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ORIGINAL ARTICLE

L-Amino acid oxidase from filamentous fungi: screening and optimization

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Abstract Twenty-seven fungal isolates recovered on medium containing L-lysine were found to have the potentiality for producing extracellular L-amino acid oxidase (L-AAO). *Aspergillus oryzae* displayed the highest yield of enzyme (2.6 U/mg protein) and antioxidants (2.3 mg/ml) followed by *Aspergillus flavipes* and *Trichoderma viride*. Upon optimization of the fermentation medium, the maximum enzyme yield (4.6 U/mg protein) was obtained on a medium containing L-lysine (80 mM), glucose (0.6%), KH_2PO_4 (0.1%), KCl (0.05%) and $MgSO_4{\cdot}7H_2O$ (0.05%), pH 7.0, under submerged conditions. Supplementing the medium with K^+, Ni^{2+} and Cu^{2+} ions increased enzyme biosynthesis by about twofold compared to the control. The activity of extracellular L-AAO from the submerged cultures of *A. oryzae* was twofold higher than that of the intracellular L-AAO. Eleven agro-industrial byproducts were tested as substrates under solid state fermentation conditions; of these, rice bran (3.3 U/mg) was the optimum solid substrate for the induction of L-AAO and antioxidants, followed by wheat bran (2.8 U/mg) and cotton seed cake (2.5 U/mg), possibly due to their higher crude protein content than ash and fiber. Glucose (1%) and L-phenylalanine were the best co-metabolic and co-inductive carbon and nitrogen source, respectively, for maximum L-AAO production by *A. oryzae* under solid state fermentation, at an initial pH of 7.0. The productivity of L-AAO by *A. oryzae* under solid state fermentation was higher than that of the submerged cultures by about 1.4-fold under optimum conditions, justifying the former fermentation processes for overproduction of this enzyme in terms of both economics and practicality.

Keywords L-Amino acid oxidase · Filamentous fungi · *Aspergillus oryzae* · Submerged · Solid state fermentation

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Introduction

L-Amino acid oxidase (L-AAO, L-amino acid: oxygen oxido-reductase, E.C. 1.4.3.2) is a flavoprotein that catalyzes the oxidative deamination of L-isomers of amino acids to give α-keto acids, hydrogen peroxide and ammonia (Kusakabe et al. 1979). L-AAOs have recently been the focus of increasing attention for their therapeutic applications as broad antibacterial (Samel et al. 2008), antiviral (Lu et al. 2002), antiprotozoal (Ciscotto et al. 2009) and antifungal (Ande et al. 2008) agents. Moreover, L-AAOs have been reported to be a powerful antitumor agent against various cell lines, including mouse leukemic cells (L5178Y) (Kusakabe et al. 1979, 1980) and human breast and acute T cell leukemia (Rodrigues et al. 2009).

The cytotoxicity of L-AAOs is attributed to the direct effect of hydrogen peroxide (H_2O_2) that evolves from the oxidation of L-amino acids (Dua and Clemetson 2002). This generation of the reactive oxygen species causes the activation of caspase-3, releasing mitochondrial cytochrome C (Zhang and Wei 2007) and causing cell apoptosis. Additionally, L-AAOs play an essential role in the resolution of racemic mixtures and the production of keto acids through the oxidation of L-amino acids.

L-AAOs are produced by various bacterial species, including *Pseudomonas tunicata* (Mai-Prochnow et al. 2004), *Rhodococcus opacus* (Geueke and Hummel 2003),

Marinomonas mediterranea (Lucas-Elio et al. 2005), *Pseudoalteromonas* sp. (Gomez et al. 2008), *Bacillus carotarum* (Brearly et al. 1994) and *Proteus rettgeri* (Duerre and Chakrabarty 1975). L-AAOs have also been comprehensively characterized in snake venoms (Rodrigues et al. 2009; Wei et al. 2009). However, the production and characterization of L-AAOs from fungi is scarcely mentioned in the literature. A few fungal species, such as *Aspergillus niger*, *A. nidulans* (Davis et al. 2005), *Neurospora* sp (Aurich et al. 1972), *Trichoderma viride* (Kusakabe et al. 1979) and *T. harzianum* (Treshalina et al. 2000), have been reported as L-AAOs producers.

Stoichiometrically, bacterial L-AAOs are relatively less substrate specific, especially towards neutral (leucine and/or methionine) and basic (L-lysine and L-arginine) amino acids (Yang et al. 2005). Unlike the low specificity of bacterial enzymes, *T. viride* L-AAOs display a higher specificity for L-lysine compared to the acidic amino acids (Yang et al. 2005). Consequently, in terms of developing affordable therapeutical applications of eukaryotic L-AAOs rather than prokaryotic ones, the search for new fungal sources of L-AAOs for the production of these enzymes is technologically justifiable.

The aims of the study reported here were (1) to evaluate the potentiality of some local fungal isolates for the production of L-amino acid oxidases; (2) to optimize the cultural conditions for maximum enzyme and antioxidant production by the potent fungal isolate under submerged conditions; (3) to overproduce this enzyme by the selected fungal isolate under solid state fermentation.

Materials and methods

Chemicals

Nessler's reagent, horseradish peroxidase and guaiacol were purchased from Sigma-Aldrich (St. Louis, MO). Folin reagent and L-lysine were obtained from LOBA Chemie (Mumbai, India). Agricultural byproducts, such as rice bran, wheat bran, broad bean cake, peanut cake, soya beans, lentil seeds and coconut seeds cake, were obtained from local Egyptian markets. Castor seed cake (CSC), cotton seed cake and sesame seed cake were provided by national oil-producing factories. Chicken feathers were collected from the local poultry breeding laboratories. All other chemicals were of analytical grade.

Isolation of fungi producing L-AAOs

Fungal isolates producing L-AAOs were isolated from different soil samples (Sharkia, Egypt) cultured on modified-Dox's medium [(g/l distilled water, pH 6.0) glucose, 5.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $ZnSO_4 \cdot 5H_2O$, $FeSO_4 \cdot H_2O$, 0.01; agar–agar 20; Ruiz-Herrera and Starkey 1969]. The filter-sterilized L-lysine (8 g/l) was added to the autoclaved basal medium and inoculated with 1 ml of soil suspension. After incubation for 6 days at 30±1°C, the developed fungal isolates were purified on the same medium prior to identification.

The purified fungal isolates were fully characterized morphologically and physiologically according to universally accepted keys for fungal identification (Raper and Fennell 1965; Rifai 1969; Booth 1971; Ellis 1971; Pitt 1979; Domsch et al. 1980; Lund 1995; Samson et al. 2007; Geiser et al. 2007).

Submerged fermentation conditions

The potentiality of isolated fungal species for L-AAO production was assessed using L-lysine–glucose liquid medium, as described above. Erlenmeyer conical flasks (250 ml), each containing 50 ml of medium, were inoculated with 1 ml of each fungal spore suspension (1 type of fungal spore suspension per flask) and incubated at 30±1°C for 6 days in a shaker incubator (130 rpm). The cultures were then filtered through Whatman No.1 filter paper, the supernatant centrifuged at 4000 rpm for 5 min, and the enzyme activity, total antioxidants and fungal biomass measured, as described below.

Solid state fermentation medium

Agricultural byproducts, such as rice bran, wheat bran, cotton seed cake, coconut seed cake, broad bean cake, peanut cake, chicken feathers, soya beans, CSC, lentil seeds and sesame seed cake were screened for L-AAO production by the potent fungal isolate under solid state fermentation conditions. Depending on the profile of chemical composition, especially those for bounded and free amino acid contents (data not shown), these natural compounds were selected as the substrate for growth and L-AAO induction by the fungal isolate. Five grams of each dried substrate in 250-ml conical flasks were moistened with 10 ml of the optimized salt solution determined in the submerged conditions, but without L-lysine. After autoclaving, the medium was inoculated with 4 ml of the fungal spore suspension of the 6-day-old cultures and incubated for 10 days at 30±1°C. The crude enzyme and antioxidants were extracted from the solid fungal cultures using 0.1 M potassium phosphate buffer, as described by El-Sayed (2009a, b).

L-AAO assay

Determination of H_2O_2

L-Amino acid oxidase was determined according to Bergmeyer et al. (1974) with slight modifications. Briefly, the reaction

mixture contained 200 μl of enzyme preparation in 0.1 M potassium phosphate buffer (pH 7.0), 0.2 mM guaiacol, 5 U/ml horseradish peroxidase and 10 mM L-lysine as substrate, in a total volume of1 ml. The reaction was incubated for 60 min at 30°C. After the reaction was frozen for 10 min, the color that developed was measured at 436 nm. One unit of the enzyme was expressed by the amount of enzyme that released 1 μmol H_2O_2 per minute under optimal assay conditions.

Activity of L-AAO = A436/min × 4/extinction coefficient of tetra-guaiacol (25.5)

Determination of ammonia

The activity of L-AAO was assessed as deaminase (Saurina et al. 1998) by the direct Nesslerization method (El-Sayed 2011). First, 0.5 ml of enzyme in 0.1 M potassium phosphate buffer and 0.5 ml of 20 mM L-lysine was incubated for 60 min at 30°C. Then, after enzyme activity was stopped by the addition of 100 μl of 1% TCA, the reaction was centrifuged for 5 min at 4000 rpm, following which 0.5 ml Nessler's reagent was added. The developed color was measured at 490 nm. One unit of L-AAO was expressed by the amount of enzyme which releases 1 μmol of ammonium per minute under standard conditions. Specific activity was expressed by the enzyme activity (units) per its protein concentration (mg).

Antioxidant concentration

Concentrations of the total antioxidants of the crude fungal extract was determined by the ferric-thiocynate method (Gupta et al. 2004) with slight modifications. In brief, 1 ml of the crude enzyme preparation was mixed with 0.2 ml of ferrous chloride (20 mM) and 0.2 ml ammonium thiocyanate (30%). After incubation for 10 min, the red color of the peroxide that had developed was measured at 500 nm. The concentration of antioxidants was calculated from the standard curve of α-tocopherol.

H_2O_2 concentration

The free H_2O_2 concentration was determined using the guaiacol (Bergmeyer et al. 1974) assay as described above, except for the addition of lysine. The amount of H_2O_2 was directly calculated as described above.

Protein determination

The concentration of L-AAO proteins was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Biomass estimation

After incubation, the fungal cultures were filtered through Whatman No. 1 filter paper; the pellets were then washed by distilled water and dried at 70°C until a constant weight.

Fractional precipitation of L-amino acid oxidase

Following the incubation of the fungal cultures under solid state fermentation, the enzymes were extracted in potassium phosphate buffer (pH 7.0) according to El-Sayed (2010). L-AAOs were fractionally precipitated in 30–60% saturated ammonium sulfate at 4°C for 12 h (Kusakabe et al. 1980). The collected protein precipitates were dissolved in 25 ml potassium phosphate buffer, dialyzed against the same buffer (100 ml) three times, then concentrated against polyethylene glycol for 3 h at 4°C. The dialysate was then used as the partially purified enzyme.

Antibiograms

Biological activity of the partially purified L-AAO was assessed against *Bacillus subtilis*. After solidification of bacterial plate cultures, 0.5 ml of the enzyme was loaded into a 5-mm hole on the plate. After 48 h of incubation under optimal bacterial conditions, the antibacterial activity was evaluated based on the diameter of the inhibition zone.

Statistical analysis

All of the experiments were carried out in triplicate, and the mean and standard error were calculated using one-way analysis of variance (ANOVA) test.

Results and discussion

Screening for fungi producing L-AAOs

Fungi producing L-AAOs were isolated from different Egyptian soil samples on solid L-lysine–glucose agar medium. In total, 27 fungal isolates (Table 1) were recovered and identified on the basis of morphological and physiological properties according to the universally accepted keys (Materials and methods). The recovered L-AAO-producing fungi belong to eight genera, namely *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Paecilomyces*, *Penicillium*, *Rhizopus* and *Trichoderma*. *Aspergillus* was the most frequently represented genus, with 15 species belonging to nine groups according to Raper and Fennell (1965): *Aspergillus niger* group (*A. awamori*, *A. carbonarius*, *A. niger*), *A. flavus* group (*A. flavus*, *A. oryzae*, *A. parasiticus*, *A. tamarii*), *A.*

ornatus group (*A. ornatus*), *A. flavipes* group (*A. flavipes*, *A. carneus*), *A. ochraceus* group (*A. ochraceus*), *A. sparsus* group (*A. sparsus*), *A. fumigatus* group (*A. fumigatus*), *A. terreus* group (*A. terreus*) and *A. nidulans* group (*A. nidulans*). In terms of the primary nitrogen sources, such as ammonium, glutamine and glutamate (Marzluf 1981), the majority of filamentous fungi are able to oxidatively deaminate most of amino acids regulated by the conserved nit-2 regulatory system (Nahm and Marzluf 1987).

The production of L-AAOs by the isolated filamentous fungal species was evaluated (Table 1) using liquid L-lysine–glucose medium. Among those fungal isolates capable of hydrolyzing amino acids, *Aspergillus oryzae* displayed the highest L-AAO (2.6 U/mg protein) and total antioxidant (2.31 mg/ml) yields, followed in decreasing order by *A. flavipes* (2.4 U/mg protein, 2.3 mg/ml), *Trichoderma viride* (2.3 U/mg protein, 1.9 mg/ml), *A. tamarii* (2.1 U/mg protein, 1.4 mg/ml), *Mucor racemosus* and *Penicillium notatum* (2.0 U/mg protein, 1.1 mg/ml). The lowest yield of L-AAO was found in the culture filtrate of *A. sparsus* (0.45 U/mg protein) and *H. fuscoatra* (0.85 U/mg protein). Based on these results, lower yields of L-AAO were correlated to the level of free H_2O_2, suggesting that H_2O_2 has a deleterious effect on the growth and metabolism of fungal cells. Interestingly, the production of L-AAOs was significantly enhanced by increasing the amount of total antioxidants, in contrast to the effect of free H_2O_2, which provides a plausible explanation for higher enzyme yield by *A. oryzae*. The deaminating activity was higher than the oxidative activity in all isolated fungal species. This observation may be explained by the fact that the enzyme preparations were crude and, therefore, all would contain amino acid oxidases with deaminating activities, resulting in the release of ammonia, but not vice versa, as well as specific deaminases.

Table 1 Screening for L-amino acid oxidase production by filamentous fungi

Number	Fungal isolate	L-Amino acid		Specific oxidase activity (U/mg protein)	Antioxidant (mg/ml)	H_2O_2 (mM)
		Deaminase (U/ml)	Oxidase (U/ml)			
1	*Aspergillus awamori* Usami	4.1	0.81	0.72	2.12±0.2	1.47
2	*Aspergillus carneus* (V. Tiegh)	5.3	1.9	1.1	1.18±0.1	1.6
3	*Aspergillus carbonarius* (Bainier) Thom	4.2	2.03	0.98	2.08±0.7	2.10
4	*Aspergillus flavipes* (Bain and Sart) Thom and Church	16.3	6.1	2.4	2.3±0.4	1.65
5	*Aspergillus fumigatus* Fersenius	4.2	1.5	1.9	2.23±0.9	1.51
6	*Aspergillus flavus* Link	6.8	2.75	1.4	1.36±0.4	2.21
7	*Aspergillus nidulans* Apinis	2.1	1.1	0.78	2.11±0.9	1.10
8	*Aspergillus niger* Van Tieghem	3.2	1.88	1.70	0.55±0.8	1.52
9	*Aspergillus ochraceus* Wilhelm	9.8	1.8	0.91	2.07±0.9	1.6
10	*Aspergillus ornatus* Raper, Fenell and Tresner	5.8	2.3	1.23	2.24±1.1	1.78
11	*Aspergillus terreus* Thom	6.3	2.8	1.9	1.92±1.8	2.1
12	*Aspergillus oryzae* (Ahlb.) Cohn	18.5	7.6	2.6	2.31±0.9	0.81
13	*Aspergillus parasiticus* Speare	2.8	1.92	1.1	1.14±0.8	1.55
14	*Aspergillus sparsus* Raper and Thom	6.2	2.25	0.45	0.91±0.4	1.93
15	*Aspergillus tamarii* Kita	4.5	1.83	2.1	1.39±0.9	1.17
16	*Fusarium nivale* (Fr.) Ces., Rabenh	6.7	1.4	1.0	2.23±1.2	0.80
17	*Fusarium oxysporum* Schlecht	5.2	2.65	0.89	1.6±0.6	2.31
18	*Fusarium solani* (Matt)	2.9	1.21	1.2	1.88±0.4	0.89
19	*Humicola fuscoatra* Traaen	5.1	1.8	0.85	2.26±1.3	1.73
20	*Mucor racemosus* Fresenius	11.8	4.1	2.0	1.08±0.8	4.4
21	*Penicillium egyptiacum* Van Beyma	5.8	3.7	1.2	1.42±1.2	2.81
22	*Penicillium citrinum* Thom	4.8	2.87	1.5	0.61±0.8	2.13
23	*Penicillium notatum* Westling	2.4	0.8	2.0	1.10±0.6	2.81
24	*Paecilomyces varioti* Bainier	3.6	1.75	1.5	1.31±0.8	1.30
25	*Rhizopus oryzae*	3.1	2.96	1.8	2.11±1.3	3.22
26	*Trichoderma koningii* Oud	8.5	3.8	1.6	1.28±1.1	1.5
27	*Trichoderma viride* Pres	7.9	3.98	2.3	1.90±0.9	1.85

A. oryzae is one of the most common strains widely used in various biotechnological processes, especially for the production of therapeutic enzymes, such as glutaminase (Nandakumar et al. 2003); it is also used in various industries as a food-fermenting agent (e.g. Miso soup, Koji steamed rice, vinegar production; Machida et al. 2008). The potentiality for growth and enzyme production by *A. oryzae* may ascribed to its specialized antioxidant system (Wardhani et al. 2010) that rapidly scavengers the released H_2O_2, compared to other fungal species.

Based on the screening profile, which depended on the amount of extracellular L-AAO and total antioxidants produced, *A. oryzae* was the most potent fungal isolate for enzyme production. Consequently, this fungal isolate was selected for further testing to optimize the enzyme yield.

Extracellular and intracellular L-AAOs from *A. oryzae*

Extracellular and intracellular forms of L-AAOs were assayed from the submerged cultures of *A. oryzae*. The intracellular enzyme was extracted from the fungal pellets (1 g) by grinding in a mortar containing 100 ml Tris–HCl buffer (pH 7.5; 20 mM EDTA, 10 mM PMSF) and 0.5 g acid-washed sterile sea sands. The pellets were homogenized for 20 min in the mortar, placed in an ice bath, then centrifuged at 5000 rpm for 10 min. The supernatant was used as the source of crude intracellular enzyme. From the obtained data (Table 2), the yield of extracellular oxidative L-AAO and amount of antioxidants was 2.6 U/mg protein and 2.31 mg/ml, respectively, compared to 1.1 U/mg and 0.58 mg/ml for the intracellular form, respectively. Thus, the yield of extracellular L-AAO was about 2.4-fold higher than that of intracellular enzyme, which may ascribed to the cellular vacuoles proteases during extraction causing proteolysis or denaturation to the intracellular enzymes. A higher activity fungal extracellular enzymes relative to intracellular ones has been comprehensively reported for various fungal enzymes (Yano et al. 1988). Therefore, we designed experiments to determine those conditions which would optimize extracellular L-AAO production from *A. oryzae*.

Initial pH of the production medium

The effect of the initial pH (3.0–9.0) on the production of L-AAO and L-lysine uptake by *A. oryzae* was studied using 50 mM potassium phosphate buffer. Based on the results (Fig. 1), the enzyme yield and L-lysine uptake by the fungal isolate is a pH-dependent process. The maximum yield of L-AAOs (2.81 U/mg protein) and L-lysine uptake (80.5%) were observed at pH 7.0. The decrease in the enzyme productivity and lysine uptake was found to be higher in acidic pHs than alkaline ones. Relative to the optimum pH, enzyme productivity was decreased by 89.1 and 7.5% at pH 3.0 and 9.0, respectively. The highest yield of L-AAOs was at neutral to slightly alkaline conditions, possibly due to the maximum balance in the ionic strength of the plasma membrane at this pH stimulating the proper enzyme posttranslational modification and subunit assembly. In accordance with our results for *A. oryzae* L-AAO, neutral conditions have also been found to be optimum for the production of this enzyme from *Proteus rettgeri* (Duerre and Chakrabarty 1975), *Chlamydomonas reinhardtii* (Piedras et al. 1992) and *Bacillus carotarum* (Brearly et al. 1994). The higher amount of L-AAO and faster growth of *A. oryzae* at neutral to slightly alkaline pHs may be somehow related to the rapid decomposition of H_2O_2 at alkaline pH and its stabilization under acidic conditions (Watts et al. 1999).

Carbon sources on L-AAO production by *A. oryzae*

The influence of various carbon sources on the production of L-AAOs and the growth of *A. oryzae* is summarized in Fig. 2. The maximum yield of L-AAO by *A. oryzae* was observed using glucose (2.9 U/mg protein), followed by sucrose (2.71 U/mg protein), fructose and maltose (2.44 U/mg protein). None of the other carbon sources had a stimulatory effect on the enzyme production by *A. oryzae*. It noteworthy that the production of L-AAO by *A. oryzae* was repressed more in medium containing polysaccharide lysine than in that containing monosaccharide lysine. For example, carboxymethyl cellulose (CMC), cellulose, chitin and pectin have an inhibitory effect on L-AAO synthesis that may be ascribed to the synthesis of polysaccharide-

Table 2 Extracellular and intracellular L-amino acid oxidase from *Aspergillus oryzae*

Enzyme	L-Amino acid				Total antioxidants (mg/ml)
	Deaminase (U/ml)	Specific deaminase activity (U/mg protein)	Oxidase (U/ml)	Specific oxidase activity (U/mg protein)	
Extracellular enzymes	18.5	6.1	7.6	2.6	2.31
Intracellular enzymes	6.28	12.5	2.5	1.1	0.58

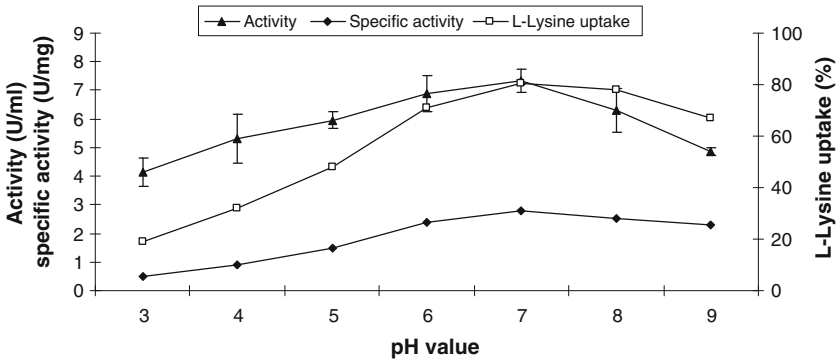

Fig. 1 Effect of pH on L-amino acid oxidase (L-AAO) productivity and L-lysine uptake by *Aspergillus oryzae*

hydrolyzing systems over that of the lysine-oxidizing system. Glucose was consistently the favored co-stimulator for the production of various fungal amino-acid deaminating enzymes (Ruiz-Herrera and Starkey 1969; El-Sayed 2009b). The preference of glucose as co-metabolic agent for oxidative deamination of amino acids could be attributed to their direct usability in various metabolic pathways as carbon and energy sources for the synthesis of the multiple antioxidant systems.

The production of L-AAO by *A. oryzae* was studied in response to different concentrations of glucose in the L-lysine production medium (data not shown). Relative to the control (0.4% glucose), the highest yield of L-AAO (3.2 U/mg protein) by *A. oryzae* was detected using 0.6% glucose. Unlike the enzyme productivity, fungal growth was proportionally related to the glucose concentration compared to the glucose-free medium. Using glucose-free medium, we observed that the enzyme production and L-lysine uptake were reduced by about 84.4 and 88.8%, respectively, compared to the control. Relative to the optimum glucose level, the yield of enzyme at the higher glucose level (1%) was repressed by about 10%. Therefore, we deduced that the induction and release of L-AAO form part of a glucose-regulated system. The lower amount of L-AAO produced by *A. oryzae* at higher glucose levels could be attributed to the interference of glucose with the amino acid transport system for lysine (System VI) or the negative effect of glucose on the plasma membrane H^+-ATPase system for active enzyme transport (Young et al. 1998).

Induction of *A. oryzae* L-AAOs by various nitrogen sources

The impact of different nitrogen sources on the induction of *A. oryzae* L-AAOs is summarized in Fig. 3. The productivity L-AAO appeared to be repressed by the complex organic nitrogenous compounds (casein, peptone, beef, malt and yeast extract) compared to inorganic nitrogen compounds and amino acids. Although the highest biomass of *A. oryzae* (6 g/L) was obtained on yeast extract–glucose medium, enzyme production on this medium was significantly repressed (69.2% relative to the control) thereby justifying the utilization of nitrogen sources and growth factors/stimulators other than L-AAO inducers. Similar results have been reported for other amino acid-hydrolyzing enzymes using yeast extract-incorporated medium (El-Sayed 2009a). The maximum yield of L-AAO (3.2 U/mg protein) was observed using lysine-containing medium, followed medium containing phenylalanine, glutamic acid (2.90 U/mg protein), L-asparagine (2.77 U/mg protein) and alanine (2.70 U/mg protein). Among the amino acids tested, the highest fungal biomass (5.3 g/L) was measured using phenylalanine-containing medium, followed medium containing L-glutamine (4.9 g/L), L-methionine and lysine (4.7 g/L). However, among the inorganic sources of nitrogen, medium containing ammonium nitrate gave the highest amount of L-AAO (1.95 U/mg protein), in contrast to the lower amount of this enzyme produced by *A. oryzae* cultured on ammonium sulphate (0.37 U/mg protein). We therefore conclude that the

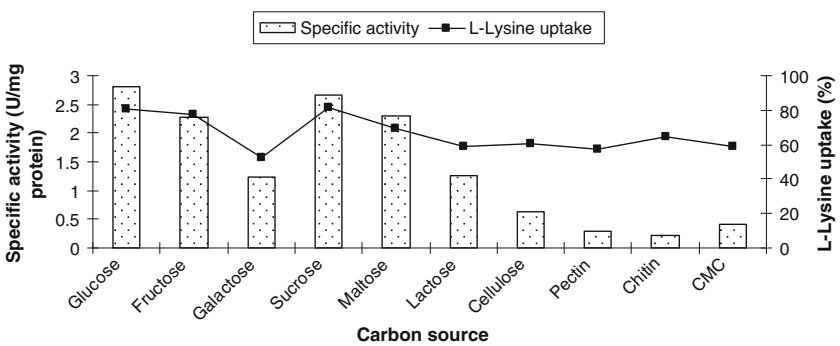

Fig. 2 Effects of different carbon sources on L-AAO production and L-lysine uptake by *A. oryzae*. *CMC* Carboxymethyl cellulose

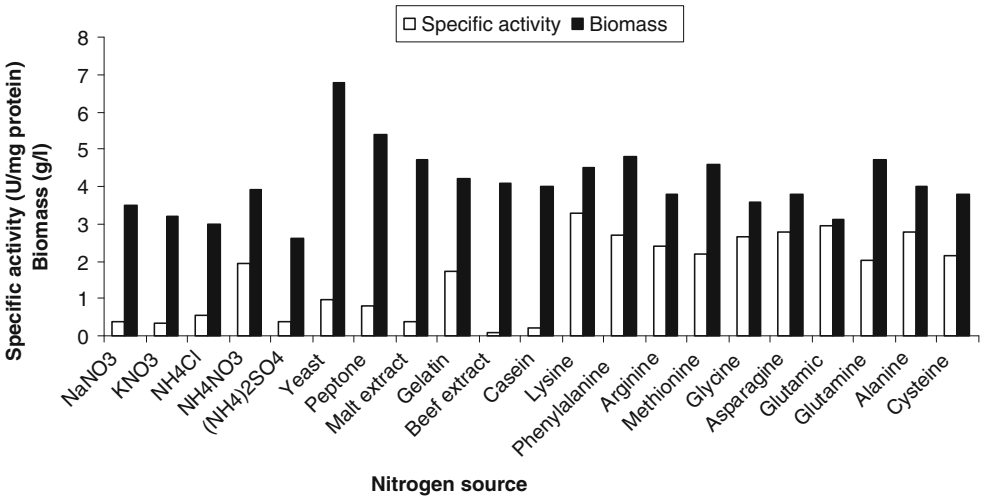

Fig. 3 Effect of different nitrogen source on L-AAO productivity and biomass of *A. oryzae*

production of L-AAO by *A. oryzae* not only relies on a nitrogen source but is also a strictly amino acid-dependent process (Papoyan et al. 2001). In other studies, L-phenylalanine was the favored inducer of L-AAOs by *Bacillus carotarum* (Brearly et al. 1994) and *Neurospora crassa* (Niedermann and Lerch 1990). When *A. oryzae* was grown on L-lysine-free medium (sodium nitrate as control), the production of L-AAOs and growth yield was reduced by about 88.2 and 23% compared to the optimum. Physiologically, the induction of L-AAO by *A. oryzae* in response to L-amino acids other than non-amino acid nitrogen sources is a a common criterion for filamentous fungi (Sikora and Marzluf 1982; Niedermann and Lerch 1990).

The effect of L-lysine concentrations (10–120 mM final concentration) on L-AAO production by *A. oryzae* was also studied (Fig. 4). The maximum yield of *A. oryzae* L-AAO (4.1 U/mg protein) was obtained at 80 mM lysine and was about 1.3-fold higher than that in the control medium (lysine 40 mM). At 140 mM lysine, the production of enzyme was about 41.5% lower than the optimal level. The lower enzyme yield at higher lysine concentrations may be due to downregulation of the nit-2 gene system for lysine assimilation, blocking of the ABC transporters or transinhibition by hyper-accumulation of the internal pool of L-lysine (Hunter and Segel 1973).

Effect of phosphorus sources on the production of *A. oryzae* L-AAOs

The influence of various phosphorus sources on L-AAO production and lysine uptake by *A. oryzae* was evaluated (data not shown). The highest enzyme yield (4.6 U/mg protein) and L-lysine uptake (83.5%) by *A. oryzae* were observed using KH_2PO_4, followed by Na_2HPO_4, NaH_2PO_4 and K_2HPO_4. While ammonium phosphate had a considerable inhibitory effect on enzyme production by *A. oryzae*, that this effect may be ascribed to the preference of ammonium ions as the nitrogen source over amino acids. The enzyme yield by the fungal isolate was reduced by about 4.6-fold using phosphorus-free medium. The preference for potassium dihydrogen phosphate as the phosphorus source is a common physiological criterion for the production of amino acid-hydrolyzing enzymes by filamentous fungi (Ruiz-Herrera and Starkey 1969) that may be ascribed to the contribution of K^+ to the regulation of cellular transport and membrane polarization, in addition to phosphorus (Jennings 1995).

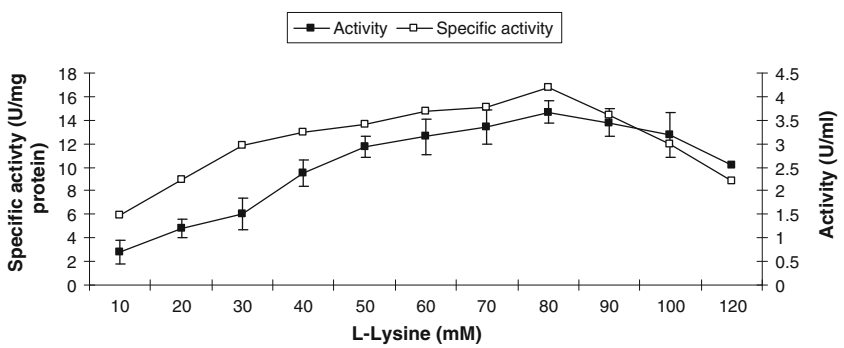

Fig. 4 Effect of different L-lysine concentrations on the productivity of L-AAO by *A. oryzae*

Effect of trace elements on the production of *A. oryzae* L-amino acid oxidase

The incorporation of various trace elements to the production medium of L-AAO by *A. oryzae* was investigated (data not shown). Compared to the trace element-free medium, the addition of Na^{+}, Fe^{2+}, K^{+}, Ca^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} and Cu^{2+} had a plausible stimulatory effect on L-AAO biosynthesis by *A. oryzae*. However, the maximum amount of enzyme (4.6 U/mg protein) and L-lysine uptake (84.1%) was detected by on medium containing the combination of $ZnSO_4$ and $FeSO_4$ (control). The positive inductive effect of Na^{+}, K^{+}, Ca^{2+}, Zn^{2} ions on enzyme synthesis may be due to their contribution as osmotic modulator/ cofactors for enzyme maturation, thereby ensuring proper fungal growth (Jennings 1995). However, both the production of L-AAO and lysine uptake were dramatically repressed by the incorporation of Co^{2+}, Li^{2+}, Cd^{2+} and Hg^{2+} to the medium of *A. oryzae*, suggesting their accumulation affected mitochondria and/or interfered with the respiratory pathways, as reported for unicellular fungi (Jennings 1995).

L-AAO production by *A. oryzae* under solid-state fermentation conditions

The production of L-AAO using natural agro-industrial byproducts as solid substrates for *A. oryzae* was investigated. Fungal submerged cultures for the production of L-AAOs have been the object of great interest using L-lysine or L-phenylalanine as substrates. However, L-lysine has been frequently reported to be one of the most common amino acids that rapidly undergoes stereo-modification via Millared reactions, forming Amadori compounds, especially in aqueous solution, by autoclaving (Kim et al. 2001), thus possibly hindering the microbial growth and their physiological processes. Physiologically, solid-state fermentation, with its low moisture content, usually provides a selective environment for the growth and production of extracellular enzymes by filamentous fungi (Jennings 1995; El-Sayed 2009b), in contrast to bacteria (Cannel and Moo-Young 1980). Moreover, bounded amino acids (solid substrates) are more chemically stable than free ones; consequently, the former are preferred for broad technological applications as substrates for the bulk production of economically valuable compounds. Given the limitations of submerged conditions, therefore, solid state fermentation would seem to be a potent cost-effective technique for upscaling the production of L-AAOs. This led us to screen for the favored solid substrate as well as optimization of the culture conditions for maximum L-AAO production by *A. oryzae*.

Several agro-industrial byproducts were screened for L-AAO production by *A. oryzae*, namely, rice bran, wheat bran, cotton seed cake, coconut seed cake, broad bean cake, peanut cake, chicken feathers, soya beans, CSC, lentil seeds and sesame seed cake. These natural substrates are insoluble and could be used as a source of carbon, nitrogen and growth factors by the fungal cells. From the screening profile (Table 3) for enzyme production, the maximum yield of L-AAO by *A. oryzae* was found using rice bran (3.3 U/mg protein), wheat bran (2.8 U/mg), milled cotton seeds (2.5 U/mg) broad bean and lentil seed (2.1 U/mg). The apparent variation on the inductivity of L-AAO by *A. oryzae* according to the type and nature of the solid substrate may be closely related to fluctuations in the chemical composition of these compounds (El-Sayed 2009b). Similar results for L-lysine oxidase production by *Trichoderma viride* were reported using wheat bran under solid state fermentation conditions (Kusakabe et al. 1979, 1980). The feasibility of rice bran and wheat bran as the favored substrate for L-AAO production by the fungal isolate may ascribed to their higher protein contents relative to their ratio of ash and fiber in comparison to other substrates (unpublished data), in addition to their unique mechanical properties (structure retention and lack of particle agglomeration) (El-Sayed 2009a, b). Therefore, rice bran was selected as the optimal solid substrate for subsequent cultural optimization to maximize the enzyme yield by *A. oryzae*. The initial moisture content was adjusted to 50% in the solution of glucose (0.6%), KH_2PO_4 (0.1%), $MgSO_4{\cdot}7H_2O$ (0.05%) and KCl (0.05%), pH 7.0, as the optimized nutritional parameters based on results from the submerged cultures.

Our data (Fig. 5) reveals the importance of fungal growth and enzyme productivity under solid state fermentation on inoculum density. The highest amounts of L-AAO (4.5 U/mg protein) and antioxidants (3.8 mg/ml) on the solid cultures of *A. oryzae* were obtained with 6 ml of inoculum (7-day-old culture) per 5 g of rice bran in a 250-ml conical flask. The amount of enzyme was decreased by about 2.2-fold, relative to the optimum value, when 1 ml of inoculum was used, possibly because there were an insufficient number of cells, thus a longer time was required for growth and substrate hydrolysis, as also reported by Kashyap et al. (2002) and EL-Sayed (2009b). The significant decrease in the enzyme yield by *A. oryzae* at higher volumes of inocula (12 ml) may be attributed to the nutrient limitation (Kashyap et al. 2002), accumulation of spore self-inhibiting substances or an increase in initial moisture contents causing substrate particle agglomeration and lower O_2 transfer, thereby enhancing the risk of bacterial growth (Hesseltine 1972).

The effect on L-AAO production of adding various carbon sources to the rice bran solid cultures of *A. oryzae*

Table 3 Screening for L-amino acid oxidase production by *A. oryzae* using different solid substrates

Experiment	Substrate	L-Amino acid oxidase (U/ml)	Specific activity (U/mg protein)	Antioxidants (mg/ml)
1	Rice bran	57.3	3.3	3.6
2	Wheat bran	43.1	2.8	3.8
3	Coconut seed cake	27.1	1.6	2.9
4	Cotton seed cake	27.1	2.5	3.4
5	Broad bean	31.0	2.1	5.1
6	Peanut cake	19.2	0.9	6.3
7	Feather	6.2	0.5	1.2
8	Soya bean seed	31.6	1.1	4.5
9	Castor seed cake	29.5	1.5	2.1
10	Lentil seed	35.9	2.1	5.0
11	Sesame seed cake	21.9	1.3	4.2

were investigated (Fig. 6). The most potent carbon sources for enzyme production under submerged conditions were experimented on under solid state fermentation conditions. Relative to the extra-carbon free-medium, all of the tested carbon sources exerted a inductive effect on the biosynthesis of the enzyme and antioxidants. Among the tested carbon sources, glucose was the best inducer fof L-AAO synthesis (4.5 U/mg protein) followed by fructose (4.1 U/mg protein) and sucrose (3.8 U/mg protein). The amount of antioxidants was fairly stable when glucose, fructose and sucrose were the carbon source, but the amount of enzyme decreased by about 2.2-fold when rice bran free of external carbon source was used. The apparent growth and enzyme production using the basal medium of rice bran reflect the usability of rice bran by the fungal isolate as a carbon, nitrogen and growth factor. The implication that D-glucose is a super stimulator of extracellualr production of L-AAO by *A. oryzae* coincides with that reported for glutaminase and L-methioninase production by fungi (El-Sayed 2009a, b), ensuring their feasibility as co-metabolic agents for the initiation of fungal growth.

The effect of supplementing the medium with amino acids, namely, L-lysine, phenylalanine, glutamic acid, L-methionine and cysteine, on the production of L-AAO and antioxidants by *A. oryzae* using rice bran as the solid substrate was evaluated (Fig. 7). Interestingly, all of the incorporated amino acids had a stimulatory effect on enzyme yield, relative to that of the rice bran medium free of amino acids. The maximum enzyme yield (6.5–6.8 U/mg protein) and maximum total antioxidant production (5.1–5.8 mg/ml) were detected with *L*-lysine and phenylalanine. Relative to L-lysine (optimum), the amount of extracellular L-AAO and antioxidants were slightly decreased by about 1.4–1.5 fold in the basal medium (free of external amino acids), revealing the ability of the fungal isolate to utilize rice bran as a sufficient amino acid source for enzyme induction. The positive effect of the additional amino acids, particularly phenylalanine and lysine, on enzyme production reveals their co-inductive effect on stimulating the gene expression of these enzymes by the fungal cells. Overproduction of L-AAO by solid cultures of *A. oryzae* with external incorporation of free amino acids is consistent with reports of many enzymes being able to hydrolyze amino acids under solid state fermentation conditions (El-Naghy et al. 1998; El-Sayed 2009a, b).

The maximum yield of L-AAO and total antioxidants was obtained using a basal medium containing a solution

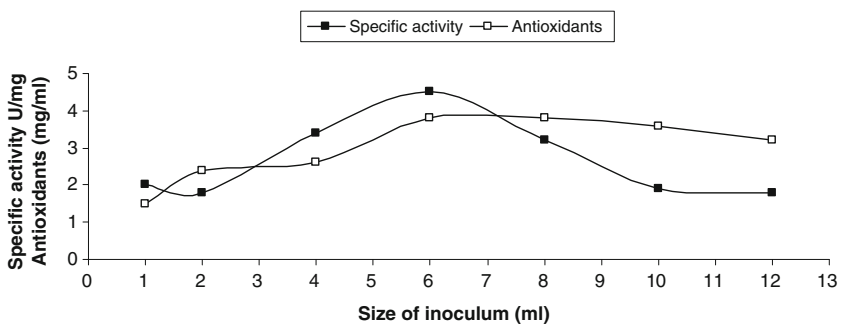

Fig. 5 Effect of inoculum density on L-AAO and antioxidants production by *A. oryzae* under solid state fermentation conditions

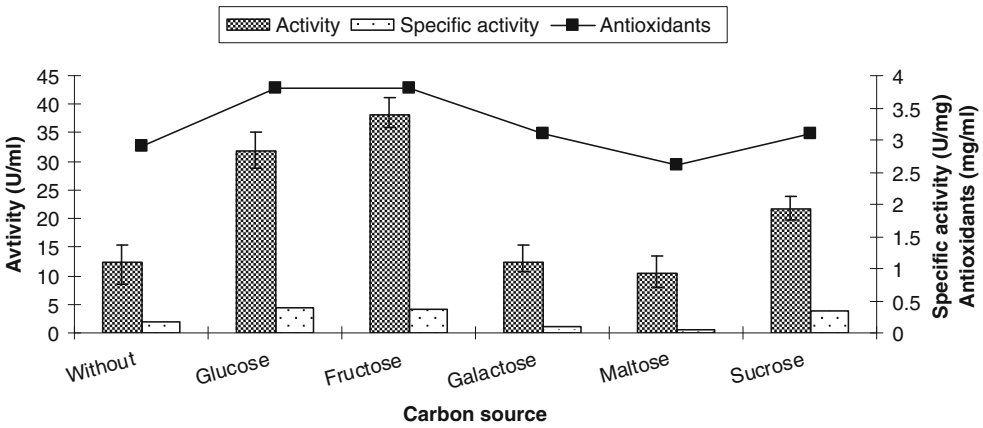

Fig. 6 Effect of different carbon sources on L-AAO and antioxidant production from *A. oryzae* under solid state fermentation conditions

salt of pH 7.5 (data not shown). The rate of enzyme repression by the solid cultures of *A. oryzae* was drastically increased at a lower pH compared to neutral and alkaline conditions. The higher yield of L-AAO at alkaline conditions suggests the rapid decomposition of H_2O_2 to water and oxygen, thus decreasing the negative effect on fungal growth. These results are consistent to those reported for deaminases, which are optimally active at neutral–alkaline pHs (Prabhu and Chandrasekaran 1995).

Antibiograms

The antibacterial activity of the partially purified L-AAO (7.4 U/mg protein) from the rice bran solid culture of *A. oryzae* against *B. subtilis* was assessed (data not shown). The diameter of the antibacterial halo zone was about 1.4 cm. The relative antibacterial activity of the enzyme ensures the release of H_2O_2 as a highly oxidizing agent against structural and energetic macromolecules. The activity of *A. oryzae* L-AAO coincides with those frequently reported for other oxidative enzymes (Lucas-Elio et al. 2005; Gomez et al. 2008).

Conclusion

The results of this study support the proposal that local isolated filamentous fungi, particularly *A. oryzae*, are able to produce L-amino acid oxidases. In terms of the potential of these enzymes to be used in biotechnological applications, their productivity was optimized under submerged culture conditions. Moreover, in an attempt to overproduce L-AAOs to satisfy their higher requirements, solid state fermentation was determined to be a reliable process for the maximum exploitation of this enzyme from the economical and practical points of view. Practically, the yield of L-AAO by the optimized solid culture of *A. oryzae* was higher than that of the submerged culture by about 1.4-fold. Therefore, purification and characterization of this enzyme from *A. oryzae* using rice bran-supplemented medium under optimized solid fermentation conditions are ongoing in our lab. To the best of our knowledge, this is the first report of a comprehensive screening of L-amino acid oxidase production by filamentous fungi locally isolated from Egyptian soils and of the optimization culture conditions for the production of AAOs under submerged and solid state fermentation conditions.

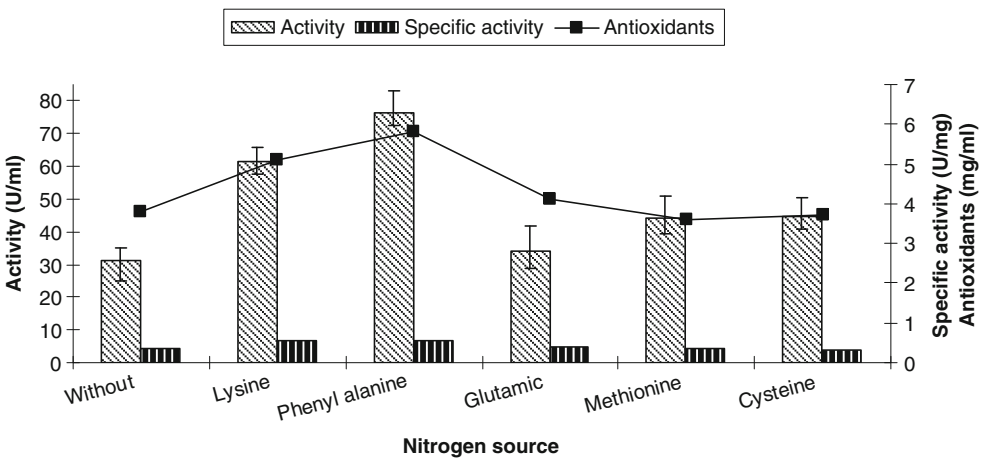

Fig. 7 Effect of different nitrogen sources on L-AAO and antioxidant production by *A. oryzae* under solid state fermentation conditions

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