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Current Commercial Perspective of *Rhizopus oryzae*: A Review

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Abstract: The present study reviews the potentiality of *Rhizopus oryzae*, a potent saprophytic and pathogenic fungus to produce a wide spectrum of metabolites, in the form of enzymes, esters, organic acids, volatile materials, polymers and bioalcohols. A number of extra and intra cellular enzymes are found to be synthesized by various strains of *Rhizopus oryzae* that includes cellulaes, hemicellulases, pectinases, tannase, phytase, amylase, lipase, protease and other enzymes of immense industrial importance. The fungus is a rich source of lactic acid and is widely studied as a commercially perspective producer of L(+)-LA. In many strains, the end product of glycolysis is channeled to ethanol by the enzyme pyruvate decarboxylase and therefore may be used for the production of alcohol. Biodiesel can also be produced by methanolysis and transesterification reactions employing specific strains of *R. oryzae* having the relevant enzymes. Dry mycelium of *Rhizopus oryzae* are proved effective for efficiently catalyzing the synthesis of different flavor esters. Since the Joint FAO/WHO expert committee on food Additives (JEFCA) and Food and Drug Administration, Department of Health and Human Services, USA certified the enzymes extracted from *Rhizopus oryzae* may be used as food additives for human consumption, this fungal group may be exhaustively used for commercial purposes.

Key words: *Rhizopus oryzae,* commercial applications of fungus, enzyme production, alcohol production, lactic-acid production

INTRODUCTION

Rhizopus oryzae is a complex of closely related, heterothallic species that are common, cosmopolitan saprotrophs in soil, dung and rotting vegetation (Zheng *et al*., 2007). Most strains of *Rhizopus oryzae* were isolated as active components in the production of oriental foods or alcoholic beverages in Indonesia, China and Japan. Many hardly distinguishable species have been described in the older literature. *Rhizopus oryzae* has long been used for enzyme production (e.g., glucoamylase and lipase), organic acid synthesis, and various fermented food applications (Mertens *et al*., 2006).

Rhizopus oryzae is one of the most economically important members of zygomycete group of fungi and represents the first sequenced fungus from the early lineages of the fungal phylogenetic tree and thus the genome sequence sheds light on the evolution of the entire fungal kingdom.

The pathogenecity of *R. oryzae* towards plants is attributed to the presence of large number of carbohydrate digesting enzymes. Apart from the cellulases and hemicellulases, several other enzymes are also found to be secreted by *R. oryzae*. Thompson and Eribo (1984) discovered protease, urease, ribonuclease, pectate lyase and polygalacturonase, at varying levels of activity, in the cultural medium of *Rhizopus oryzae*. However, other than enzymes, *R. oryzae* is found to be equipped with the efficacy of producing a number of organic acid, alcohol and esters. The unique capabilities of the strains of *R. oryzae* warrants thorough review that may open newer avenues for better utilization of these strains in food and pharmaceutical industries and ameliorate the future prospect of bio-diesel production. The bounty of enzymes, polymers, organic acids, alcohols and other factors produced by various strains of *Rhizopus oryzae,* are stated below:

ENZYMES

Cellulases: Cellulose is a major polysaccharide constituent of plant cell walls and is one of the most abundant organic compounds in the biosphere (Murai *et al*., 1998; Hong *et al*., 2001). Cellulases have a wide range of enormous potential applications in biotechnology and many thermo stable endoglucanase appeared to have a great potentiality for industrial use

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(Karmakar and Ray, 2010a). Some of the most important applications of cellulases are in food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture and for research purposes. (Karmakar and Ray, 2011a).

The conversion of the cellulosic biomass is accomplished by the enzyme cellulase, which is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Himanshu, 2008). The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1, 4)-β-D-glucanase (endoglucanase or CMCase [EC 3.2.1.4]. exo-(1, 4)- β-D-glucanase (avicalase [EC 3.2.1.91]), and β-glucosidase (cellobiase [EC 3.2.1.21]). New extracellular endoglucanases, designated RCE1 and RCE2, produced by *Rhizopus oryzae* isolated from the soil, were purified to apparent homogeneity from the culture supernatant by Murashima *et al.* (2002). Extra cellular endoglucanase produced by *Rhizopus oryzae* from both LSF and SSF of various agro wastes could be used for rapid and commercial production of cellulase (Karmakar and Ray, 2010b). Karmakar and Ray (2011b) also found that cellulosic wastes could be easily and rapidly converted into glucose with the help of this endoglucanase secreted without the requirement of alkali or acid pretreatments. The same strain was also reported to produce extra cellular exoglucanase (Mukherjee *et al.*, 2011). FPase, a measure for total cellulase was also produced from the LSF and SSF of the same strain of *Rhizopus oryzae* (Karmakar and Ray, 2011c). Cellulases have also been derived both *in vivo* and *in vitro* by *R. oryzae* (Amadioha, 1993).

Xylanase: Xylanase is an enzyme that catalyzes the hydrolysis of 1, 4-beta-D-xylosidic linkages in xylans that are constituents of hemicellulose, a structural component of plant cell walls. Endo-xylanase (β-1,4-D-xylan-xylanohydrolase, EC 3.2.1.8) is the key enzyme for xylan depolymerization and was produced by *Rhizopus oryzae* fermentation from different xylan-containing agricultural byproducts such as wheat straw, wheat stems, cotton bagasse, hazelnut shells, corn cobs and oat sawdust (Bakir *et al.*, 2001). According to them the partially purified xylanase was with a molecular weight of about 22 kDa and a K_m and V_{max} values of 18.5 mg xylan mL^{-1} and 90 IU/mg protein, respectively. Maas *et al.* (2008) reported that Rhizopus oryzae converts both glucose and xylose under aerobic conditions into chirally pure L (+)-lactic acid with by-products such as xylitol, glycerol, ethanol, carbon dioxide and fungal biomass. They demonstrated that complete xylose utilisation required a significantly lower C/N ratio (61/1) compared to glucose (201/1), but decreased oxygen transfer rate resulted in decline of xylose consumption rates. The fungal strain *R. oryzae* CBS 112.07 utilized xylose via the two-step reduction/oxidation route.

Pectinase: Pectinolytic enzymes catalyzing the degradation of pectic substances are of great industrial importance (Spagna *et al.*, 1995). The pectinases are required for extraction and clarification of fruit juices and wines, extraction of oils, flavors and pigments from plant materials, preparation of cellulose fibers for linen, jute and hemp manufacture (Castilho *et al.*, 1999), coffee and tea fermentations (Taragano *et al.*, 1997) and in the production of oligogalacturonides as functional food components (Hang and Dornenburg, 2000).

The pectinolytic enzyme from the solid-state culture of *Rhizopus oryzae* NBRC 4707 was purified to homogeneity by column chromatography on CM-Toyopearl 650 M and hydroxylapatite. The molecular weight of the enzyme was estimated by SDS-polyacrylamide gel electrophoresis to be 31,000 and was reduced to 29, 700 after treatment with endoglycosidase H. Maximal activity was observed near pH 4.5 at 45°C. The enzyme was shown to be endopolygalacturonase, as judged from the formation of oligogalacturonides as its reaction products. The addition of purified enzyme, as expected, enhanced the formation of lactic acid and ethanol in potato pulp grown with *R. oryzae* (Saito *et al.*, 2004).

Potentiality of *Rhizopus oryzae* to utilize orange peels under solid state fermentation conditions to produce macerating fluid with high cellulolytic and pectinolytic activities were confirmed in the study of Hamdy (2005). He also purified the Pectin Lyase (PL) secreted by *R. oryzae* to electrophoretic homogeneity, using ammonium sulfate fractionation and 2-step-column chromatography and Values of K sub (m), V sub (max), K sub (cat) and molecular mass of the purified enzyme were found to be 3.87 mg mL^{-1}, 297 U mL^{-1}, 5.94 mg U min^{-1} and 31 kDa, respectively (Hamdy, 2006).

Fungal pectinase enzyme was produced by *Rhizopus oryzae* on a solid culture containing citrus peel of orange (35% w/v) and the crude extract with maximum pectinase activity of 1,360 μ mL^{-1} was used to clarify orange juice (Kareem and Adebowale, 2007). Orange finisher pulp, a juice processing by-product, was investigated by Hart *et al.* (1991) as a substrate for Poly Galacturonase (PG) production by Rhizopus oryzae (ATCC 24563) via solid state fermentation., where little or no pectinesterase, pectin or pectate lyase activities were detected; therefore, potential for direct utilization of the PG exists in the citrus industry.

The crude extract of the fungal culture grown in pectin liquid medium, which macerated the roots of mulberry, showed only the activity of three pectic enzymes (polygalacturonase, pectate lyase and pectin lyase). Polygalacturonase may play an essential role in the maceration of mulberry roots by *R.oryzae* (Yoshida *et al*., 2003).

Amylase: *Rhizopus oryzae* being a potent pathogen of rice plant possesses the starch breaking ability and is therefore showing amylolytic activities. Scientists are trying to exploit this efficacy and employ it in industrial production of various amylases, namely- β-, gluco and isoamylases. Amylase production from a potent raw starch-digesting strain of *Rhizopus oryzae* was maximum when the fungus was grown on wheat bran medium for 3 days at 30°C with an initial pH of 4 (Kim *et al*., 1987). *Rhizopus oryzae* produced extracellular amylase (3.8 units mL^{-1}) when grown on a liquid medium containing 2% (WN) soluble starch or cassava starch residue as the sole carbon source (Ray, 2004). Amadioha (1998) reported a strain of *Rhizopus oryzae* achieved a peak amylase production within 6 days of culture from starch yeast extract medium at 30°C and pH 6. *Rhizopus oryzae* isolated from cassava dried chips (Etoa *et al*., 2005) was found to produce extracellular amylase using cassava starch. The fungus as a prolific amylase producer could be of industrial use in some fermentation processes. In another report, *Rhizopus oryzae* NRRL 395 grown on different agricultural commodities produced much higher enzyme activity from barley, corn, bats and rice than from cassava, (optimal temperature 30°C). The rate of enzyme production was greatly enhanced by neutralization with $CaCO_3$. Nitrogen supplementation of cassava resulted in higher enzyme yields (Yu and Hang, 1990). Production of amylase by *Rhizopus oryzae* was found to be inhibited by fungicides namely, Brassicol, Captan, Dithane M-45, Fytolan, Parasan, Sulfex and Thiram (Chaurasia, 1992).

Rhizopus sp. A-11 produced glucoamylase (GA, EC 3.2.1.3) to a high concentration in a basal liquid medium supplemented with zinc and calcium ions (Fujio and Morita, 1996). *Rhizopus*-RFF screened from seventeen *Rhizopus* isolates from Bangladesh was selected as the potential producer of glucoamylase and the highest enzyme activity was observed at 45°C and pH 4.5 (Nahar *et al*., 2008) studied the fermentation conditions of glucoamylase production by *Rhizopus oryzae* R.SCLG 0319 and found the optimum temperature of 30°C, pH 6.0 and fermentation time 40 h and the catalytic ability of ethanol-dependent *Rhizopus oryzae* R.SCLG 0319 occurred in ethanol of low-concentration, with Ca^{2+} and Cu^{2+} probably enhancing the starch-hydrolying enzymatic activation.

Rhizopus oryzae PR7 MTCC 9642 has been reported to produce extra cellular isoamylase, a potent enzyme used in food industries (Ray, 2011), under submerged conditions at 28 °C, pH 8.0 and after 72 h of growth (Ghosh and Ray, 2010a). The strain could utilize native starch molecules from waste food stuffs, of which rice extract followed by bread dust showed best growth and isoamylase inducing activity (Ghosh and Ray, 2010b). The temperature and pH optima of isoamylase from *Rhizopus oryzae* PR7 MTCC 9642 were found to be at 55 °C and 5, respectively. The enzyme showed stability at 55°C for 10 min and at a broad pH range of 4-8 (Ghosh and Ray, 2010c). The enzyme was found to saccharify soluble potato starch and various native raw starches collected from domestic effluents, of which arrow root, tamarind kernel, tapioca and oat were highly promising (Ghosh and Ray, 2010a). This saccharifying ability of the enzyme would definitely increase its applicability in industries related to sugar production. The isoamylase was also found to be adsorbed onto various raw starch molecules, the rate of adsorption was highest toward pure potato starch, followed by arrowroot, corn flour and rice powder starch; while desorption was highest in case of corn flour and rice powder (Ghosh and Ray, 2011) which might make the purification process more convenient industrially and also provide with an economically less expensive method.

Carbohydrate-active enzymes (CAZy): Carbohydrate Active enzyme (CAZy) annotation of the *R. oryzae* identified a low number of Glycoside Hydrolases (GHs) and a high number of Glycosyl Transferases (GTs) and Carbohydrate Esterases (CEs). The specific genomic and growth features for degradation of easily digestible plant cell wall mono and polysaccharides (starch, galactomannan, unbranched pectin, hexose sugars), chitin, chitosan, β-1, 3-glucan and fungal cell wall fractions suggest specific adaptations of *R. oryzae* to its environment (Battaglia *et al*., 2011).

Tannase: Tannase (tannin acyl hydrolse, E.C.3.1.1.20) catalyses the hydrolysis of ester and depside bonds of hydrolysable tannins as tannic acid, methygallate, ethylgallate, n-propylgallate and isoamylgallate releasing glucose and gallic acid (Barthomeuf *et al*., 1994). Few reports are available on tannase production by *Rhizopus oryzae*. Production of tannase by *Rhizopus oryzae* from the powdered fruits of *Terminalia chebula* and *Caesalpinia digyna* has been reported by Madhavakrishna *et al*. (1960). A strain isolated locally and identified as *Rhizopus oryzae* (RO, IIT KGP) was found to synthesise an extracellular enzyme, tanin acyl hydrolase, showing its degradability of tannic acid to gallic acid

(Hadi *et al*., 1994). An attempt has been made to optimize the production of enzyme tannase by Solid State Fermentation (SSF) using the organism *Rhizopus oryzae*. The best favourable conditions for enzyme production include initial pH-5 with 4 days of incubation period at 40°C, 72% humidity and 10 g wheat bran soaked in 2.5% tannic acid (Chatterjee *et al*., 1996). Hota *et al*. (2007) reported production of tannase using different tannin rich agro-residues, like sal seed, fruit of myrobalan and tea-leaf were used as carbon source by *Rhizopus oryzae* RO IIT RB-13, NRRL-21498 and the maximum enzyme production of 17.7 U/mL was obtained in sal seed powder incubated for 48 h at 30°C. The enzymatic conversion of these agro-residues was carried out using tannase immobilized by entrapment method using sodium alginate and calcium chloride. The maximum bioconversion (90 and 87%, respectively) was achieved with sal seed and tea leaf as substrate at 40°C and initial pH 4.5. In case of myrobalan, the maximum bioconversion was 90.2% at 50°C and initial pH 5.0. Moreover, optimization of the pH and temperature largely reduced the incubation time to 36 h. The immobilized tannase was stable for 7 cycles. The kinetic properties of immobilized enzyme revealed that there was a decrease in maximal reaction velocity (Vmax) and increase in Michaelis constant (Km) when compared to its free native counterpart. The highest yield of tannase and gallic acid was obtained after 60 h at optimum initial pH and temperature of 4.5 and 30°C, respectively from solid-state fermentation of Rhizopus oryzae (Mukherjee and Banerjee, 2004). A few colonies from the co-culture of *Aspergillus foetidus* and *Rhizopus oryzae* were screened after mutagenesis by UV, heat and NTG for tannase study and the strain SCPR 337 was selected as the best mutant that could produce 53.6 U mL^{-1} (44.2 U mL^{-1} after 48 h by wild strain). The mutant was sensitive to tetracycline and was also an over-producer of protease and amylase (Purohit *et al*., 2006). Banerjee and Mukherjee, 2006) developed a process for the preparation of gallic acid using co-culture comprising providing a tannin-rich mixed substrate and a culture medium in fluid communication with each other, adding an induced innoculum comprising the fungi *Rhizopus oryzae* and *Aspergillus foetidus*.

Phytase: Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) is a major storage form of phosphorus and the source of inositol in plant seeds (Bhavsar *et al*., 2008) and phytases (EC 3.1.3.8 for 3-phytase and EC 3. 1.3.26 for 6-phytase) hydrolyses phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates (Mullaney *et al*., 2000). Ramachandran *et al*. (2005) reported that commercially available Coconut Oil Cake (COC), Sesame Oil Cake (SOC), Palm Kernel Cake (PKC), Groundnut Oil Cake (GOC), Cottonseed Oil Cake (CSC) and Olive Oil Cake (OOC) were used as substrates for phytase production in solid-state fermentation using three strains of *Rhizopus* spp., namely *Rhizopus oligosporus* NRRL 5905, *Rhizopus oryzae* NRRL 1891 and *R. oryzae* NRRL 3562, of which mixed substrate fermentation of COC and SOC (1:1 w/w) further enhanced enzyme production by *R. oryzae* NRRL 1891 to 35U/gds). On the other hand, Ghosh (2011) tested the feasibility of various agro-industrial residues; individually as well as in combinations for the production of newly identified phytase from *Rhizopus oryzae* and optimized the culture parameters for maximum phytase production followed by the characterization of the enzyme.

Proteases: Proteases are a group of enzymes that conducts proteolysis by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain and are the most valuable commercial enzymes that account for 60% of the total enzyme market Rao *et al*. (1998). They find increased application in food, pharmaceutical, detergent, leather, tanning industry and to some extent in silver recovery and peptide synthesis (Godfrey and West, 1996; Kumar and Takagi, 1999; Oberoi *et al*., 2001).

A locally isolated species of *Rhizopus oryzae* was found to secrete alkaline serine protease of industrial importance. The kinetic parameter V (reaction velocity) of the purified fractionated enzyme was evaluated under different environmental conditions and substrate to enzyme ratios. The K_m and V_{max} values were also estimated (Banerjee and Bhattacharyya, 1993). The protease showed high pH stability within 3.0-6.0 and poor thermo-stability, which might lose its activity when incubated at 50°C for 30 min. Production of proteolytic enzyme was also carried out in a bubble column bioreactor using the microorganism *Rhizopus oryzae* (Prasad *et al*., 1995). Aikat *et al*. (2001) partially purified activated charcoal decolorized protease from a crude extract of solid state fermentation of wheat bran by *Rhizopus oryzae*. The protease showed over a 3-fold purification and SDS-PAGE showed that it consisted of two sub-units (about 22 and 24 kDa). A secretory aspartic protease (also termed as rhizopuspepsin), a non glycoprotein, was purified from *Rhizopus oryzae* NBRC 4749 by ion exchange chromatography with a yield of 45% (Chen *et al*., 2009). Extra cellular aspartate protease from *Rhizopus oryzae* was purified 91 times with 26% recovery using $(NH_4)_2SO_4$ fractionation, ion-exchange and size-exclusion chromatographic techniques (Kumar *et al*., 2005).

Purification of the crude proteolytic enzyme produced by *Rhizopus oryzae* was done using converging-diverging foam fractionators which provided a cost effective purification method (Banerjee *et al*., 1993). *Rhizopus oryzae* was found to possess a secreted aspartic proteinase gene family *(sap)* of at least four members *(sapl-sap4)* (Farley and Sullivan, 1998). According to a report by Chen *et al.* (2009) N-terminal sequence and LC-MS/MS analyses revealed that this rhizopuspepsin corresponded to the hypothetical protein RO3G-12822.1 in the *R. oryzae* genome database. Comparison of genomic and cDNA genes demonstrated that the rhizopuspepsin contained two introns, whereas only one intron was reported in other rhizopuspepsin genes. The use of the mixture of selected enterococci and *R. oryzae* proteases were considered as a potential tool to decrease gluten concentration in foods (M'hir *et al*., 2009). A thermostable and alkaline protease from *Rhizopus oryzae* was found to be potential for several industrial uses viz., cleaning agents, food additives, pharmaceuticals and confectionaries (Banerjee and Bhattacharyya, 1992).

Lipase: Lipase derived from *R. oryzae* has been consumed for many years as digestive aids without apparent adverse reactions. Lipases (triacylglycerol ester hydrolases, E.C.3.1.1.3) have been classified as enzymes that hydrolyze fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol (Jaeger *et al*., 1999; Woolley and Petersen, 1994). During the last decade, lipases have gained a great interest in biotechnology applications. This interest arises from the ability of these enzymes to catalyze synthetic reactions occurring in non-aqueous media (Gandhi, 1997). The lipases of the *Rhizopus species* family are important and versatile enzymes that are mainly used in fat and oil modification due to their strong 1, 3-regiospecificity.

Due to their commercial availability, low cost, high stereo selectivity and the possibility of use at large range of pH and temperature; lipases are among the most used biocatalysts in organic synthesis (Dalla-Vecchia *et al*., 2005). They have been employed for direct esterification and transesterification reactions in organic media to produce esters having potential applications in fine chemicals, pharmaceuticals and agrochemicals industries. Moreover, numerous works reported the aptitude of lipases to catalyze the synthesis of short chain fatty acids and alcohols used as additives for a variety of perfumes and flavours (Abbas and Comeau, 2003; Karra-Chaabouni *et al*., 2006; Bezbradica *et al*., 2007), biosurfactants (Sabeder *et al*., 2006; Chen *et al*., 2005) and biofuels (Salis *et al*., 2005; Royon *et al*., 2007). These enzymes occur extensively in nature in animals, plants and microorganisms. Fungal lipases are commercially important and find use in diverse range of industries like detergents, pharmaceuticals, beverages, dairy etc. (Mateo *et al*., 2007).

The fungus *Rhizopus oryzae* synthesizes an extracellular lipase precursor bearing N-terminal pre and pro-sequences (Beer *et al*., 1996). There are also reports of lipase from thermophilic *Rhizopus oryzae* strain isolated from Palm oil. Exploration of fungal diversity for improved production of lipases using statistical models for novel bioprocess development makes it as a high-profile area for novel discovery with enormous potential of massive returns (Shukla *et al*., 2007). Lipase from *R. oryzae* has been successfully immobilized on inorganic supports-aluminium oxide and silica gel (Nekliudov *et al*., 1981). Immobilization of the enzyme on cellulose substrate enhanced the tolerance of the enzyme to the temperature and pH (Karra-Chaabouni *et al*., 2008).

A new enzymatic process to obtain polyglycerol polyricinoleate (PGPR) has been developed by successful immobilization of lipase from *Rhizopus oryzae* by Bodalo *et al.* (2009). Inexpensive synthetic medium was used for the production of *Rhizopus oryzae* lipase in the methylotrophic yeast *Pichia pastoris* (Minning *et al*., 2001). Studies have shown that the stereo preference of ROL toward triradylglycerol substrates can be predicted by rational designs (Scheib *et al*., 1998).

ROL can synthesize methyl esters from plant oil and methanol in a solvent-free reaction system (Kaieda *et al*., 1999) and has also been reported to catalyse the condensation of siloxane bond formation (Abbate *et al*., 2010). Studies show that lipase activity of *R. oryzae* is active at high pH but decrease in the presence of alkanes, acetone, ethers and chloroalkanes (Essamri *et al*., 1998). Membrane-bound lipase plays a crucial role in the methanolysis activity of *R. oryzae* cells which can be used for bio-fuel production (Hama *et al*., 2006). Abbate *et al.* (2010) experimentally demonstrated that three enzymes, *Rhizopus oryzae* lipase (ROL), lysozyme and phytase catalysed the condensation of trimethylsilanol and residue-specific modification of the key-amino acids believed to participate in the ROL catalysis also had a significant effect on the silicon bio-catalysis.

Other enzymes: Few other enzymes are reported to be synthesized by rhizopus oryzae strains, which are as follows:

Steroid 11 α-hydroxylase: Petric *et al.* (2010) have reported for the first time the expression of a fungal 11 α-steroid hydroxylase from *Rhizopus oryzae* which can

be employed to perform the 11 α-hydroxylation of the steroid skeleton, thereby significantly simplifying steroid drug production.

Ribonuclease: *Rhizopus oryzae* IFO 4697 was found to produce intracellular ribonuclease (RNase) in a metal ion-regulated liquid medium, with Ca^{2+} and Mo^{6+} stimulating RNase production (Morita *et al*., 2002).

Tyrosinase and peroxidase production: *Rhizopus oryzae* strain ENHE isolated from contaminated soil was found to be capable of tolerating and removing PCP (pentachlorophenol). This fungus produced extra and intracellular tyrosinase and extracellular lignin peroxidase (Leon-Santiesteban *et al*., 2008). According to their study, the enzymes tyrosinase and lignin peroxidase were probably involved in the PCP removal process.

Urease: Urease (EC 3.5.1.5) is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia. Microbial ureases hydrolyze urea to ammonia and carbon dioxide. Extraction, purification and characterization of intra cellular urease of 172 kDa from *Rhizopus oryzae* was carried out by Geweely (2006). Farley and Santosa (2002) investigated the regulation of intracellular urease and uricase activities in *Rhizopus oryzae* that differs from that found in *N. crassa* and *A. nidulans*.

Pyruvate decarboxylase: According to Acar (2004), pyruvate decarboxylase was purified and characterised for the first time from *R. oryzae* and the purified enzyme had a Hill coefficient of 1.84 and the Km of 8.6 mM for pyruvate at pH 6.5. The enzyme was inhibited at pyruvate concentrations higher than 30 mM. The optimum pH for enzyme activity shows a broad range from 5.7 and 7.2. The monomer molecular weight was estimated as 59±2 kDa by SDS-PAGE analysis. The metabolic flux analysis of *R. oryzae* has shown that most of the pyruvate produced at the end of the glycolysis was channelled to ethanol, acetyl-CoA and oxaloacetate production and therefore the enzyme pyruvate decarboxylase was of immense significances.

POLYMER

Chitosan: Chitosan is a natural biopolymer which stimulates growth and increases yield of plants as well as induces the immune system of plants (Boonlertnirun *et al*., 2008). Chitosan was successfully produced as a second valuable product from *Rhizopus oryzae* mycelia which had been previously used to make L (+) lactic acid. The highest yield of extractable chitosan was 700 mg L^{-1} (Hang, 1990a). Some plant growth hormones, viz., gibberellic acid, indole-3-acetic acid, indole-3-butyric acid, and kinetin have been found to enhance chitin deacetylase activity of *R. oryzae* by 1.067-1.267-fold and may be one of the reasons for increased chitosan production (Chatterjee *et al*., 2008). Potato chip processing waste of trimmed potato, potato peel and substandard (low-quality) potato chips, obtained from a potato chip processing plant, were used as substrates for chitosan production from *Rhizopus oryzae* (Kleekayai and Suntornsuk, 2011). Chitosan was produced by Rhizopus oryzae 00.4367 in shake flask culture and a stirred tank fermenter where synthetic medium, treated and untreated beet molasses were used as cultivation media (Goksungur, 2004). He found that the chitosan concentration reached its maximum value at the late exponential growth phase of *R. oryzae*.

Chitin: Liu *et al*. (2008) proposed an innovative process for lactic acid and chitin co-production using pelletized Rhizopus oryzae NRRL 395 to improve both fermentation yield and productivity with cull potatoes as nutrient source. *Rhizopus oryzae* ATCC 20344 with high chitin content was used (Liao *et al*., 2008) for the production of chitin making the utilization of manure more efficient and more profitable by a three step fermentation process yielding 0.21gchitin/gbiomass.

Biopolymer: The thermotolerant strain *Rhizopus oryzae* ST29 produced biopolymer during cultivation in palm oil mill effluent (POME) at 45°C, but not in the three synthetic media, which could be recovered by filtration, centrifugation, and precipitation by adding 4 volumes of 95% ethanol, then freeze-drying (Suyala *et al*., 2008).

ORGANIC ACIDS

Organic acid production had been studied extensively in *R. oryzae*. Takahashi and Sakaguchi (1925) and Takahashi *et al*. (1926) studied the production of fumaric acid and lactic acid and found that *Rhizopus* spp. could be divided into fumaric acid producers, lactic acid producers and producers of both fumaric and lactic acid, but they did not comment on the taxonomic importance of acid production. According to Ayumi (2007), *Rhizopus oryzae* strains were divided into two groups, LA (lactic acid producer) and FMA (fumaric-malic acid producers) according to organic acid production.

Oda *et al*. (2003) conducted an experiment where two groups of 15 *Rhizopus oryzae* strains were grown in a liquid medium to analyse metabolic products such as lactic, fumaric, malic and other organic acids and ethanol of which the first group produced a large amount of lactic acid and the second group produced fumaric and malic

acids. Amongst other acids namely, myristic, palmitic, palmitoleic, oleic, stearic, linoleic and γ-linolenic acids group A contained high amounts of unsaturated fatty acids and differed from group B in the proportion of palmitic and γ-linolenic acids. The scientists presumed that the physiological differences in *R. oryzae* strains may depend on the productivity of organic acids, reflecting the composition of fatty acids.

Fumaric acid: Fumaric acid is widely used as a food additive for flavor and preservation. Cao *et al.* (1996) described the production of fumaric acid by a *Rhizopus oryzae* strain from glucose in a Rotary Biofilm Contactor (RBC) coupled with an adsorption column. That fermentation system is capable of producing fumaric acid with an average yield of 85 g/liter from 100 g of glucose per liter within 20 h under repetitive fed-batch cycles. *Rhizopus oryzae* ATCC 20344 was known for good fumaric acid production from the manure fiber (Liao *et al.*, 2008).

Lactic acid: Lactic acid, also known as milk acid, is a chemical compound that plays a role in several biochemical processes. Lactic acid and its salts can be used as components of food, pharmaceuticals, cosmetics, and agrichemicals (Liaw, 2005). Skory *et al.* (1998) found during aerobic growth, *Rhizopus oryzae* produced L-lactic acid from lactate dehydrogenase mediated reduction of pyruvate, while oxygen limiting conditions yield primarily ethanol. They isolated a mutant that expressed almost a ten-fold increase in lactic acid production when compared to the parent strain. A L (+)-lactic acid over-producing mutant, *Rhizopus oryzae* R1021, was isolated by mutagenizing the parent strain (*R. oryzae* R3017) with UV, diethyl sulfate (DES) and Co by Bai *et al.* (2004). Park *et al.* (2004) investigated the production of L (+)-lactic acid using an enzymatic hydrolysate of waste Office Automation (OA) paper in a culture of the filamentous fungus *Rhizopus oryzae* of 4 day culture.

The fungus *Rhizopus oryzae* is widely studied as a commercially perspective producer of L (+)-LA (Miura *et al.*, 2003; Yin *et al.*, 1997), because the fungus cells possess better resistance to high concentrations of accumulated LA (Hamamci and Ryu, 1994; Schepers *et al.*, 2003) and lower content of nutrient requirements compared to the commonly used bacterial producers (Hujanen *et al.*, 2001; Kwon *et al.*, 2000). The use of *R. oryzae* in immobilised form is one of the most efficient approaches to improve the LA production process that facilitates multiple reuses of fungal cells for long-term LA production (Sun *et al.*, 1999; Tay and Yang, 2002; Xuemei *et al.*, 1999; Nedovic and Willaert, 2004). Ward *et al.* (1938) have produced dextro-Lactic acid by fermentation of glucose 25 solutions with *Rhizopus oryzae* L. (+)-lactic acid is produced in high yield and purity by a direct one step fermentation of renewable biomass such as ground corn using *Rhizopus oryzae* in the presence of calcium carbonate. L (+)-lactic acid and its salts will find increased use as components of foods, 30 pharmaceuticals and as monomer in the preparation of biodegradeable polymers (Hang, 1990b). Lactic Acid has also been produced by fermentation of potato pulp and cassava pulp by *Rhizopus oryzae* (Thongchul *et al.*, 2010). It may be used as an inoculant for ensiling potato pulp and other agricultural by-products containing starch (Oda *et al.*, 2002). According to Thongchul and Yang (2003), a high lactic acid concentration of 137 g L^{-1} with a high yield of 0.83 g^{-1} g and reactor productivity of 2.1 g L^{-1} h was obtained with the Rotating Fibrous Bed Bioreactor (RFBB) in repeated batch fermentations. The process was stable and can be used for an extended operation period.

A new immobilized biocatalyst based on *Rhizopus oryzae* fungal cells entrapped in poly (vinyl alcohol)-cryogel was evaluated in both the batch and semi-batch processes of L(+)-Lactic Acid (LA) production, when glucose, acid hydrolysates of starch or gelatinized potato starch were used as the main substrates (Efremenko *et al.*, 2006). Skory (2003) found some advantages of using *Rhizopus* species as an alternative to lactic acid bacteria include production of optically pure lactic acid, which is preferred for many applications and the ability of the fungus to grow in a chemically defined minimal medium without the need for complex components such as yeast extract that may reduce cost and complication of the purification of the final product. In Skory (2004) opined *Rhizopus oryzae* was capable of producing high levels of lactic acid by the fermentation of glucose. In order to increase lactate dehydrogenase (LDH) activity, three plasmids, namely pLdhA71X, pLdhA48XI and pLdhA89VII, containing various lengths of the *ldhA* gene fragment, were transformed into *R. oryzae*. He found the greatest levels of *ldhA* transcript and enzyme production occurred with isolates transformed with plasmid pLdhA89VII and also found the production of lactic acid by these transformants along with higher amounts of ethanol, fumaric and glycerol. Pritchard (1973) stated that the NAD-dependent lactate dehydrogenase of *Rhizopus oryzae*, like some bacterial lactate dehydrogenases, catalyses reduction of pyruvate by NADH but not the reverse reaction. It will, however, catalyse reduction of the NAD^+ analogue, 3-acetylpyridine adenine dinucleotide ($APAD^+$) by L (+)-lactate; NAD^+ does not inhibit the rate of $APAD^+$ reduction in this reaction.

The filamentous fungus *Rhizopus* is an obligate aerobe that is often used for industrial production of L-(-) lactic acid, which currently has an estimated global market in excess of 100,000 tons per year (Hester, 2000). Approximately 75% of the lactic acid is used in the food industry as an acidulant for flavor or as an antimicrobial agent. Liaw (2005), developed a novel, temperature-resistant strains of *Rhizopus oryzae* and mutants thereof. These novel strains were used to produce lactic acid at high temperatures.

However, the shortcoming of using *Rhizopus* is that the production efficiency is still generally considered low compared to bacterial fermentations. In an effort to better understand the fermentation control mechanisms of *Rhizopus*, Skory (2003) cloned an NAD-dependent lactate dehydrogenase (LDH; EC1.1.1.27) that appears to be primarily responsible for the conversion of pyruvate to lactic acid. *R. oryzae* contains an NAD-dependent l-lactate dehydrogenase enzyme, RO-LdhA, which is primarily responsible for production of lactic acid, while both organisms contain another enzyme, LdhB that is thought to be involved in lactic acid production only under certain growth conditions (Skory *et al*., 2009).

Thitiprasert *et al*. (2011) checked the *in vivo* regulation of alcohol dehydrogenase and lactate dehydrogenase in *Rhizopus oryzae* to improve L-Lactic acid fermentation where he found the highest lactic acid yield of 0.47 g g^{-1} glucose was obtained when 0.01 mM 2, 2, 2-trifluoroethanol was present during the production phase of the pregrown *R. oryzae* which represented about 38% increase in yield.

ESTERS

Esters are usually derived from an inorganic acid or organic acid in which at least one -OH (hydroxyl) group is replaced by an -O-alkyl (alkoxy) group and most commonly from carboxylic acids and alcohol. A kinetic model for the biocatalytic synthesis of esters using *Rhizopus oryzae* resting cells is proposed by Mendez *et al*. (2009) using *Rhizopus oryzae* resting cells in a range of 30-50 °C and atmospheric pressure where the kinetics corresponding to the esterification of palmitic acid and propanol in MTBE catalyzed by *Rhizopus oryzae* resting cells were suggested to agree with a Ping Pong Bi Bi mechanism.

The use of dry mycelium of *Rhizopus oryzae* as biocatalyst for ester production in organic solvent has been studied by Gandolfi *et al*. (2001). Dry mycelium of four strains of *Rhizopus oryzae* proved effective for efficiently catalysing the synthesis of different flavour esters (hexylacetate and butyrate, geranylacetate and butyrate) starting from the corresponding alcohol and free acid, including acetic acid. The esterification of the racemic mixture of 2-octanol and butyric acid proceeded with high enantioselectivity (R-ester produced with enantiomeric excess> or = 97%) when *Rhizopus oryzae* CBS 112.07 and *Rhizopus oryzae* CBS 260.28 were employed. A new lipase preparation from *Rhizopus oryzae* was reported to catalyze the esterification reaction between acetic acid and butanol to produce butyl acetate ester (pineapple flavor) by Salah *et al*. (2007) who claimed that this flavor compound can be used in food, cosmetic and pharmaceutical industries. Microbial production of different alipathic esters with flavour characteristic has been studied by Molinari *et al*. (1995) and lyophilized whole cells of *Rhizopus oryzae* CBS 112-07 were found to be particularly suitable to catalyse the synthesis of different flavour esters (hexyl acetate, propionate, butyrate, caprylate; geranyl acetate, propionate, butyrate and 2 and 3-methylbutyl acetate, butyrate) in n-heptane. To select the best biocatalysts for ethanol acylations with phenylacetic and 2-phenylpropionic acids, lyophilized mycelia of, *Rhizopus oryzae* CBS 11207, CBS 39134, CBS 26028 and CBS 32847 were tested by Torre *et al*. (2007). The carboxylesterase activities of *R. oryzae* 11207, was revealed to be the best biocatalysts to catalyze the hydrolysis or the synthesis of ethyl esters of these acids.

ALCOHOL AND BIODIESEL

Ethanol is a main by-product in the fermentation broth of *Rhizopus oryzae* during the production of high-optical purity L-lactic acid (Zheng *et al*., 2009). In *Rhizopus oryzae* cultivations, lactate production decreased and ethanol and biomass productions increased as inoculated spore concentration increased (Buyukkileci *et al*., 2006). In the study of Zheng *et al*. (2009), a high-producing lactic acid mutant HBF-12 of *R. oryzae* As3.3461 was screened out with YPD medium containing allyl alcohol. Higher ethanol yield and productivity were observed from fermentation by *Rhizopus oryzae* NRRL 395 when the acid or enzymatic hydrolysates of cassava pulp were used as the carbon source (Thongchul *et al*., 2010) than that obtained from fermentation with glucose in which lactic acid was the main product. Ethanol production from rice straw by simultaneous Saccharification and Fermentation (SSF) with *Mucor indicus*, *Rhizopus oryzae* and *Saccharomyces cerevisiae* was investigated by Karimi *et al*. (2006). They found an average of 2-3 days were usually enough to achieve the maximum ethanol yield. All the strains were able to produce ethanol from the pretreated rice straw of

which *R. oryzae* had the best ethanol yield. Rice straw was also successfully converted to ethanol by separate enzymatic hydrolysis and fermentation by *Mucor indicus*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*, of which *R. oryzae* produced lactic acid as the major by-product with yield of 0.05-0.09 gg^{-1}. This fungus had ethanol, biomass and glycerol yields of 0.33-0.41, 0.06-0.12 and 0.03-0.04 gg^{-1}, respectively (Abedinifar *et al*., 2009). Among the major metabolites of *Rhizopus oryzae* ME-F01 ethanol was the main metabolic byproduct and the formation of ethanol competed with the biosynthesis of fumaric acid for the cofactor NADH (Fu *et al*., 2010). Ethanol is the main by-product in the fermentation broth of *Rhizopus oryzae* As 3.3461 for the production of high-optical purity L-lactic acid. Alcohol Dehydrogenase (ADH) was the branch pathway enzyme that catalyzed the transformation of ethanol from pyruvate in *Rhizopus oryzae*, which decreased the conversion rate of glucose to L-lactic acid. (Pan *et al*., 2006). Fu *et al*. (2010) isolated one mutant of *Rhizopus oryzae* ME-F01, ME-UN- 8, which produced 21.1% more fumaric acid than wild type with the corresponding byproduct of ethanol.

Biodiesel fuel, as fatty acid methyl ester is produced by esterification of plant oil or animal fat with methanol. This renewable fuel resource is an attractive alternative for the replacement of petroleum based fuels (Pazouki *et al*., 2010). Jin *et al*. (2008) compared the yield of biodiesel from separate hydrolysis and methanolysis and transesterification reactions where both hydrolysis and methanolysis reactions occur in the same reactor. About 90% of biodiesel could be obtained when a transesterification reaction using methanol was followed by one hydrolysis and one ethanolysis reaction. Production of biodiesel fuel from plant oils from cells of *Rhizopus oryzae* (*R. oryzae*) IFO4697 (with a 1, 3-positional specificity lipase) immobilized within biomass support particles (BSPs) were investigated for the methanolysis of soybean oil by Ban *et al*. (2001). Olive oil or oleic acid was found to be significantly effective for enhancing the methanolysis activity.

Utilization of the whole cell as biocatalyst instead of free or immobilized enzyme is a new approach to reduce the catalysts costs in lipase-catalyzed biodiesel production. Kim *et al*. (2007) optimized biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. The immobilized cell of *Rhizopus oryzae* (PTCC, 5174) in Biomass Support Particles (BSPs) was used for the methanolysis of Used Cooking Oil (UCO). After filtration and heating at 100°C, the pretreated UCO was converted to biodiesel. BSPs of the immobilized cells of *R. oryzae* were added for 72 h to allow the free acid to methyl esters conversion (Pazouki *et al*., 2010). According to Li *et al*. (2008), the application of *R. oryzae* whole cell in biodiesel production from triglycerides is restrained as the intracellular lipase was found to have 1, 3-positional specificity when used to catalyze methanolysis of triglycerides. They found that in a *tert*-butanol system, *R. oryzae* IFO4697 whole cell exhibited both better methanol endurance and better stability than that in a solvent-free system. Molecular sieves (3 Å) were added into the reaction mixture to online remove the produced water and a much higher biodiesel yield could be achieved (biodiesel yield reached 90% at 48 h).

Lipase from *Rhizopus oryzae* efficiently catalyzed the methanolysis of soybean oil in the presence of 4-30 wt% water in the starting materials; however the lipase was nearly inactive in the absence of water. The kinetics of the reaction appears to be in accordance with the successive reaction mechanism. That is, the oil is first hydrolyzed to free fatty acids and partial glycerides and the fatty acids produced are then esterified with methanol. Although *R. oryzae* lipase is considered to exhibit 1(3)-regiospecificity, a certain amount of 1, 3-diglyceride was obtained during the methanolysis and hydrolysis of soybean oil by *R. oryzae* lipase solution. Therefore, the high ME content in the reaction mixture is probably attributable to the acyl migration from the sn-2 position to the sn-1 or sn-3 position in partial glycerides. (Kaieda *et al*., 1999). It was reported that *R.oryzae* lipase, a 1,3-specific lipase, mainly converts fatty acids from oils into biodiesel and takes over 40 h for to finish biodiesel production. Lee *et al*. (2006) successfully developed a new process for biodiesel production using a mixture of *Rhizopus oryzae* and *Candida rugosa* lipases where as *C. rugosa* lipase does not require acyl migration; the production does not require much time. In another study (Lee *et al*., 2008), the enzymatic process for biodiesel production was optimized using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. The optimal temperature and agitation speed for biodiesel production were 45°C and 300 rpm, respectively. The optimal ratio of *R. oryzae* and *C. rugosa* lipases in the mixture was 3:1 (w:w).

In their recent most study Lee *et al*. (2011), determined the effects of temperature, pressure, agitation speed, water content and the concentration and ratio of immobilized lipases from *Rhizopus oryzae* and *Candida rugosa* for the efficient enzymatic production of biodiesel using a supercritical carbon dioxide process, where they found that highest production at 130 bar pressure, 45°C temperature, 250 rpm agitation speed, 10% water content, and 20% immobilized ROL and CRL (1:1). Moreover, the conversion yield of biodiesel produced by

the repeated recycling of immobilized lipase in the stepwise reactions was 85% after 20 reuses.

In *Rhizopus oryzae*, when cells were cultivated with various substrate-related compounds, such as olive oil and oleic acid, the intracellular methanolysis activity strongly correlated with the relative amounts of the membrane-bound lipase, which suggests that ROL31 localized in the membrane plays a crucial role in the methanolysis activity of *R. oryzae* cells (Hama *et al*., 2006). Yeast whole-cell biocatalysts for lipase-catalyzed reactions were constructed by intracellularly overproducing *Rhizopus oryzae* lipase (ROL) in *Saccharomyces cerevisiae* MT8-1. The gene encoding lipase from R. oryzae IFO4697 was cloned and intracellular over production systems of a recombinant ROL with a pro-sequence (rProROL) were constructed (Matsumoto *et al*., 2001).

The *R. oryzae* cells easily became immobilized within the BSPs during batch operation. To enhance the methanolysis activity of the immobilized cells under the culture conditions used, various substrate-related compounds were added to the culture medium. Among the compounds tested, olive oil or oleic acid was significantly effective. The process developed by Ban *et al*. (2001) might be considered to be promising for biodiesel fuel production in industrial applications.

Whole cell-mediated methanolysis of renewable oils for biodiesel production has drawn much attention in recent years since it can avoid the complex procedures of isolation, purification and immobilization required for the preparation of immobilized lipase. Whole cell (*R.oryzae* IFO 4697) adopted directly as biocatalyst could catalyze the transesterification (Zeng *et al*., 2005) and methanolysis of vegetable oils for biodiesel production (Zeng *et al*., 2006). It was found that different oils contained in the cultivation medium had varied effects on the whole cell-catalyzed transesterification and methanolysis of oils for biodiesel production; with some specified oil as the carbon source for cell cultivation.

Sun *et al*. (2010) reported that *Rhizopus oryzae* IFO4697 whole cell could catalyze the methanolysis of renewable oils for biodiesel production effectively and Glutaraldehyde (GA) cross-linking treatment on whole cell catalyst could improve its stability in the repeated uses. Treated cells expressed higher methanol tolerance and high catalytic activity could be maintained with higher ratio of methanol to oil; the operational stability of whole cell catalyst and methanol utilization rate was also considered in optimization of methanol addition strategy. Whole cell *Rhizopus oryzae* IFO4697 immobilized within Biomass Support Particles (BSPs) was used as catalyst for biodiesel production in tert-butanol. Refined, crude and acidified rapeseed oils were adopted further for biodiesel production in tert-butanol system and it was found that when acidified rapeseed oil was used as feedstocks, the reaction rate and final Methyl Ester (ME) yield were significantly higher than that of refined and crude rapeseed oil (Li *et al*., 2007). They found that in a *tert*-butanol system, *R. oryzae* IFO4697 whole cell exhibited both better methanol endurance and better stability than that in a solvent-free system (Li *et al*., 2008).

VOLATILE COMPOUNDS

Numerous microorganisms are capable of synthesizing potentially valuable flavour compounds and enzymes used in flavour manufacturing. Tropical agro-industrial substrates were used by *Rhizopus oryzae* for production of volatile compounds. *Rhizopus oryzae* grown on medium containing cassava bagasse and soybean meal, CO_2 production was at its highest after 20 h, but when amaranth grain was used as substrate, highest volatile metabolites were produced, amongst which ethanol was the most abundant compound (more than 80%) followed by acetaldehyde, 1-propanol, ethyl acetate, ethyl propionate and 3-methyl butanol within 36 h (Bramorski *et al*., 1998).

Christen *et al*. (2000) cultivated four edible *Rhizopus* strains on eight combinations of solid agro-industrial wastes (cassava bagasse, apple pomace), soyabean, amaranth grain and soyabean oil. *Rhizopus oryzae* ATCC 34612 was found to be the best producer of volatiles. The amaranth medium with mineral salts solution produced the highest amount of Volatile Compounds (VC) within the first day of culture and the aromas of the cultures were light and rather pleasant.

OTHER FACTORS

The aqueous extracts of the fungus *Rhizopus oryzae* U-1(Aq-ROU) Aq-ROU had anti proliferative activity and could induce apoptosis in a human promyelocytic leukemia cell line, HL-60. Moreover, it produced one or more water-soluble factor that can reliably and efficiently induc e apoptosis in human cells via activation of caspase-3 (Suzuki *et al*., 2007).

CONCLUSION

Strains of the *Rhizopus oryzae* complex have been used for centuries as fermented food starters for the production of tempeh and other Asian foods and being a producer of so many extra and intra cellular enzymes of industrial importance, it could easily be called a beneficial one. Soy tempeh being low in price was eaten mostly by

poor people. Mycelium biomass from *Rhizopus oryzae*, can partly substitute high-quality fishmeal in diets to rainbow trout without causing any major short-term adverse effects on growth, nitrogen and amino acids digestibility. For humans, it is also considered as food additive to improve flavor, fat binding and more recently as a replacement for animal protein in the diet (Jamal *et al*., 2007).

According to Evaluations of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Carbohydrase from *Rhizopus oryzae, var.* prepared at the 15th JECFA (1971), published in NMRS 50B (1972) and in FNP 52 (1992), commercial enzyme preparations namely, 1, 4-alpha D-Glucan glucanohydrolase (EC 3.2.1.1), Poly (1, 4-alpha D-galactouronide) glycanohydrolase (EC 3.2.1.15) and 1, 4-alpha D-Glucan glucohydrolase (EC 3.2.1.3), produced by the controlled fermentation of *Rhizopus oryzae*, var. can be used as food additives and for enzyme preparation .

Moreover, according to 21 CFR-Code of Federal Regulations(Title 21): Food and Drugs, Carbohydrase from *Rhizopus oryzae* may be safely used in the production of dextrose from starch in accordance with the following prescribed conditions: The strain of *Rhizopus oryzae* is nonpathogenic and nontoxic, the carbohydrase is produced under controlled conditions to maintain nonpathogenicity and nontoxicity, including the absence of aflatoxin and is produced by a process which completely removes the organism *Rhizopus oryzae* from the carbohydrase product and is maintained under refrigeration from production to use and is labeled to include the necessity of refrigerated storage. Therefore carbohydrase derived from *Rhizopus oryzae* can be used as food and drugs as certified by Food and Drug Administration, Department of Health and Human Services, USA.

Moreover, the discovery of presence of one or more water soluble factors that can induce apoptosis in a human cancer cell line may develop a new way for treatments of human cancer (Suzuki *et al*., 2007).

Therefore strains of *Rhizopus oryzae* with its bounty of different enzymes are of ample use for the benefit of human society and can no more be considered as only an opportunistic pathogen.

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