JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 1767-1778

Xanthophyllomyces dendrorhous as a New Source of Enzymes and its Enzymatic Potential in Non-Carotenoid Related Applications

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(Received: 11 April 2015; accepted: 09 June 2015)

***Xanthophyllomyces dendrorhous* is a red-pigmented, fermentative basidiomycetous yeast which was well documented for the production of astaxanthin for over three decades. Astaxanthin is economically important because it is the most expensive aquaculture feed component for artificial pigmentation of crustaceans, fish and poultry. It also contributes tremendously in human health as it exhibits antioxidant and anti-inflammatory properties. So far, *X. dendrorhous* has been biotechnologically exploited as a natural source of astaxanthin. Thus, considerable literature has accumulated in the field of astaxanthin production in *X. dendrorhous*. However, there are no reviews on its enzymatic capabilities. Focus of this review is to examine enzymatic activities that have been briefly reported for *X. dendrorhous*. The potential applications of these enzymes were also discussed, unveiling the prospective biotechnological use and value of this yeast. These include β-fructofuranosidase for neo-FOS production, α-glucosidase for the production of prebiotic IMOs, β–amylase mainly for starch saccharification, endo-β-1,3(4)-glucanase for the removal of anti-nutritive β-glucan in animal feed, aspartic protease for biocontrol properties against pathogenic fungi and plant diseases and carboxypeptidase for ochratoxin A decontamination. The information presented indicates that besides producing astaxanthin, *X. dendrorhous* is able to contribute in other aspects especially with its enzymatic capabilities.**

Key words: *Xanthophyllomyces dendrorhous*, β-fructofuranosidase, α-glucosidase, β–amylase, endo-β-1,3(4)-glucanase, aspartic protease, carboxypeptidase.

The basidiomycetous yeast *Xanthophyllomyces dendrorhous* (teleomorphic state of *Phaffia rhodozyma*) is one of the few microorganisms that can synthesize astaxanthin (3,3'-dihydroxy-β-β'-carotene-4,4'-dione) as the main carotenoid[1]. It has become the most promising microbial source of astaxanthin for the poultry and aquaculture industry since farmed animals like salmons and trouts are unable to synthesize this compound *de novo*. Astaxanthin belongs to the family of xanthophylls, the oxygenated derivatives of carotenoid. It is one of the most important natural pigments responsible for the orange–red color of animals such as invertebrates, fish and birds. It also has considerable potential and promising applications in human nutrition and health[2]. Numerous studies have shown that astaxanthin has potential health-promoting effects in the prevention and treatment of various diseases[3,4]. The beneficial roles of astaxanthin in biological systems like light protection, immunoenhancement, protection against carcinogens and powerful antioxidant property has generated extensive interest in *Xanthophyllomyces* research.

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Considerable effort has been put into the development of effcient *X. dendrorhous* mutant strains and fermentation processes for astaxanthin production. In our laboratory, an astaxanthin hyperproducing mutant of *X. dendrorhous* was obtained through NTG mutation and its pigment production was further enhanced by the optimization of the medium composition and cultural conditions[5]. Our subsequent feeding trial with *Tilapia* sp. showed that the incorporation of the dried *X. dendrorhous* biomass in fish diet exhibited enhanced colouration in the fish and good growth comparable to the control diet. Other than effort to improve the productivity of astaxanthin, investigators have also begun to look into other potential applications of *X. dendrorhous*. There is an increasing trend in exploring the production of potential enzymes in *X. dendrorhous* for example carboxypeptidase and aspartic protease[6,7]. In terms of the use of carbon sources, enzymes such as endo-β-1,3(4)-glucanase, β-amylase, α-glucosidase and β-fructofuranosidase have been reported for *X. dendrorhous* mainly in biochemical and molecular aspects[8-11]. These enzymes are of great significance due to their wide area of potential applications in industrial processes such as in the food, textiles, paper and fine chemicals industries.

So far, no review article covering these enzymes and their potential applications has been published. This review highlights the possibility of using *X. dendrorhous* as a source for the production of alternative compounds other than astaxanthin, i.e. enzymes, focusing mainly on their potential and possible applications in various industries. It demonstrates the huge potential of this yeast to become an interesting enzymes producer, an aspect of *X. dendrorhous* study which has not been thoroughly addressed before.

β-fructofuranosidase

β-fructofuranosidases are key hydrolytic enzymes involved in the uptake and incorporation of carbon sources. They catalyze the release of β-fructose from the non-reducing termini of various β-D-fructofuranoside substrates and also the synthesis of short-chain fructooligosaccharides (FOS)[12]. Fructooligosaccharides (FOS) are non-digestible carbohydrates that possess interesting physiological and functional attributes like low sweetness, low caloric value, low glycemic index, non-cariogenicity, prebiotic, hypolipidemic and hypocholestrolemic properties[13]. They are one of the most favourable ingredients for functional foods since they function as prebiotics to stimulate the proliferation of bifidobacteria in the intestine[14]. They can also lower blood cholesterol, enhance dietary calcium absorption and give positive effects in the prevention of diabetics, colon cancer, osteoporosis and cardiovascular diseases[15].

The commercially available FOS are produced through the enzymatic synthesis from sucrose by microbial β-fructofuranosidase. FOS produced from sucrose falls into three categories: inulin-, levan- and neo-type of oligosaccharides. Among these oligosaccharides, only the inulin-type FOS is currently available and the main industrial FOS producers are enzymes from *Aspergillus*[16]. Structurally, this inulin-type FOS are short-chain oligomers of monosaccharide units consisting 1-kestose (GF2), nystose (GF3) and fructofuranosyl nystose (GF4) in which fructosyl units are bound by β(2→1) position of sucrose with the last one linked to a terminal glucose moiety (Fig. 1). However, several studies also showed the production of neo-FOS in which fructosyl units are bound at β(2–6) position of sucrose forming neokestose and 6-kestose, respectively, depending on the type of linkage between the monosaccharide units (Fig. 2). Neokestose is formed when β-fructofuranosidase catalyzes the transfer of a fructose residue from 1-kestose to the C6 of the glucose moiety of sucrose.

β-fructofuranosidases from *X. dendrorhous* produces neokestose (6G-FOS), a novel bifidogenic trisaccharide, as the main transglycosylation product, unlike other microbial β-fructofuranosidases that produce mainly 1F-FOS and 6F-FOS, with little or no 6G-FOS[19-21]. The isolated and sequenced *X. dendrorhous* β-fructofuranosidase gene encodes a putative mature polypeptide of 595 amino acids and it shares significant identity with other yeast, fungal and plant β-fructofuranosidases. Other than transfructosylating activity, the β-fructofuranosidase purified from *X. dendrorhous* can hydrolyze fructosyl-β-(2→1)-linked carbohydrates (sucrose, nystose and 1-kestose) and palatinose [α-D-Glc-(1→6)-D-Fru], although its catalytic efficiency indicated that it hydrolyzes sucrose approximately 4.2 times more efficiently than 1-kestose. Analysis deduced the purified

enzyme as a glycoprotein with a content of 59–67% N-linked carbohydrate and an estimated molecular mass of 160 kDa that was derived from a 66-kDa unglycosylated monomer[22].

Study by Linde *et al*. showed that maximum level of β-fructofuranosidase activity (approximately 2 to 4 U/ml) was detected in the culture filtrate of *X. dendrorhous* at the beginning of the stationary phase and this level was maintained for at least 80 h of growth and only low level of activity (0.8 U/ml) was found in the cell-associated fraction during this period[23]. The enzyme displayed optimum activity at pH 5.0 to 6.5 and its thermophilicity (maximum activity at 65 to 70°C) and thermostability (T50 in the range of 66 to 71°C) is higher than that exhibited by other microbial β-fructofuranosidases. Production of FOS was much higher at 70°C than lower temperatures. Meanwhile β-fructofuranosidases in bacteria are generally unstable above 50°C, like the β-fructofuranosidase of *Bifidobacterium infantis* ATCC 15697 with an optimum temperature of 37°C[24].

For maximum production of FOS at an industrial level, development of more β-fructofuranosidases with high activity and stability is required. Other than progressive improvement of prominent processes like optimization, immobilization, separation and purification, the improvement of FOS yield can be achieved by the use of novel strains as microbial sources of β-fructofuranosidase. Screening and selection of microorganisms for enzymatic biotransformation of sucrose to FOS has been investigated and about 30 microorganisms have been accounted for the enzymatic activity but only a few of them have been exploited for the production of FOS at industrial level[18]. There is also interest in the development of novel molecules that may have better prebiotic and physiological properties compared to existing commercial FOS. Neo-FOS, consists of neokestose and neonystose, is a bifidogenic substance with prebiotic effects that may surpass those of commercial FOS[25]. Moreover the branched structure of the neo-FOS confers enhanced chemical and thermal stability in comparison to conventional FOS[26]. β-fructofuranosidase from *X. dendrorhous* could serve as a better alternative as it shows its uniqueness in producing neokestose as its main transglycosylation product besides its thermostability which is favourable in industrial applications.

α-glucosidase

α-glucosidase is a heterosubunit protein, in which the two hetero subunits are composed of residues 26-252 and 267-985 of aglu respectively and the two hetero subunits have a very tight interaction[27]. It is usually found in association with other amylolytic enzymes to accomplish complete degradation of starch. In nature, there are more than 150 starch assimilating yeasts, but only a few of them secrete a combination of enzymes that can cleave the α-1,4 and α-1,6 linkages of the complex starch molecule[28]. Among them is *X. dendrorhous* which make possible the utilization of the polysaccharide as its carbon source. A study by Marin *et al*. showed that *X. dendrorhous* grown in different media exhibited amylolytic activity[10]. Their results showed that amylolytic ability of *X. dendrorhous* is due to an extracellular exo-acting α-glucosidase which was able to hydrolyze only α-glycosidic bonds, producing glucose from malto-oligosaccharides and soluble starch. It exhibited optimum activity at pH 5.5 and 45°C with thermostability of up to 50°C.

α-glucosidase from *X. dendrorhous* not only exhibits hydrolytic activity but also transglycosylating activity. It has the ability to catalyze both the hydrolysis of α-D-gluco-oligosaccharides and transfer of the glycosyl group to other glycosyl residues resulting in the synthesis of prebiotic isomalto-oligosaccharides (IMOs)[29]. IMOs are of special interest as functional oligosaccharides as they have physiological functions such as the improvement of intestinal microflora due to the selective proliferation of bifidobacteria and lactic acid bacteria[30]. They are associated with a lower risk of infections and diarrhea and an improvement of the immune system response[31]. In food industry, these oligosaccharides have a great potential to improve the physiochemical quality of many foods. For example, it functions as anti-fading agent for food pigments, as food antioxidant and as a sweetener. In addition, IMOs are also utilized in animal feed to increase dry matter and calcium digestibility[32].

A study by Fernandez-Arrojo *et al*. showed that transglycosylation activity of α-glucosidase from *X. dendrorhous* was able to

synthesize oligosaccharides with α-(1→2), α-(1→4) and α-(1→6) bonds when using maltose as glycosyl donor[33]. Their results indicated that *X. dendrorhous* α-glucosidase is able to transfer glycosyl moieties to the 2-OH, 4-OH and 6-OH of a glucose residue. *X. dendrorhous* α-glucosidase gives a notable yield approximately 40% of total transglycosylating products. The transglycosylatying yield of α-glucosidase from *X. dendrorhous* was 3.6 times higher than that observed with the α-glucosidase from *S. cerevisiae*. In addition, compared with other microbial α-glucosidases that synthesize mainly disaccharides, the α-glucosidase from *X. dendrorhous* yields a final transglycosylation product enriched in trisaccharides and tetrasaccharides[33]. Meanwhile it has been shown that α-glucosidases from different sources have varied substrate specificities. Interestingly, the α-glucosidase from *X. dendrorhous* has an remarkable substrate specificity, being capable of hydrolyzing malto-oligosaccharides and starch at a high catalytic efficiency, calculated by the relationship Vcat/Km for malto-oligosaccharides, such as maltotriose (873 mM^{-1} min^{-1}), or maltoheptose (698 mM^{-1} min^{-1})[28]. There is little information on the secretion from yeast and yeast-like fungi of α-glucosidases that are active on higher malto-oligosaccharides and starch, thus making *X. dendrorhous*'s ability to hydrolyze the polysaccharide a remarkable feature.

α-glucosidase has a number of other potential applications especially in fundamental and clinical research. It has emerged as a new molecular target in drug development studies for the screening of α-glucosidase inhibitors. α-glucosidase is a very important enzyme responsible for the hydrolysis of dietary disaccharides into absorbable monosaccharide in microbial system and small intestine of animal digestive system. Besides carbohydrate digestion, it is also very important for the processing of glycoproteins and glycolipids and is involved in a variety of metabolic disorders and diseases such as diabetes and obesity[34]. α-glucosidase inhibitors are prospective therapeutic agents for the control of these carbohydrates dependent diseases through their antidiabetic and antihyperglycemic mechanism by inhibiting carbohydrate metabolism. These inhibitors can retard the liberation of glucose from dietary complex carbohydrates in the small intestine and delay glucose absorption from the intestine into the blood, resulting in reduced postprandial plasma glucose level and suppress postprandial hyperglycemia.

α-glucosidases can also be used to screen novel inhibitors that specifically target the folding of viral proteins. Many viruses contain an outer envelope which is composed of one or more viral glycoproteins. These glycoproteins are often essential proteins in that they are required in the viral life cycle, either in virion assembly and secretion and/or infectivity[35]. α-glucosidases are endoplasmic reticulum-resident enzymes that are essential for viral envelope glycoprotein processing and folding and thus are important host factor-based antiviral targets in combating viral infection. α-glucosidase inhibitors can be used to prevent these processes and their anti-viral activity may be especially promising in cases where the virus buds through the endoplasmic reticulum. By causing the misfolding of only a small number of envelope glycoproteins, these inhibitors can sufficiently prevent proper virus envelopment in the endoplasmic reticulum and hence, secretion of virions. α-glucosidase inhibitors have been demonstrated to inhibit woodchuck hepatitis virus in chronically infected woodchucks, several flaviviruses in mice and hepatitis C virus (HCV) in human in a phase II clinical trial[36-38]. α-glucosidase inhibitors with potent antiviral efficacy hold promise for the development into a therapeutic agent for the treatment of chronic viral infection.

β-amylase

β-amylase is of great interest for biotechnological and industrial applications because of its widespread use in starch saccharification and also in the textile, food, brewing and distilling industries. It also has potential applications in the pharmaceutical and fine-chemical industries if enzymes with suitable properties can be prepared.

β-amylase has been partially purified extracellularly from *X. dendrorhous* CECT 1690 and this is the first time they have been characterized in a yeast[9]. This enzyme has a molecular weight of 240 kDa and an isoelectric point of 8.6. β-amylases with a similar range of molecular weight have been described in *B. megaterium* (209 kDa)[39] and

Clostridium thermosulfurogenes (222 kDa) as a tetramer with identical subunits but this molecular weight is significantly higher than that assigned to most microbial β-amylases[40]. β-amylase from *X. dendrorhous* hydrolyzed soluble starch but it did not yield glucose as the final reaction product and was unable to hydrolyze maltotriose. The values of the kinetic parameters, apparent Michaelis constant (Km) and maximum velocity (Vmax), for starch were 1.35 mg/ml and 0.68 nmol/min respectively. The enzyme was active in the pH range of 4–6.5, pH 5.5 being the optimum. This correlates with the optimum pH values that had been reported for most bacterial amylases and yeast amylases ranging from 4.5-6.2[41]. The enzyme retained 50% of its activity after 30 min at a sub-optimum pH of 7.5 and this activity was reduced to 25% after 1 h. Total inactivation of the enzyme occurred after it had been incubated for 120 min at pH 7.5. For temperature, the enzyme was active in a range of 20–60°C, 50°C being the optimum, a widely reported attribute of β-amylase. For thermal stability, the enzyme retained 35% activity at a temperature of 60°C for 30 min, and 25% after 1 h with total inactivation occurring after 90 min.

β-amylase has generally been obtained from plant source and this has been limited to edible plants, particularly in the seeds, such as barley, wheat and soybean so far. Considering the current worldwide food crisis, the exploration for alternative sources for a stable and constant enzyme supply is indeed imminent. Unlike other members of the amylase family, only a few attempts have been made to study β–amylases particularly of plant origin while not much work has been done on the production of β-amylase using microorganisms. Thus effort to develop new sources for microbial β-amylase as an alternative to the plant source is really welcomed and *X. dendrorhous* can be a promising novel source of β-amylase. Among the amylases, β-amylase and α-glucosidase have been briefly studied in *X. dendrorhous*, but there is no report yet on α-amylase production in this microorganism. The level of α-amylase activity in various human body fluids is of clinical importance e.g. in diabetes, pancreatitis and cancer research[42-43]. So far, α-amylases from fungal and bacterial sources have dominated applications in industrial sectors. Thus, there is a need to further study amylases in *X. dendrorhous* to uncover its full potential.

Endo-β-1,3(4)-glucanase

Due to their habitats and consumption of plant material as a significant source of energy, certain microorganisms like *X. dendrorhous* secrete enzymes capable of degrading plant cell wall components[44]. β-glucan is one of the major components of the cell wall in the main cereal grains such as barley and oats. As β-glucan forms highly viscous solutions, high concentration of β-glucan lead to high viscosity of product, formation of gelatinous precipitate and reduction in the extract yield during the production process[45]. Its solubilization in the gut increases the viscosity of the intestinal digesta. In poultry, the effects include a reduced rate of digesta movement, a decrease in digestion efficiency and absorption of nutrients, the development of undesirable intestinal microflora and the excretion of wet, sticky droppings which adhere to eggs, feathers and bedding[46,47]. Incorporation of exogenous β-1,3(4)-glucanase into animal feed is common practice to catalyze the hydrolysis of β-glucan into low molecular weight glucose polymers, thus overcoming the anti-nutritive effects of β-glucan and increase the efficiency of nutrition absorption[48]. The commercial β-1,3(4)-glucanase products currently used in animal feed are derived mainly from species such as *Trichoderma* and *Rhizomucor*. Such β-1,3(4)-glucanase was not initially developed specifically for the use in animal feed and hence may not be ideally suited for this application in terms of their physicochemical properties such as its thermolability. For example, the endo-β-1,3(4)-glucanase purified from *R. miehei* retained only 34% of its maximum activity at avian physiological temperature (40°C)[49]. Thermal stability of an enzyme is essential for animal feed application, as animal feed is heat treated to control microbial growth and prevent transmission of pathogens. Thus, there is always a need to look further for more alternative sources of endo-β-1,3(4)-glucanase.

Bang *et al.* has described the identification and expression cloning of a novel β-glucanase in *Phaffia rhodozyma* CBS 6938, the anamorph of *X. dendrorhous*[8]. The endo-acting β-glucanase secretion is controlled by glucose-mediated

catabolite repression and induced mainly by media containing cell-wall materials. Subsequently, a full-length cDNA encoding an endo-β-1,3(4)-glucanase (bg1) was cloned by expression cloning in *S. cerevisiae* W3124. The bg1 cDNA encodes a 424-residue precursor protein with a putative signal peptide. Endo-β-1,3(4)-glucanase belongs to glycoside hydrolase family 16, members of which share a β-jelly roll structure and catalyze the hydrolysis of 1,3 or 1,4 glycosidic bonds in β-D-glucans[50]. The reported molecular weight of endo-β−1,3(4)-glucanases was in similar range, including those from *R. miehei* (39.7 kDa), *Talaromyces emersonii* CBS 814.70 (33.8 kDa) and *Phanerochaete chrysosporium* (36 kDa)[49,51,52]. Isoelectric point (pI) of *P. rhodozyma* endo-β-1,3(4)-glucanase was approximately 6.7 and this is different from the pI reported for *R. miehei* (pI 3.6), *Candida albicans* (pI 3.6) and *Bacillus pumilus* (pI 3.7)[49,53,54]. There is no other literature report on the production, purification or characterization of endo-β−1,3(4)glucanase from *X. dendrorhous* so far. The suitability of this enzyme in term of its optimum pH and temperature and whether its stability is in agreement with the normal environment of digestive tract has yet to be determined.

Despite that, endo-β-1,3(4)-glucanase from *X. dendrorhous* still shows its excellent potential to be developed into an enzyme supplement in animal feed. The incorporation of *X. dendrorhous* in animal feed will not only serve as a source for endo-β-1,3(4)-glucanase but also the pigment astaxanthin. The enzyme endo-β-1,3(4)-glucanase will help to degrade and remove the anti-nutritive β-glucan while astaxanthin can be incorporated to improve the colouration of the flesh, skin and egg yolk naturally. Furthermore, the biomass of *X. dendrorhous* could be incorporated directly in animal feed without many further processes of extraction and purification, consequently simplifying the feed enzyme and pigment supplementation procedure. This makes its application less time-consuming and cost effective.

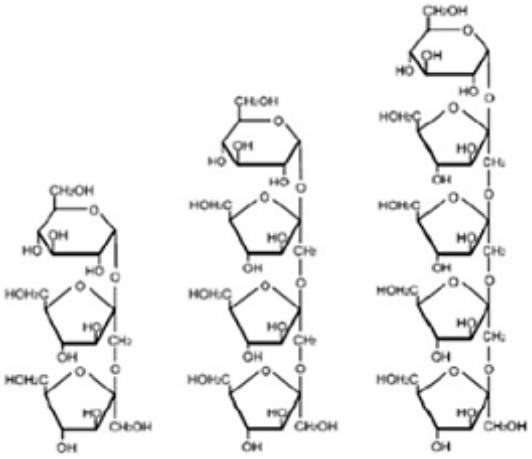

Fig. 1. Chemical structure of FOS produced from sucrose by β-fructofuranosidase :1-kestose (GF2, left), nystose (GF3, center) and fructofuranosyl nystose (GF4, right)[17]

Aspartic protease

Killer or mycocinogenic yeasts produce proteinous toxins that kill other yeasts of the same genus and, less frequently, those of different genera[55]. The toxin-producing killer phenomenon is indeed widespread among yeasts especially ascomycetes and basidiomycetes. A study by Baeza *et al* has identified an aspartic protease, a protein of ~33 kDa to render *X. dendrorhous* its mycocinogenicity[7]. Ecological studies have suggested that mycocin (killer toxin) production could be a mechanism of interference competition, giving the mycocinogenic yeasts an advantage over sensitive competing microorganisms[56]. For yeast strains such as *X. dendrorhous* living in their natural habitat, it has been shown that toxin production can confer a marked advantage in the competition with sensitive yeast strains for limited nutrients available[57]. This mycocinogenic characteristic could help to establish the organism's dominance during the habitat colonization. It could also be explained by the natural environment of *X. dendrorhous*, which is slime exudates from deciduous trees. In early spring these exudates have a high content of sugars and therefore nitrogen will most likely be the limiting growth factor. Secreting a protease under glucose-rich conditions could be a way for the yeast to ensure the availability of peptides, which can be assimilated by the yeast and used as a source of nitrogen[8].

The killer toxin (aspartic protease) of *X. dendrorhous* showed greater activity in rich media at pH 4.6 to 5.0, which is within the range of 3 to 5.5 reported for most killer toxins. Killer toxins are stable and act only within a narrow range of acidic pH

values. Meanwhile Bang *et al*. has constructed the aspartic protease cDNA from *P. rhodozyma* that encodes a 405-residue prepropolypeptide with an 81-residue leader peptide[8]. The aspartic protease was purified and characterized as an aspartic endopeptidase with a molecular mass of 36 kDa, an isoelectric point of 7.5, optimum pH of 4.0-6.0 and optimum temperature around 40°C. This optimum temperature is in contrast to most yeast killer proteins which exhibit their cytotoxic activity only at temperatures between 20°C and 30°C. Most of the killer toxins studied are heat-labile macromolecules which is unstable at temperatures above 25°C. Therefore, yeast toxins of *X. dendrorhous* with an optimum temperature of 40°C are probably not only suitable for topical applications in the treatment of superficial lesions, oral and intravenous administration might well be possible with its derivation of killer toxin-like antibodies or killer peptides[58].

Research on killer yeasts and their toxins for industrial application is relatively new and is becoming increasingly interesting as they have many potential applications in medical, industrial and environmental biotechnology. They have been used as model systems to study the mechanisms of regulation in eukaryotic protein processing, secretion and toxin interaction with sensitive cells. They can be applied to combat undesired contaminants which can occur during beverage and food fermentations other than prevention of aerobic spoilage of silage[59]. Killer yeasts have also been used as biocontrol agents in the preservation of foods thus reducing the use of chemical preservatives[60]. In taxonomy studies, killer toxin sensitivity patterns may be indicative of phylogenetic affiliation. According to the different sensitivities to killer toxins, it is possible to group yeasts into different categories and help in the bio-typing of medically important pathogenic yeasts and yeast-like fungi[61]. Secreted killer toxins produced mainly by non-*Saccharomyces* yeasts show a broad spectrum of killing activity against a great number of plant and human pathogens. *X. dendrorhous*, with the ability to produce aspartic protease as its killer toxin, is a potential candidate in the search for more environmentally efficacious and toxicologically safe fungicides for the treatment of human fungal infections, particularly in immunocompromised patients. For plants, killer toxins could serve as sustainable and environmentally acceptable biological control agent based on their antifungal and zymocide activities against plant pathogenic fungi.

However further pharmacological studies are needed to demonstrate their antifungal potential. Better understanding on the range of toxin activity, regulating mechanisms, compatibility and level of toxin production in *X. dendrorhous* will allow its killer activities to be better exploited for a wide range of industrial applications.

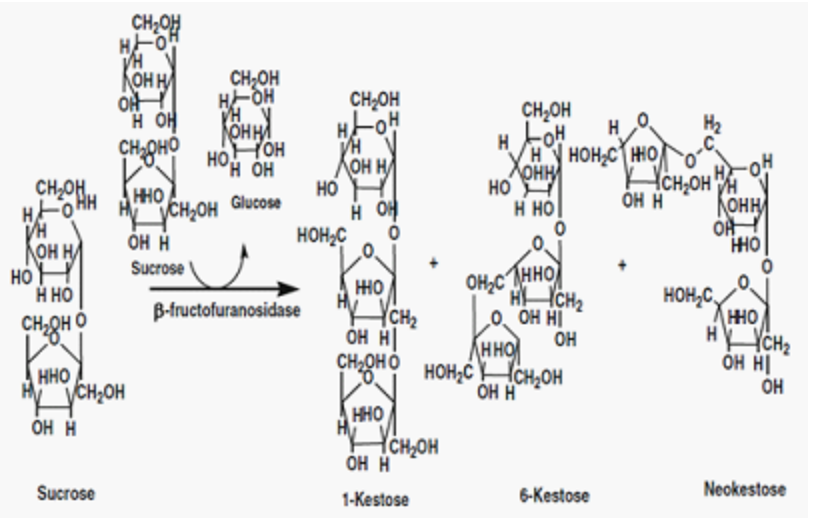

Fig. 2. Structure of FOS formation of various linkages[18]

Carboxypeptidase

Ochratoxin A (OTA) is the most toxic of the known ochratoxins and is the predominant compound detected in agriculture commodities making it the most relevant to food and feed safety. It is a potent mycotoxin produced by species of two genera *Aspergillus* and *Penicillium* with mutagenic, nephrotoxic, hepatotoxic, teratogenic and carcinogenic properties[62]. This secondary metabolite of fungi may cause diseases in animals and humans other than creating health hazard through the contamination of agricultural products. It is considered to be a cumulative toxic compound since it is easily absorbed through the stomach and the small intestine but hardly eliminated through the biliary and urinary routes. Strategies available for the removal or detoxification of mycotoxins can be classified into physical, chemical and biological approaches. Biological methods use microorganisms and their enzymes, which can biodegrade, transform or adsorb OTA to prevent the toxic effects of ingested mycotoxins. Microbes or their enzymes may be a more promising alternative for mycotoxin detoxification as physical and chemical methods showed varying degrees of success and these strategies might lead to significant loss in nutritive value and palatability of decontaminated products. Various studies on biological approaches have led to the identification of several microbes or cell cultures and their enzymes capable of detoxifying OTA including filamentous fungi, protozoa, bacteria, yeasts and plant cell cultures[63-67]. OTA hydrolytic enzymes substantially hydrolyzed ochratoxin A into a less toxic ochratoxin α (OTα) and these include carboxypeptidase in fungi, predominantly *Aspergillus* species, α-chymotrypsin, pancreatin from porcine pancreas, lipase and protease A from *A. niger*[63,68,69]. Meanwhile adsorption mechanism has been suggested for OTA removal by lactic acid bacteria and yeasts[70-71].

Astaxanthin-producing yeast strains, *P. rdodozyma* and *X. dendrorhous* converted OTA to a less toxic OTα and also facilitated the adsorption of OTA into its viable and heat-treated cells. *P. rhodozyma* CBS 5905 degraded more than 90% of 7.5 mg/ml OTA after 15 days at 20°C[6]. The degradation of OTA was made possible by the production of a cell-bound metallo carboxypeptidase. The optimum temperature for this enzyme was found to be above 30°C, which is much higher than the optimum temperature for the growth of *P. rhodozyma* cells, which is around 20°C. The enzyme remains active at up to 60°C. Besides, both viable and heat-treated (dead) *P. rhodozyma* cells were also found to be able to adsorb significant amounts (up to 250 ng/ml) of OTA. Heat treatment enhanced OTA adsorbing activities of the cells as heat treated cells of *P. rhodozyma* was found to be more effective than viable cells. As the dead cells do not lose their binding ability, the removal of mycotoxins is most probably by physical adhesion to cell wall components rather than a metabolic process. Numerous physico-chemical changes take place in the cell wall during the heat treatment resulting in exposing more binding sites. Heating may cause denaturation of proteins or formation of Maillard reaction products. These released products could offer more adsorption sites than viable cells and may increase surfaces for OTA binding. The decrease in wall thickness of peptidoglycan and/or the increase in its pore size under heat treatment could probably make available other sites from yeast cells for OTA adsorption[72]. Moreover, heating may also increase permeability of the external layer of the cell wall due to the dissolution of some of the mannans from the cell surface leading to the increased availability of the otherwise hidden binding sites[73]. Dried yeast cell wall fractions were reported to be the most efficient at adsorbing OTA, may be due to yeast β-D-glucans, glucomannans and mannan oligosaccharide[74-76]. Since the nature of cell wall components involved in OTA binding is still not very clear, systematic studies with the intact cells and isolated cell walls are still needed to further understand the chemistry of OTA binding.

Further studies on characterization of the OTA hydrolytic enzyme present in *X. dendrorhous* and its ability to hydrolyze OTA should also be carried out to evaluate its feasibility for industrial applications. It will be a technology of choice for decontamination purposes because enzymatic reactions present several advantages in the way that they are very specific, efficient, environmentally friendly and they preserve nutritive quality. Moreover GRAS (generally regarded as safe) status of *X. dendrorhous* and its historical and extensive use in the food and feed

industry make it a good candidate for the decontamination of OTA as the toxicological safety of the final product after treatment can always be guaranteed. The bioremediation of OTA contaminated products with *X. dendrorhous* and its enzymes could be an environmentally-sound and practical option to reduce the levels of contaminants and lead to safer food and feed.

CONCLUSION

So far *X. dendrorhous* was mainly recognized and studied for the production of the pigment astaxanthin and the microorganism is largely unexplored for its other biotechnological capabilities. It exhibits great potential in producing value-added enzymes and it is worthwhile to explore its enzymatic potential for new applications. With the advent of new frontiers in biotechnology, the spectrum of enzyme application has expanded into many other fields and enzyme-producing strains can always be improved to achieve desirable characteristics. This presents opportunities for further investigation to exploit *X. dendrorhous* for the simultaneous production of carotenoids and enzymes, thus enhance its industrial prospect as a unique resource in various industrial applications.

ACKNOWLEDGEMENTS

Authors would like to thank Universiti Sains Malaysia and the Fundamental Research Grant Scheme (FRGS) from The Ministry of Education Malaysia (203/CIPPM/6711337) for their support in this work.The second author would also like to thank The Ministry of Education Malaysia for the MyMaster scholarship awarded.

REFERENCES

1. Johnson, E.A.*Phaffia rhodozyma*: colorful odyssey. *Int. Microbiol.*, 2003; **6**(3):169-174.
2. Higuera-Ciapara, I., Felix-Valenzuela, L., Goycoolea, F.M. Astaxanthin: a review of its chemistry and applications. *Crit. Rev. Food Sci. Nutr.*, 2006; **46**: 185–196.
3. Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H. Astaxanthin, a carotenoid with potential in human health and nutrition. *J. Nat. Prod.*, 2006; **69**:443–449.
4. Yuan, J.P., Peng, J., Yin, K., Wang, J.H. Potential health-promoting effects of astaxanthin: A high-value carotenoid mostly from microalgae. *Mol. Nutr. Food Res.,* 2011; **55**:150–165.
5. Chew, A.L., Ibrahim, C.O. Cultural conditions optimization for the production of astaxanthin by a stable mutant of *Xanthophyllomyces dendrorhous* DSM5626. *Annual Report IC Biotechnology,*1998; **21:**935-946.
6. Peteri, Z., Teren,J., Vagvolgyi, C., Varga, J. Ochratoxin degradation and adsorption caused by astaxanthin-producing yeasts. *Food Microbiol*., 2007; **24**: 205-210.
7. Baeza, M., Flores, O., Carrasco, M., Rozas, J.M., Oviedo, V., Barahona, S., Cifuentes, V.The inter-generic fungicidal activity of *Xanthophyllomyces dendrorhous.The Journal of Microbiology*, 2010; **48**(6): 822-828.
8. Bang, M.L., Villadsen, I., Sandal, T. Cloning and characterization of an endo-β-1,3(4)glucanase and an aspartic protease from *Phaffia rhodozyma* CBS 6938. *Appl. Microbiol. Biotechnol.,*1999; **51**: 215-222.
9. Diaz, A., Sieiro, C.,Villa, T.G.Production and partial characterization of a β-amylase by *Xanthophyllomyces dendrorhous*. *Letters in Applied Microbiology*, 2003; **36**(4):203–207.
10. Marin,D., Linde, D.,Lobato, M.F.Purification and biochemical characterization of an α-glucosidase from *Xanthophyllomyces dendrorhous*. *Yeast*, 2006; **23**:117–125.
11. Persike, D.S., Bonfim, T.M., Santos, M.H., Lyng, S.M., Chiarello, M.D., Fontana, J.D. Invertase and urease activities in the carotenogenic yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*). *Bioresour. Technol*., 2002; **82**:79–85.
12. Antosova, M., Polakovi, M. Fructosyltransferases: the enzymes catalyzing production of fructooligosaccharides. *Chem. Pap-Chem. Zvesti.,* 2001; **55**: 350-358.
13. Katapodis, P., Kalogeris, E., Kekos, D., Macris, B.J., Christakopoulos, P. Production of β-fructofuranosidase from *Sporotrichum thermophile* and its application in the synthesis of fructooligosaccharides. *Food Biotechnology*, 2003; **17**(1):1-14.
14. Rao, A.V. Dose-response effects of inulin and oligofructose on intestinal bifidogenesis effects. *J. Nutr.*, 1999; 129 Suppl **7**:1442S–1445S.
15. Kaur, N., Gupta, A.K. Applications of inulin and oligofructose in health and nutrition. *J. Biosci*. 2002; **27**:703–714.
16. Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Plou, F.J., Ballesteros, A. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus. J. Biotechnol*., 2007;

128:204–211.

17. Dominguez, A.L., Rodrigues, L.R., Lima, N.M., Teixeira, J.A. An overview of the recent developments on fructooligosaccharide production and applications. *Food Bioprocess Technol.*, 2014; **7**: 324–337.
18. Ganaie, M.A., Lateef, A., Gupta, U.S. Enzymatic trends of fructooligosaccharides production by microorganisms. *Appl. Biochem. Biotechnol*., 2014; **172**: 2143–2159.
19. Sheu, D.C., Chang, J.Y., Chen, Y.J., Lee, C.W. Production of high-purity neofructooligosaccharides by culture of *Xanthophyllomyces dendrorhous*. *Bioresource Technology,* 2013; **132**:432-435.
20. Chen, J., Chen, X., Xu, X., Ning, Y., Jin, Z., Tian, Y. Biochemical characterization of an intracellular 6G-fructofuranosidase from *Xanthophyllomyces dendrorhous* and its use in production of neo-fructooligosaccharides (neo-FOSs). *Bioresource Technology,* 2011; **102**:1715–1721.
21. Hayashi, S., Yoshiyama, T., Fujii, N., Shinohara, S. Production of a novel syrup containing neofructooligosaccharides by the cells of *Penicillium citrinum. Biotechnol. Lett.,* 2000; **22** :1465-1469.
22. Linde, D., Rodriguez-Colinas, B., Estevez, M., Poveda, A., Plou, F.J., Fernandez Lobato, M. Analysis of neofructooligosaccharides production mediated by the extracellular β-fructofuranosidase from Xanthophyllomyces dendrorhous. *Bioresource Technology,* 2012; **109**:123-130.
23. Linde, D., Macias, I., Fernandez-Arrojo, L., Plou, F.J., Jimenez, A., Fernandez-Lobato, M. Molecular and biochemical characterization of a *b*-fructofuranosidase from *Xanthophyllomyces dendrorhous*. *Appl. Environ. Microbiol*., 2009; **75**(4):1065–1073.
24. Warchol, M., Perrin, S., Grill, J.P., Schneider, F. Characterization of a purified beta-fructofuranosidase from *Bifidobacterium infantis* ATCC 15697. *Lett. Appl. Microbiol*., 2002; **35**(6):462-7.
25. Kilian, S., Kritzinger, S., Rycroft, C., Gibson, G., du Preez, J. The effects of the novel bifidogenic trisaccharide, neokestose, on the human colonia microbiota. *World J. Microbiol Biotechnol*., 2002; **18**:637–644.
26. Lim, J.S., Lee, J.H., Kang, S.W., Park, S.W., Kim, S.W. Studies on production and physical properties of neo-FOS produced by co-immobilized *Penicillium citrinum* and neo-fructosyltransferase. *Eur. Food Res. Technol*., 2007; **225**:457–462.
27. Kita, A., Matsui, H., Somoto, A., Kimura, A., Takata, M., Chiba, S. Substrate specificity and subsite affinities of crystalline alpha-glucosidase from *Aspergillus niger. Agric. Biol. Chem*.,1991; **55**(9):2327–2335.
28. Ramachandran, N., Pretorius, I.S., Cordero Otero, R.R. Amylolytic enzymes from the yeast *Lipomyces kononenkoae*. *Biologia (Bratislava)*, 2005; **60**:103—110.
29. Nakakuki, T. Development of functional oligosaccharides in Japan. *Trends in Glycoscience and Glycotechnology*, 2003; 15:57-64.
30. Thitaram, S.N., Chung, C.H., Day, D.F., Hinton, A., Bailey, J.S., Siragusa, G.R. Isomaltooligosaccharide increases cecal *Bifidobacterium* population in young broiler chickens. *Poultry Science*, 2005; **84**:998-1003.
31. Mizubuchi, H., Yajima, T., Aoi, N., Tomita, T., Yoshikai, Y. Isomalto-oligosaccharides polarize Th1-like responses in intestinal and systemic immunity in mice. *Journal of Nutrition,* 2005; **135**: 2857-2861.
32. Li, Y.J., Zhao, G.Y., Du, W., Zhang, T.J. Effect of dietary isomaltooligosaccharides on nutrient digestibility and concentration of glucose, insulin, cholesterol and triglycerides in serum of growing pigs. *Anim. Feed Sci. Technol*., 2009; **151**(3-4):312–315.
33. Fernandez-Arrojo, L., Marin, D., Gomez de Segura, A., Linde, D., Alcalde, M., Gutierrez-Alonso, P., Ghazi, I., Plou, F.J., Fernandez-Lobato, M., Ballesteros, A. Transformation of maltose into prebiotic isomaltooligosaccharides by a novel alpha-glucosidase from *Xantophyllomyces dendrorhous*. *Process Biochemistry*, 2007; **42**:1530-1536.
34. Hamada, Y., Nagasaki, H., Fuchigami, M., Furuta, S., Seino, Y., Nakamura, J., Oiso, Y. The alpha-glucosidase inhibitor miglitol affects bile acid metabolism and ameliorates obesity and insulin resistance in diabetic mice. *Metabolism*, 2013; **62**(5):734–742.
35. Mehta, A., Zitzmann, N., Rudd, P.M., Block, T.M., Dwek, R.A. α-glucosidase inhibitors as potential broad based anti-viral agents. *FEBS Letters*, 1998; **430**:17-22.
36. Block, T.M., Lu, X., Mehta, A.S., Blumberg, B.S., Tennant, B., Ebling,M., Korba, B., Lansky, D.M., Jacob, G.S., Dwek, R.A. Treatment of chronic hepadnavirus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. *Nat. Med*., 1998; **4**:610–614.
37. Wu, S.F., Lee, C.J., Liao, C.L., Dwek, R.A., Zitzmann, N., Lin, Y.L. Antiviral effects of an iminosugar derivative on flavivirus infections. *J.*

Virol.,2002; **76**: 3596–3604.

38. Durantel, D. Celgosivir, an alpha-glucosidase I inhibitor for the potential treatment of HCV infection. *Curr. Opin. Investig. Drugs*, 2009; 860–870.
39. Ray, R.R. Purification and characterization of extracellular *b*–amylase of *Bacillus megaterium* B (6). *Acta Microbiology and Immunology*,2000; **47**: 29-40.
40. Reddy, P.R.M., Swamy, M.V., Seenayya, G. Purification and characterization of thermostable β-amylase and pullulanase from high yielding *Clostridium thermosulfurogenes* SV2. *World Journal of Microbiology Biotechnology*,1998; **14**:89-94.
41. Madi, E., Antranikan, G., Ohmiya, K., Gottschu, G. Thermostable amylolytic enzymes from a new *Clostridium* isolate. *Applied and Environmental Bacteriology*, 1987; **53**:1661-1667.
42. Shigemura, M., Moriyama, T., Endo, T., Shibuya, H., Suzuki, H., Nishimura, M., Chiba, H., Matsuno, K. Myeloma cells produce sialyl salivary-type amylase. *Clin. Chem. Lab. Med*., 2004; **42**(6):677-80.
43. Abou-Seif, M.A., Youssef, A.A. Evaluation of some biochemical changes in diabetic patients. *Clin. Chim. Acta*., 2004; **346**(2):161-70.
44. Linton, S.M., Greenaway, P. A review of feeding and nutrition of herbivorous land crabs: adaptations to low quality plant diets. *J. Comp. Physiol. B*, 2007; **177**:269-286.
45. Bamforth, C. β-glucan and β-glucanases in malting and brewing: practical aspects. *Brew Dig*.,1994; **69**(5):12-16.
46. Classen, H. Cereal grain starch and exogenous enzymes in poultry diets. *Anim. Feed Sci. Technol*.,1996; **62**:21–27.
47. Choct, M.:Non-starch polysaccharides : effect on nutritive value. In: Poultry feedstuffs, supply, composition and nutritive value (McNab J, Boorman K, eds). Poultry Science Symposium Series, **26**. CABI Publishing, Oxon UK, 2002; pp 221–235.
48. Choct, M.:Enzyme supplementation of poultry diets based onviscous cereals. In: Enzymes infarm animal nutrition (Bedford M, Partridge G, eds). CABI Publishing, Oxon UK, 2001; pp 145–160.
49. Boyce, A., Walsh, G. Production, purification and application-relevant characterisation of an endo-1,3(4)-β-glucanase from *Rhizomucor miehei*. *Appl. Microbiol. Biotechnol*., 2007; **76**:835–841.
50. Coutinho, P., Henrissat, B., Gilbert, H., Davies, G., Henrissat, B., Svensson, B.(eds): Carbohydrate-active enzymes: an integrated database approach. Recent advances in carbohydrate bioengineering. The Royal Society of Chemistry, Cambridge UK,1999; pp 3–12.
51. McCarthy, T., Hanniffy, O., Savage, A., Tuohy, M. Catalyticproperties and mode of action of three endo-β-glucanases from *Talaromyces emersonii* on soluble β-1,4-and β-1,3;1,4-linked glucans. *Int. J. Biol. Macromol*., 2003; **33**:141–148.
52. Kawai, R., Igarashi, K., Yoshida, M., Kitaoka, M., Samejima, M. Hydrolysis of β-1,3/1,6-glucan by glycoside hydrolase family 16 endo-1,3(4)-beta-glucanase from the basidiomycete *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol*., 2006; **71**(6):898–906.
53. Pitson, S., Seviour, R., McDougall, B. Noncellulolytic fungal β-glucanases: their physiology and regulation. *Enzyme Microb. Technol*., 1993; **15**:178–192.
54. Christakopoulos, P., Hatzinikolaou, D., Fountoukidis, G., Kekos, D., Claeyssens, M., Macris, B. Puification and mode of action of an alkali-resistant endo-1,4-β-glucanase from *Bacillus pumilus*. *Arch. Biochem. Biophys*., 1999; **364**(1):61–66.
55. Marquina, D., Santos, A., Peinado, J.M. Biology of killer yeasts. *Int. Microbiol*., 2002; **5**: 65-71.
56. Conti, S., Cantelli, C., Gerloni, M., Fisicaro, P., Magliani Bertolotti, W,, Mozzoni, P., Sullivan, D., Coleman, D., Polonelli, L. Killer factor interference in mixed opportunistic yeast cultures. *Mycopathologia*, 1996; **135**:1-8.
57. Starmer, W.T., Ganter, P.F., Aberdeen, V., Lachance, M.A., Phaff, H.J. The ecological role of killer yeasts in natural communitiesof yeasts. *Can. J. Microbiol.* 1987; **33**: 783-796.
58. Theisen, S., Molkenau, E., Schmitt, M.J. Wicaltin, a new protein toxin secreted by the yeast *Williopsis californica* and its broad-spectrum antimycotic potential. *J. Microbiol. Biotechnol*., 2000; **10**: 547–550.
59. Javadekar, V.S., SivaRaman, H., Gokhale, D.V. Industrial yeast strain improvement: construction of a highly flocculent yeast with a killer character by protoplast fusion. *J. Indust. Microbiol*., 1995; **5:** 94–102.
60. Palpacelli, V., Ciani, M., Rosini, G. Activity of different 'killer' yeasts on strains of yeast species undesirable in the food industry. *FEMS Microbiol. Lett*., 1991; **68**:75–78.
61. Buzzini, P., Martini, A. Discrimination between *Candida albicans* and other pathogenic species of the genus *Candida* by their differential sensitivities to toxins of a panel of killer yeasts. *J. Clin. Microbiol*., 2001; **39**:3362–3364.

62. Pfohl-Leszkowicz, A., Manderville, R.A. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res*., 2007; **51**(1):61–99.
63. Abrunhosa, L., Serra, R., Venancio, A. Biodegradation of ochratoxin A by fungi isolated from grapes. *J. Agric. Food Chem*., 2002; **50**: 7493–7496.
64. Ozpinar, H., Bilal, T., Abas, I., Kutay, C. Degradation of ochratoxin A in rumen fluid in vitro. *Med. Biol*., 2002; **9**(1):66–69.
65. Wegst, W., Lingens, F. Bacterial degradation of ochratoxin A. *FEMS Microbiol. Lett*., 1983; **17**(1-3):341–344.
66. Schatzmayr, G., Heidler, D., Fuchs, E., Mohnl, M., Taubel, M., Loibner, A.P., Braun, R., Binder, E.M. Investigation of different yeast strains for the detoxification of ochratoxin A. *Mycot. Res*., 2003; **19**:124–128.
67. Ruhland, M., Engelhardt, G., Wallnofer, P.R. Transformation of the mycotoxin ochratoxin A in plants. 2. Time course and rates of degradation and metabolite production in cell-suspension cultures of different crop plants. *Mycopathologia*, 1996; **134**(2):97–102.
68. Varga, J., Rigo, K., Teren, J. Degradation of ochratoxin A by *Aspergillus* species. *Int. J. Food Microbiol*., 2000; **59**:1–7.
69. Stander, M.A., Bornscheuer, U.T., Henke, E., Steyn, P.S. Screening of commercial hydrolases for the degradation of ochratoxin A. *J. Agric. Food Chem*., 2000; **48**(11):5736–5739.
70. Piotrowska, M., Zakowska, Z. The biodegradation of ochratoxin A in food products by lactic acid bacteria and baker's yeast. *Food Biotechnol*., 2000; **17**:307–310.
71. Bejaoui, H., Mathieu, F., Taillandier, P., Lebrihi, A. Ochratoxin A removal in synthetic and natural grape juices by selected oenological *Saccharomyces* strains. *J. Appl. Microbiol*., 2004; **97**: 1038–1044.
72. El-Nezami, H., Polychronaki, N., Salminen, S., Mykkanen, H. Binding rather than metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenone and its derivative a-zearalenone. *Applied and Environmental Microbiology*, 2002; **68**: 3545–3549.
73. Zlotnik, H., Fernandez, M.P., Bowers, B., Cabib, E.*Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *Journal of Bacteriology*, 1984; **159**: 1018–1026.
74. Yiannikouris, A., Andre, G., Poughon, L., Francois, J., Dussap, C.G., Jeminet, G., Bertin, G., Jouany, J.P. Chemical and conformational study of the interactions involved in mycotoxin complexation with β-D-glucans. *Biomacromolecules*, 2006; **7:** 1147–1155.
75. Raju, M.V.L.N., Devegowda, G. Esterified-glucomannan in broiler chicken diets-contaminated with aflatoxin, ochratoxin and T-2 toxin: Evaluation of its binding ability (in vitro) and efficacy as immunomodulator. *Asian Australas J. Anim. Sci*., 2002; **15**:1051–1056.
76. Oguz, H., Parlat, S.S. Effects of dietary mannanoligosaccharide on performance of Japanese quail affected by aflatoxicosis. *S. Afr. J. Anim. Sci*., 2004; **34**:144–148.