J. Indian bot. Soc. (2023) Vol. 103 (2): 127-132

e-ISSN:2455-7218, ISSN:0019 - 4468

https://doi: 10.5958/2455-7218.2023.00011.6

www.indianbotsoc.org

ORIGINAL ARTICLE

Factors influencing production of L-asparaginase by three thermophilic fungi

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Abstract

Influence of different factors such as pH, temperature, carbon and nitrogen source on production of asparaginase by *Thermomyces lanuginosus, Talaromyces luteus* and *Rhizomucor pusillus* was investigated. All three fungi under investigation preferred modified Czepek Dox (medium E) and 8 days incubation, temperature of 40-45˚C and pH 6.0–7.0 for maximum production of asparaginase. Production of asparaginase varied with the carbon and nitrogen source present in the medium. Asparaginase production by all the three fungi was adaptive in nature.

Keywords: asparaginase, medium, temperature, pH, carbon and nitrogen source

Introduction

L-asparaginase (L-aspargine amido hydrolase) catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia. Colaspase, crasnitin, porton and asparaginase are synonyms, while Elspar, Oncaspar, Erwinase and kidrolase are trade names of L-asparaginase. Commercial production of L-asparaginase was considered desirable only after Mashburn & Wriston (1964) demonstarted that L-asparaginase from *E. coli* inhibited tumors in mice and was used in treating acute lymphoblastic leukemia. It functions by reducing the availability of circulatory L-asparagine to tumor cells. L-asparaginase has also been used for making diagnostic biosensor as the amount of ammonia produced by the action of the enzyme directly correlates to the level of L-asparagine in patients blood (Verma *et al.* 2007). Asparaginases obtained from bacteria have been categorized as type I and type II enzymes based on their sub-cellular location and their kinetic properties. Type I asparaginases are constitutively expressed cytoplasmic enzyme with characteristic specificity for L-asparagine, while type II enzymes are regulatory and periplasmic with wider substrate specificity (Yun *et al.* 2007, Sanchez *et al.* 2007).

To date asparaginases from *E. coli* (Ec AII) and *Erwinia chrysanthemi* (ErA) have been used for clinical purposes. However, these enzymes have an associated glutaminase activity, which causes side effects. Further, this enzymes has low stability and reduced half-life in the blood, requiring multiple dose administration for effective treatment (Li *et al.* 2007).

Recent advances in food technology have demonstrated that fried and baked food (particularly fried potato) contains significant amount of acrylamide (a carcinogenic) formed by the reaction of L-asparagine with reducing sugars (Amrein *et al.* 2004). A pretreatment of potato slices and bread dough with asparaginase from *Aspergillus oryzae* and *A. niger* before frying or baking prevents acrylamide formation (Pedreschi *et al.* 2008), asparaginase used in baking industries (Morales *et al.* 2008).

Asparagine which is extensively active optimally at 40–60˚C and at pH 6.0-7.0 is extensively use in baking (Kotzia *et al.* 2007). Archael asparaginase from *Thermus aquaticus* (Taq A), *T. thermophilus* (Tth A), *Tetrahymena pyriformis* and

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How to cite this article: Kumar RR, Reddy ER, Girisham S, Reddy SM (2023). Factors Influencing Production of L-asparaginase by Three Thermophilic Fungi. *J. Indian bot. Soc.103(2):127-132.* Doi: 10.5958/2455-7218.2023.00011.6

Source of support: Nil

Conflict of interest: None.

Received: 01/12/2022 **Accepted:** 05/01/2023 **Published online**: 15/ 06/2023

Bacillus stearothermophilus appears to be promising because of their inherent thermostability and substrate specificity (Triantafillou *et al.* 1988).

Though L-asparaginase production by mesophilic fungi was investigated by large number of workers including Gulati *et al.*(1997), Seriquis *et al.*(2004), Lee *et al.*(2005) and Ferrara *et al.*(2006), only limited information is available on thermophilic fungi (Richardson,2008 and Powell *et al.*2012). therefore, prodction of L-Asparaginase by three thermophilic fungi was investigated for assessing optimal conditions and discussed in this communication.

Materials and Methods

To find more effective system for the production of *L-asparaginase,* thermophilic fungi from different substrates were isolated by using dilution plate method. Thus total of 28 species covering 9 genera were screened for their potential to produce L-asparaginase. Out of these, three fungi with varying potential (*Thermomyces lanuginosus, Talaromyces luteus* and *Rhizomucor pusillus*) were selected for detailed studies for assessing optimal conditions for production of asparaginase.

Twenty five ml of modified Czepek Dox medium was portioned in to 100 ml Erlenmeyer conical flasks and sterilized by steaming for 30 min 3 consecutive days. They were inoculated by 1ml of spore suspension of each fungus at fixed time and incubated for 15 days at 37±2°C so as to get 4,8and 12 days old culture at the end of incubation. They were harvested on pre weighed Whatman filter paper No.42 for determination of biomass produced. The filter paper along with mycelial mat was dried at 60-70˚C for 48hrs and weighted to a constant weight after cooling to room temperature in a desiccator. Average of three replicates was taken as a criteria for determining the mycelial growth. The culture filtrate thus obtained was subjected to centrifuge at 2000 for 10 min and then dialysed against distilled water for over night and taken as enzyme sample.

Influence of different media on growth and asparaginase production was studied by growing the organism on the following media

1:Yeast Extract Starch medium (A): Yeast extract 5.0 g, Starch 15 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.5 g, agar agar 20 g and distilled water 1 liter,

2: Yeast Extract Starch medium (B): Yeast extract 5.0 g, Starch 15 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.5g, agar agar 20 g and distilled water 1 liter).+ 0.1 % L- asparagine,

3 :Yeast Extract Glucose medium (C): Yeast extract 5.0 g, Glucose 15 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.5g, agar agar 20 g, and distilled water 1 liter,

4: Yeast Extract Glucose medium (D): Yeast extract 5.0 g, Glucose 15 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.5g, agar agar 20 g and distilled water 1 liter)+0.1 % L-asparagine,

5: Modified Czepek dox (E): Glucose 2.0g, L-asparagine 10.0g, K_2HPO_4 1.52, KCl 0.52g. $MgSO_4$. $7H_2O$ 0.52g, $CuNO_3$. 3 H_2O trace, $ZnSO_4$ trace, agar agar 20g and distilled water 1 liter,

6: Malt Extracat Media (F): Yeast extract 5.0 g, Malt extract15 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.5 g, agar agar 20 g and distilled water 1 liter.

Effect of pH was studied by adjusting the pH of the medium by adding 6N HCL/NaOH so as to get the basal medium pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Similarly, effect of incubation temperature was studied by incubating the inoculated cultures at 35, 40, 45, 50 and 55˚C. Influence of carbon and nitrogen source was studied by substituting glucose/nitrate source separately so as to get equal amount of carbon and nitrogen concentration.

Table 1: Thermophilic fungi producing *L-asparaginase*

Name of the fungi	*Area colouration zone (in mm)*			
	0.03	*0.06*	*0.09*	*0.1*
Absidia corymbifera	-	2.0	4.0	4.0
Acremoniun thermophilum	2.0	4.0	6.0	0.8
Aspergillus fumigatus	2.0	3.0	4.0	0.6
Aspergillus nidulans	2.0	4.0	8.0	0.9
A. terreus	1.0	2.0	3.0	0.4
A. flavus	2.0	6.0	7.0	9.0
A. niger	-	-	4.0	6.0
Chaetomium thermophile V.caprophile	1.0	2.0	6.0	8.0
C. thermophile V. dissitum	1.0	2.0	3.0	5.0
Chrysosporium fergusii	2.0	4.0	6.0	8.0
Emericella nudulans	-	2.0	3.0	3.0
Humicola grisea	5.0	8.0	9.0	10.0
H. fuscoatra	2.0	4.0	8.0	9.0
H. insolens	-	2.0	3.0	5.0
H. stellata	4.0	6.0	8.0	9.0
Rhizomucor miechei	2.0	4.0	5.0	6.0
R. pusillus	4.0	9.0	12.0	14.0
Rhizopus arrhizus	2.0	4.0	5.0	6.0
R. microspores	4.0	8.0	10.0	8.0
R. rhizopodiformis	1.0	2.0	3.0	4.0
Malbranchea pulchella	1.0	1.0	4.0	6.0
Myriococcum albomyces	2.0	4.0	5.0	8.0
Penicillium duponti	3.0	4.0	6.0	8.0
P. purpurogenus	2.0	6.0	8.0	5.0
Talaromyces luteus	5.0	10.0	12.0	12.0
Thermoscus aurantiacus	1.0	2.0	5.0	9.0
Thermomyces lanuginosus	4.0	-	7.0	13.0
T.thermophila	2.0	4.0	8.0	8.0

The asparaginase was assayed following the method as described by Peterson and Ciegler (1969). Took 0.1 ml of enzyme, 0.9 ml of 0.1 M sodium chlorate buffer (pH 8.5) and 1 ml of 0.04 mol L-asparagine were added and incubated for 20 min at 37˚C. The reaction was stopped by addition of 0.5 ml of 15% trichloroacetic acid (TCA) and subjected to centrifugation at x 1800. 0.1 ml of the reaction mixture thus obtained was taken in a test tube consisting of 0.1 ml, and 9.9 ml distilled water and 1.0 ml of Nessler's reagent was added and incubated for 15 min. The intensity of the colour thus developed was read at 500 nm. The uninoculated medium in place of enzyme was served as blank. The amount of ammonium liberated was read from standard cuve prepared from a solution of ammonium sulphate. One international unit (IU) of asparaginase is that amount of enzyme which liberated 1µ mol of ammonia at 37˚C.

Results and Disscussion

Out of 28 thermophilic fungi screened were positive for asparaginase production on modified Czepek dox medium containing phenol red dye as pH indicator. *R.pusillus, T. lanuginosus* and *T. luteus* and *Humicola grisea* produced good amount of L-asparaginase, while *H.stellata, H.fuscoatra, A. flavus* and *T. aurantiacus* were less efficient in the production of L-asparaginase (Table 1).

L-asparaginase production by three thermophilic fungi on different synthetic media was assessed. The modified Czepek Dox (medium E) was the best for the asparaginase production by all the three thermophilic fungi under study, while Yeast extract of glucose medium (medium C) was poor substratum. The enzyme production increased in the presence of L-asparagine (table 2). Similarly Bhaskar and Ranganathan (2009) have recorded good amount of L- asparaginase by *Aspergillus terreus* studied by them. Modified Czepek dox medium was reported to be the best substratum for asparaginase production by *F.oxysporum* (Gulati *et al.* 1997).

T.luteus could grow in the pH range of 5.0 to 8.0. It failed to grow at pH 4.0 and 9.0. It opted pH 7.0 for maximum asparaginase production as well as mycelial growth. *R.pusillus* failed to produce asparginase at alkaline pH 9.0, while pH 6.0 was optimum for asparaginase production (table 3). Almost similar trend was observed in the production of asparginase by *T.lanuginosus*. Eight days incubation period was optimum for asparaginase production at the pH range tried.

Table 2: Influence of different media on mycelial growth (in µg), ph,changes and asparaginase* production (in eight days) by three thermophilic fungi

Medium	*T. lanuginosus*			*T. luteus*			*R. pusillus*		
	Dry wt (µg/ml)	*pH*	*Aspara ginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Aspara ginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*
Yeast extract Starch medium [A]	202.4	5.9	54.0	176.2	5.8	48.0	245.9	6.4	44.0
Medium A + 0.1 % Aspargine [B]	218.0	5.2	67.0	197.2	6.4	59.0	270.2	6.3	51.0
Yeast extract Glucose Medium [C]	198.5	6.4	47.0	181.2	5.4	39.0	244.5	6.2	44.0
Yeast extract Glucose Medium 0.1% Aspargine [D]	210.0	5.7	55.0	172.3	6.1	52.0	235.8	5.1	55.0
Modified Czepek dox [E]	217.8	5.8	72.0	202.5	5.5	64.0	274.5	6.3	65.0
Malt Extract Media [F]	182.1	5.9	55.0	154.2	6.4	48.0	232.7	5.7	48.0

* Enzyme activity is expressed in unit of asparaginase enzyme equal to amount of liberated 1µ mol ammonia aspargine.

Table 3: Effect of pH on growth and asparaginase* production (in eight days) by three thermophilic fungi

	T.lanuginosus			*T.luteus*			*R.pusillus*		
pH	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*
4.0	177.2	4.3	24.0	--	--	--	213.7	3.5	38.0
5.0	210.8	5.2	35.0	155.2	5.7	39.0	247.5	4.7	42.0
6.0	217.8	6.3	62.0	172.3	5.3	56.0	265.8	5.5	53.0
7.0	210.2	5.7	55.0	189.2	5.8	59.0	233.6	6.3	25.0
8.0	187.2	7.6	44.0	165.2	5.3	21.0	210.2	7.5	27.0
9.0	171.2	7.3	25.0	--	--	--	155.6	7.6	16.0

*Enzyme activity is expressed in unit of asparaginase enzyme equal to amount of ammonia liberated 1 µ mol asparagine.

Incubation temperature of 45˚C was preferred over 40˚C and 50˚C all the three fungi under investigated for the asparaginase production (table 4). The vegetative growth of all the three thermophilic fungi under investigation was maximum at 45˚C incubation temperature. On the other hand, *A.terreus* strain was reported to produce maximum asparaginase at 26˚C (Seriquis *et al.* 2004).

D-glucose was the best carbon source for production of asparaginase by *T.lanuginosus* and *T.luteus*. Dhevagi and Poorani (2006) also reported that the glucose was the best carbon source for asparaginase production by *F.oxysporum*. D-mannose followed by maltose and starch were next preferred carbon source for the production of asparaginase by *T.lanuginosus*. Rest of the carbon sources induced varying amount of L-asparaginase. *R.pusillus* preferred mannitol for the production of asparaginase (table 5), while maltose, sucrose and starch were the next preferred carbon sources. L-asparagine followed by L-arginine and yeast extract supported maximum production of asparaginase by *T.lanuginosus*, while ammonium salts, L-methionine, L-histidine and L-tyrosine were poor nitrogen sources for the production of asparaginase. When asparaginase production by *T.lanuginosus* showed increasing trend with the progress of incubation period on L-aspartic acid, other nitrogen sources were responsible for decreased asparaginase production after eight days incubation period.

T. luteus secreted comparatively more amount of asparaginase during its growth in L-asparagine, yeast extract and L-tyrosine. Asparaginase production was low in medium containing ammonium compounds as nitrogen source

Table 4: Effect of temparature on growth and asparaginase* production (in eight days) by three thermophilic fungi

Temperature	*T. lanuginosus*			*T. luteus*			*R. pusillus*		
	Dry wt (µg/ml)	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*
35	177.2	6.4	38.0	158.3	6.6	42.0	213.7	6.1	30.0
40	186.3	6.5	39.0	164.4	5.7	48.0	244.2	6.3	42.0
45	217.8	6.3	66.0	182.3	5.3	59.0	268.3	6.4	53.0
50	172.3	6.4	57.0	155.2	5.8	43.0	245.3	6.3	47.0
55	153.8	7.6	38.0	144.3	5.3	14.0	231.2	6.5	38.0
9.0	171.2	7.3	25.0	--	--	--	155.6	7.6	16.0

*Enzyme activity is expressed in unit of asparaginase enzyme equal to amount of ammonia liberate1 µ mol aspargine

Table 5: Influence of different carbon sources on growth and asparaginase* production (in eight days) by three thermophilic fungi

Carbon source	*T. lanuginosus*			*T. luteus*			*R. pusillus*		
	Dry wt (µg/ml)	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*
D-glucose	197.4	6.2	68.0	180.7	6.2	62.0	245.3	6.2	48.0
D-fructose	187.5	6.7	51.0	186.4	6.0	48.0	245.2	6.4	43.0
D-galactose	178.4	6.8	42.0	152.6	6.4	43.0	223.0	5.9	38.0
D-mannose	178.3	6.3	63.0	183.5	6.3	56.0	235.7	5.7	44.0
L-sorbose	162.1	6.3	37.0	172.5	6.3	44.0	212.4	6.2	32.0
D-ribose	155.8	6.2	37.0	162.4	6.2	41.0	218.3	5.8	34.0
D-xylose	158.2	5.9	28.0	155.6	5.4	48.0	224.6	6.2	38.0
Sucrose	188.3	6.3	45.0	172.6	6.4	48.0	238.6	5.8	58.0
Maltose	178.2	6.2	59.0	189.5	6.2	55.0	243.6	6.1	62.0
Lactose	178.3	6.1	42.0	178.6	5.2	45.0	223.6	5.8	49.0
Citric acid	--	--	--	--	--	--	186.3	3.6	31.0
Succinic acid	135.0	3.8	22.0	144.3	4.7	24.0	191.6	3.7	32.0
Mannitol	189.2	6.4	53.0	188.3	6.4	52.0	158.3	6.2	72.0
Glycerol	159.3	6.2	45.0	168.4	6.3	41.0	243.2	5.7	48.0
Starch	178.3	6.6	52.0	184.3	6.2	48.0	247.9	6.3	58.0

Table 6: Influence of different nitrogen sources on growth and asparaginase*production (in 8 days) by three thermophilic fungi

Nitrogen source	*T. lanuginosus*			*T. luteus*			*R. pusillus*		
	Dry wt (µg/ml)	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*	*Dry wt (µg/ml)*	*pH*	*Asparaginase (in units)*
Ammonium chloride	154.2	6.3	31.0	143.2	5.4	23.0	232.1	6.2	28.0
Ammonium nitrate	154.2	5.9	26.0	155.4	5.5	21.0	236.2	5.6	23.0
Ammonium sulphate	154.2	5.5	32.0	166.3	5.4	24.0	213.4	5.8	32.0
L-arginine	189.2	5.7	58.0	174.8	5.3	31.0	265.8	6.4	58.0
L-aspargine	209.3	6.3	66.0	189.6	6.4	58.0	255.7	6.1	55.0
L-aspartic acid	178.3	5.6	38.0	178.3	6.2	37.0	262.8	6.4	44.0
L-glutamine	183.2	6.2	41.0	165.3	5.4	26.0	236.4	5.6	41.0
L-glutamic acid	216.4	6.2	34.0	172.3	6.3	38.0	221.3	5.5	34.0
L-Glycine	210.6	6.2	43.0	167.3	6.7	44.0	237.4	5.8	47.0
L-methionine	167.3	5.5	18.0	154.2	5.6	16.0	202.4	5.5	37.0
L-histidine	154.2	5.2	26.0	176.2	6.4	39.0	211.7	6.3	32.0
L-lysine	165.2	6.3	32.0	162.2	6.1	34.0	232.4	6.2	39.0
L-tryptophan	155.3	6.2	42.0	145.2	5.2	36.0	241.2	6.0	44
L-tyrosine	172.4	5.8	18.0	164.2	4.8	45.0	227.3	5.5	41.0
Yeast extract	211.3	6.2	57.0	189.2	6.3	51.0	254.3	6.4	48.0

and L-methionine. Rest of the nitrogen sources supported intermediate degree of asparaginase production (table 6). Eight days incubation period was optimum for production of asparaginase. However, in medium containing methionine the enzyme production showed increasing trend till the end of incubation period tried.

R.pusillus could secrete good amount of asparaginase during its growth in L-arginine and L-asparagine, while it was low during its growth on ammonium nitrogen source (table 6). Rest of the nitrogen sources supported intermediate degree of asparaginase activity. *T.luteus* opted L-asparagine and yeast extract for its vegetative growth, while ammonium nitrogen sources were inferior. Eight days incubation period was optimum for its vegetative growth of all the present three thermophilic fungi.

From the present studies it can be concluded that thermophilic fungi can be exploited for asparaginase production. However, more detailed studies involving many strains are desired.

Acknowledgement

Thanks are due to Head, Department of Microbiology, Kakatiya University, Warangal and UGC, New Delhi for providing facilities and financial assistance respectively for the current research work

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