β-GLUCOSIDASE FROM *Trichoderma harzianum* T12 AS GREEN FUNGICIDE AGAINST *Macrophomina phaseolina* IN SOYBEAN (*Glycine max* L.)

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Specially dedicated to:
My family for their endless support and motivation.

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ABSTRACT

Macrophomina phaseolina (Tassi) Goid remains the prevailing causal agent of charcoal rot disease that can significantly suppress yields of a variety of crops. Its wide host range and survivability under arid conditions as well as the ineffectiveness of fungicides have spurred scientific endeavors in search of alternative avenues to control this phytopathogen. The present study is aimed to provide empirical evidence on the efficacy of β-glucosidase from *Trichoderma harzianum* T12 as a biological control agent against *M. phaseolina*. *In-vitro* pathogenicity tests on 60 isolates of *M. phaseolina* and 30 isolates of *T. harzianum*, collected from different areas of the Mazandaran province in Iran revealed the isolates, M2 of *M. phaseolina* and the T12 of *T. harzianum* were the most virulent and effective in inhibiting growth of *M. phaseolina*, respectively. The present study showed that biochemical and phylogenetic analyses and BIOLOG results confirmed the fungal antagonists and phytopathogen were *T. harzianum* (Rifai) and *M. phaseolina* (Tassi) Goid, respectively. Purified extracellular β-glucosidase of *T. harzianum* inhibited the growth of *M. phaseolina* as seen from the large halo zones, indicating its possible application as a green fungicide against *M. phaseolina*. The β-glucosidase had an optimum pH (7) and temperature 45˚C, respectively, remarkably stable up to 240 min with a half-live of $t_{1/2}$ = 210 min at 40 ˚C to 60 ˚C. Zn^{2+}, Mn^{2+}, and Tween 80 enhanced its activity while was substantially inhibited by Fe^{3+}. Enzyme activity was the highest when wheat bran and $(NH_4)_2SO_4$ were used as carbon and nitrogen sources respectively. The kinetic parameters for β-glucosidase T12, K_m, V_{max} and k_{cat} were estimated as 0.79 mM, 8.45 mM min^{-1} mg^{-1} protein and 10.69 s^{-1}, respectively, to give a turnover number of 10.69 s^{-1}. Optimization by the Box-Behnken Design (BBD) based on: temperature, carbon sources, inoculum size and pH (7), exhibited the highest β-glucosidase activity (1260 U/mL) at 45˚C, pH 7, using a carbon source 10 % (w/v) and inoculum size of 5 % (w/v). The BBD optimization for the application of the β-glucosidase formulation from *T. harzianum* to control infestation of *M. phaseolina* M2 was carried out on soybean plants grown under a greenhouse condition. Under an optimized condition, the lowest plant disease index (PDI) of 4.32% (R^2 = 0.9676) was attained using 10 mM Zn^{2+}, Tween 80 at 2 % (w/v) an enzyme concentration at 15 mg/L and an irrigation frequency of 2 times/week. A comparative study showed the developed formulation gave the lowest PDI (4.14 %) ($p < 0.05$) followed by the antagonist *T. harzianum* Rifai (26.13 %) and the commercial fungicide, Carbendazim (32.45 %). The assessments cost revealed that the enzyme formulation only costs at USD34/acre as compared to Carbendazim at USD240/acre. Hence, the findings affirmed that the novel use of crude β-glucosidase from the growth supernatant of *T. harzianum* was efficient in combating charcoal rot disease. Since the enzyme formulation was substantially cheaper and its application combines the practicality of an *in-situ* spraying for rapid control of *M. phaseolina* infestation, the technique proposed here was prospectively feasible to control such disease in crops.

ABSTRAK

Macrophomina phaseolina (Tassi) Goid merupakan penyebab penyakit reput arang yang memberi kesan ketara kepada penghasilan pelbagai tanaman. Kepelbagaian perumah dan keupayaan untuk terus hidup di bawah keadaan tandus, ditambah pula dengan ketidakberkesanan fungisid telah merangsang kajian ini untuk mencari alternatif bagi mengawal fitopatogen ini. Kajian ini bertujuan memberi bukti secara empirikal ke atas keupayaan β-glukosida daripada *Trichoderma harzianum* T12 sebagai agen kawalan biologi terhadap penyakit reput arang. Ujian kepathogenenikan secara *in-vitro* ke atas 60 isolat *M. fhaseolina* dan 30 isolat *T. harzianum*, yang dikutip dari kawasan yang berlainan di wilayah Mazandaran di Iran mendedahkan bahawa isolat M2 *M. phaseolina* ialah yang paling patogenik manakala isolat T12 *T. harzianum* paling efektif merencat pertumbuhan *M. phaseolina* M2. Ujian biokimia, analisa filogeni dan keputusan BIOLOG masing-masing mengesahkan bahawa kulat antagonistik dan patogenik tersebut adalah *T. harzianum* (Rifai) dan *M. phaseolina* (Tassi) Goid. β-glukosida ekstrasel *T. harzianum* didapati menghalang pertumbuhan *M. phasaolina* seperti yang dilihat dari zon halo yang besar, menunjukkan kemungkinan penggunaannya sebagai fungisida hijau terhadap *M. phaseolina*. β-glukosida tersebut mempunyai pH dan suhu optimum masing-masing pada pH 7 dan 45˚C, sangat stabil pada 240 minit dengan separuh hayat $t_{1/2}$ = 210 min pada suhu 40 ˚C to 60 ˚C. Ion Zn^{2+}, Mn^{2+} and surfaktan Tween 80 didapati dapat meningkatkan aktiviti manakala Fe^{3+} merencat aktivitinya dengan ketara. Aktiviti enzim adalah pada tahap tertinggi apabila dedak gandum dan amonium sulfida digunakan sebagai sumber karbon dan nitrogen masing-masing. Parameter kinetik K_m, V_{max} dan k_{cat} β-glukosida T12 yang dijangka masing-masing pada 0.79 mM, 8.45 mM min^{-1} mg^{-1} protein and 10.69 s^{-1}, unuk memberi jumlah perolehan sebanyak 10.69 s^{-1}. Pengoptimum menggunakan Design Box-Behken (BBD) berdasarkan empat parameter: suhu, sumber karbon, saiz inokulum, pH (7) mendedahkan keadaan optimum. Oleh itu, penghasilan aktiviti β-glukosida (1260 U/mL) adalah pada suhu 45˚C, pH 7, dengan sumber karbon 10 % (w/v) dan saiz inokulum 5 % (w/v). Indeks penyakit tumbuhan terendah (PDI) sebesar 4.32% (R^2 = 0.9676) dicapai dengan menggunakan Zn^{2+}(10 mM), Tween 80 pada 2% (w/v), 15 mg/L kepekatan enzim dan kekerapan pengairan sekurang-kurangnya 2 kali/minggu. β-glukosida dari *T. harzianum* digunakan untuk mengawal serangan *M. phaseolina* M2 dilakukan pada penggunaan pokok soya yang tumbuh di dalam persekitaran rumah hijau. Pengoptimuman BBD untuk penggunaan formula indeks penyakit tumbuhan yang paling rendah (PD1) ialah pada 4.32%. Kajian komparatif menunjukkan formula β-glucosidase memberikan PDI paling rendah (4.14%) ($p < 0.05$) berbanding antagonis *T. harzianum* Rifai (26.13%) dan racun kulat komersial, Carbendazim (32.45%). Penilaian kos menunjukkan kos formula yang dibangunkan hanya USD34/ekar berbanding Carbendazim USD 240/ekar. Oleh itu, hasil kajian mengesahkan bahawa β-glucokida yang diperoleh dari supernatant pertumbuhan *T. harzianum* adalah cekap dalam memerangi penyakit reput arang. Memandangkan formula β-glukosida yang dibangunkan lebih murah dan penggunaannya yang praktikal dengan hanya semburan secara *in-situ* untuk kawalan segera *M. phaseolina*. Oleh itu, teknik yang dicadangkan di sini adalah berpotensi dan boleh digunakan untuk mengawal penyakit arang reput pada tanaman.

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LIST OF ABBREVIATIONS

˚C	-	Centigrade
A_{nm}	-	Absorption Spectroscopy at nm Light Source
ANOVA	-	Analysis of Variance
APS	-	Ammonium Per Sulfate
atm	-	Airborne Thematic Mapper
BLASTn	-	Basic Local Alignment Search Tool – Nucleotide
bp	-	Base Pairs
BSA	-	Bovine Serum Albumin
Ca	-	Calcium
$CaCl_2$	-	Calcium Chloride
CHR	-	Charcoal Rot
cm	-	Centimetre
$CoCl_2$	-	Cobalt Chloride
CRD	-	Completely Randomized Design
Cu	-	Copper
DMRT	-	Duncan's Multiple Range Test
DNA	-	Deoxyribonucleic Acid
dNTPs	-	Deoxynucleotide Triphosphates
E.q	-	Equation
ECe	-	Electrical Conductivity
EDTA	-	Ethylene Diamine Tetraaceti Cacid
EtBr	-	Ethidium Bromide
Fe	-	Iron
$FeSO_4$	-	Ferrous Sulfate

$FeSO_4 \bullet 7H_2O$	-	Ferrous Sulfate Heptahydrate
GIP	-	Growth Inhibition Percentage
h	-	Hour
H_2O	-	Water
HCl	-	Hydrogen Chloride
ITS	-	Internal Transcribed Space
K	-	Potassium
kDa	-	kilodalton
kg	-	Kilogram
KH_2PO_4	-	Assium Dihydrogen Phosphate
KNO_3	-	Potassium Nitrate
Li	-	Lithium
M	-	Molarity (Molar)
MEGA6	-	Molecular Evolutionary Genetics Analysis Software
mg	-	Milligram
Mg	-	Magnesium
$MgCl_2$	-	Magnesium Chloride
$MgSO_4.H_2O$	-	Magnesium Sulfate
$MgSO_4$	-	Magnesium Sulfate
min	-	Minutes
mL	-	Milliliter
mM	-	Millimolar
mm	-	Millimetre
Mn	-	Manganese
Na	-	Sodium
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
$NaNO_3$	-	Sodium Nitrate
NCBI	-	National Center for Biotechnology Information
$(NH_4)_2\ SO_4$	-	Ammonium Sulfate
NH_4Cl	-	Ammonium Chloride

NH_4NO_3	-	Ammonium Nitrate
OVAT	-	One-Variable-at-A-Time
Pb	-	Lead
PCR	-	Polymerase Chain Reaction
PDA	-	Potato Dextrose Agar
PDB	-	Potato Dextrose Broth
PDI	-	Plant Disease Index
pKa	-	Acid Dissociation Constant
pNPG	-	P-nitrophenyl-β-D-glucopyranoside
R^2	-	Coefficient of Determination
R7	-	Yellowing of the Leaves and Yellow Pods at 50% Growing stage
rpm	-	Revolution Per Minute
rRNA	-	Ribosomal Ribonucleic Acid
RSM	-	Response Surface Methodology
s	-	Second
$MnSO_4 \cdot H_2O$	-	Manganese Sulfate Dihydrate
$ZnSO_4$	-	Zinc sulfate
SDS- PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	-	Tetra Methyl Ethylene Diamine
TNV	-	Total Neutralization Value
U	-	Unite
US	-	United States Dollars
UV	-	Ultraviolet
v/v	-	Volume Percentage per 100mL volume
W	-	Watt
w/v	-	Weight per 100mL Volume Percentage
xg	-	Times Gravity
Zn	-	Zinc
µ	-	Mikro
µg	-	Microgram
µL	-	Microliter

U/ml	-	Unit Per Millilitre

DNA BASES

A	-	Adenine
C	-	Cytosine
G	-	Guanine
M	-	Amino; represented by either A or C
N	-	Any base; A or C or G or T
R	-	Purine; Represented by Either G or A
T	-	Thymine
W	-	Pyrimidine; Represented by Either C or T

AMINO ACIDS

A or Ala	-	Alanine
C or Cys	-	Cysteine
D or Asp	-	Aspartic Acid
E or Glu	-	Glutamic Acid
F or Phe	-	Phenylalanine
G or Gly	-	Glycine
H or His	-	Histidine
I or Ile	-	Isoleucine
K or Lys	-	Lysine
L or Leu	-	Leucine
N or Asn	-	Asparagine
P or Pro	-	Proline
Q or Gln	-	Glutamine
R or Arg	-	Arginine
S or Ser	-	Serine
T or Thr	-	Threonine
V or Val	-	Valine
W or Trp	-	Tryptophan
Y or Tyr	-	Tyrosine

APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Among the plant diseases, soil-borne diseases are regarded to be more devastating than air-borne or seed-borne diseases accounting for 10-20% of annual yield losses (Kumar *et al*., 2016). Such diseases are mainly caused by phytopathogenic fungi that constitutes to approximately 10,000 species of such fungi. Among the phytopathogenic fungi, *Macrophomina phaseolina* (Tassi) Goid is one of the most ubiquitous ones which causes the fatal charcoal rot disease and capable of infecting approximately 700 plant species belonging to over 100 families of monocots and dicots. Such extraordinary adaptability of *M. phaseolina* originates in its significant physiological, morphological and pathogenic diversity that has resulted in its wide distribution across diverse climatic conditions, ranging from arid to tropical regions (Ambrosio *et al*., 2015).

The fungus is also an exceptionally robust soil-borne pathogen that devastate a myriad of major agricultural crops *viz*. grains, legumes, oil seeds, jute and cotton (Ma *et al*., 2010; Rayatpanah *et al*., 2012; Sun *et al*., 2016) as well as some vegetables and fruits (Ambrosio *et al*., 2015). The fungus can survive for long periods in soil, as long as 15 years as resistant structures (sclerotia and chlamydospores) without relying on a host (Sanchez *et al*., 2016). Charcoal rot caused by *M. phaseolina* is also disseminated by wind, soil and contact with infected plant material (Gaige *et al*., 2010). Formation of the 'black leg' in infected plants is symptomatic of the charcoal rot disease caused by the formation of sclerotia in the plant crown. Infected plants generally have weak appearances and decolourized leaves with fewer black secondary roots (Santos *et al*., 2016). The infected plants eventually die from exposure to phytotoxic metabolites released by *M. phaseolina*, such as phaseolinone, as well as other complications, such as vascular blockages that compromise nutrient transport (Santos *et al*., 2016).

Various types of agricultural management strategies to control plant diseases caused by *M. phaseolina* using cultural, biological and chemical techniques have been investigated for decades. Cultural practices such as using crop rotation, soil solarization and tolerant cultivars are not sufficiently efficacious on its own and are usually combined with other controls (biological agents, low doses of pesticides) to be more effective. For such technique to work, a higher level of technological support system is needed hence resulting in higher costs of implementation (Patel *et al.*, 2014). So far, the use of fungicides and fumigants to control *M. phaseolina* infestation in crops has been the main choice in the agricultural sector. However, these approaches have been found ineffective and uneconomical. Recent studies have shown that pesticide use per hectare, had generally increased more than proportionally with crop output per hectare. A 1% increase in crop output per hectare was matched with 1.8 % increase in pesticide use per hectare (Patel *et al.*, 2014). This is a particularly worrying trend in agricultural practices.

The indiscriminate use of pesticides has incurred serious concerns over the long term deleterious effects of pesticides on the ecology and human health (Chamorro *et al*., 2015; Pastrana *et al*., 2016). Agricultural run-offs containing concentrated amounts of pesticides are highly mobile and can pollute nearby water bodies. These toxic compounds are naturally bioaccumulated through our food chain and ingested by mammals and bird populations (Chua *et al*., 2014; Edbeib *et al*., 2016). In one hand, biological techniques using biological control agents and agri-biotechnology i.e. genetically modified crops developed using recombinant DNA technology appears more promising and ecologically friendlier to combat plant diseases i.e. charcoal rot. But both techniques have been subjected to much debate, due to concerns over new risks on food security as well as the emergence of superweeds and superpest. Even so, the former lacks the consistency in the field studies (Gerbore *et al.*, 2014) which limits its application. Aside from the unknown long-term and other unintended effects on the environment due to the loss of biodiversity, the issue pertaining to food allergies has also been mentioned (Maghari *et al.*, 2011, Arancibia *et al.*, 2013).

In view of the limitations in current management strategies to control charcoal rot disease in crops caused by *M. phaseolina*, the study believes the answer to solving this problem may lie in a simple but effective technique based on a biological control approach using bioactive secretions produced by a well-known antagonistic fungus, *T. harzianum*. For decades, extensive research carried out on the fungus was solely focused on the isolation and *in-situ* cultivation of the fungus to combat *M. phaseolina* infestation in crops. The antagonistic efficacy of the fungus against *M. phaseolina* is attributable to several antagonistic mechanisms such as nutrient competition, antibiotic production and mycoparasitism (Pastrana *et al.*, 2016). However, the study believes the need for *in-situ* propagation of this antagonistic is rather slow and, possibly inadequately effective to confer a comprehensive protection on the crops throughout the different growth stages. But the study notes that the *T. harzianum* secretes an enzyme *via* the mycoparasitism mechanism. It is a well-known fact that the β-glucosidase produced by this fungus explicitly hydrolyzes the β-linkage of the amorphic β-1, 3-glucan filling material in the chitin-based cell wall of *M. phaseolina* (Gajera *et al.*, 2012). This antagonistic mechanism is well-reported for species of *T. harzianum* whereby the β-glucosidase facilitates the penetration of *T. harzianum* into the cytoplasm of the target pathogenic fungi (Kavitha *et al.*, 2012; Andersen *et al.*, 2016). This interesting antagonistic feature of *T. harzianum* to win the biological war against another competing fungus, i.e. *M. phaseolina* seems agronomically useful and scientifically interesting.

1.2 Problem Statement

In view of the limitations and ineffectiveness of current management strategies to control charcoal rot disease caused by the soil borne nature of *M. phaseolina*, and the environmental hazards associated with the use of pesticides, the search for alternative ecologically benign and sustainable strategy, preferably cost-effective, may prove timely and merits agronomical consideration. Herein, a simple but possibly effective bio-based technique of *in-situ* application of the extracellular lytic enzyme i.e. β-glucosidase secreted by a well-known antagonistic fungus, *T. harzianum* is proposed. The technique is justified based on a well-known fact that the *Trichoderma* spp. such as *T. harzianum* has evolved in such a way to specifically secrete a specific β-glucosidase adapted to hydrolyzing the amorphic β-1, 3-glucan cell wall components in *M. phaseolina.* Driven by this fact, the direct spraying of a formulation containing the growth supernatant of the extracellular β-glucosidase would instantaneously destroy the *M. phaseolina*. Hence, further dissemination of the fungal material by wind, soil or by physical contact could be averted and the charcoal disease is halted.

It is hypothesized the extracellular enzyme β-glucosidase isolated from the highly antagonistic *T. harzianum* T12 strain can be effectively used as the bioactive ingredient over the chemically formulated ones for an environmentally benign fungicide formulation. The study strongly believes the enzyme can be formulated as an effective greener fungicide for *in-situ* protection against charcoal rot disease due to *M. phaseolina* infestation for crops at various growth stages.

Although the direct use of the β-glucosidase would probably be cheaper, a comprehensive characterization of its vulnerability against other possible additives in the formulation may prove necessary for this study. This is to ensure its hydrolytic activity is boosted, hence maximizing the efficacy in inhibiting *M. phaseolina* infestation. Furthermore, the specific use of β-glucosidase from *T. harzianum* as the active ingredient in fungicide formulation for agricultural management of *M.*

phaseolina infestation has not been reported. The feasibility of this proposed technique remains to be seen.

1.3 Objectives of Research

This study highlights upon four main research objectives:

1. To collect, isolate and identify the most pathogenic *M. phaseolina* and most effective *T. harzianum* isolates.
2. To purify and characterize the physicho- and biochemical properties of the β-glucosidase.
3. To optimize the growth conditions of *T. harzianum* for maximum activity of the produced β-glucosidase.
4. To optimize the application of the β-glucosidase-loaded formulation for maximum inhibition on *M. phaseolina*.

1.4 Aim of Study

The study aimed to prepare a cheaper and greener fungicide using the crude β-glucosidase from *T. harzianum* as the bioactive ingredient over the toxic chemically formulated fungicide for inhibiting the growth of the phytopathogen *M. phaseolina*.

1.5 Scopes of the Study

This study first isolated and screened for fungal strains of the most effective *T. harzianum* and the most pathogenic *M. phaseolina* done under laboratory settings. These *M. phaseolina* strains were isolated from infected plants and *T. harzianum* isolates were isolated from soil samples from different agricultural locations in the Mazandaran province of Iran. The most pathogenic (most prevalent) *M. phaseolina* was identified using pathogenic variability test. It was necessary for the study to isolate the most virulent strain of *M. phaseolina* as the model. If the *T. harzianum* isolate/β-glucosidase was effective against the most virulent strain of the fungal pathogen, the efficacy of former to inhibit other less virulent ones was highly possible. Similarly, selection of the most effective *T. harzianum* isolate was based on the most prevalent fungus strain found in the soil. This fungus was identified using, dual culture test, hyper parasitism test, and volatile metabolites production. Both fungi were then identified using morphological and biochemical tests.

Prior to formulation, the β-glucosidase in the growth supernatant of the *T. harzianum* was purified using ammonium sulfate precipitation and dialyzed. The enzyme was sequenced to ascertain its classification as a β-glucosidase and subsequently assessed for physicochemical susceptibilities to environmental conditions and possible additives in the fungicide formulation. The molecular weight of the enzyme was evaluated using SDS-PAGE and the assessed physicochemical properties included optimal pH and temperature as well as the effects of metal ions, surfactants, carbon and nitrogen sources, and inoculum size. Since, this was a novel enzyme, the kinetic parameters of the purified β-glucosidase was also evaluated.

The study went on to optimizing the physical and nutritional parameters for batch cultivation of *T. harzianum* to produce the β-glucosidase using the method of Response Surface Methodology (RSM) by the Box-Behnken Design (BBD). A cheap agro-industrial substrate i.e. banana wastes was used as the carbon source for batch cultivation of the *T. harzianum* to produce crude β-glucosidase. The response of this optimization was the highest activity of the harvested β-glucosidase. This was based on the largest inhibition zone surrounding a paper disc soaked with β-glucosidase, carried out on potato dextrose assay plates.

The study finally optimized the preparation and application of the β-glucosidase loaded fungicide formulation using soybean plants. The plants were grown in soil infected with *M. phaseolina* and the work was evaluated over a period of 8 weeks under a controlled condition in a greenhouse located within the grounds of the Faculty of Bioscience and Medical Engineering, UTM. The efficacy of the crude β-glucosidase to control charcoal rot disease was assessed based on the lowest plant disease index (PDI). Since this study is a pioneering work using β-glucosidase as the fungicide, RSM was again used to evaluate the effects of multiple factors *viz.* metal ions, surfactants, enzyme concentration and irrigation frequency of the formulation on the response i.e. PDI. Consequently, a controlled study comparing the optimum protocol of β-glucosidase, a commonly use pesticide i.e. carbendazim and the antagonist *T. harzianum* (Rifai) to result in the lowest PDI in growing soy bean plants sown on *M. phaseolina* infected soil was carried out. Cost assessment on the use of carbendazim and β-glucosidase based on per hectare of cultivated soybean was also performed.

1.6 Significance of the Study

The strategy proposed here is potentially more rapid and sustainable to control the spread of charcoal rot disease due to *M. phseolina* infestation in crops. The approach used in this study is possibly a more practical and cost-effective means to avert charcoal rot disease in other agronomically relevant crops, too.

1.7 Operational Framework of the Research

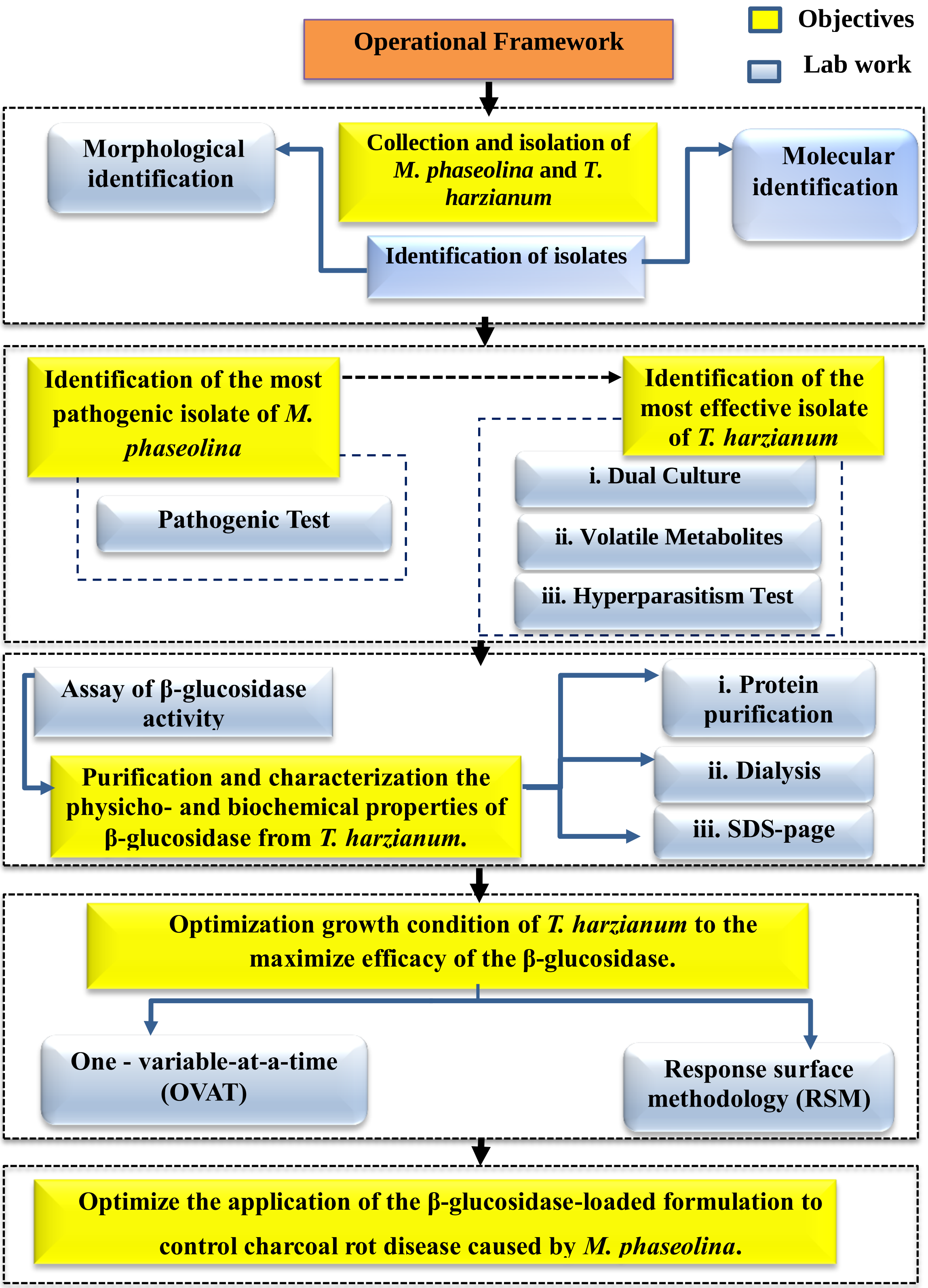

Figure 1.1 Operational framework of the research methodology.

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