.371	43247.7
6	10.00	10.00	0.20	0.20	4.409	200.734	539.284
7	10.00	40.00	1.50	0.20	2.813	567.549	2192.248
8	25.00	25.00	0.85	0.85	3.767	275.730	873.793
9	40.00	40.00	0.20	0.20	3.411	575.717	737.048
10	25.00	25.00	0.85	-0.07	2.562	576.459	1740.984
11	40.00	40.00	0.20	1.50	3.866	605.913	822.507
12	40.00	40.00	1.50	0.20	5.784	678.435	1403.658
13	25.00	25.00	0.85	0.85	3.058	535.867	1799.553
14	46.21	25.00	0.85	0.85	3.704	606.408	769.771
15	40.00	10.00	0.20	1.50	2.258	364.622	729.245
16	10.00	40.00	1.50	1.50	2.402	608.636	3445.109
17	25.00	25.00	0.85	1.77	2.618	553.688	1894.750
18	10.00	40.00	0.20	0.20	8.511	383.151	1306.197
19	10.00	10.00	1.50	1.50	7.849	372.260	4589.513
20	40.00	10.00	1.50	1.50	3.160	55.133	1162.171
21	25.00	25.00	0.85	0.85	2.533	572.251	1290.793
22	40.00	10.00	1.50	0.20	2.800	597.993	1655.980
23	10.00	40.00	0.20	1.50	7.298	495.275	1375.763
24	25.00	25.00	1.77	0.85	2.980	532.154	1565.160
25	25.00	25.00	0.85	0.85	3.296	585.617	1488.857
26	25.00	25.00	0.85	0.85	3.016	558.391	1570.474
27	25.00	3.79	0.85	0.85	7.096	446.020	1338.059
28	25.00	25.00	0.85	0.85	2.898	505.918	1512.711
29	40.00	10.00	0.20	0.20	1.987	540.322	1061.769
30	25.00	25.00	-0.07	0.85	3.869	433.149	790.738

The responses were verified using ANOVA for each factor (Table 4.5). The *P* values ($P<0.1$, statistically significant) indicated that the models were significant at a high confidence level for

each response. The lack of fit values (1.91 for CDW; 0.25 for protease activity; and 3.22 for specific protease activity) were not significant with respect to their corresponding pure error.

The relationship between the response and the experimental variables were illustrated graphically by plotting the perturbation and the three-dimensional response surface plots in accordance with two factors while the two others remained constant at their mean level. The simultaneous effect of all the factors on responses can be compared by the perturbation plots. CDW was not clearly affected by changing the substrates. A decreasing rate of molasses concentration was important for biomass generation. CSL concentrations at low and high values had a significant effect on CDW. Manganese had no effect on CDW, but the concentration of magnesium at low and high values was associated with a slight difference in CDW (Figure 4.21a). On the other hand, magnesium showed no effect on protease activity. However, CSL and manganese showed a strong effect on protease activity by changing molasses (Figure 4.21b). In literature, the metal ions, Ca^{2+}, Mn^{2+} and Mg^{2+}, have provided superior effect on alkaline proteases produced different *Bacillus* sp.. In our previous one variable a time studies, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and Ca^{2+} ions have been tested on enzyme activity, however the alkaline protease was stimulated by Mn^{2+} and Mg^{2+}. Exact molasses and manganese concentrations were important for specific protease activity. The changing of CSL and magnesium had a lower influence than molasses and manganese on specific protease activity (Figure 4.21c).

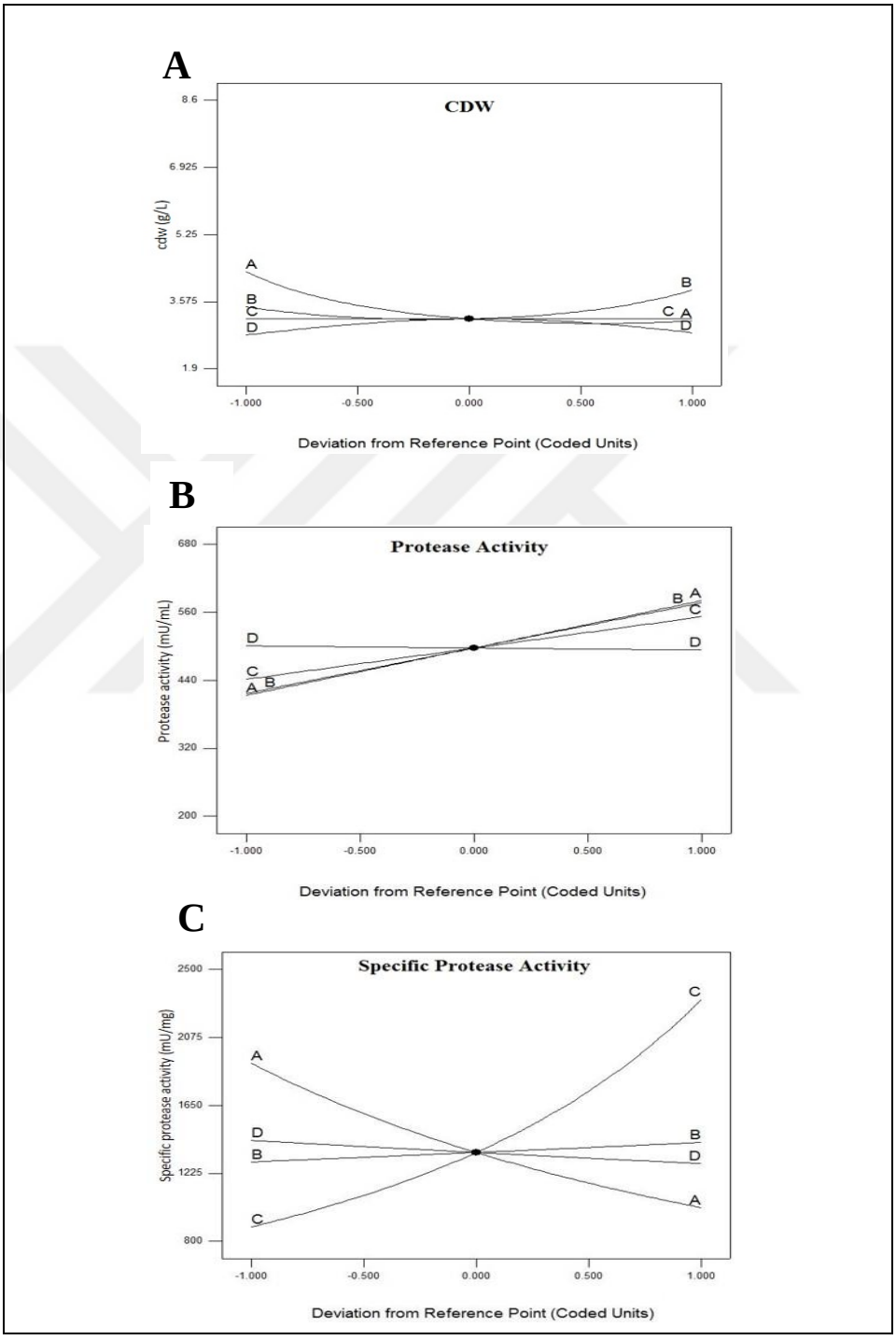
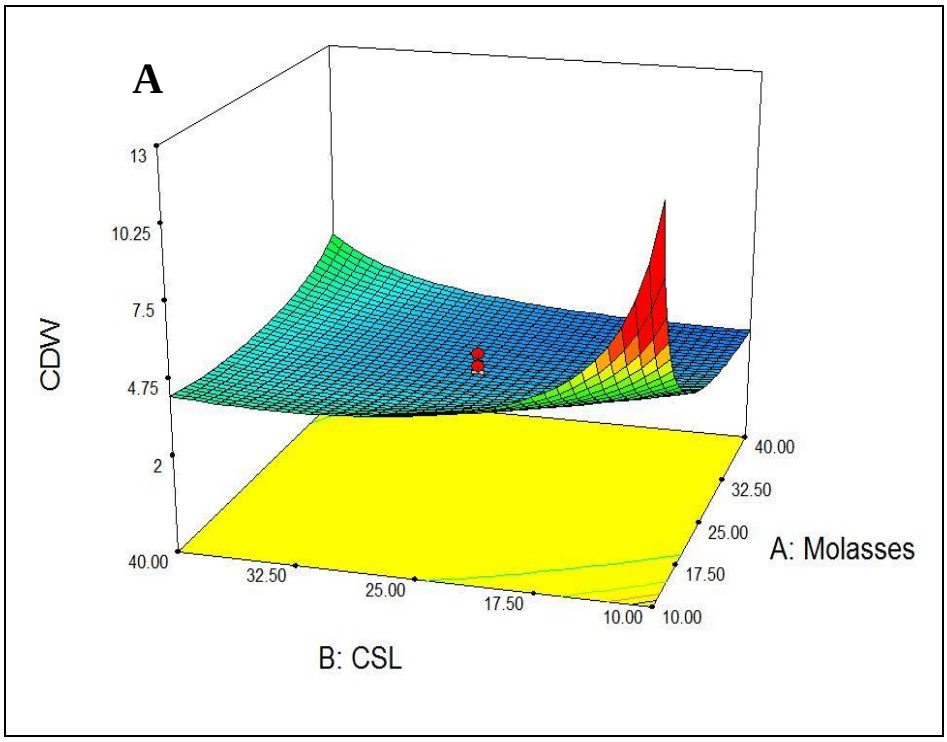


Figure 4.21. Perturbation graphs of A) CDW, B) protease activity, and C) specific protease activity showing the effect of variables on responses (A is molasses at 25 %; v/v, B is the CSL at 25 %; v/v, C is the $MnSO_4$ at 0.85 %; w/v, and D is the $MgSO_4$ at 0.85 %; w/v).

The interactions between the factors visualised in the three-dimensional (3-D) response contour and surface plots graphs (Figure 4.22) have a statistically significant effect on at least one of the responses. According to observations of substrates effect on CDW, protease and specific protease activities, Figure 4.22a and 4.22b were prepared for CDW and protease activity by molasses and CSL, and 2c was demonstrated for specific protease activity by molasses and $MnSO_4$. It can be clearly observed that the concentration of molasses and CSL at low levels had high CDW values (Figure 4.22a). On the other hand, the activity of protease was at higher values under increasing molasses and CSL concentrations (Figure 4.22b). Specific protease activity was strongly influenced by increasing concentrations of manganese, and slightly changed by molasses (Figure 4.22c). The data showed that manganese may play a role as a cofactor for protease. The combination of CSL and molasses was important for protease production because of enriched protein and energy source contents, respectively; however, they did not have a strong influence on specific protease activity.



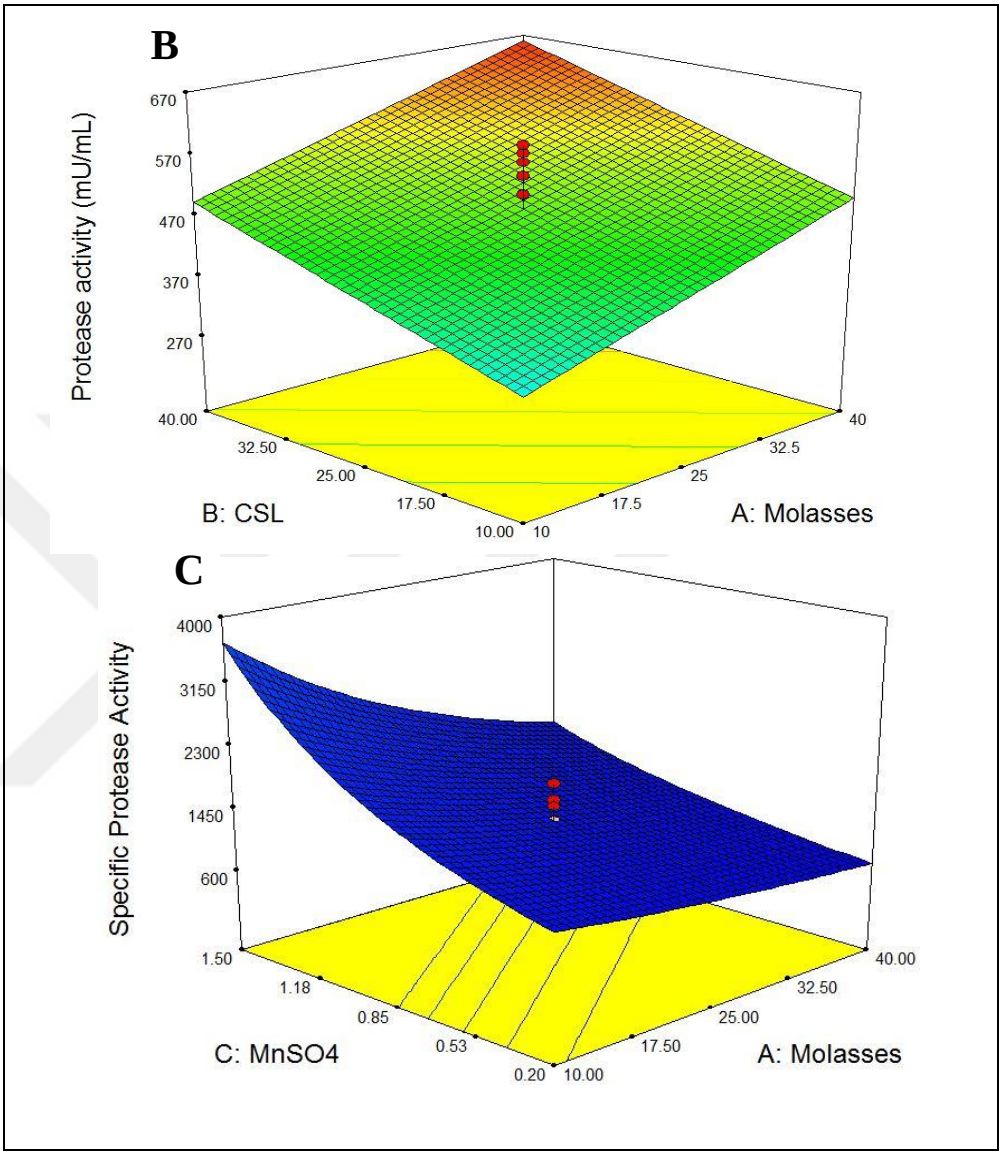


Figure 4.22. Three-dimensional surface plots of A) CDW (g/L) by molasses and CSL, B) Protease activity (mU/mL) by molasses and CSL, C) Specific protease activity (mU/mg) by molasses and $MnSO_4$ showing the effect of the interactions ($MnSO_4$ (0.85 %; w/v) and $MgSO_4$ (0.85 %; w/v) were constant in a) and b); CSL (25 %; v/v) and $MgSO_4$ (0.85 %; w/v) were constant in c)).

Numerical optimization was carried out to set goals for each response at maximum, while the other variables were in the different perspectives, such as constant range, minimisation or maximisation of each parameter (Table 4.6).

Propositions of optimal design values on molasses and CSL were not as different in each response as were CDW and specific activity of protease. The preferable concentrations of molasses and CSL were different for CDW, protease activity and specific protease activity. Higher values of CDW, protease activity and specific protease activity were observed at lower concentrations of molasses and CSL, higher concentrations of molasses and CSL and lower concentration of molasses with higher concentrations of CSL, respectively. Mineral concentrations had a strong influence on CDW and protease activity. However, even the effect of manganese was observed in perturbation and 3-D plots, and the concentration of CSL was important for optimization on specific protease activity (Table 4.6a).

Specific protease activity and protease activity values are normally changed in a similar pattern. However, their optimised values, especially molasses, were totally different as minimum and maximum values for specific activity and activity of protease, respectively. We concluded that molasses has significant importance for the production of proteinaceous substances. In the commercial production of enzymes, the aim is to increase enzyme activity in the fermentation step. The perturbation plots demonstrated the importance of substrates for the production of biomass and protease. RSM analysis was adjusted by minimisation and maximisation using this information to obtain higher values (Table 4.6b).

Table 4.5. Analysis of variance (ANOVA) of the CCD experiment for the calculated responses

Model	**CDW (g/L)**			**Protease activity (mU/mL)**			**Specific Protease activity (mU/mg)**		
Transformation	Power (Lambda:-1.74)			None			Inverse square root (Lambda:-0.5)		
ANOVA for RSM	Reduced Quadratic			Linear			Linear		
Source	**Sum of Squares**	**F Value**	**p-value Prob > F[1]**	**Sum of Squares**	**F Value[2]**	**p-value[3] Prob > F**	**Sum of Squares**	**F Value**	**p-value Prob > F**
Model	0.1232	8.228	< 0.0001*	32950.24	15.20	< 0.0001	0.00120	8.303	0.0002
A-Molasses	0.0192	16.702	0.0009*	13996.23	25.82	< 0.0001*	0.00037	10.256	0.0037*
B-CSL	0.0022	1.886	0.1886	12812.78	23.64	< 0.0001*	0.00001	0.207	0.6528
C-$MnSO_4$	0.0000	0.004	0.9530	6113.28	11.28	0.0025*	0.00081	22.456	< 0.0001*
D-$MgSO_4$	0.0002	0.179	0.6778	271.96	0.05	0.8246	0.00001	0.294	0.5927
AB	0.0328	28.507	< 0.0001*						
AC	0.0220	19.060	0.0005*						
AD	0.0008	0.682	0.4210						
BC	0.0188	16.357	0.0009*						
BD	0.0023	1.989	0.1776						
CD	0.0008	0.715	0.4103						
A^2	0.0070	6.043	0.0257*						
B^2	0.0091	7.902	0.0126*						
D^2	0.0110	9.519	0.0071*						
Residual	0.0184			13552.1			0.00090		
Lack of Fit	0.0130	0.082	0.4988	6813.8	0.2528	0.9883	0.00084	3.2250	0.0989
Pure Error	0.0055			6738.4			0.00007		
Core Total	0.1417			46502.4			0.00210		

[1] *Statistically significant (P<0.1).
[2] Calculated value from a hypothesis test.
[3] Probability level

Table (continued)

Std. Dev.	0.034			73.627			0.00601		
Mean	0.12			497.083			0.02716		
C.V. %[4]	28.24			14.812			22.1378		
PRESS[5]	0.070			18198.81			0.00142		

[4] Coefficient of Variance %.
[5] Prediction error sum of squares.

Table 4.6. Possible optimised values (a) and simultaneous optimization values via a different approach on variables (b) of biomass and protease

a)

Response	Number of Possibilities	Variables				Predicted values	Desirability
		Molasses	CSL	$MnSO_4$	$MgSO_4$		
CDW (g/L)	1	10.31	10.38	0.22	0.96	12.218	1.00
	2	10.20	10.01	1.21	1.04	10.259	1.00
	3	10.22	10.11	0.76	0.71	9.978	1.00
Protease Activity (mU/mL)	1	39.76	39.15	1.43	0.88	704.5	1.00
	2	36.54	39.50	1.42	0.78	687.6	1.00
	3	39.79	38.74	1.16	0.53	681.5	1.00
Specific Protease Activity (mU/mg)	1	10.00	38.12	1.50	0.21	4309.5	0.73
	2	10.01	29.66	1.50	0.20	4124.9	0.72
	3	10.00	18.15	1.50	0.20	3887.9	0.71

b)

Parameter	Optimization via[1]	Variables (%)				Predicted values		Changing (%)[2]
		Molasses	CSL	$MnSO_4$	$MgSO_4$	CDW (g/L)	Protease activity (mU/mL)	
CDW	Minimization of molasses and CSL	10.00	10.00	0.27	0.90	15.263	-	+ 24.9
Protease activity	Maximization of molasses, CSL and $MnSO_4$	40.00	40.00	1.50	0.44	-	718.4	+ 1.9
CDW and Protease activity	Concentrations of substrates were in range	40.00	40.00	1.50	0.76	5.540	716.5	- 54.7 for CDW; + 1.7 for Protease activity
	Concentrations of substrates were in range weighted to production of biomass	12.46	39.99	0.20	0.61	8.511	453.2	- 30.3 for CDW; - 35.7 for Protease activity

The predicted values of CDW and protease activity were increased 24.9 % and 1.9 % compared to optimization by concentration of substrates in ranges via minimisation of molasses and CSL and maximisation of molasses, CSL and $MnSO_4$, respectively (Figure 4.23a and 4.23b). However, simultaneous optimization values were lower than optimization by each response in every possible scenario. Even when a 1.7 % increase in protease activity was observed, the CDW value was drastically decreased. The optimised values for biomass production were 10 % of molasses, 10 % of CSL, 0.27 % of $MnSO_4$ and 0.90 % of $MgSO_4$, to yield 15.263 g/L CDW.

[1] Desirability values were 1.00, 1.00, 0.95 and 0.73 for minimization of molasses and CSL, maximization of molasses, CSL and MnSO4, concentrations of substrates were in range and concentrations of substrates were in range weighted to the production of biomass, respectively.

[2] Changing ratios were calculated based on maximum values of CDW and protease activity that were 12.218 g/L and 704.5 mU/mL in Table 4a, respectively.

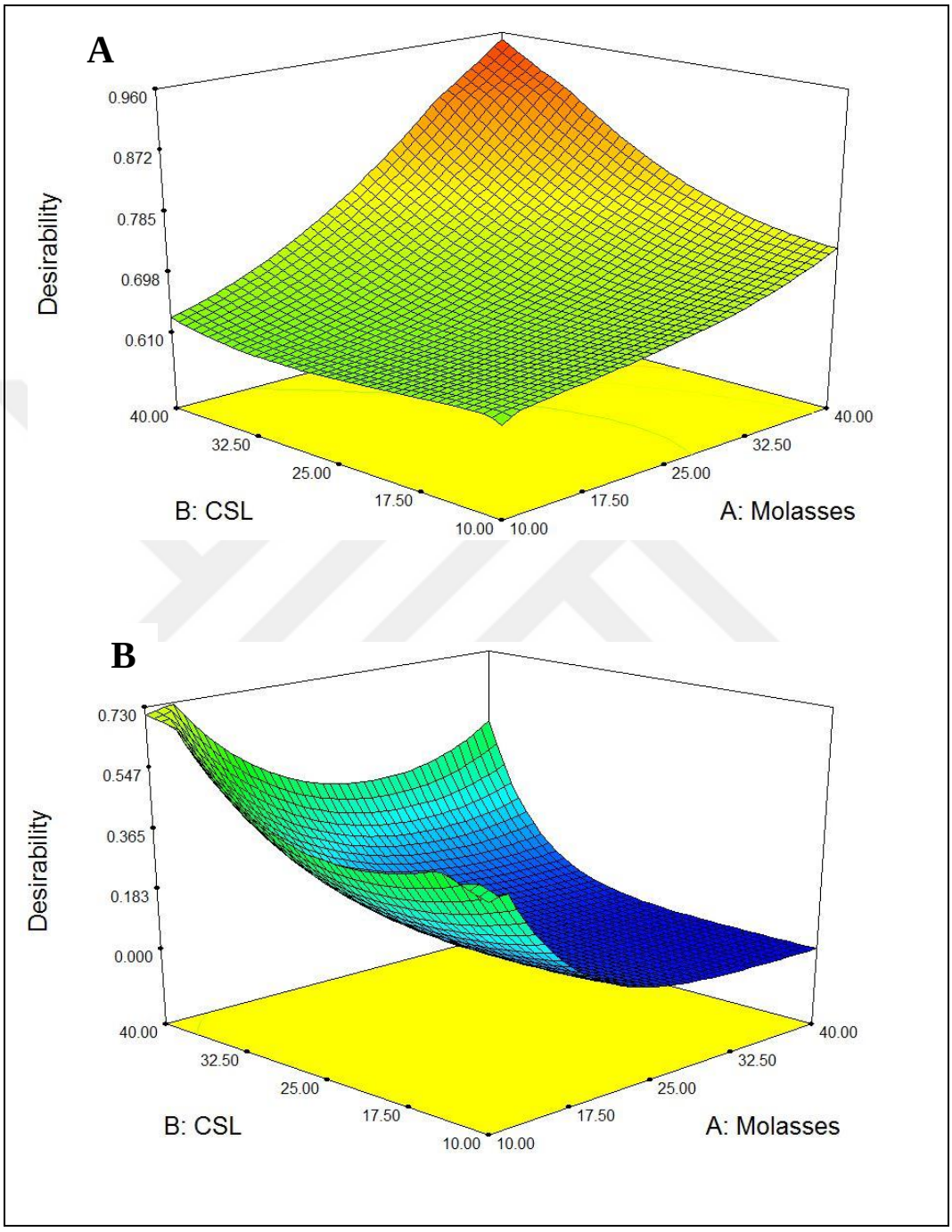


Figure 4.23. Three-dimensional surface plots of the simultaneous optimization of CDW and protease activity via A) concentration of the substrate in range and B) concentrations of the substrate in range weighted to the production of biomass, showing the desirability of the model according to the calculated responses.

For protease activity of 718.4 mU/mL, the optimised values were 40 % of molasses, 40 % of CSL, 1.5 % of $MnSO_4$ and 0.44 % of $MgSO_4$.

According to optimization parameters for batch fermentation of biopesticide, the best medium composition was identified to increase the cell dry weight amount. This medium was designed using waste product (Table 4.7).

Table 4.7. Optimized medium to large scale production of biopesticide based on cell dry weight

	Component (%)			
Medium	**MPhys**	**CSL**	**$MnSO_4$**	**$MgSO_4$**
	10.00	10.00	0.27	0.90

4.5 SCALE-UP

Medium optimization for batch production of biopesticide by Bs MBI5 was completed via optimization tools, second step is focusing on large scale production by bioprocess engineering activities. The volumetric power drawn (P_g/V_L) was the criterion used for scaling up the culture process from shaking flasks to 3-L and 30-L bioreactor. The selected procedure for scale up is translated between two scales of operation according to constant power consumption per unit volume of liquid.

Initial P_g/V_L in the shake flask was determined from the large experimental data for unbaffled flasks. The viscosity and gravity of liquid are 0.0032 kg/ms and 1140 kg/m^3, respectively. Calculated P_g/V_L value was 0.225 kW/m^3 for 150 rpm using 150 mL liquid volume. The large scale studies were assessed at aeration rates of 0.1, 0.5, 1 vvm and different speeds were evaluated to reach P_g/V_L value of shake flask.

Table 4.8. Mixing rate, gas flow and aeration number comparisons for bench scale and large scale bioreactors

Working Volume (L)	Air Rate (vvm)	Total Air Rate (L/min)	Mixing Rate (rpm)	N (rps)	Di (m)	Na (Q/ND_i^3)
3	0.1	0.3	275	4.583	0.06	0.005
	0.5	1.5	275	4.583	0.06	0.025
	1	3	300	5	0.06	0.046
30	0.5	15	250	4.166	0.105	0.051
	1	30	275	4.583	0.105	0.094

Table 4.9. Scale-up based on constant power per unit volume for Rushton radial flow impellers

Working Volume (L)	N (rps)	*Re*	P_0 (W)	P_g/P_0	P_g/V_L (kW/m^3)	Number of cultivation
3	4.583	5.88E+03	0.795	0.979	0.26	1
	4.583	5.88E+03	0.78	0.816	0.212	2
	5	6.41E+03	1.043	0.73	0.254	2
30	4.166	1.64E+04	9.472	0.71	0.224	1
	4.583	1.80E+04	12.608	0.57	0.24	1

Finally, in order to scale up this culture to 30L bioreactor using the same P_g/V_L (0.225 kW/m^3), an agitation of 250 rpm is required, maintaining an air flow rate of 0.5 vvm (Table 4.8 and 4.9). Figure 4.24 shows the calculation steps of scale up both shake flask and large scale bioreactor. To obtain the power consumption in erlen, Reynolds number and modified Newton number was used. After determination of the power rate in erlen, to calculate the power value for bioreactor, aeration number, Reynolds number, Power number, power number without aeration, and gassed power were used respectively.

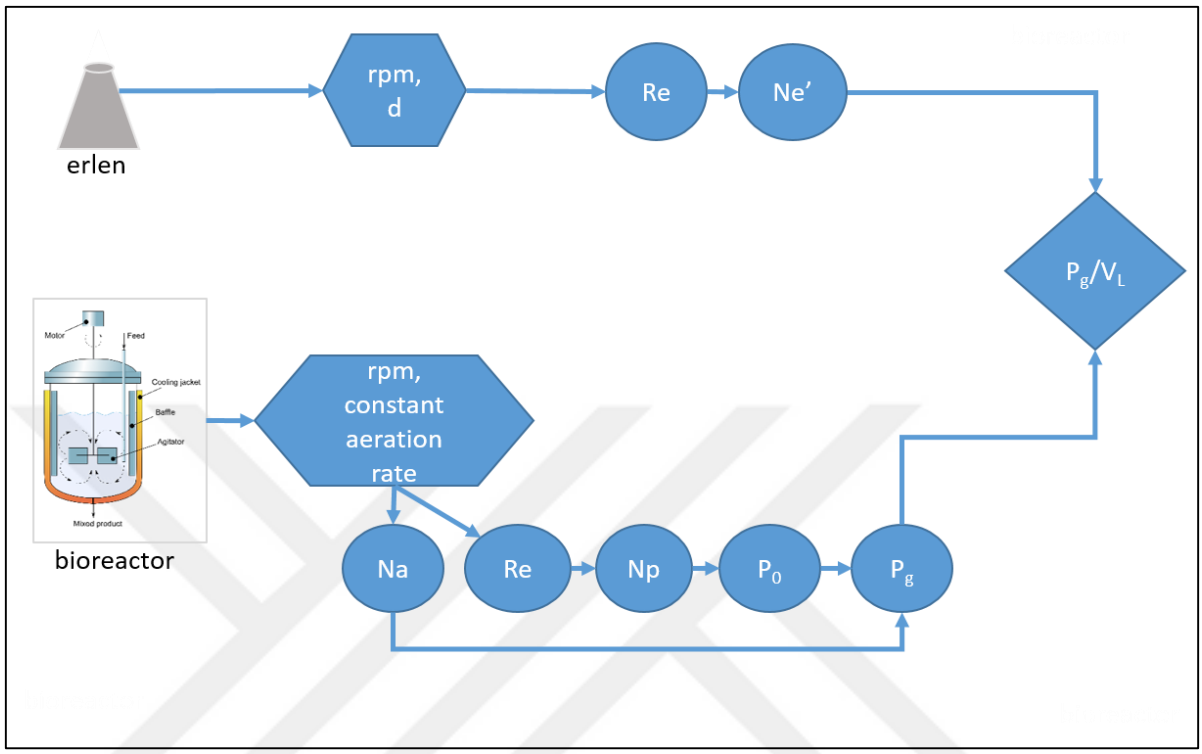


Figure 4.24. A summary of systematically building the scale up process from erlen to bioreactor.

4.6 PILOT SCALE PRODUCTION

Mixing is a very crucial aspect to get the maximum productivity in microbial fermentations. It could be achieved by means of aeration and agitation. The power consumption approach showed that the mixing rate and aeration rate for Bs MBI5 cultivation at different scales. It is important to provide optimum combination of aeration and agitation to avoid shear stress and cell disruption. The first starting set according to the aeration rate was studied at 3L bioreactors. The agitation speed and aeration rate were 275 rpm and 0.1 vvm. Second experimental set was 275 rpm agitation rate and 0.5 vvm airflow rate. Thirdly, speed of impeller was adjusted to 300 rpm at 1 vvm airflow rate. The aeration rate differences of whole experiments were evaluated by growth density of cells, viable cell amount, sporulation amount and total protein amount.

The effects of aeration rate showed that 1 and 0.1 vvm airflow increased the cell density at 3L bioreactor. 0.5 vvm airflow diminished a bit Bs MBI5 cell density (Table 4.25A). However, when evaluated the vegetative cell amount, 0.5 vvm and 1 vvm have higher effect compared to 0.1 vvm airflow rate. 0.5 vvm and 1 vvm aeration rate increase the cell amount up to $5.28x10^8$ cfu/mL and $1.09x10^9$ cfu/mL. 0.1 vvm air flow showed that a repressing effect of viable cell amount (Table 4.25B). To find effect of air rate on sporulation were followed spore amount. Biopesticide sporulation displayed quite similar pattern when it was aerated 0.1, 0.5 and 1 vvm (Table 4.25C). The other main point in biopesticide production was toxin protein amount. Bs MBI5 produced high protein with 1 vvm air flow rate at 12h. 0.1 vvm air rate was detected to reduce total protein amount (Table 4.25D).

When compared the effect of aeration rate on cell density, viable cell amount, sporulation rate, and total protein amount, it illustrated that scale up trials should be continue with 0.5 and 1 vvm aeration rate. According to first 3L bench scale bioreactor working, two aeration rates were tested in 3L and 30L as parallel studies.

To evaluate the scale up yield in 3L and 30L at 0.5 vvm air flow rate, bioreactors had started at the same time. The results showed that growing cell density, viable cell amount, and spore amount were not any significant difference between 3L and 30L bioreactors while they were aerated with 0.5 vvm air flow rate (Table 4.26A, B, C). The total protein amount was produced higher in 30L bioreactor than 3L bioreactor during 12h and 24h (Table 4.26D).

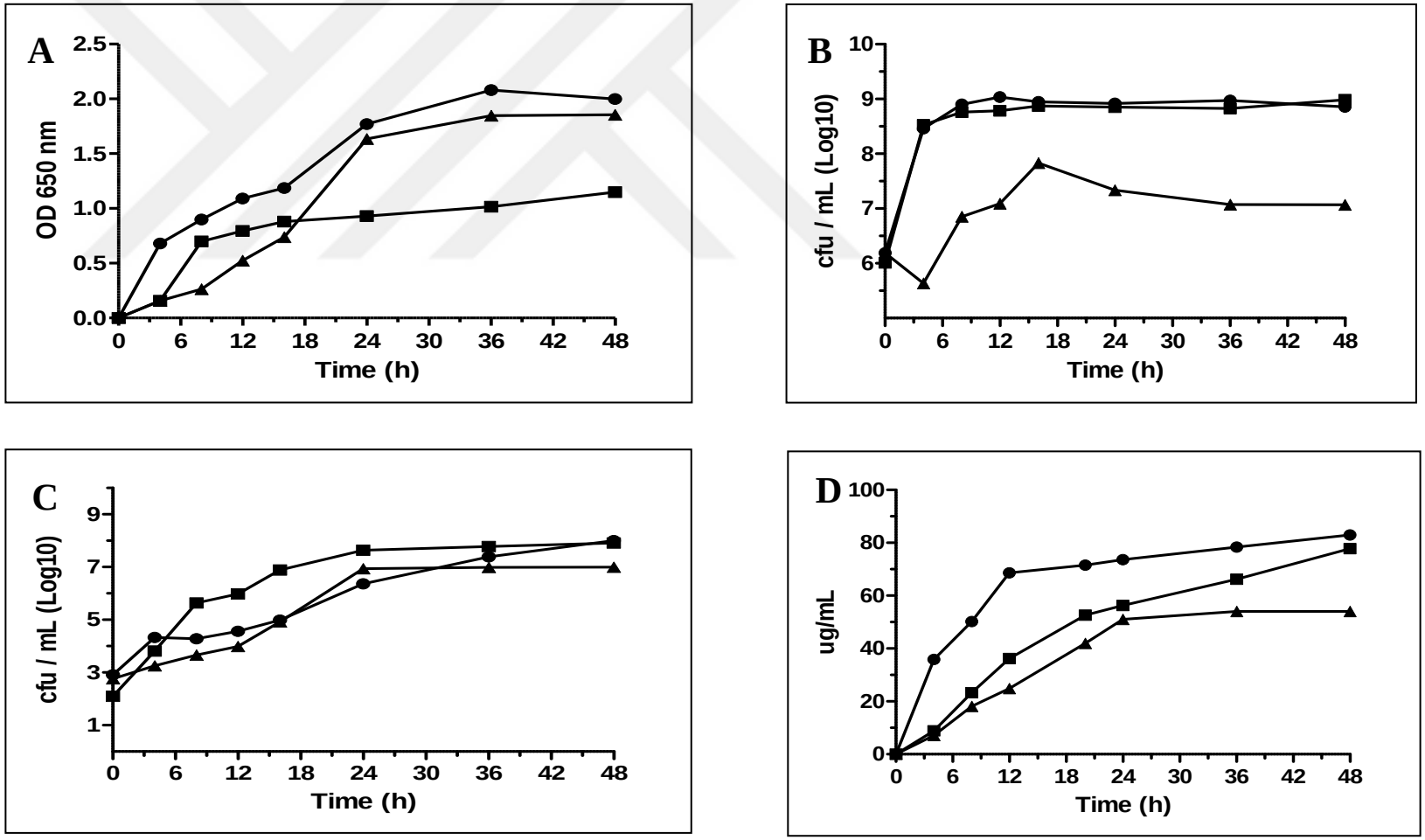


Figure 4.25. Different growth parameters at different aeration rate in 3L bioreactor. A) Growth curves; B) Vegetative cell amount; C) Spore amount; D) Total protein amount. (▲, 0.1vvm; ■, 0.5vvm; ●, 1vvm)

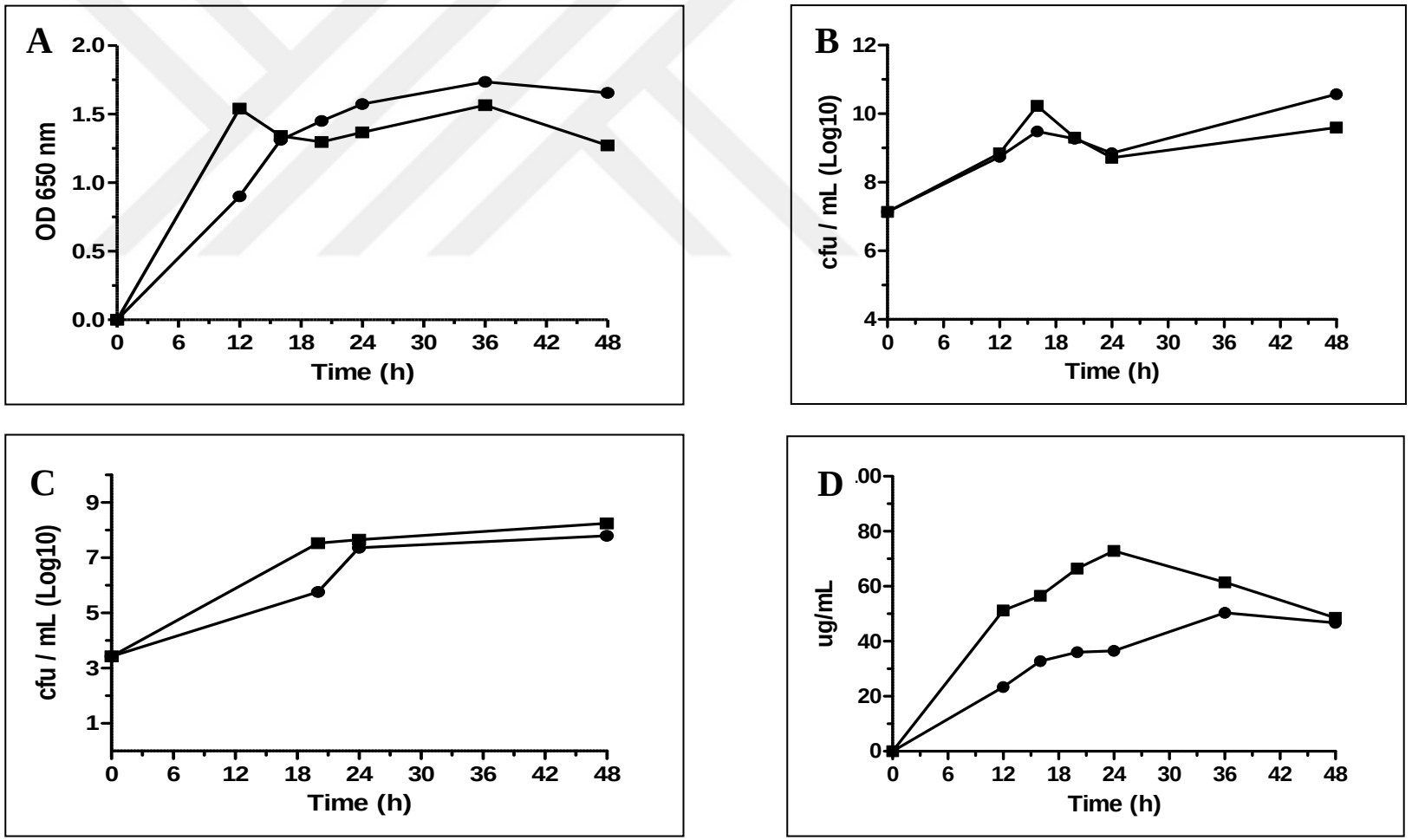


Figure 4.26. Growth curves of 0.5 vvm gas flow rate cultivation at 3L and 30L bioreactors. A) Growth curves; B) Vegetative cell amount; C) Spore amount; D) Total protein amount. (●, 3L; ■, 30L)

The other fermentation batch was carried out at 1 vvm in 3L and 30L volume with 300 and 275 rpm, respectively. Cell density was higher in 30L bioreactor than 3L during 36h. End of cultivation, the cell density was reached same level (Table 4.27A). The viable cell amount has near value both vessels during cultivation. However, the higher cell density in 30L provided a bit higher cell amount compared to 3L (Table 4.27B).

The sporulation rate was very similar between 3L and 30L under1 vvm aeration (Table 4.27C). Protein amount was found to be higher in 30L reactors related to much viable cell amount than 3L bioreactor (Table 4.27D).

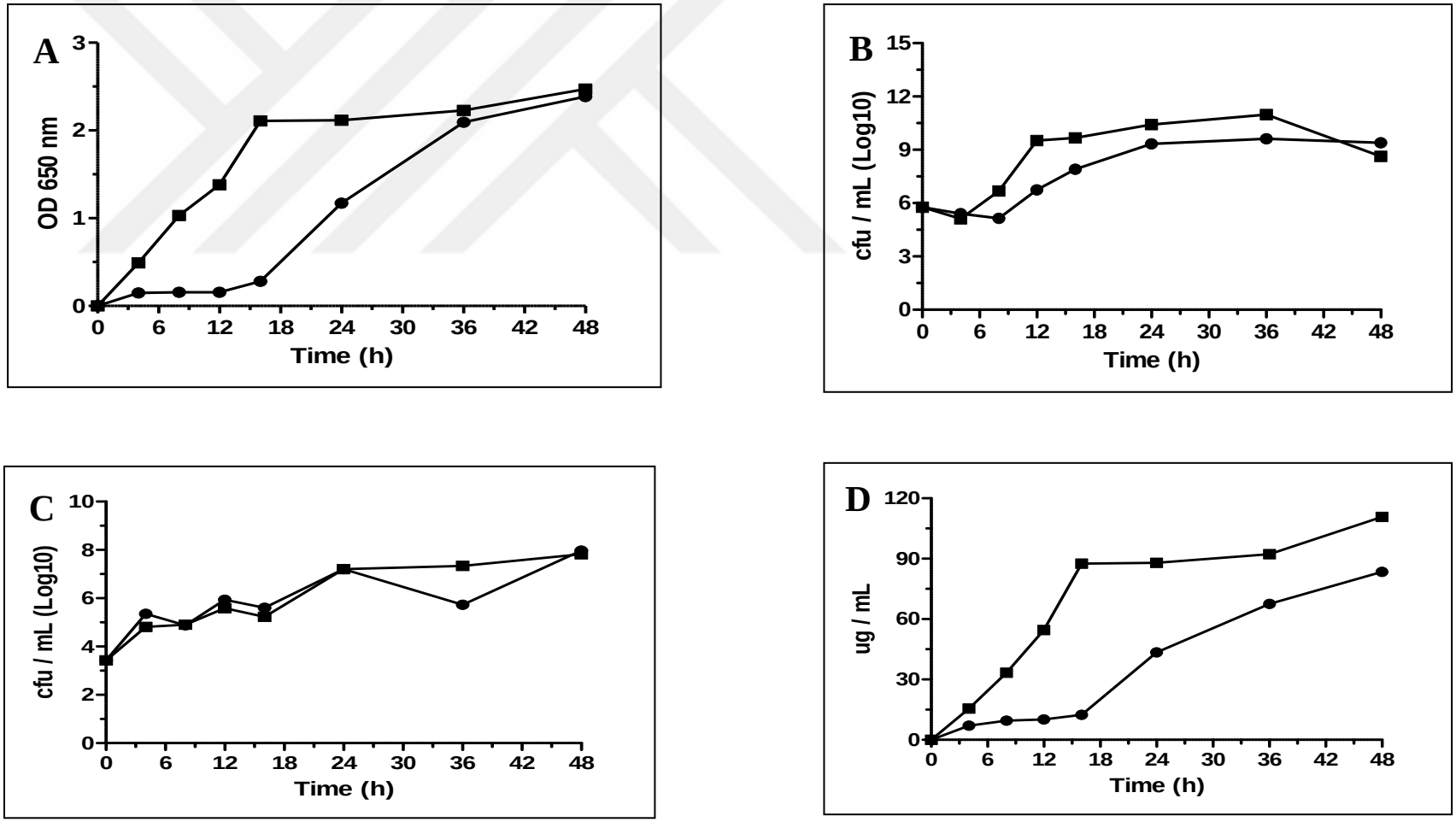


Figure 4.27. Growth curves of 1 vvm gas flow rate cultivation at 3L and 30L bioreactors. A) Growth curves; B) Vegetative cell amount; C) Spore amount; D) Total protein amount. (●, 3L; ■, 30L)

4.7 IN VIVO LARVAL TOXICITY

The biomass was produced by different aeration rate in 3L and 30L vessels. After lyophilization, all of the samples were kept in room temperature. The larval toxicity tests were performed with Bs MBI5 which was produced in bioreactor. The lethal concentrations were defined in nanogram of toxins per milliliter. The laboratory reared mosquito larvae of *Culex* spp. were used for bioassays. The comparative toxicities of Bs MBI5 samples produced from different aeration rate at different vessel size are shown in Figure 4.28. The all cultivation biomass had similar toxic effects against larvae. Bs MBI5 toxin produced from all four fermentation progress were more lethal than commercial Bs toxins (Vectolex WG).

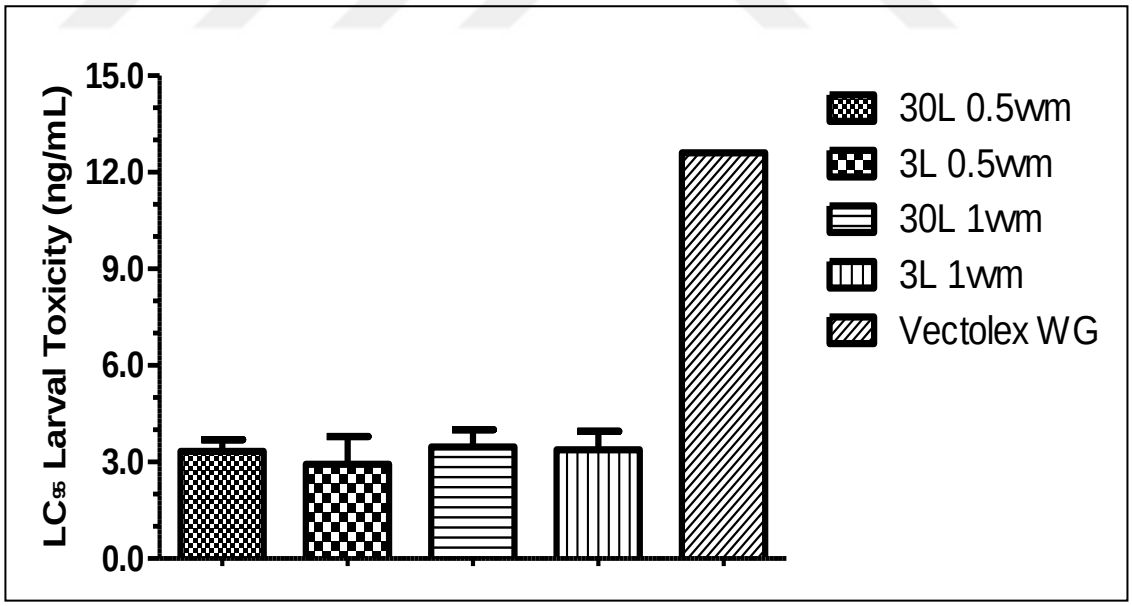


Figure 4.28. Toxicity of Bs MBI5 produced from different aeration rate in 3L and 30L bioreactors.

5. DISCUSSION

The mosquito-borne diseases causes lots of terrible epidemic invasion over the world. Mosquitoes is the starting point a lot of viral and parasitic disease such as malaria, yellow fever, dengue fever, filariasis, St. Louis encephalitis and West Nile virus between humans and animals [115–117]. The World Health Organization (WHO) publishes every year the estimated risk of malaria infection threated population who are nearly 250 million people. 1 million of this infection cases result in death [115]. Especially in African geography, under 5 years old child are suffer from outbreak of mosquito transmitting diseases [117,118]. Yellow fever and dengue fever have become a major public health problem. They cause nearly 50 million cases worldwide every year [1,119,120]. At present, after a person has been infected by mosquito-borne diseases, treatment of disease is either too expensive or owing to a lack of vaccines to prevent. The control of mosquito larvae is seen as the most effective way to reduce the incidence of these transmission [121]. The mosquito-transmitting disease will increase in the future. The main reasons are declining of vector control activities, international human traffic, insufficient political regulations, low funding, and greater levels of urbanization without enough infrastructure [24,115,122]. Early mosquito control efforts were carried on by some synthetic chemical insecticides such as organochlorines (DDT, dieldrin), organophosphates (malathion, temephos, and chlorpyrifos) [29,57,122]. Until 15 years ago, there is no a big concern to avoid using chemical insecticides. Because of mosquito resistance against chemical agent and their harmful effects on non-target species, including humans, livestock, and bees has been necessary to find alternative strategy for mosquito control [99,123–125]. To overcome the resistance factor and the public concerns about adverse environmental effect of chemicals has been started to asses biologically based insecticides.

Biopesticides have considered much attention respect to lack of adverse effect on mammalian and ecological opportunities [126]. The last 10 years, development of molecular biology, genetic and protein engineering has improved the novel and more effective biopesticides production. The developing in biotechnology provides superior characteristics of biopesticides and its application had been replaced the highly toxic pesticides in the market [8,14,127,128]. All over world, the spore forming bacteria *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis israelensis*

(Bti) have been used as the most promising biopesticide against the mosquito control. After some vector related to critical disease began to show signs of resistance to chemical agents, they showed their savior activity to control the resistance mosquito larvae. The Bti activity depends on their hundreds different crystal proteins named cry toxin gene. These genes are specific to different insect species [129,130]. Bs also has some crystallized proteins to contribute sporulation and higher toxicity effect. On the other hand using biopesticide instead of chemical insecticide has depend on many factors effecting efficacy of action in environment. Influencing parameters can be listed as feeding rate of larvae, larval age, water temperature, density of larvae. These factors affect directly biopesticides treatment efficacy [58,131]. Biopesticides' mode of action based on eating, digesting and activating in gut of larvae. For example, larvae does not feed in mature instar stage [2,132]. When water temperature decreases, the feeding regime become less. Finally the high number of larvae provide the competition for food increases. Some other extrinsic environmental problems are ultraviolet and water organic composition. These two parameters have reduced Bti efficacy [116,133]. Bti was described and used as biopesticide for mosquito larvae in 1977 [134]. Bti based a lot of biological agents have been used for mosquito control worldwide. Bti shows larvacidal activity on *Culex, Aedes, Anopheles, Mansonia*, and *Coquillettidia* [44,135,136]. Bti is more sensitive to sunlight and organic pollution in water which are decreased its activity after 2-3 days under acceptable levels. After 1week, it is unable to be detected in treatment water area. Because of low potency, it should be applied large amounts [137,138].

Bs was initially described by Kellen and Meyers in 1964 to use as pathogen for mosquito [43]. Nowadays, more 150 strains have been isolated by various researchers to use in mosquito larvae control. Bs has a highly toxic effect on genus *Culex* and *Anopheles* [139–144]. When compared the Bs with Bti, it is more resistance and longer remain activity in the field. The residual activity of Bs was reported as 3 weeks in Australia, 4 weeks in the USA, and 6 weeks in Africa [140,145,146]. Long term residual efficacy of Bs provides lower dose of product applied. The residual activity is gaining from replicating of spores in death larvae body and then cytolysis of gut membrane, being released into the water [146,147]. Currently, two highly toxic activity strains are known as 2362 and 1593 which are utilized in most studies [148,149]. The resistance development to Bs is of higher problem than Bti, related to a single protein binding to one

specific receptor. Recent studies have been focused on the toxin resistance mechanisms on *Culex* and *Anopheles*. Hire was reported relationship between the Bin toxin and receptor of Culex and *Anopheles* larvae. The Bin toxin binds the glucosidase receptor, Cpm1, in *Culex*; its orthologue Agm3 in *Anopheles* mosquitoes. After binding the Bin toxin, it internalizes the cell membrane and induces vacuolation and cells death [99]. The resistance to Bs in *Aedes* larvae is related to BinA and BinB toxin protein unable to reach the cytoplasm. BinB functions is a receptor binding subunit and attributes translocation of BinA and BinB into the cytosol. In susceptible larvae, these toxin form complex with membrane receptor and this complex is internalized into cytosol. BinA evokes the autophagy or induces apoptosis. The insusceptible larvae, *Aedes*, BinA and BinB are able to bind cell membrane, they are not introduce the cytosol and it means the lack of toxicity in this host [150,151].

Bs media production studies based on two approaches which are decrease the cost of production using some wastes and increase the toxicity level adding some critical carbon, nitrogen and mineral sources. For example, production of mosquitocidal Bs by solid state fermentation was aimed to most efficient for production of Bs mosquitocidal toxin against *Culex pipiens* using twelve agricultural wastes as main carbon, nitrogen and energy sources [152]. Prabarakan has attempted to develop a cost effective medium for Bs production using soybean flour and peanut cake which are locally available raw materials. They have reported that soybean culture medium reached highest spore count and maximum toxicity against *Culex quinquefasciatus* [153]. Some studies have focused on proteinaceous substrates to enhance the biomass of Bs due to the lack of key enzyme in utilizing carbohydrates. However, protein based substrates are always expensive than carbohydrates. To overcome this costing problem, some waste protein sources have been used in fermentation of Bs as a mosquitocidal activity. The locally available egg yolk was used as fermentation medium to produce Bs biopesticide. This medium has provided significantly shortened fermentation time to 15h and yielded high toxic activity compared to conventional medium against *Culex* larvae [45]. To enhance toxicity, peptonized milk medium with yeast extract and mineral supplements were used to cultivate the Bs 1593 and Bs 2362 separately. End of the fermentation, biomass was separated by centrifugation and then resuspended in lactose solution and precipitated with acetone. These final formulation has showed highly insecticidal activity to *Culex quinquifasciatus* larvae [148]. In another study, five

different media formulated from dried cow blood, mineral salts and four different legume seeds were used to produce the Bs 1593 production. All tested media have showed good activity in growth, sporulation, and toxin activity of Bs 1593 [154]. Using CSL as a replacement of yeast extract increased the biomass, sporulation and toxicity of Bs 2362 [155].

In the current study, Bs MBI5 has been produced based on power input scale up strategies. In literature, there is no working about Bs production as a biopesticide using some waste materials in fermentation media. This current study has successfully achieved the large scale production keeping same yield in shake flasks cultivation.

6. CONCLUSIONS

In the world, there must be used integrated pest management (IPM) system to more sustainable protection of human, animals, agriculture and environment from harmful organisms. The IPM concept is harmonizing different ways such as biological, cultural, physical, and chemical to minimize the economic, health and environmental risks. Biological control is using natural enemies to reduce harmful insects especially mosquito. Using biopesticides in mosquito control is a highly effective strategy for preventing mosquito borne disease transmission such as dengue, malaria, and fevers.

In this thesis, we have focused on fermentation development of novel Bs MBI5 strain producing highly effective biopesticide. The research achieved significant success in media and process optimization, process scale up and large scale production. This study is the first Bs cultivation research that applies using power input way to scale up of fermentation, and high yield fermentation using waste product.

In conclusion, this thesis work successfully completed the research objectives listed in section 1.8. It presented new and basic principle that will benefit future Bs fermentation research and commercial development.

We have demonstrated first key points in this thesis;

- Providing a novel highly mosquitocidal product into the biopesticide market.
- Statistical optimization is used for the production of Bs as a biopesticide.
- Investigation of scale up strategy, power input, to production of Bs as a biopesticide.
- Applied successful large scale production of Bs as a biopesticide.

7. FUTURE PERSPECTIVES

The researched presented in this thesis was focused on the development a new strain as using a biological control agent and their large scale production methods such as cheaper growth media via optimization tools, scale up way via bioengineering tools, and effective large production success. In the relation with that aim, some important results that provide new very high mosquitocidal toxin capacity bacterium have been achieved.

Based on the results of this thesis, it is conceived that there are a number of future research directions focusing on this novel strain. Below these points are presented.

- This study was demonstrated a bacterium which has highly effective toxicity against mosquito larva. The proposed higher activity bacterium should be formulated based on application area in nature, protection of biomass survival, and bettering the natural performance. The end-formulation of microbial biopesticides are crucial to obtain ultimate efficacy in the field.
- This developed bacterium was used only mosquito control studies in the thesis. This entomopathogenic activity should further be investigated the activity against other pests which situated in order of Diptera, Lepidoptera, and Coleptera.
- The studies constructed in this thesis was focused on the production process of novel biopesticide. However, during research studies have been realized that this bacterium has a good protease activity. For further studies, building a dual production model can be evaluated.
- In designed production media were used cheap carbon sources especially molasses as sugar source. Yet, the bacterium of Bs has a reputation for unable to use sugars as carbon source. In this view, the glycolysis pathway of this bacterium should detailed be examined.
- This potential biopesticide was studied as wild type strain. This higher activity comes from its nature genomic profile. There can be some more advantageous on assessing some genetic modification tools for higher activity.

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