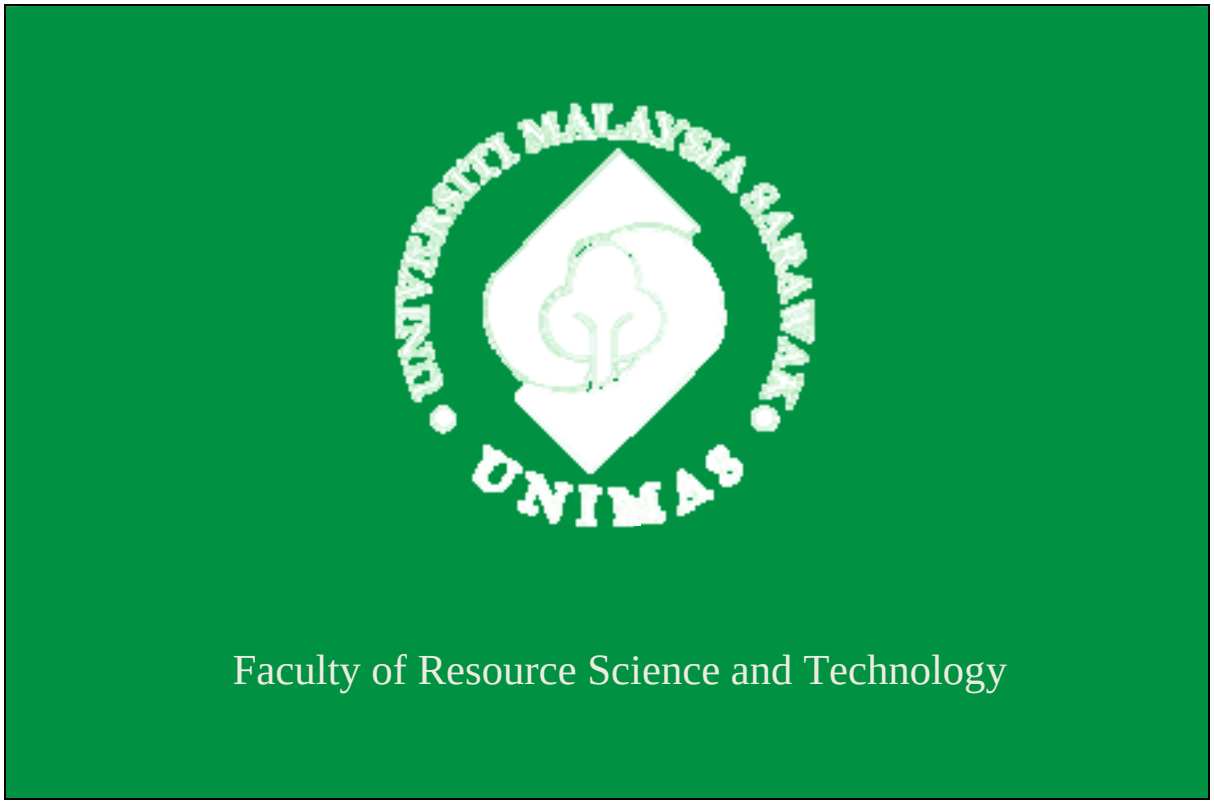

SCREENING AND ISOLATION OF AMYLOLYTIC FUNGUS AND CRUDE ENZYME CHARACTERIZATION FROM SAGO INDUSTRIAL WASTE

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"Vast learning, perfect handicraft, a highly trained discipline, and always speaking pleasant. This is the highest blessing." --- *The Discourse of Blessings (Mangala Sutta)*

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Micro gram
µl	Micro liter
µM	Micro molar
α	Alpha
ß	Beta
bp	Base pair
BSA	Bovine serum albumin
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicyclic acid
dNTP	Deoxynucleoside-5'-triphosphate
EDTA	Ethylene diamine tetraacetic acid
kb	Kilo base pair
kD / kDa	Kilo Dalton
M	Molar
MEA	Malt extract agar
ml	Milliliter
MW	Molecular weights
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium-dodecyl-sulfate
U	Unit(s)
U/mg	Unit per milligram
UV	Ultra violet
w/v	Weight over volume

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ABSTRACT

Five best amylolytic fungus from 15 successful by isolates from sago industrial waste were selected and studied for crude enzyme starch digesting ability, enzyme properties, enzyme production as well as fungal morphological and molecular identification. This study had revealed that *Ceratocystis paradoxa* is the best amylolytic fungus from all the isolates. Starch-iodine assay was use to determine the enzyme activity. The kinetic constant (K_m), maximum velocity (V_{max}) and catalytic constant (k_{cat}) was recorded at 1363.5 mg/ml, 144.9 U/ml, 8.17 mg/ml minutes^{-1} respectively for *Ceratocystis paradoxa.* The crude enzyme is thermostable on all assayed temperature where the optimum temperature is 60^{o}C, and maintained high relative activity (80 % and above) in pH 4.0 to 8.0 from studies performed, as well as remaining stable in the presence of metal ions (Cu^{2+}, CO^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}) studied. The genetic related study between the 5 isolates via ITS primers and DNA sequencing was also performed. Further study should be emphasizing on the optimization of fungal crude enzyme production, solid state fermentation, cloning of the enzyme expression gene, as well as further molecular studies on the development of expressed sequence tag (EST) and random sequence tags (RSTs) in the future.

Keywords: Starch digesting enzymes, *Ceratocystis Paradoxa*, enzyme kinetics, enzyme characterization, fungal ITS sequences.

ABSTRAK

Lima kulat amillolitik terbaik dari 15 isolasi yang berjaya dicari dalam bahan pembuangan industri sagu telah dipilih dan dikaji untuk enzim dalam penghadaman kanji, enzim fungsi, penghasilan enzim dan juga morfologi kulat dan identifikasi secara molekular. Kajian ini menunjukkan Ceratocystis paradoxa ialah amilolitik kulat yang terbaik daripada semua isolasi. Pengujian kanji-iodin telah digunakan dalam penentuan aktiviti enzim. Pemalar kinetik (K_m), halaju maksimum (V_{max}) dan pemalar katalitik (k_{cat}) adalah masing-masing 1363.5 mg/ml, 144.9 U/ml, 8.17 mg/ml per minit untuk Ceratocystis paradoxa. Enzim ini adalah thermostabil dalam semua suhu yang telah dikaji di mana suhu optimum adalah dalam 60^{o}C dan mengekalkan aktiviti bandingan (80 % ke atas) dalam pH 4.0 hingga 8.0 daripada kajian yang telah dijalankan, dan juga mengekalkan kestabilan dalam kehadiran ion-ion logam (Cu^{2+}, CO^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}). Kajian hubungan genetik lima isolasi melalui primer ITS dan pengurutan DNA juga telah dilakukan. Kajian seterusnya boleh mengutamakan penghasilan enzim kulat secara optimum, fermentasi SSF, pengklonan gen ekspresi enzim, dan juga kajian molekular yang lebih terperinci dalam pembangunan "expressed sequence tag" (EST) dan "random sequence tags" (RSTs) dalam masa hadapan.

Kata kunci: Enzim penghadaman kanji, Ceratocystis Paradoxa, enzim kinetik, perwatakan enzim, pengurutan ITS kulat.

CHAPTER 1

INTRODUCTION

Development in technologies aimed at improving the quality of life of a community of people. (DaSilva *et al.,* 1992) Thus, this leads to developing countries looking for technologies that enable sustainable resource, reducing health risk and achieving higher economical growth. This is exactly why the trend of urbanization should be "reverse" and make farming more attractive, and not just only to detoxify the results of the industrial and green revolutions (Doelle, 1989).

Agriculture industries produce significant amount post processing waste and residue (Vikineswary, 2006) particularly in Malaysia. Such waste used to be discarded or burn, or allowed to decay naturally in fields and causes environment hazards (Vikineswary, 2006). Some of the agricultural wastes were used as fertilizers, animal feed, burial, stock piling and land filling (Vikineswary, 2006). Agricultural waste can be use as energy or value added product, thus making the waste and environmental management becomes crucial role to reduce environmental pollution as well as boost up the economy. This is the reason whereby research in agricultural waste is urgent and important for economy boosting and cultivating for developing countries, and would drive nation becoming stronger participant in agriculture trade globally (Vikineswary, 2006).

As sago industry in Malaysia had become an important industry especially in the state of Sarawak, the daily sago waste production has become a major environment concern.

Agro-residues from sago *hampas* can be use as bulk substrate for enzyme production (Vikineswary, 2006). Sago *hampas* is industrial by-process waste from sago palm (*Metroxylon sagu*) starch processing. This can be used as a potential substrate for microbial conversion via solid state fermentation (SSF) into enzymes production (Vikineswary, 2006). This shows the significance of sago research findings are valuable for local agriculture industry and for sustainable waste management.

Fungal carbohydrases have wide applications in food industries besides in ecological bioremediation recycling of celluloses and starchy biomass materials (Marlida *et al.*, 2000c). Starch degrading amylases represents the largest industrial potential usage, especially in baking, brewing and glucose syrups production. The screening for indigenous amylolytic fungus from sago industrial waste is therefore required to utilize for the purpose of bioremediation usage as well as produce significant revenue for a country's economical growth.

The main objective for this study is to isolate the best amylase producing indigenous fungus from sago industrial waste. In addition, this study also aims to characterize and perform kinetic studies on the fungal amylase.

In this study, the best crude amylolytic enzyme producing fungus were determined based on the following criteria: First, the enzyme must be very effective in starch degrading; Second, the enzyme must be able to degrade large amount of starch in a given period of time; Third, the enzyme must be able to tolerate wide range of environment factor which

includes pH, metal ions and temperature; Forth, the fungus must be able to produce large amount of enzymes. However, the substrate specificity and total digestibility of various starches are not taken into count in this study. This is because fungus might produce different amount of crude enzyme and different contain of crude enzyme under different condition and environment. (Marlida *et al.,* 2000c)

CHAPTER 2

LITERATURE REVIEW

2.1 Amylases

Amylases are hydrolyses which widely available in microorganisms, plants and animals. It degrades polyglucosides with α-1,4 glucosidic bonds, such as glycogen, starch and related polysaccharides and oligosaccharides in a random manner (Lombrana *et al*., 2005). This is the reason amylase is known for its ability to convert starch or starch-based substrates (Forgarty, 1983; Okolo *et al*., 1995). By using starch as substrate, most α-amylases produce glucose or maltose as major product (Yang and Liu, 2004).

2.2 The industrial applications of amylases

Amylase hydrolyses starch to form glucose for the usage of metabolism. Amylases have many industrial applications, this including glucose syrup preparation, brewing, bread making (Muralikrishna and Nirmala, 2005), pharmaceuticals, detergents (Gupta *et al*., 2003). Downstream processing for pure enzyme production is crucial and will affect the overall industrial production especially if end purity requirement are stringent (Amritkar *et al.*, 2004; Sommers *et al*., 1989). The studies of amylase characteristics is therefore important for the industrial usage and for the countries depend on starch as energy source, and could reduce the level of competition for starches in the developing countries, and will lower the cost of the starch production (Abu *et al*., 2005).

There are many industrial applications of amylases. According to Aiyer (2005), amylases can be use in liquefaction. Liquefaction is a process of dispersion of insoluble starch granules in aqueous solution and then followed by partial hydrolysis using thermostable amylases (Aiyer, 2005). Thermostable α-amylase from *B. licheniformis* and *B. amyloliquefaciens* are used in maltose manufacturing (Aiyer, 2005). High fructose containing syrups preparation is also involved amylases. Oligosaccharides mixture manufacture (maltooligomer mix) is obtained by digestion of corn starch with α-amylase, β-amylase as well as pullulanase (Aiyer, 2005). High molecular weight branched dextrins manufacturing are prepared by corn starch hydrolysis with α-amylase. Treatment of starch processing waste water is also applying amylases in treatment process. (Aiyer, 2005) Alkaline amylase was also used in making detergents (Aiyer, 2005).

2.3 Starch

Native starch is a semi-crystalline material synthesized as roughly spherical granules in many plant tissues (Tester *et al.,* 2004). Commercially starch which extracted in pure form consists of varieties of sources (Tester *et al.,* 2004). Starch is the nature carbohydrate reserve in major plants (Aiyer, 2005). Starches are commercially produced from the plant seeds such as corn, wheat, sorghum or rice; or from the plants tubers and roots like cassava, potato, arrowroot and the pith of sago palm (Aiyer, 2005). As cited by Aiyer (2005), Berkhout (1976) has mentioned that commercial source of corn starch is extracted by a wet milling process.

Starch is a heterogeneous polysaccharide which composed of two high molecular weight entities known as amylose and amylopectin which both of these two polymers have different structures and physical properties (Aiyer, 2005). In simple, the difference is that amylose structure is essentially linear and amylopectin had a branched structure. Such difference in the structures makes them have differ properties overall.

The starch hydrolysis can be done by using either acid or enzyme as catalyst. Enzyme hydrolysis has several advantages (Aiyer, 2005). It is more specific, and therefore there are fewer byproducts formed, and hence higher and purer yields comparatively (Aiyer, 2005). Downstream refining stages for ash and color removal are minimized (Aiyer, 2005). As cited by Aiyer (2005), Underkofler *et al.* (1965) and Barfoed (1976) had mentioned that industrial scale of starch hydrolysis had gradually switched to enzymatic method.

2.4 Sago palm

In the region of Southeast Asia, the main staple of food crops include: rice, cassava, and sago. Of these, sago palm (*Metroxylon sagu*) and cassava were used as starch sources. The sago palm grows well in swampy areas, and is very suitable to grow in a humid tropical low lands. *Metroxylon Sagu* is an easily found palm in Southeast Asia especially in Malaysia. It is now become an important source of income for Malaysia. It is commercially grow, as it can produce sago starch and/or conversion to food, fuel ethanol, bioremediation and other purpose as well. Besides, it can be used as heavy metal absorption which absorbs lead and cooper, as it industrial waste largely composed of celluloses and lignins (Vikineswary *et al*., 1994) and thus makes it as a biosorbent (Quek *et al.*, 1998).

2.5 Importance of sago palm in socio-economic

According to Doelle (1998), the sustainability is defined as *"a future mean of a society to be able not only to feed themselves but also to be independent from imports for their basic requirements, which means utilizing their own natural renewable resources to furnish them with food, feed, fertilizer, fuel and energy"* in line with the socio-economical concept.

Sago palm grows well in swampy areas and contains an average of 160 kg starch. Sometimes the starch contains can also be increased to 275 kg in a well attended farm which means an average of 25 tons starch per hectare could be obtained (Doelle, 1998). According to Doelle (1998), a comprehensive socio-economic integrated biosystem will enable sago palm farm to supply:

1. House building material
2. Energy through gasification
3. Mushroom production
4. Starch flour
5. Ethanol for biofuel
6. Methane or biogas for energy
7. Aquaponics and fish production for food
8. Microbial protein for animal feed
9. Compost or other residual effluent for organic fertilization

Such biosystem would increase the farm self-efficiency and clean environment through reprocessing of the industrial waste into value-added products. This will greatly increase the income of the farming community. However, it depends on which and how the strategies are adopted in order to take advantage of biotechnologies based on their needs and situation as well as constraints.

2.6 Sago processing wastes

According to Vikineswary *et al.* (1994), sago palm which is 10 meter in height is cut down into log sections, where each section consists of 75 to 90 centimeters. The fibrous bark is stripped off during process, treated river water is then added to rasped pith in large amount, and "repos" to wash out starch granules, and the starch granules is then passed through a series of vibrating sieves to separate starch and pith residues. The waste products were bark, wastewater (sago effluent) and pith residue "hampas".

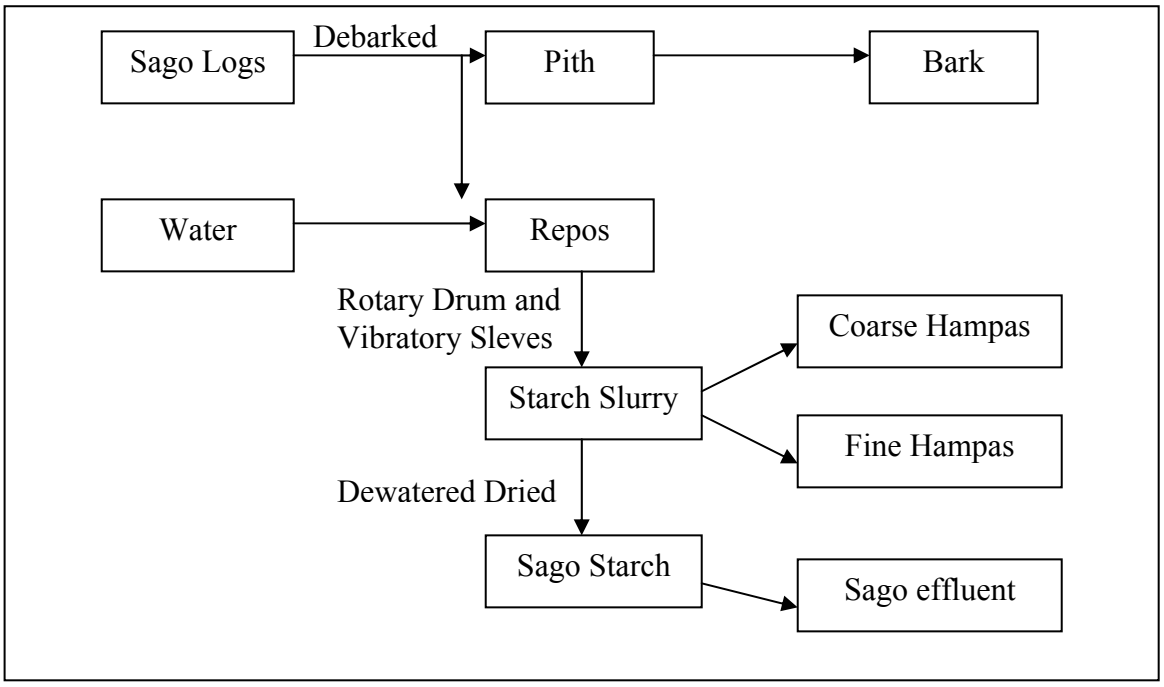

Figure 2.1: Adapted and edited diagrammatic of sago processing from Vikineswary *et al.* (1994).

2.7 Sago *hampas*

The pith residue of the industrial waste is called sago *hampas* and is usually wash off into the drain, and about 66% of a high proportion starch can be found in the *hampas* (Vikineswary *et al*., 1994).

The sago *hampas* contains a lot of low molecular weight carbohydrate which consist of hemicelluloses, celluloses (Kram, 2004), starch, fiber, and a fair amount of minerals (Apun *et al.,* 2000). As cited by Apun *et al.* (2000), Wina *et al.* (1986) mentioned the crude starch and fiber contents range from 41.7 % to 65.0 % and 14.8 %, respectively. As cited in Vikineswary *et al.* (2006), Chew and Shim (1993) had reported the *hampas* contains approximately 66 % starch and 14 % fiber on a dry weight basis. According to Kram (2004), holocellulose in the "hampas" is higher concentrate than in the sago palm bark, and the *hampas* itself still contains high value of starch. This is the main reason some fungi species like *Aspergillus* and *Chalara* (Vikineswary, 2006) were able to grow on sago *hampas* and digesting the starch for metabolism. Both cellulose and starch components have good potential for bioconversion into value-added products through a biotechnological approach where microbial strains are applied to degrade the sago waste (Apun *et al.,* 2000). Such breakdown of these polysaccharides components produces simple sugars that useful in the feed and fermentation industries (Apun *et al.,* 2000). For this reason, the sago industrial waste can be utilize later for more further usage as another alternative source of renewable energy by make use of amylase producing fungus.

According to Bujang *et al*. (1996), in Sarawak the sago factories had produced approximately 7 tons of sago pith waste every day. According to recent article by Vikineswary *et al.* (2006), which is nine years later, the Sibu division alone of Sarawak had produced 50-110 tonnes of sago pith waste daily. This means that the sago *hampas* had significantly increased from years to years. Such plentiful amount of sago *hampas* is wasted as it is not yet fully utilize. If such huge amount of sago *hampas* were use to convert into reusable energy source or products, significant revenue can be obtain from such bioremediation industries.

Studies were done on bacterial (amylolytic *Bacillus*) isolates of sago *hampas* (Apun *et al.,* 2000), while screening and isolation of cellulolytic and amylolytic microorganisms from sago wastes was done as well by Apun *et al.* (1996). However the focus of current studies is to search for microorganisms that are both cellulolytic and amylolytic from sago pith residue. Screening for amylolytic fungus was not carried out on the other wastes like debarked barks and fruits and the processed effluent. This experiment intended to isolate and identify the best amylolytic fungus from such indigenous sago industrial waste.

2.8 Internal transcribe spacer (ITS) region

Ribosomal RNA gene cluster are most probably the most widely used DNA region in studies of systematics and evolution as well as molecular diagnostics development (Bridge, 2002). This region consists of 3 major genes which responsible for coding the large, small and highly conserved 5.8S ribosomal subunits (Bridge, 2002). These genes are separated by internal transcribe spacer region (ITS) (Bridge, 2002). This gene cluster is repeated many times along a chromosome and is known as multiply repeated DNA (Bridge, 2002). It had been used to determine the phylogenetic relationships between fungal species like Ascomycetes, Basidiomycetes and Chytridiomycetes (Bowman *et al.,* 1992).

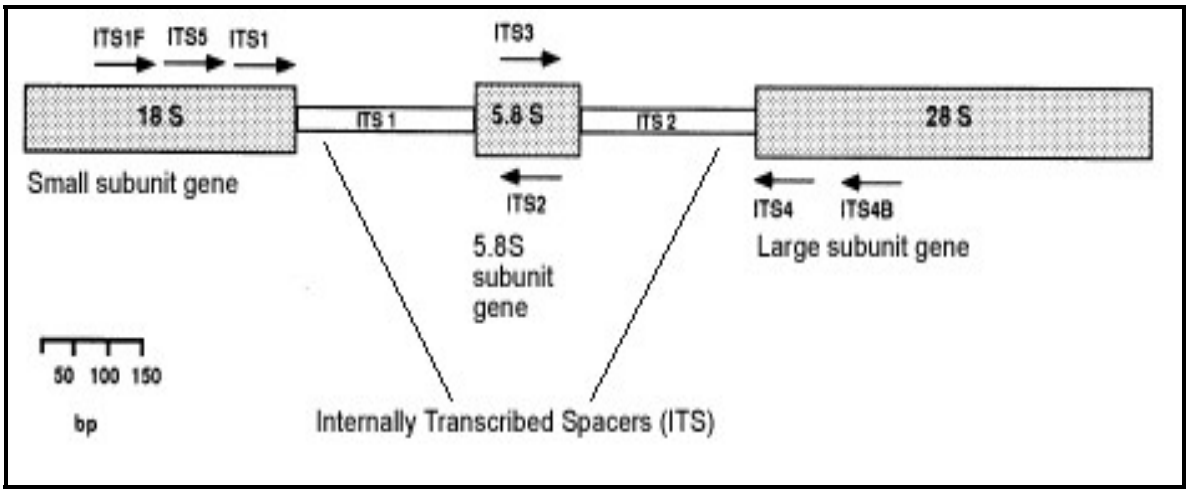

Figure 2.2: Schematic representation of internally transcribed spacers (ITS) region. Arrows denote the PCR sequencing primers position. Copied and edited from Boysen *et al.*, 1996

Based on the study done by Gardes and Bruns (1993), ITS primers especially ITS1F which is the fungal specific primer with ITS4 had efficiently amplified ascomycetous and basidiomycetous fungus. ITS1F/ITS4 and ITS5/ITS4 primers were both used in this study.

There are several features that make ITS region convenient target for fungi molecular identification. According to Gardes and Bruns (1993), (1) the entire fungi ITS region is often between 600 to 800 bp and readily be amplified by universal primers within the rRNA genes (White *et al.,* 1990), (2) Multicopy nature of the rDNA repeats makes amplification of ITS region easy, (3) ITS region is often highly variable among morphological distinct fungal species.