STUDIES ON THE BIOSYNTHESIS OF L- ALANINE DEHYDROGENASE BY *Thielaviopsis paradoxa*

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ABSTRACT

Cell free extracts of *Thielaviopsis paradoxa* grown on L- alanine as the sole source of nitrogen contained L- alanine dehydrogenase that catalyzes the oxidative deamination of L- alanine to equimolar amounts of pyruvate and ammonia . L-alanine dehydrogenase formation takes place during the logarithmic phase of growth .Maximal growth and enzyme formation were obtained at the 4th day of growth .The optimum pH for growth and enzyme synthesis was 4.0 . L- alanine dehydrogenase was induced by a great variety of nitrogen sources , but L –alanine , DL- alanine, L-theronine and ammonium phosphate were the most potent inducers . L-alanine concentration of 2.1 g/ l was found to be optimum for both growth and enzyme synthesis . L- alanine dehydrogenase formation was increased with increasing concentrations of ammonium phosphate in the growth medium ; the optimum concentration was 2.6 g/ l . The effect of different carbon sources and metal salts on growth and enzyme production was studied .

Keywords : *Thielaviopsis paradoxa* , L- alanine dehydrogenase synthesis .

INTRODUCTION

L- Alanine dehydrogenase (L – alanine : NAD $^+$ oxidoreductase , EC 1.4.1.1) is widely distributed in nature and a voluminous literature has appeared in the past fourty years on the enzyme from microorganisms .The enzyme has been extensively studied in several bacteria (Freese and Oosterwyk ,1963 ; Yoshida and Freese ,1964 ; Germano and Anderson , 1968 ; McCowen and Phibbs , 1974 ; Epstein and Grossowicz , 1976 Elimora *et al.*, 1997), actinomycetes (Roszkowski *et al .,* 1969 ; Aharonowitz and Friedrich , 1980) , algae (Rowell and Stewart , 1976) , and fungi (El – Awamry and El – Rahmany, 1988) . Freese and Oosterwyk (1963) showed that the formation of L- alanine dehydrogenase in *B . subtilis* was induced by L- alanine and D - alanine as well as by certain other L- and D- amino acids . More information pertaining to such study was given by Berberich *et al* . (1968) who demonstrated that L-alanine dehydrogenase in *B . subtilis* is inducible by its substrate L-alanine . In addition to L-alanine ,10 other L-amino acids as well as D - alanine and 11 other D- amino acids are also inducers . Kenealy *et al.* (1982) reported that L-alanine dehydrogenase activity and growth yield of *Methanobacterium thermoautotrophicum* were high when the organism was cultured with excess ammonia . Aharonowitz and Friedrich (1980) demonstrated that *Streptomyces clavuligerus* L-alanine dehydrogenase was induced by L-alanine and ammonia . Roszkowski *et al* . (1969) reported that L-alanine dehydrogenase in *St . erythreus* is a substrate inducible enzyme and the induction is not specific ; L-alanine , D - alanine and D , L- glutamate being equally good inducer . El – Awamry and El – Rahmany (1988) found that L-alanine dehydrogenase of *Cunninghamella elegans* was produced during logarithmic phase of growth

,and that maximum enzyme synthesis occurred at pH 5-7 . The enzyme was induced by L-alanine, DL – alanine, L-aspartic acid, L-asparagine and ammonium phosphate, but L-alanine was the best inducer .

The present investigation deals with the biosynthesis of L- alanine dehydrogenase in *Thielaviopsis paradoxa* under different physiologyical conditions . Such studies have not been reported before in *Thielaviopsis paradoxa* .

MATERIALS AND METHODS

Fungal organism

Thielaviopsis paradoxa was obtained from Plant Prevenient Department , College of Agriculture , King Saud University .

Media and culture

The organism was grown on glucose - Czapek – Dox liquied medium with L – alanine replacing $NaNO_3$ on nitrogen equivalent basis to induce the formation of L-alanine dehydrogenase . Five ml aliquots of spore suspension of *Thielaviopsis paradoxa* were used to inoculate 250 ml Erlenmeyer flasks , each containing 50 ml sterile medium . The inoculated flasks were incubated at 25 ^{o}C for 4 days , then the mycelia were harvested by filtration , washed thoroughly with distilled water, and finally blotted dry with absorbent paper .

Preperation of cell - free extract

The harvested mycelia were ground with cold sand in a cold mortar and extracted with cold distilled water . The obtained slurry was then centrifuged at 5000 r.p.m. for 10 min and the supernatant was used as the crude enzyme preparation .The crude extracts were dialysed against 200 volumes of distilled water for 24 hr at 4 ^{o}C .

Chemical methods

Pyruvate was estamited by the method of Friedmann and Haugen (1943) . Ammonia was estamited by using Nessler's reagent . Protein was determined according to the method of Bradford (1976) .

Assay of L-alanine dehydrogenase

L-Alanine dehydrogenase activity was routinely assayed by following the formation of pyruvate from alanine (oxidative deamination). One unit of enzyme activity is defined as the amount of protein which catalyzes the formation of one µmole pyruvate in 6 min at 60 ^{o}C .

Chromatographic identification of pyruvate

Pyruvate was identified by ascending paper chromatogaphy of its hydrazone using Whatman No.I filter paper and two solvent systems .

Solvent 1 consisted of n - butanol- ethanol-water (40 : 10 : 20) (Germano and Anderson ,1968) and solvent II consisted of n- butanolethanol –0.5 N NH_4OH (70 : 10 : 20) (Seakins *et al* ., 1976) . The identified pyruvate was compared with an authentic sample . Under the conditions mentioned pyruvate hydrazone gave R_f values 0.55 and 0.77 in solvent I and 0.49 and 0.66 in solvent II . All data were statisticaly analysed using Person coefficient (Marija , J and Norusis / spss Inc . , 1990) .

RESULTS AND DISCUSSION

Catabolism of L-alanine into pyruvate and ammonia by L-alanine dehydrogenase of extracts of *T. paradoxa*

Fig 1 shows the rate of formation of both pyruvate and ammonia when the dialyzed extracts were incubated with L - alanine ***.***

L - Alanine is oxidatively deaminated , by the action of L-alanine dehydrogenase,to equimolar amounts of pyruvate and ammonia. Maximum formation of both products was reached after 6 min incubation . It is assumed that the reaction did not proceed to completion because of the reverse reaction . However , the level of pyruvate decreased afterward . This suggests that pyruvate may be pulled through other metabolic reactions .

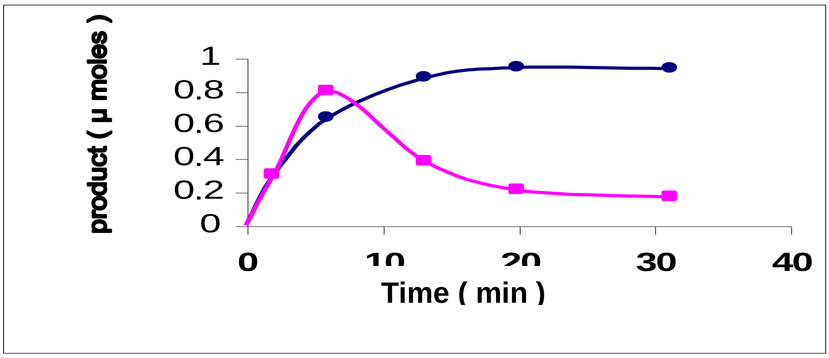

Fig. 1 : Catabolism of L-alanine into pyruvate and ammonia by L-alanine dehydrogenase of extracts of *T. paradoxa* .
Reaction mixture contained : L-alanine ,60 µmoles ; NAD^+, 18 µmoles; Na_2CO_3 - $NaHCO_3$ buffer at pH 9.5 , 480 µmoles ; extract protein ,5.4 mg ; total volume ,6 ml ; time , as indicated , temp., 60 ºC .
(●) Ammonia .
(■) Pyruvate .

L-alanine dehydrogenase activity at different stages of growth of *T.paradoxa*

The growth was measured by the dry weight of the mycelium , and L-alanine dehydrogenase activity in extracts of the experimental fungus was determined at different periods of incubation . Fig. 2 shows that the highest specific enzyme activity was obtained at the 4th day of growth after which the enzyme activity decreased . Maximal growth was also obtained after 4th day incubation .

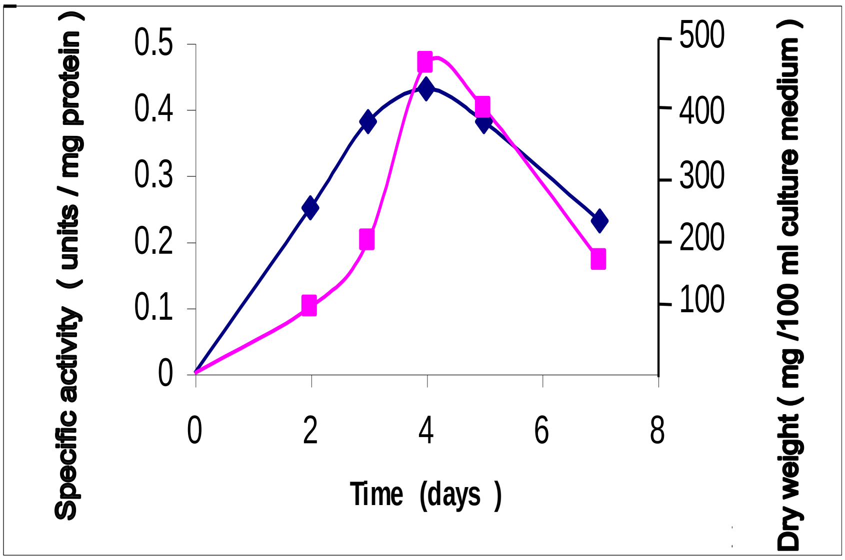

Fig . 2 : L-Alanine dehydrogenase activity at different stages of growth of *T. paradoxa* .
(◆) Specific activity .
(■) Dry weight .

Effect of different pH values on growth and L-alanine dehydrogenase synthesis of *T.paradoxa*

To study the effect of the original pH value of L-alanine-containing medium on the intensity of growth and the activity of L-alanine dehydrogenase in *T.paradoxa* , 6 pH values were chosen, one is pH 2.0 which lies below the $pká_1$ of alanine (2.35) and the others rang from 3.0 to 10 exceeding the $pká_1$ of this amino acid . It was found that no growth occurred at pH 2.0. The data obtained are presented in table 1 . It is clear that *T.paradoxa* could grow and synthesize L-alanine dehydrogenase within a wide pH range (3 - 10) .It is clear that pH 3.0 supported weak growth , which was associated with low level of L-alanine dehydrogenase . Maximum growth and enzyme formation were obtained at pH 4. These results may be attributed to the form of L-alanine prevailing in the culture medium (El-Awamry and El- Rahmany , 1988) .

Table 1. Influence of different pH values on L -alanine dehydrogenasesynthesis and growth of *T . paradoxa* .

pH	Specific activity (units / mg protein)	Mycelia dry weight (mg / 100 ml culture medium)
2	0	0
3	0.38	129
4	0.5	468
5	0.44	342
8	0.43	248
10	0.34	180

In addition, the pH values of the culture media were measured at the end of incubation .No significant change in the pH values 3,4 and 5 of these media was detected, while there was significant change in the pH values 8 and 10 of these media . The acid production by the fungus causes decrease in the pH values of the medium . These results are in close agreement to those reported for another microorganisms (Booth , 1985) .

Growth and formation of L-alanine dehydrogenase of *T. paradoxa* on various nitrogen sources

Table 2 shows that *T.paradoxa* can grow with a great variety of nitrogen nutrients . In most cases , the rate of growth was more or less equal to that of cultures grown on sodium nitrate . However , growth on L –alanine , L-serine , DL- alanine , DL – valine was superior as nitrogen sources for sodium nitrate , while growth on arginine and L-threonine was suppressed as compared with that on sodium nitrate.

Results shown in Table 2 indicate that the synthesis of *T.paradoxa* L – alanine dehydrogenase was induced by L- alanine , DL – alanine L-threonine and ammonium phosphate as compared with that of nitrate - grown cultures . L-alnine was the best inducer . It caused about 209 % increase in specific enzyme activity .DL- alanine , L- threonine and ammonium phosphate resulted in192, 168 and 136 % increase , respectively, in specific activity over that of nitrate- grown cultures . There was no significant induction by L- asparagine , ammonium chloride , L- serine , DL-valine. Moreover , cultures grown on medium with L-arginine and L- glutamic acid contained only low levels of L – alanine dehydrogenase .

These results agree with those repoted by Berberich *et al.* (1968) who demonstrated that L–alanine dehydrogenase of *B. subtilis* was catalyzed by a great variety of amino acids ,but L-alanine was the most potent inducer . Roszkowski *et al.*(1969) stated that L-alanine dehydrogenase of *St . erytherus* was induced by D-alanine,D,L glutamic acid, in addition to L-alanine , and that all were equally good inducer . Epstein and Grossowicz (1976) reported that D-alanine was as effective as L-alanine in induction of L- alanine dehydrogenase of thermophilic bacillus .

They explained their results by the presence of alanine racemase in this thermophilic bacterium . Aharonowitz and Friedrich (1980) found that L-alanine dehydrogenase of *Streptomyces clavuligerus* was induced by L-alanine and ammonia , no another amino acid,or amide induced enzyme synthesis . El-Awamry and EL-Rahmany (1988) demonstrated that L- alanine dehydrogenase of *Cunninghamela elegans* was stimulated by L-alanine , DL-alanine, L-asparagine , aspartic acid and ammounium phosphate .

Table 2.Effect of different nitrogen sources on L-alanine dehydrogenase formation and growth of *T.paradoxa* .

Nitrogen Source	Specific activity (Units/mg protein)	Mycelial dry weight (mg/100 ml culture medium)
$NaNO_3$	0.25	234
L- Alanine	0.5230	468
L- Threonine	0.42	122
DL- Alanine	0.48	342
DL- Valine	0.12	324
Arginine	0.08	158
L- Glutamic acid	0.05	272
NH_4CL	0.14	178
$(NH_4)H_2 PO_4$	0.34	200
L- Serine	0.12	344
L- Asparagine	0.156	278

Each nitrogen source was added in amounts equivalent, on nitrogen basis to the amount of nitrogen in sodium nitrate in Czapek-Dox medium.

Dependence of *T. paradoxa* growth and L- alanine dehydrogenase formation on L-alanine concentration

L-alanine was added to the basal medium as the sole nitrogen source in concentrations ranging from 0.52 to 4.2 g/l . Fig .3 demonsrtrates that maximum concentration of L-alanine for growth and L- alanine dehydrogenase production was 2.1 g /L . Further increase in L-alanine concentration above 2.1 g /l showed no further increase neither in growth nor in enzyme synthesis.

Dependence of *T. paradoxa* growth and L-alanine dehydrogenase formation on ammonia concentration

Ammounium dihydrogen phosphate was added to the basal medium of *T.paradoxa* as the sole source of nitrogen in concentrations ranging from 0.65 to 5.2 g/l. As shown in Fig. 4 the optimum concentration of ammonia for growth was 2.6 g/L. The mount of nitrogen in this concentration is equivalent to its amount in 2 g sodium nitrate . The specific activity of L-alanine dehydrogenase was increased with increasing ammonia concentration in the medium , i.e the level of induction was dependent on the extra- cellular concentration of ammonia .Optimum concentration of ammonia for enzyme synthesis was 2.6 g/l . This result agreed with that reported for alanine dehydrogenase of *St . clavuligerus* (Aharonowitz and Friedrich , 1980) , *M . thermoautotrophicum* (Kenealy *et al.* ,1982) *,C . elegans* (El – Awamry *El* – Rahmany *,1988) and Halobacterium salinarium (*Elfimova , *et al.,1997)* **.**

fig3,4

Influence of different carbon sources on L-alanine dehydrogenase production and growth of *T. paradoxa.*

Table 3 demonstrates that the growth of *T . paradoxa* was not significantly affected when glucose was replaced by starch, fructose and sucrose , whereas maltose and lactose supported weak growth . None of the tested carbon sources induced the synthesis of L-alanine dehydrogenase by *T . paradoxa* over that of of glucose-grown cultures . However, starch resulted in 46 % repression in enzyme synthesis . Moreover , the enzyme formation was greatly suppressed by fructose , lactose , sucrose and maltose .

Table 3 . Influence of different carbon sources on L-alanine dehydrogenase formation and growth of *T.paradoxa* .

Carbon source	Specific activity (Units/mg protein)	Mycelial dry weight (mg/100 ml culture medium)
Glucose	0.48	468
Fructose	0.136	212
Sucrose	0.112	226
Maltose	0.08	158
Lactose	0.12	176
Starch	0.26	370

Each carbon source was added at a concentration of 3 g / l .

Influence of some metal salts on L-alanine dehydrogenase production and growth of *T. paradoxa*

Table 4 shows that L- alanine dehydrogenase synthesis of *T . paradoxa* was greatly affected by the type of metal in the medium . The metal salts $FeSO_4$ and $CuSO_4$ showed lower effect on L- alanine dehydrogenase synthesis. $CoCl_2$, $CaCl_2$ and $MnCl_2$ stimulated enzyme synthesis about 19 % ,14 % and 13 % respectively , while fungal growth showed no appreciable effect by addition of metals to the growth medium .

Table 4 . Influence of some metal salts on L-alanine dehydrogenase synthesis and growth of *T.paradoxa* .

addition	Specific activity (Units/mg protein)	Mycelial dry weight (mg/100 ml culture medium)
None	.5	200
$CaCl_2$	0.7	210
$MnCl_2$	0.68	252
$FeSO_4$	0.6	178
$CuSO_4$	0.58	218
$CoCl_2$	1	242

Each metal salt was added at concentration of 10 mg / l .

CONCLUSIONS

L-alanine dehydrogenase of *T . paradoxa* was produced during the logarithmic phase of growth , maximal growth and enzyme formation were obtained after 4th day of incubation . The optimum pH for growth and enzyme synthesis was 4.

T. paradoxa could grow and synthesize L-alanine dehydrogenase with agreat variety of nitrogen sources , the rate of growth was more or less equal to that cultures grown on sodium nitrate . However, growth on L – alanine, L-serine , DL- alanine , DL – valine was superior as nitrogen sources for sodium nitrate. L-alanine dehydrogenase was induced by L- alanine , DL – alanine L-threonine and ammonium phosphate as compared with that of nitrate - grown cultures . L-alanine was the best inducer .

None of the tested carbon sources induced the growth of *T. paradoxa* and synthesis of L-alanine dehydrogenase over that of glucose-grown cultures.

Fungal growth showed no appreciable effect by addition metals to the growth medium, while production of L- alanine dehydrogenase by *T . paradoxa* was greatly affected by the type of metal in the medium .

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دراسات علىالتكوين الحيوي لإنـزيم ل- ألانـين ديهيـدروجينيزفي فطرة ثيلافيوبسـيس برادوكسا

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لقد أوضحت النتائج التي تم الحصول عليها أن خلاصات خلايا فطرة ثيلافيوبسيس برادوكسا المنماة على ل – ألانين كمصدر وحيد للنتروجين قد أنتجت إنزيم ل- ألانين ديهيدروجينيزالمحفز لتفاعل هدم ل- ألانين عن طريق - تفاعل نزع المجموعة الأمينية بالأكسدة – إلى كميات متساوية جزيئياً من البيروفات و الأمونيا .
وقد تبين أن التكوين الحيوي للإنزيم يتم خلال الطور اللوغاريتمي لنمو الفطرة ، وأن أعلى معدل لنموالفطرة قد تم الحصول علية في اليوم الرابع للنمو .

وقد وجد أن الرقم الهيدروجيني ٤ هو الأمثل لنمو الفطرة والتكوين الحيوي للإنزيم .
كما أوضحت النتائج أن النمو و التكوين الحيوي للإنزيم قد أستحث بواسطة العديد من المصادر النيتروجنية , ولكن ل- ألانين , ل– ثريونين , دل- ألانين وفوسفات الأمونيوم أكثر هذه المواد حثاً لإنتاج الإنزيم .

وقد وجد أن تركيز ل-ألانين الأمثل لنمو الفطرة وتكوين الإنزيم هو ٢,١ جم / لتر .
واتضح أن معدلي نمو الفطرة وإنتاجها لإنزيم ل- ألانين ديهيدروجينيز قد أزداد بزيادة تركيز فوسفات الأمونيوم في بيئة النمو . وكان التركيز الأمثل ٢,٦ جم / لتر.

كذلك تمت دراسة تاثير المصادر الكربونية المختلفة والأملاح المعدنية على النمو وعلى إنتاج الإنزيم .

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