African Journal of Microbiology Research Vol. 6(20), pp. 4365-4376, 30 May, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/ AJMR-11-1470
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Production and optimization of D-amino acid oxidase which is involved in the biosynthesis of β-lactam antibiotics

A. Saleem[1*], A. M. Moharram[2] and N. Fathy[3]

[1]Department of Botany, Faculty of Science, South Valley University, Qena, Egypt.
[2]Department of Botany, Faculty of Science, Assuit University, Assiut, Egypt.
[3]Department of Microbiology, Hebi Company for Pharmaceutical Industries, Egypt.

Accepted 10 January, 2012

A total of sixty seven fungal isolates representing 40 fungal species related to 14 genera were screened for their abilities to produce endocellular D-amino acid oxidase enzyme. The most active fungal isolates were *Fusarium heterosporum* and *Nectria haematococca*, producing 210.41 and 207.94 units/ml, respectively. Maximum activity of D-amino acid oxidase produced by *F. heterosporum* and *N. haematococca* was obtained after 7 days of incubation at 30°C with pH 7 culture medium containing glucose and ammonium sulphate as carbon and nitrogen sources, respectively. Inoculation of cultures by 3 discs of fungi and incubation of cultures at 160 rpm shaking condition improved the enzyme production. Among seven amino acids tested, D-alanine was the best inducer for D-amino acid oxidase production by *F. heterosporum*; however L-asparagine was the best by *N. haematococca*. High-performance liquid chromatography (HPLC) analysis indicated that the purified D-amino acid oxidase produced by both fungi was active for the conversion of cephalosporin C to glutaryl-7-aminocephalosporanic acid.

Key words: D-amino acid oxidase, antibiotic biosynthesis, *Fusarium heterosporum*, *Nectria haematococca.*

INTRODUCTION

D-amino acid oxidase is a flavin adenine dinucleotide (FAD)-containing enzyme that catalyzes oxidative deamination of D-amino acids yielding hydrogen peroxide and imino acid. The later is further non-enzymatically hydrolyzed to α-keto acids and ammonia. A major characteristic of all D-amino acid oxidase is their high specificity towards D-isomers of amino acids. They are almost inactive towards the corresponding L-isomers (Khoronenkova and Tishkov, 2008; Kuan et al., 2008). D-amino acid oxidase enzyme is participating in the transformation of cephalosporin C into glutaryl-7-aminocephalosporanic acid for the production of β-lactam antibiotics. It catalyzes one of the key steps in the production of semisynthetic cephalosporins (Conlon et al., 1995; Singh et al., 2001; Kuan et al., 2008).

In eukaryotic cells, D-amino acid oxidase supports the physiological level of D-amino acids that play an important role in the regulation of many processes such as aging, neural signaling, hormone secretion, etc. D-amino acid oxidase is finding more practical applications such as D-amino acid detection in biological samples, diagnostics and prophylaxis of psychosomatic diseases and cancer, preparation of unnatural L-amino acids, analysis of enantiometric purity of amino acids, synthesis of α-keto acids and 7-aminocephalosporanic acid preparation from cephalosporin C (Khoronenkova and Tishkov, 2008; Rosini et al., 2009). D-amino acid oxidase was first detected in mammalian tissue by Kreps in 1935. For many years the enzyme from hog kidney was probably most commonly employed, until the early eighties

*Corresponding author. E-mail: abdosaleem@yahoo.com.

(Schrader and Andreesen, 1993), when articles describing characterization, induction and purification of D-amino acid oxidase enzymes were found in fungi including *Aspergillus niger*, *Neurospora crassa* and many species of *Penicillium* and in the Yeasts such as *Rhodotorula gracilis* and *Trigonopsis variabilis* (Huber et al., 1992; Pollengioni et al., 1993; D'Acunzo et al., 1996; Alonso et al., 1998; Vikartovska-Welwardova et al., 1999; Singh et al., 2001; Zheng et al., 2006).

D-amino acid oxidase plays a very important role in biotechnology and pharmacology due to its high stereoselectivily and variety of D-amino acids used as substrate. It can be employed in a number of applications such as production of α-Keto acid, which can be used for chronic uremia therapy (Fischer, 1998). The β-lactam nucleus 7-aminocephalosporanic acid is a key inter-mediate used in making semisynthetic cephalosporins from cephalosporin C (Binder et al., 1994). The primary method for producing 7-aminocephalosporanic acid industrially has been a chemical deacylation of cephalosporin C.

This method of the enzyme production has a number of disadvantages. In the chemical synthesis pathway a number of toxic and hazardous chemicals are needed, Thus creating safety and environ-mental problems. Another advantage of a biological route is that the process can be conducted in water instead of organic solvents (Conlon et al., 1995). This article was designed to study the potential of different fungal genera and species to produce D-amino acid oxidase under different environmental and nutritional conditions. Documentation of the conversion of cephalosporin C to glutaryl-7-aminocephalosporanic acid by the action of D-amino acid oxidase produced by *Fusarium heterosporum* and *Nectria haematococca* using high-performance liquid chromatography (HPLC) technique.

MATERIALS AND METHODS

Screening of fungi for production of D-amino acid oxidase

A total of sixty seven fungal isolates recovered from soil collected from Qena were screened for their ability to produce intracellular D-amino acid oxidase. These isolates comprised 10 species of *Aspergillus*, 7 species of *Fusarium*, 9 species of *Penicillium*, 3 species of *Gibberella* and one species from fungal genera of *Emericella*, *Trichoderma*, *Cochliobolus*, *Phoma*, *Geotrichum*, *Necteria* and *Neurospora*, in addition to some species of yeast.

Production medium

D-amino acid oxidase was tested according to the method described by Gabler and Fischer (1999). The standard medium contained the following constituents (g/L): Glucose, 18; K_2HPO_4, 4; $(NH_4)_2SO_4$, 4; yeast extract, 4 and metal salts $MgSO_4.7H_2O$, 1; $CaCl_2.2H_2O$, 0.5; $NaMoO_4$, 0.04; $ZnSO_4.7H_2O$, 0.04; $CuSO_4.7H_2O$, 0.045; $FeSO_4.7H_2O$, 0.025; H_3BO_3 0.1. The pH was adjusted to 7.0.

Cultivation and culture conditions

The medium was distributed in Erlenmeyer flasks (250 ml). Each flask contains 100 ml of the medium. The flasks were sterilized at 121°C for 20 min. Each flask inoculated with 10 mm mycelial disc cut out from 5 days fungal colony grown on glucose-Czapek's agar medium for filamentous fungi and on Sabouraud dextrose agar medium in case of yeasts. The inoculated flasks were incubated under shaking condition at 30°C for 7 days. At the end of the incubation period, the content of each flask was filtrated in case of filamentous fungi but centrifuged at 13000 rpm for 10 min in case of yeasts.

Determination the activity of D-amino acid oxidase enzyme

Three grams of wet biomass was weighed and suspended in a 6 ml mixture of 50 mM potassium phosphate buffer (pH = 8.0), 0.1 M sucrose and 3 mM EDTA. 0.5 g of glass beads was added and the microtubes were placed in a bead mill at 4°C for 25 min, followed by centrifugation at 13000 rpm for 5 min. 0.1 ml of the clear supernatant was added to the mixture of the following components at 37°C (Ballagi, 1999). 1.5 ml, 50 mM phosphate buffer, pH 8, air saturated. 1 ml from a mixture of 210 mg O-Dianisidine, 3000 unit peroxidase, 50 ml H_2O and 50 ml glycerin. 1.4 ml from D-alanine solution (20 mg/ml). The reaction was stopped by addition of 2 ml 30% H_2SO_4. O-dianisidine turns brown due to the oxidation caused by H_2O_2. As a result of the H_2SO_4 addition the O-dianisidine changes color from brown to red. Red color was measured at 540 nm using a spectrophotometer. A set of standard mixtures of H_2O_2 enabled the successful establishment of a reference correspond-dence between H_2O_2 concentration and absorption at 540 nm (Billy, 1999).

Effect of incubation periods on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

F. heterosporum and *N. haematococca* were employed because they were found to be the most active D-amino acid oxidase producers. The medium described by Gabler and Fischer (1999) which was previously described was also employed. The influence of different incubation periods (2, 4, 6, 7, 8, 10 and 12 days) on the production of D-amino acid oxidase and fungal dry weight were tested. The medium was distributed in 250 ml flasks, containing 100 ml each. The flasks were sterilized at 121°C for 20 min, inoculated with 10 mm disc cut out from 5 days fungal colony grown on glucose-Czapek's agar medium. Two replicates were used for each treatment. Inoculated flasks were incubated under shaking condition at 30°C. At the end of different incubation periods, fungal mycelium was separated from the growth medium by suction filtration through pre-weighted Whatman #1 filter paper placed in a buchner funnel. The filter paper with mycelium was dried in an oven at 85°C for 24 h and the mycelial dry weight was recorded. Three grams of wet biomass was used for determination the activity of D-amino acid oxidase.

Effect of temperatures on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The influence of various temperature values (15, 20, 25, 30, 35 and 40°C) on the activity of D-amino acid oxidase and fungal dry weight were tested by the incubation of tested fungi at different temperatures in liquid synthetic medium for 7 days under shaking condition. After the incubation period, fungal dry weight and D-amino acid oxidase were measured. Two replicates were used in each treatment.

Effect of pH values on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The influence of different pH values (2, 4, 6, 7, 8, 10 and 12) on fungal growth and D-amino acid oxidase production were measured by incubating *F. heterosporum* and *N. haematococca* at 30°C in liquid synthetic medium previously adjusted to different pH values for 7 days. After the incubation period, fungal dry weight and D-amino acid oxidase were measured. Two replicates were used for each treatment.

Effect of different carbon sources on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The two fungal species were grown in Erlenmeyer flasks (250 ml) containing 100 ml liquid medium. Nine different carbon sources were added individually to the basal medium as follow (g/L): Glucose, 18; Fructose, 18; Maltose, 7.2; Lactose, 7.2; Sucrose, 7.6; Cellulose, 18; Starch, 18; Dextrin, 18 and Glycerol, 7. The flasks were sterilized at 121°C for 20 min, inoculated with 10 mm disc cut out from 5 days fungal colony grown on glucose-Czapek's agar medium. Two replicates were used for each carbon source.

Inoculated flasks were incubated under shaking condition at 30°C for 7 days. At the end of the incubation period, the mycelial dry weight was recorded. Three grams of wet biomass was used for determination the activity of D- amino acid oxidase.

Effect of different nitrogen sources on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

F. heterosporum and *N. haematococca* were grown in Erlenmeyer flasks (250 ml) containing 100 ml of liquid medium. Eight different nitrogen sources were added individually to basal medium as follow (g/L): NH_4Cl, 1.9; $(NH_4)_2SO_4$, 4; NH_4NO_3, 2.4; $(NH_4)_2HPO_4$, 4; Na NO_3, 5.1; KNO_3, 6.06; Ca $(NO_3)_2.4H_2O$, 7.08 and CH_3COONH_4, 4.62. The flasks were sterilized at 121°C for 20 min, inoculated with 10 mm disc cut out from 5 days fungal colony grown on glucose-Czapek's agar medium. The inoculated flasks were incubated under shaking condition at 30°C for 7 days. At the end of the incubation period, the mycelial dry weight and the activity of D-amino acid oxidase were determined in two replicates.

Effect of different amino acids on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The tested fungi were grown in Erlenmeyer flasks (250 ml) containing 100 ml of liquid synthetic medium. Seven different amino acids which act as inducers were added individually to the basal medium for D-amino acid oxidase production. These amino acids were as the following (g/L): D-alanine, 2.673; Dl-alanine, 2.673; L-alanine, 2.673; L-asparagine, 4.5; L-aspartic acid, 3.99; DL-methionine, 4.47 and cephalosporin C, 14.7. The flasks were sterilized at 121°C for 20 min, inoculated with 10 mm disc cut out from a 5 days colony of the fungus grown on glucose-Czapek's agar medium. The inoculated flasks were incubated under shaking condition at 30°C for 7 days. After the incubation period, the activity of D-amino acid oxidase was determined in two replicates as well as for fungal dry weight.

Effect of inoculum size on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The influence of inoculum size (1, 2 and 3 discs, each disc was 10 mm diameter) was tested. The discs were cut out from 5 days colony of fungi which were grown on glucose-Czapek's agar medium. The flasks were inoculated with different numbers of fungal discs and incubated with shaking at 30°C in liquid synthetic medium for 7 days. After the incubation period, D-amino acid oxidase was measured in two replicates as well as for fungal dry weight.

Effect of shaker speed on D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The production of D-amino acid oxidase enzyme by tested fungi was estimated by using different speed of rotary shaker (160, 180 and 200 rpm). The inoculated flasks were incubated under different shaking speeds for 7 days at 30°C. After the incubation period, the activity of D-amino acid oxidase and fungal dry weight were determined in two replicates.

Effect of aeration on the D-amino acid oxidase enzyme production by *F. heterosporum* and *N. haematococca*

This experiment was done by using 2 volumes of liquid synthetic medium (50 and 100 ml) in 250 ml Erlenmeyer flasks. The flasks were inoculated with 10 mm disc of tested fungi and incubated with shaking at 30°C for 7 days. After the incubation period, D-amino acid oxidase enzyme was measured in two replicates as well as for fungal dry weight.

Purification and activity of D-amino acid oxidase produced by *F. heterosporum* and *N. haematococca* using HPLC technique

The two species of fungi were grown in liquid synthetic medium under optimized conditions. After 7 days of incubation at 30°C, the cells of fungus were harvested by centrifugation at 4°C and 10000 rpm for 20 min and used immediately for preparation of the cell-free extracted or permeated cells. Cells were then suspended in chilled (50 ml) 100 mM potassium phosphate buffer (pH = 8) containing 2.5% (V/V) toloune in ethanol (40 ml) at 0°C for 30 min. The permeated cells were washed twice with chilled 100 mM phosphate buffer and then used for conversion of cephalosporin C. The D-amino acid oxidase activity toward cephalosporin C was monitored by HPLC. The reaction mixture contained 0.25 ml of cephalosporin C (250 mM), 6.416 ml of potassium phosphate buffer (100 mM at pH = 8) and 10 ml of permeated cells in a final volume of 16.6 ml. After incubation at 25°C for 2 h, 0.166 ml of H_2O_2 (3.5%) was added to the solution and reaction proceeded for another 10 min at 25°C. The product of conversion in the supernatant was then analyzed by HPLC. The mobile phase was 20% acetonitril in 0.1N sodium acetate buffer (pH = 4.8). A Nova pack C-18 column (water material synthesis facility, Taunton), 3.9 × 150 mm, was used UV detector was set at 254 nm. The retention times for cephalosporin C and glutaryl-7-aminocephalosporanic acid at flow rate of 2 ml/minute were 4.6 and 1.3 min, respectively.

RESULTS AND DISCUSSION

Production of D-amino acid oxidase enzyme by fungi

Screening of fungal isolates for production of D-amino acid oxidase enzyme showed that, seven isolates represent 10.45% of total isolates showed high enzyme activity. Thirteen isolates contribute 19.40% of total isolates were found to be moderate activity. Twenty nine

Table 1. Screening of fungi for their abilities to produce D-amino acid oxidase enzyme.

Fungi	Enzyme activity
Aspergillus flavus	141.1 H
A.flavus	-
A.fumigatus	51.98 M
A.fumigatus	-
A.galaucus	-
A.niger	32.18 L
A.niger	-
A.ochraceus	5.90 L
A.sydowii	3.54 L
A.tamarii	-
A.tamarii	-
A.terreus	42.80 L
A.terreus	27.23 L
A.ustus	42.80 L
A.versicolor	-
Candida albicans	61.88 M
C. albicans	64.36 M
C. albicans	66.83 M
C. krusei	81.70 M
Cochliobolus spicifer	37.13 L
Emericella nidulans	8.60 L
E. nidulans	-
E. nidulans	-
E. nidulans	-
Fusarium chlamydosporum	9.45 L
F.compactum	37.13 L
F.heterosporum	210.41 H
F.mersmipoides	29.70 L
F.oxysporum	51.98 M
F.oxysporum	34.65 L
F.sambucinum	56.93 M
F.tricinctum	113.87 H
Geotrichum candidum	96.54 M
Gibberella avenacea	153.48 H
G. fujikuroi	32.18 L
G. fujikuroi	94.06 M
G.fujikuroi	133.67 H
G.intricans	44.55 L
G.intricans	59.41 M
Nectria haematococca	47.03 L
N. haematococca	207.94 H
N. haematococca	49.50 L
N. haematococca	42.08 L
N. haematococca	29.70 L
Neurospora crassa	14.17 L
Penicillium aurantiogriseum	-
P.brevicompactum	-
P.chrysogenum	56.93 M
P.chrysogenum	-
P.chrysogenum	-
P.chrysogenum	-
P.duclauxii	143.57 H
P.oxalicum	47.03 L
P.purpurogenum	-
P. roqueforti	27.23 L
P.rugulosum	11.75 L
P.verrucosum	44.55 L
Phoma herbarum	44.55 L
Rhodotorula sp.	54.46 M
Rhodotorula sp.	64.30 M
Rhodotorula sp.	49.50 L
Rhodotorula sp.	44.55 L
Rhodotorula sp.	29.70 L
Saccharomyces sp.	37.13 L
Trichoderma harzianum	24.75 L

High activity (H) > 100, Moderate activity (M) = 50 -100, Low activity (L) < 50, No activity (-).

isolates represent 43.28% of total isolates were low producers of D-amino acid oxidase. Eighteen isolates represent 26.87% had no activity. The most active producer isolates of these fungi were *F. heterosporum* and *N. haematococca.* These fungi produced 210.41 and 207.94 units/ml, respectively. *Aspergillus flavus*, *Fusarium tricinctum*, *Gibberella avenacea*, *Gibberella fujikuroi* and *Penicillium duclauxii* relatively produced considerable amounts (141.1, 113.87, 153.48, 133.67 and 143.57 units/ml, respectively) of D-amino acid oxidase enzyme. The remaining isolates were low producers or had no activity for D-amino acid oxidase (Table 1). Although several microorganisms were screened for their abilities to produce D-amino acid oxidase, including *Escherichia coli*, *Pseudomonas species*, *Aerobacter species*, *Candida tropicalis*, *Penicillium roqueforti*, *Aspergillus flavus* and *A. niger*, *N. crassa*, *Nocardia*, *Citrobacter* and *T. variabilis*. Only *T. variabilis* and *Citrobacter* could deaminate cephalosporin C to keto adipic-7-aminocephalosporanic acid. The activity of *Citrobacter* was very low and appeared to be membrane bound while the enzyme from *Trigonopsis* was present in the cytoplasm at much higher level (Szwajcer and Mosbach, 1985). *T. variabilis* has been found to be one of the most potent sources of D-amino acid oxidase which could act on most of amino acids (Brodelius et al., 1981; Kubicek-Pranz and Rohr, 1985; Singh et al., 2001). Other investigators also have shown that different microorganisms have the ability to produce D-amino acid oxidase enzyme including *C. tropicalis*, *N. crassa*, *P. chrysogenum*, *Fusarium oxysporum*, *Fusarium solani*, *R. gracilis*, *Saccharomyces pasteurianus*, *Rhodosporidium toruloides* and *T. variabilis* (Simonetta et al., 1989; Gabler and Fischer 1999; Kujan et al., 2001).

Table 2. Effect of incubation periods on growth and D-amino acid oxidase production by *Fusarium heterosporum* and *Nectria haematococca.*

Incubation periods (days)	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
2	-	-	-	-
4	9.61	35.00	6.04	30.05
6	203.13	65.31	199.35	62.04
7	213.94	73.10	211.44	70.15
8	209.14	73.04	205.40	70.13
10	174.28	74.01	169.15	71.42
12	112.98	69.81`	111.15	65.45

Table 3. Effect of temperatures on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*.

Temperature	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
15	-	-	-	-
20	74.20	70.30	76.70	77.40
25	89.12	87.30	101.49	90.50
30	215.36	102.00	227.75	120.00
35	61.88	60.30	64.36	65.20
40	-	-	-	-

Effect of incubation periods on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

F. heterosporum and *N. haematococca* exhibited maximum D-amino acid oxidase production at 7 days of incubation (213.94 and 211.44 units/ml, respectively). At 6 and 8 days of incubation the two tested fungi produced relatively high amounts of D-amino acid oxidase enzyme, however decreasing of incubation period to 2 or 4 days had negative effect on the enzyme production by the two tested fungi. Increasing of incubation period to 10 or 12 days decreased the production of D-amino acid oxidase enzyme by *F. heterosporum* and *N. haematococca*. The results recorded for the effect of incubation time on mycelial growth of *F. heterosporum* and *N. haematococca* was nearly similar to those reported for D-amino acid oxidase enzyme (Table 2). These findings are similar to those reported by Gabler and Fischer (1999). They found that *F. oxysporum* achieved maximum activity of D-amino acid oxidase at 30°C after 7 days of incubation period, but *R. gracilis* and *T. variabilis* recorded maximum activity after 48 h (Cambiaghi et al., 1994; Ballagi 1999).

Effect of temperatures on fungal growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

Temperature greatly influenced the production of endocellular D-amino acid oxidase by *F. heterosporum* and *N. haematococca*. Maximum production of the enzyme was recorded at 30°C by the two tested fungi (215.36 and 227.75 unit/ml, respectively). Decreasing of incubation temperature to 20 or 25°C inhibited the production of D-amino acid oxidase by the two fungal species. Also increasing of temperature to 35°C decreased D-amino acid oxidase production. No amino acid oxidase enzyme was recorded at 15 and 40°C. This means that the enzyme biosynthesis was sensitive to the incubation temperature with optimum at 30°C. The results obtained for the effect of temperatures on mycelial growth of *F. heterosporum* and *N. haematococca* was similar to those reported for D-amino acid oxidase enzyme (Table 3). Gabler and Fischer (1999) reported that *Fusarium oxysporum* achieved maximum activity of D-amino acid oxidase and mycelial growth at 30°C after 7 days of incubation; however *R. gracilis* achieved good activity of the enzyme at 37°C (Simmonetta et al., 1989). *R. gracilis* in other investigation showed maximum activity of D-amino acid oxidase at 50°C (Kuan et al., 2008).

Effect of pH values on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The pH value greatly affected the production of endocellular D-amino acid oxidase by *F. heterosporum* and *N. haematococca*. Maximum enzyme production was recorded at pH 7 by the two fungal species (185.6 and

Table 4. Effect of pH values on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca.*

pH value	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
2	8.66	45.30	4.95	40.00
4	61.88	70.10	49.50	65.10
6	64.36	71.10	61.88	69.00
7	185.6	90.00	180.70	85.20
8	84.16	75.00	79.20	73.10
10	-	-	-	-
12	-	-	-	-

180.7 unit/ml, respectively). Decreasing of pH value towards the acidic medium exhibited an inhibitive effect to D-amino acid oxidase production. Also increasing of pH value to 8, decreased the enzyme production by the two tested fungi. No enzyme production was recorded at pH 10 and 12 by the two fungal species. Data recorded for the effect of pH value on mycelial growth of *F. heterosporum* and *N. haematococca* was similar to those reported for D-amino acid oxidase enzyme (Table 4). As mentioned by Glaber and Fischer (1999), *F. oxysporum* recorded maximum D-amino acid oxidase production at pH 7 with good mycelial growth. Simonetta et al. (1989) proved that the optimal pH for *R. gracilis* was at pH 5.6. Another investigation found that to achieve good production of the enzyme by *R. gracilis*, the pH of the medium must maintained between 4 and 6.5 preferably at 5 (Cambiaghi et al., 1994). On the other hand, Maximum activity of *R. gracilis* D-amino acid oxidase was achieved with pH 8 to 9 and pH values above 9 decayed the enzyme activity (Pollegioni et al., 1992 and Kuan et al., 2008). The basic properties of purified D-amino acid oxidase from *T. variabilis* revealed that the optimum pH of the activity was between 8.5 and 9 (Schrader and Andreesen 1993). The enzyme from *R. toruloides* was active over a wide pH range of 6.0 to 11.0 with an optimum at 8.5 (Lee and Chu 1996).

Effect of different carbon sources on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

Carbon source is one of the most essential components in the microbial medium. The effect of different carbon sources on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca* was variable. Monosaccharides (glucose and fructose) and disaccharides (maltose, dextrin, lactose and sucrose) were more favorable carbon sources for D-amino acid oxidase production than polysaccharides (cellulose and starch). Maximum production of D-amino acid oxidase enzyme was achieved by incorporation of glucose in the culture medium for enzyme production (216.35 and 205.40 unit/ml for *F. heterosporum* and *N. haematococca*, respectively). Addition of other mono- and disaccharides in the fungal cultures exhibited considerable amounts of D-amino acid oxidase by *F. heterosporum* and *N. haematococca*. However the addition of cellulose or starch in the culture medium decreased the enzyme production by the two fungal species. This means that mono-and disaccharides may be easier than polysaccharides for metabolic assimilation. Results obtained for the effect of carbon sources on mycelial growth of *F. heterosporum* and *N. haematococca* was nearly similar to those recorded for D-amino acid oxidase enzyme (Table 5). These results support the previous data reported by Gabler and Fisher (1999). They found that *F. oxysporum* had good activity of D-amino acid oxidase and mycelial growth when glucose was used as a sole carbon source and ammonium sulphate as nitrogen source. Also *T. variabilis* achieved good production of the enzyme and mycelial growth when glucose was used as carbon and energy source (Horner et al., 1996; Ballagi 1999; Prell et al., 2001).

Effect of different nitrogen sources on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

Nitrogen source is one of the most essential components in the microbial medium because the nitrogen element participates in the formation of amino and nucleic acids and protein in microbial cells. The effect of different nitrogen sources on fungal growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca* was variable. Among eight nitrogen sources studied, ammonium sulphate was the most suitable nitrogen source for D-amino acid oxidase production. Maximum production of D-amino acid oxidase was recorded by the incorporation of ammonium sulphate in the culture medium for enzyme production (207.94 and 205.40 unit/ml for *F. heterosporum* and *N. haematococca*, respectively). Addition of ammonium hydrogen phosphate to the fungal cultures exhibited considerable amount of D-amino acid oxidase to some extent. However, the amount of the enzyme was better

Table 5. Effect of different carbon sources on growth and D-amino acid oxidase production by *F. heterosporum* and *N.haematococca*.

Carbon sources	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
Glucose	216.35	75.10	205.40	72.20
Fructose	210.34	66.20	202.98	60.20
Maltose	199.52	60.30	198.15	60.30
Dextrin	203.18	65.10	200.65	61.40
Lactose	186.30	57.40	193.31	59.50
Sucrose	180.29	55.30	187.27	50.40
Cellulose	108.17	35.50	108.74	40.60
Starch	114.18	35.50	111.15	40.10

Table 6. Effect of different nitrogen sources on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*.

Nitrogen sources	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
$(NH_4)_2SO_4$	207.94	85.30	205.40	82.20
$(NH_4)_2HPO_4$	111.39	66.10	195.56	75.10
KNO_3	64.36	50.20	76.70	55.30
CH_3COONH_4	61.88	45.40	54.46	40.10
$Ca(NO_3)_2$	49.50	40.60	37.10	30.20
NH_4NO_3	69.30	55.10	37.10	25.10
$NaNO_3$	47.30	35.30	37.10	20.20
NH_4Cl	4.95	1.00	6.18	2.00

for *N. haematococca* (195.56 unit/ml) than *F. heterosporum* (111.39 unit/ml). The other nitrogen sources induced an inhibitive effect for the enzyme production (Table 6). Gabler and Fisher (1999) demonstrated that the ammonium sulphate was essential nitrogen source in the media specific for the production of D-amino acid oxidase by *F. oxysporum* in a good manner. Moreover, Maximum production of D-amino acid oxidase and growth of *T. variabilis* were achieved by using ammonium sulphate as nitrogen source in liquid synthetic media as reported by some workers (Horner et al., 1996; Ballagi 1999; Prell et al., 2001).

Effect of different amino acids on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

Among seven amino acids used as inducers for endocellular D-amino acid oxidase production, D-alanine and L-asparagine were the best inducers for D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*, respectively. The highest production of D-amino acid oxidase was achieved by *F. heterosporum* (313.20 unit/ml) when D-alanine was added to the culture medium, whereas L-asparagine achieved maximum production of D-amino acid oxidase by *N. haematococca* (259 unit/ml). DL-methionine, Cephalosporin C and DL-alanine was good inducers for D-amino acid oxidase production. Considerable amounts (ranging from 193 to 289.1 unit/ml) of the enzyme were produced by *F. heterosporum* and *N. haematococca* when these amino acids were added to the fungal cultures. L-alanine and L-aspartic acid were less favorable amino acids for D-amino acid oxidase production. Data recorded for the effect of amino acids on mycelial growth of *F. heterosporum* and *N. haematococca* was similar to those reported for D-amino acid oxidase enzyme (Table 7). Glaber and Fisher (1999) revealed in their investigation that the best inducers for mycelial growth and D-amino acid oxidase activity in *F. oxysporum* were D-alanine and D-3-aminobutyric acid. Compared to the activity in cells cultivated without any inducer, the specific D-amino acid oxidase was increased about 13-fold. Screening of 10 new D-amino acids as inducers for the induction of D-amino acid oxidase by *T. variabilis* showed that the highest D-amino acid oxidase activities were obtained when N-carbamoy-D-alanine, N-acetyl-D-tryptophan or N-chloroacetyl-DL-aminobutyric acid were used as inducing compounds. The activity of the enzyme with N-carbamoyl-D-alanine was 4.2 times higher than that with D-alanine (Horner et al., 1996). Three types of inducers (D-alanine, D-valine and cephalosporin C) were used for induction of D-amino acid oxidase by *R. gracilis*.

Table 7. Effect of different amino acids on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca.*

Amino acids	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
L-asparagine	240.90	70.30	259.00	119.00
D-alanine	313.20	99.40	252.90	114.00
DL-methionine	289.10	89.50	243.30	109.00
Cephalosporin C	225.20	84.20	205.40	106.00
DL-alanine	240.90	85.10	193.00	100.00
L-aspartic acid	168.33	54.00	180.70	77.50
L-alanine	193.00	63.30	180.70	74.00

Table 8. Effect of inoculum size on the growth and D-amino acid oxidase production by *F. heterosporum* and *N.haematococca.*

Inoculum size	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100ml)
1 disc	183.20	74.30	178.20	70.40
2 discs	277.00	99.20	225.30	90.70
3 discs	321.80	102.00	228.80	99.00

Maximum production of the enzyme was achieved with cephalosporin C (Pilone et al., 1995). However *R. gracilis*achieved maximum activity of the enzyme with D-alanine as inducer (Kuan et al., 2008). Different amino acids had different effect on the biomass yiled of *T. variabilis* under the same physical and chemical conditions. Highest specific growth rate was achieved with DL-alanine and DL- methionine followed by DL-phenylalanine and DL-glutamine (Singh et al., 2001). The action of D-amino acids as inducer for enzyme activity may be due to one of two reasons; either these compounds are easily metabolized or they do not fit into the binding site of the
protein which is responsible for expression of the D-amino acid oxidase gene (Horner et al., 1996).

Effect of inoculum size on the growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The inoculum size greatly influenced D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*. Maximum yield of D-amino acid oxidase by *F. heterosporum* was achieved by inoculating liquid synthetic medium with 3 discs (10 mm diameter) of fungi. *F. heterosporum* produced 321.8 corresponding to 228.8 unit/ml for *N. haematococca*. Decreasing of the inoculum size to 2 or 1 disk decreased the production of D-amino acid oxidase enzyme by the two fungal species. Results recorded for the effect of inoculum size on mycelial growth of *F. heterosporum* and *N. haematococca* was similar to those reported for D-amino acid oxidase enzyme (Table 8). There is no information about the effect of inoculum size on D-amino acid oxidase production. Some investigators present data on the relation between inoculum size and enzyme activity other than D-amino acid oxidase. The effect of different inoculum size on lipase production by *F. solani* was studied. Lipase produced by *F. solani* increased with the increasing rate of inoculum size until it reaches 290 U/ml (Shafei and Abd-El Salam, 2005).

Effect of shaker speed on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

Speed of shaker had an effect on growth and endocellular D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*. *F. heterosporum* and *N. haematococca* showed maximum D-amino acid oxidase production (185.6 and 180.7 units/ml, respectively) at 160 rpm. Increasing of shaker speed to 180 or 200 rpm decreased the production of the enzyme. Vice versa was recorded for mycelial growth of tested fungi. Increasing of shaker speed to 180 or 200 rpm stimulated the mycelial growth of tested fungi (Table 9). Gabler and Fisher (1999) found that *F. oxysporum* recorded maximum D-amino acid oxidase production and mycelial growth at 100 rpm shaker speed, however *R. toruloides* recorded maximum enzyme production and mycelial growth at 150 rpm (Lee and Chu, 1996). *T. variabilis* recorded maximum enzyme production and

Table 9. Effect of shaker speed on growth and D-amino acid oxidase production by *F. heterosporum* and *N.haematococca.*

Shaker speed (rmp)	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight (mg/100 ml)	Enzyme activity	Dry weight (mg/100 ml)
160	185.60	77.40	180.70	70.00
180	178.20	89.00	168.33	80.20
200	143.60	110.00	133.60	100.00

Table 10. Effect of aeration on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca.*

Aeration	*F. heterosporum*		*N. haematococca*	
	Enzyme activity	Dry weight	Enzyme activity	Dry weight
50 ml	101.49	45.01	97.90	37.00
100 ml	211.54	89.00	207.94	85.00

Table 11. The activity of D-amino acid oxidase on cephalosporin C.

Cephalosporin C before treating with D-amino acid oxidase	7970.93 µg/ml
Cephalosporin C after treating with *F. heterosporum* D-amino acid oxidase	3521.39 µg/ml
Cephalosporin C after treating with *N. haematococca* D-amino acid oxidase	3566.01 µg/ml

mycelial growth at shaker speed ranged from 100 to 200 rpm (Ballagi 1999; Singh et al., 2001).

Effect of aeration on growth and D-amino acid oxidase production by *F. heterosporum* and *N. haematococca*

The effect of aeration on growth and D-amino acid oxidase was produced by *F. heterosporum* and *N. haematococca* was studied by using different volumes of cultivation medium. *F. heterosporum* and *N. haematococca* produced higher amounts of D-amino acid oxidase (211.54 and 207.94 unit/ml, respectively) in flask containing 100 ml of medium. Decreasing of the amount of medium to 50 ml decreased the enzyme production by nearly half of the amount (101.49 and 97.9 unit/ml, respectively). Data recorded for the effect of aeration on mycelial growth of *F. heterosporum* and *N. haematococca* was similar to those reported for D-amino acid oxidase enzyme (Table 10). Schrader and Andreesen (1993) reported that D-Amino acid oxidase could slightly be protected by the absence of oxygen. Although D-leucine had a protective effect under aerobic conditions, a full protection could only be achieved under anaerobic conditions in the presence of a substrate amino acid. This might indicate that the reactive cysteine residue is located near or at the substrate-binding site, which is completely blocked only under the latter conditions where no substrate was converted.

Activity of D-amino acid oxidase produced by *F. heterosporum* and *N. haematococca* using HPLC technique

Analysis of cephalosporin C and glutaryl-7-aminocephalosporanic acid by HPLC before and after the addition of D-amino acid oxidase enzyme indicated that the enzyme produced by two fungi tested was active in hydrolyzing cephalosporin C to glutaryl-7-aminocephalosporanic acid and successfully transformed cephalosporin C into glutaryl-7-aminocephalosporanic acid as shown in Table 11 and Figures 1,2,3. D-amino acid oxidase produced by *R. gracilis* and *T. variabilis* have seemed to be the only microbial D-amino acid oxidase producers with satisfactory yields for commercialpurposes (Perotti et al., 1991; Horner et al., 1996). Although the gene of pig kidney D-amino acid oxidase was expressed in *E. coli* recently (Setoyama et al., 1996) and the three-dimensional structure of the enzyme was solved by two groups (Mizutani et al., 1996), pig kidney D-amino acid oxidase is not appropriate for biotechnological processes due to its low binding constant for flavin adenine dinucleotide and its operational instability (Fischer, 1998). Gabler and Fischer (1999) reported that, *F. oxysporum* produced a new D-amino acid oxidase in a medium containing glucose and ammonium sulphate as carbon and nitrogen sources, respectively. The specific D-amino acid oxidase activity was increased up to 12.5 fold with various D-amino acids or their corresponding derivatives as inducers. The best

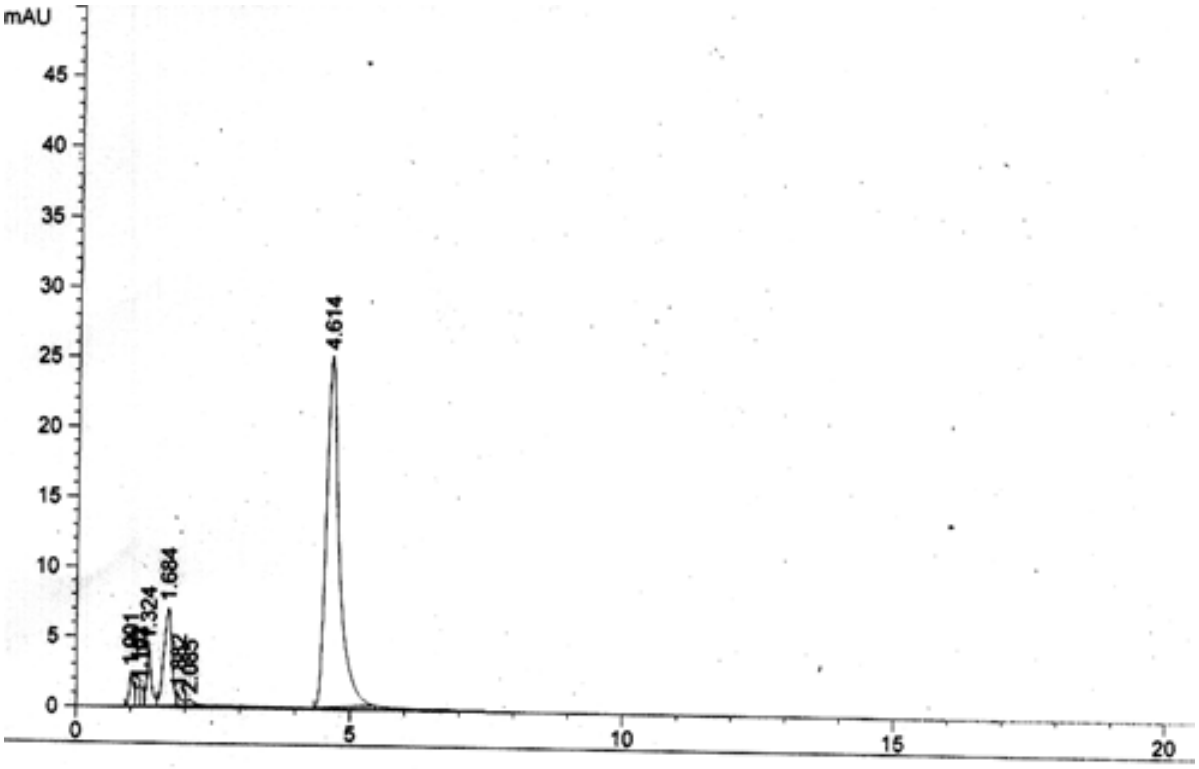

Figure 1. Conversion of cephalosporin C by D-amino acid oxidase produced by *Fusaruim heterosporum* using HPLC chromatography analysis.

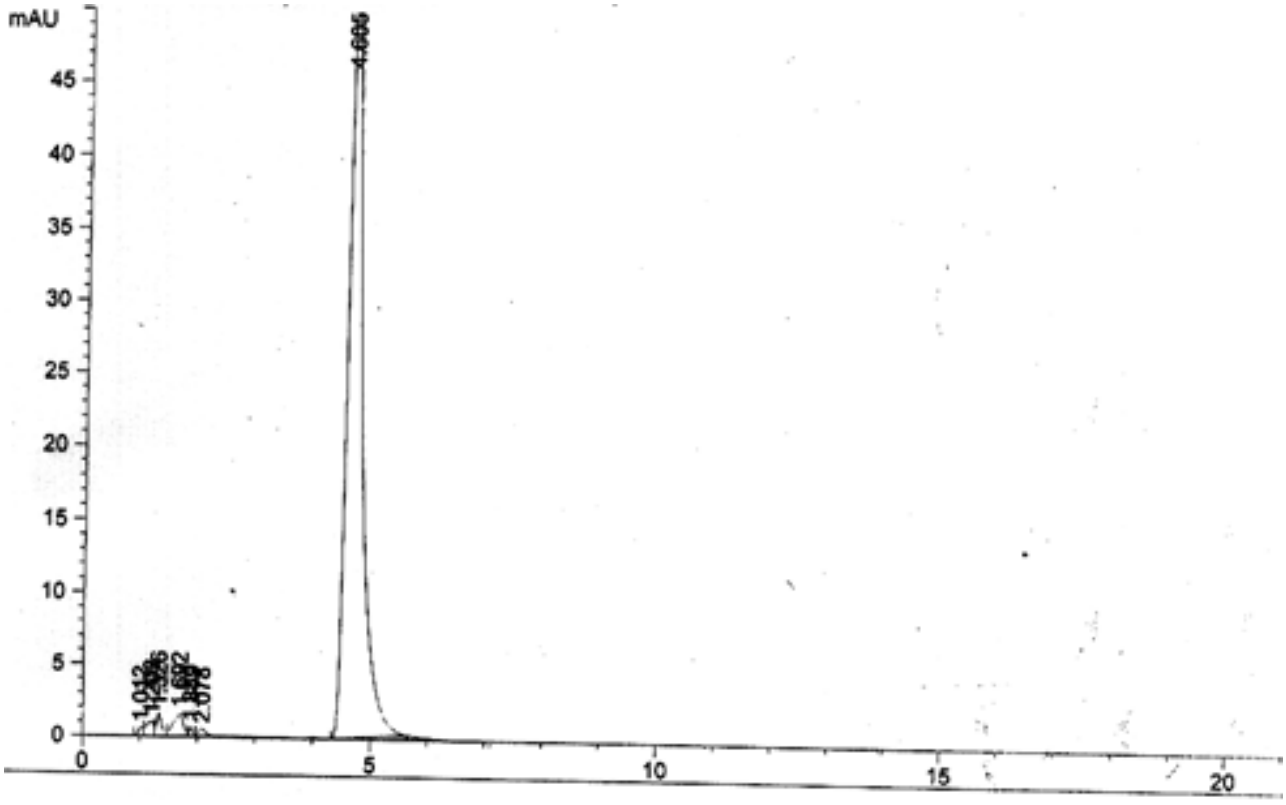

Figure 2. Cephalosporin C without treating with D-amino acid oxidase enzyme using HPLC analysis.

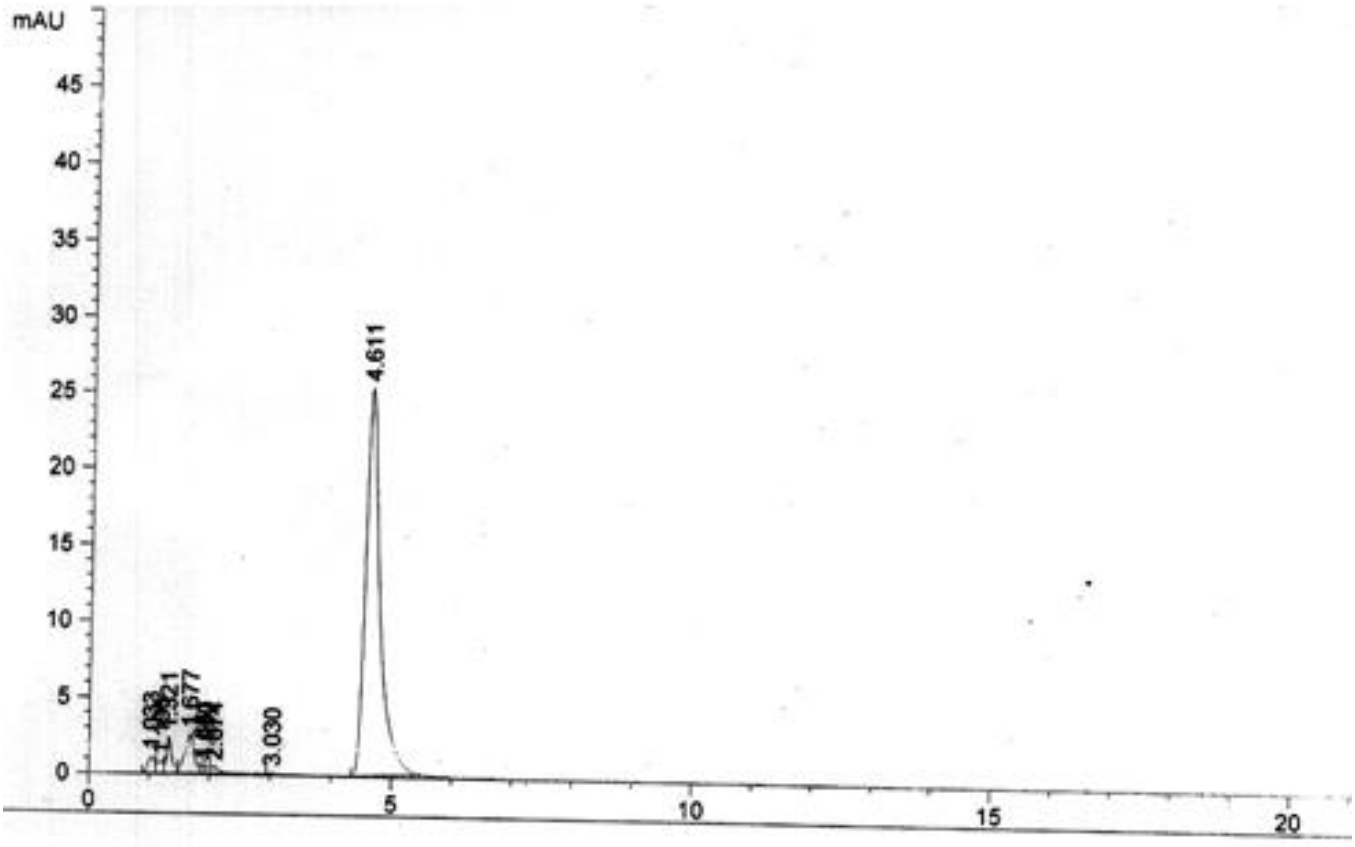

Figure 3. Conversion of cephalosporin C by D-amino acid oxidase produced by *Nectria haematococca* using HPLC chromatography analysis.

inducers for the enzyme production were D-alanine and D-3-aminobutyric acid.

Conclusion

This work would contribute to the findings of the best sources of D-amino acid oxidase production and as a result will lead to the development of some antibiotic manufacture.

REFERENCES

Alonso J, Barredo JL, Diez B, Mellado E, Salto F, Garcia JL, Cortes E (1998). D-Amino-acid oxidase gene from *Rhodotorula gracilis* (*Rhodosporidium toruloides*) ATCC 26217. Microbiol., 144: 1095-1101.

Ballagi A (1999). High cell density fermentation of *Trigonopsis variabilis* with D-amino acid oxidase production. M.Sc. Thesis, Biochemistry and Biotechnology Department, The Technical University of Braunschweig.

Billy L (1999). High cell density fermentation of *Trigonopsis variabilis* with D-amino acid oxidase production. Molecular Biotechnology Programme Uppsala University, School of Engineering, pp. 46.

Binder R, Brown J, Romancik G (1994). Biochemical characterization of a glutaryl-7-aminocephalosporanic acid acylase from *Pseudomonas* strain BL072. Appl. Environ. Microbiol., 60: 1805-1809.

Brodelius P, Nillson K, Mosbach K (1981). Production of α-ketoacids: Part I. Immobilized cells of *Trigonopsis variabilis* containing D-amino acid oxidase. Appl. Biochem. Biotechnol., 6: 293-308.

Cambiaghi S, Tomaselli S, Verga R (1994). Enzymatic process for preparing 7-amino cephaloporanic acid and derivatives. United States Patent, 5: 424, 196.

Conlon HD, Baqai J, Baker K, Shen YQ, Wong BL, Nolies R, Rausch CW (1995). Two step immobilized enzyme conversion of cephalosporin C to 7-aminocephalosporanic acid. Biotechnol. Bioeng., 46: 510-513.

D`Acunzo A, De Alteriis E, Maurano F, Battistel E, Parascandola P (1996). D-amino acid oxidase from *Trigonopsis variabilis*, Immobilization of whole cells in Natural polymeric Gels for glutaryl-7-amino cephalosporanic acid production. J. Fermentation Bioengineer., 81: 138-142.

Fischer L (1998). D-amino acid oxidases in biotechnology. Recent Res. Dev. Microbiol., 2: 295-317.

Gabler M, Fisher L (1999). Production of a new D-amino acid oxidase from the fungus *Fusarium oxysporum*. Appl. Environ. Microbiol., 65: 3750-3753.

Horner R, Wagner F, Fisher L (1996). Induction of the D-amino acid oxidase from *Trigonopsis variabilis*. Appl. Environ. Microbiol., 62: 2106-2110.

Huber FM, Vicenzi, JT, Tietz AJ (1992). High yielding culture conditions for the biosynthesis of D-amino acid oxidase by *Trigonopsis variabilis*. Biotechnol. Lett., 14: 195-200.

Khoronenkova SV, Tishkov VI (2008). D-amino acid oxidase: Physiological Role and Applications. Biochem., 73: 1511-1518.

Kreps HA (1935). Metabolism of amino acids. III- Deamination of amino acids. Biochem. J., 29: 1620-1644.

Kuan I, Liao R, Hsieh H, Chen K, Yu C (2008). Properties of

Rhodotorula gracilis D-amino acid oxidase immobilized on magnetic beads through His-Tag. J. Biosci. Bioeng., 105: 110-115.
Kubicek-Pranz E M, Rohr M (1985): D-amino acid oxidase from the yeast *Trigonopsis variabilis*. J. Appl. Biochem., 7: 104-113.
Kujan P, Prell A, Safar H, Holler P, Plhackova K, Sobotka M (2001): D-amino acid oxidase an improved production of the enzyme by the yeast *Trigonopsis Variabilis* in a laboratory fermentor. Folia Microbiol., 46: 427-431.
Lee Y, Chu W (1996). Purification and characterization of a D-amino acid oxidase active against cephalosporin C from *Rhodosporidium toruloides*. Process Biochem., 33: 461-467.
Mizutani H, Miyahara I, Hirotsu K, Nishina Y, Shiga K, Setoyama C, Miura R (1996). Three-dimensional structure of porcine kidney D-amino acid oxidase at 3.0 A resolution. J. Biochem., 120: 14–17.
Perotti ME, Pollegioni L, Pilone MS (1991). Expression of D-amino acid oxidase in *Rhodoturula gracilis* under induction conditions: a biochemical and cytochemical study. Eur. J. Cell Biol., 55: 104 - 113.
Pilone MS, Buto S, Pollegioni L (1995): A process for bioconversion of cephalosporin C by *Rhodotorula gracilis* D-amino acid oxidase. Biotechnol. Lett., 17: 199-204.
Pollengioni L, Buto S, Tischer W, Ghisla S, Simonneta PM (1993). Chracterization of D-amino acid oxidase from *Trigonopsis variabilis*. Biochem. Mol. Biol. Internat, 31:709 -717.
Pollegioni L, Falbo A, Pilone MS (1992). Specificity and Kinetics of *Rhodotorula gracilis* D- amino acid oxidase. Biochimica et Biophysica Acta, 1120: 11-16.
Prell A, Kujan P, Safar H, Holler P, Plhackova K, Sobotka A (2001). D-amino acid oxidase an improved production of the enzyme by the yeast *Trigonopsis variabilis* in laboratory fermentor. Folia Microbiol., 46: 427- 431.
Rosini E, Pollegioni L, Ghisla S, Orru R, Molla G (2009). Optimization of D-amino acid oxidase for low substrate concentrations towards a cancer enzyme therapy. FEBS J., 276: 4921-4932.
Schrader T, Andreesen JR (1993). Evidence for the functional importance of Cys 298 in D-amino acid oxidase from *Trigonopsis variabilis*, Eur. J. Biochem., 218: 735-744.
Setoyama C, Miura R, Nishina Y, Shiga K, Mizutani H, Miyahara I, Hirotsu K (1996). Crystallization of expressed porcine kidney D-amino acid oxidase and preliminary X-ray crystallographic characterization. J. Biochem., 119: 1114–1117.
Shafei MS, Abd-Elsalam IS (2005). Role of some fermentation parameters affecting lipase production by *Fusarium solani.* Acta Pharmaceutical Turcica, 47: 209-223.
Simonetta P M, Verga R, Fretta A, Hanozet GM (1989). Induction of D-amino acid oxidase by D-alanine in *Rhodotorula gracilis* grown in identified medium. J. Gen. Microbiol., 135: 593-600.
Singh P, Marwaha S, Verma N (2001). Role of amino acids in production of D-amino acid oxidase. J. Microbiol., 39: 229-231.
Szwajcer E, Mosbach K (1985). Isolation and parietal characterization of a D-amino acid oxidase active against cephalosporin C from the yeast *Trigonopsis variabilies*. J. Biotechnol. Lett., 7: 1-7.
Vikartovska-Welwardova A, Michalkova E, Gemeiner P, Welward L (1999). Stabilization of D-amino acid oxidase from *Trigonopsis variabilis* by manganese oxide. Folia Microbiol., 44: 380-384.
Zheng H, Wang X, Chen J, Zhu K, Zhao Y, Yang Y, Yang S, Jiang W (2006). Expression, purification, and immobilization of his-tagged D-amino acid oxidase of *Trigonopsis variabilis* in *Pichia pastoris*. Appl. Microbial. Biotechnol., 70: 683-689.