Regular Research Article

Amylase Production by Thermotolerant Isolates of *Bacillus licheniformis*

[1]Dawood E. S*. and [2]Ibrahim S. A.

[1]Department of life Science, Faculty of Education, Nile Valley University
[2]Department of Botany, Faculty of Science Khartoum University
* Author for Correspondence
Email: elhamdawood@hotmail.com (Elham)

Received: 19/09/2011; Received in Revised form: 14/11/2011; Accepted: 20/11/2011

Abstract

Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery. Amylases have a quarter of the world enzyme market and thermostable amylases possess extensive commercial applications. Since little work has been done on strain isolation, growth and enzyme yield optimization, the level of thermophilic enzyme production remains relatively low. Therefore, large scale exploitation of thermophiles requires further intensive and integrated work. The present study describes isolation of four amylase producing *bacilli* from Sudanese soil. The isolates were identified and named as *Bacillus licheniformis* ($SUDK_1$, $SUDK_2$, $SUDK_4$ and SUDO) . These isolates were tested for the production of amylase enzymes as they recorded the largest zone of activity. Amylase activity was determined using DNS method. The strain was cultured in liquid media to produce amylases. The enzyme production conditions of the newly isolated *bacillii* revealed that the maximum enzyme production after 24 h of cultivation at alkalophile pH and 50°C. 5m M Mg++ ions, 1% soluble starch in production medium enhanced the enzyme productivity for all isolates . 1% Peptone was the best nitrogen source for all isolates except $SUDK_2$ which needed malt extract. The results showed that *B. licheniformis* ($SUDK_1$, $SUDK_2$, $SUDK_4$ and SUDO) is a good producer of extracellular amylase at high temperatures which could be an indication that amylase produced would be thermostable and suitable for application in starch processing and food industries.

Key word: *Bacillus licheniformis,* thermo-stable amylase, culture conditions,

Introduction

Sudanese soil is rich with many sources of starch such as wheat, sorghum, maize, potato, sweet potato and cassava remains. In order to be useful for any microbial transformation, these starch resources must be converted into maltose and glucose.

Amylases constitute one of the most important groups of industrial enzymes and account for nearly 25% of the total sale of enzymes [1]. They are used in many commercial biotechnological processes including starch degradation, detergent, foodstuff, pharmaceutical, textile, and paper manufacturing [2, 3,1]. In addition, they play an important role in the biogeochemical cycle of carbon [4]. Amylolytic enzymes can be divided into three groups: α-amylase, β-amylase, and glucoamylase, which are capable of hydrolyzing starch and glycogen). Amylases may be obtained from animal, plant and microbial sources. In comparison with animal and plant enzymes, microbial amylases are cheap, simple to produce and can be improved by genetic manipulation. Amylases are produced by a variety of microorganisms such as bacteria and fungi [5]. Although there are many microbial sources available for producing amylases, only a few such as *Bacillus subtilis, Bacillus licheniformis* and *Bacillus amyloliquifaciens* are recognized as comercial producers [6]. The capacity of *Bacillus* strains to produce large quantities of enzymes has placed them among the most important enzyme producers. Indeed, they produce about 60% of commercially available enzymes [1]. Other bacilli are also recorde as amylases producers, such as *Bacillus circulans* [7 ,8] and *B.cereus* MTCC130 [9 ,10] . *B. licheniformis* is known as The best producer of thermostable amylases [11 ,12]. In this study four isolates of *Bacillus licheniformis* ($SUDK_1$, $SUDK_2$, $SUDK_4$ and SUDO) have been isolated from Sudanese soils and are found to be amylase producers which may play important roles in industry.

The objective of this study is screening of thermostolerent amylase producers.

Materials and methods

Bacterial strains and growth media

The strains used in this study were isolated from soil samples, collected from east and middle parts of Sudan. Cultures for experiments were streak-plated once a week and inoculated into nutrient agar media from a single colony and incubated for 24 h. *Bacillus* cultures were maintained on nutrient agar slants and stored at 4ºC. Before use, the *Bacillus* cultures were transferred twice into the nutrient medium and incubated at 37ºC. All chemicals used in experiments were of analytical grade or equivalent. (3,5) Dinitrosalycilic acid was obtained from Hexrode Research Park Zone, H.R. R.C. Leuven 6172, Belgium, and all growth media were purchased from Difco.

Bacterial strain isolation and identification

Soil samples were collected from different areas in Sudan and 10 grams from each sample were suspended in a saline phosphate buffer (pH 7.5). The suspension was heated at 80 ºC for 10 minutes to kill the vegetative forms of bacteria. One ml. of this suspension was then added to 20 ml. of melted nutrient agar medium in a Petri – plate. This medium was prepared by dissolving 1 g of meat extract, 2 g yeast extract, 10 g peptone, 5 g sodium chloride and 15 g agar in 1 litre distilled water. The plates were incubated at 37 ºC for 48 hours. Basic morphological and biochemical tests were carried out to identify the bacterial isolates [13].

Inoculum preparation and Enzyme Production

Amylase producing colonies were selected by flooding the plates with iodine solution (1% iodine in 2%potassium iodide w/v). The clearance zone-forming ability on starch nutrient agar plates was used for amylase production [1, 6].Eight Erlenmeyer flasks (500 ml capacity) were prepared, each containing 100 ml of a nutrient broth which consisted of (g/l): peptone 10 g, dipotassium hydrogen phosphate 3 g, magnesium sulphate.7H2O 1 g and starch 0.5 g The medium was sterilized by autoclaving at 15 lbs/in2 pressure, 121ºC for 20 min. After cooling, a loopful of bacterial culture (48h old) was aseptically transferred and rotated at 200 rpm (37°C) in a shaking incubator (Model: for 24 h. Ten ml of these vegetative inocula were

ACT-Biotechnology Research Communications 2:1 (2012) 100-103

Open Access: http://www.act.sd/act-brcs
ISSN: 1858-5965

then inoculated into a 100 ml of fresh medium and incubated at different temperatures 25, 30, 35, 40 ,45, 50 and 55ºC in shaking incubator for 24 hrs. 20 ml of each fermented broth was spun at 16000×*g* for 10 min., 4ºC in a cold centrifuge (Model: D-37520, Osterodeam-Harz, Germany) and amylase activity was determined at each temperature.

Analytical Methods

Amylase activity was determined at 40ºC (pH 7.5) by measuring the reducing sugars produced from soluble starch by the dinitrosalicylic acid (DNS) procedure using maltose as standard [14]. One unit of enzyme is defined as 1 mg reducing sugar equivalent released /ml of supernatant /min under the assay conditions. The protein content of the enzyme solution was measured by the protein – dye binding method [15] with bovine serum albumin as the protein standard. The specific activity of amylolytic enzyme was calculated by dividing the enzyme activity by its protein content.

Optimization of amylase production

Different factors were tested for optimization of amylase production. These were temperature (25-55ºC), pH (5-10), substrate concentrations, different carbon and nitrogen sources and different metal ions.

Result and Discussion

Isolation and Identification

Based on the index of amylolytic activity four *Bacillus* isolates were chosen for further investigation. These isolates were designated as SUDK1, SUDK2, SUDK4 and SUDO. These isolates were tentatively identified by standard biochemical and bacteriological tests as Bacillus isolates, resembling *Bacillus licheniformis* [13].

Amylase Production

Effect of temperature

The results illustrated in table (1) showed a positive correlation between the bacterial growth ,enzyme and the incubation temperature up to 50˚C. The effect of temperature on enzyme production was related to the bacterial growth. Similar results were recorded using different *Bacillus* isolates [16 ; 17; 18; ,19]. In contrast, maximum enzyme production was obtained at 60˚C by *Bacillus* sp. [6], whereas thermotolerant *Bacillus subtilis* produced highest α-amylase activity at 40 ˚C [20].

Table 1: Effect of temperature on amylase production by different isolates of *Bacillus licheniformis*

Bacillus isolates	Enzyme specific activity at different temp (u/mg protein)						
	25 ºC	30 ºC	35 ºC	40 ºC	45 ºC	50 ºC	55 ºC
B. licheniformis SUDK1	0.51	0.58	0.68	1.31	2.48	3.52	0.79
B. licheniformis SUDK2	0.23	0.62	0.66	0.88	1.32	1.95	0.30
B. licheniformis SUDK4	0.11	0.76	0.82	1.31	1.40	3.86	0.47
B.licheniformis SUDO	0.30	1.11	1.32	1.75	1.87	1.94	0.54

Effect of pH

The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes [21] . The production of amylase was investigated at different pH values ranging from 5.0 to 10.0. The results presented in Fig. (1) shows that pH 9.0 was the optimum pH value for amylase production and bacterial growth. This result agrees with that obtained by different *Bacillus* sp. which recorded maximum amylase production between pH 7.5 and 8.5 [22], although *Bacillus* No.A-40-2 recorded maximum amylase production at pH 10 [23] and *Bacillus subtilus* JS-2004 has the ability to produce an optimum amylase value at pH 7 [24].

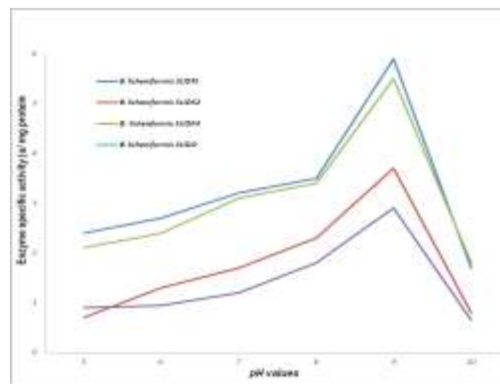

Figure 1: Effect of pH on amylolytic production by different isolates of *Bacillus licheniformis*

Effect of different Carbon sources

A number of carbon sources (1% w/v) were tested in order to determine their effect on growth and amylase production. The results suggest that amylase was an inducible enzyme and generally induced in the presence of carbon sources (Fig.2).

Since starch is considered to be one of the best inducers for amylase production, different carbon sources were tested, namely sucrose, maltose, lactose, fructose and glucose. Figure (2) shows higher preference for soluble starch as compared to the other carbon sources. There were clear differences among the four isolates in specific activity due to their different responses to the carbon source . The same finding was recorded by different *Bacillus* strains which respond differently to the various carbon sources [5].

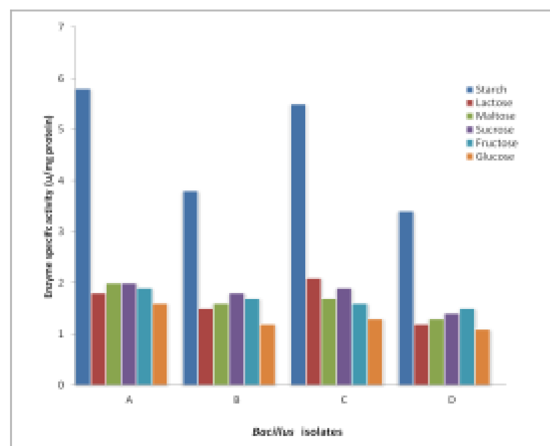

Fig 2: Effect of different organic carbon sources on amylase production of by different isolates of *Bacillus licheniformis.*

A= *B.licheniformis* SUD-K1 B = *B.licheniformis* SUD-K2
C = *B.licheniformis* SUD-K4 D = *B.licheniformis* SUD-O

Effect of different organic nitrogen sources:

The amylase synthesis by several microorganisms has been correlated to the presence or absence of various

Open Access: http://www.act.sd/act-brcs
ISSN: 1858-5965
ACT-Biotechnology Research Communications 2:1 (2012) 100-103

10X400.XX2.C, SANYO Gallenkamp, PLC, UK) amino acids and complex nitrogenous sources in the culture medium [25, 26,27]. In this investigation the growth medium was supplemented with
with 1%(w/v) of each of the following nitrogen sources peptone, tryptone, yeast extract, malt extract and meat extract (Fig. 3), Better enzyme production was obtained in the medium supplemented with peptone, while the isolate SUDK2 recorded highest enzyme production in the presence of malt-extract . It was found that several strains of *Bacillus spp.* synthesized high amount of amylase in a medium supplemented with yeast extract and peptone [28] . In contrast, high amylase production was obtained from a combination of peptone with ammonium hydrogen phosphate [29].

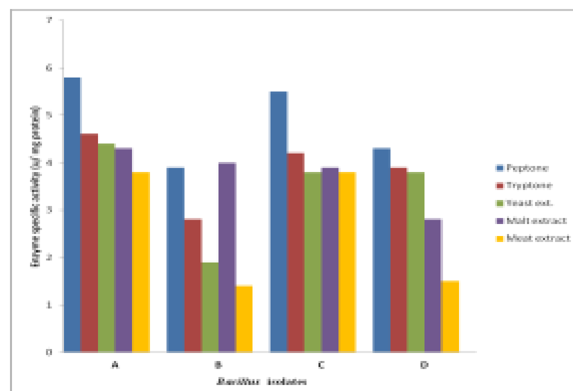

Fig 3: Effect of different organic nitrogen sources on amylase production by different isolates of *Bacillus licheniformis*.

A=*B.licheniformis* SUD-K1 **B = *B.licheniformis* SUD-K**
C= *B.licheniformis* SUD-K4 **D = *B.licheniformis* SUD-O**

Effect of metal ions
Five metal ions were supplemented to the growth medium separately. These were Mg^{++}, Ca^{++}, Ba^{++}, Mn^{++} and Fe^{++} in a concentration of 5 mM. Best enzyme production was obtained in the medium containing Mg^{++} (Table 2). Similar results were detected by *Citrobacter* sp. [30] . In contrast other strains of *Bacillus* prefer Ca^{++} ions as the best inducer for amylase production among the metal ions tested whereas Mg^{++}, Ba^{++} ,Fe^{++} and Mn^{++} have no effect [24].

Table 2: Effect of different metal ions on amylase production by different isolates of B. licheniformis

Bacillus isolates	Amylase activity (u/mg protein) (5 mM/liter)				
	Mg++	Ca++	Ba++	Mn++	Fe++
B. licheniformis SUDK1	5.87	3.92	3.31	3.28	2.98
B licheniformis SUDK2	3.85	3.75	3.10	2.52	1.89
B licheniformis SUDK4	5.61	3.52	3.52	3.34	3.27
B licheniformis SUDO	4.35	2.95	2.53	1.00	1.42

Conclusion
The use of amylases in starch based industries has been prevalent for many decades and a number of microbial sources exist for the efficient production of this enzyme, but only a few selected strains of bacteria meet the criteria for commercial production. In this paper four *Bacillus* isolates were isolated from Sudanese soil and recorded as alkaline, thermostable amylase producers (PH9.0 , 50ºC) after 24h. These amylase producers may be promising for extensive commercial applications.

Authors' contributions
Dawood E.S. performed the practical work, wrote the paper, Ibrahim, S. A. supervised the practical work and the final preparation of manuscript. All authors read and approved the final manuscript.

References

[1] A. Burhan, U. Nisa, C. Gokhan, C. Omer , A. Ashabil, and G. Osman, “Enzymatic properties of a novel thermophilic, alkaline and chelator resistant amylase from an alkalophilic *Bacillus* sp. Isolate ANT- 6”. Proc. Biochem. 38 , (2003) 1397-1403.
[2] A. Ghosh, B. Chatterjee and A. Das. Production of glucoamylase by 2- deoxy-D-glucan resistant mutant of *Aspergillus terreus* . *Biotech. Letts.* 13 (7), (1991) 515-520.
[3] E. Carlos de Souza Teodoro and L. Meire Lelis Martins*. Culture condition for the production of thermostable amylase by *Bacillus*. *Brazil J. Microb.* 31 , (2000) 298-302.
[4] A. Pandey, P. Nigam, C.R. Soccol, V.T. D. Soccol Sing, and R. Mohan. Advances in microbial amylases. *Biotech. Appl. Biochem*., 31, (2000) 135- 152.
[5] A.N Oliveira, L.A Oliveira , J.S. and A.F. Andradeand Júnior . *Rhizobia* amylase production using various starchy substances as carbon substrates. *Brazil. J. Microbiol.*, 38 , (2007) 208-216.
[6] R. K. Saxena , K. Dutt, L. Agarwal and P. Nayyar. A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. *Bioresour Technol* 98 (2) , (2007) 260- 265.
[7] B. E. Norman . The application of polysaccharides degrading enzymes in the starch industry. In: "Microbial saccharides and Polysaccaharrase". Berkley, R.C.W., Goodways, G.W. and Ellwood, D.C. (eds.) (1979) pp. 3 Academic Press London.
[8] Y .Takasaki , H. Shinohora , M. Tsuruhisa., S. S. Yayesh, and K. Imada. Maltotetrose producing amylase from *Bacillus sp.* producing MGU. *Agr and Biol. Chem.* 55 (7) , (1992) 1715-1720.
[9] A. Hema., T. Ujjval and P.l . Kamlesh . Alpha amylase production by *Bacillus cereus* MTCC 130 5 *Ferment. Food Tech. Biotech.* 44 (2) , (2006) 241– 245.

Open Access: http://www.act.sd/act-brcs
ISSN: 1858-5965
ACT-Biotechnology Research Communications 2:1 (2012) 100-103

[10] M. A. Atiyeh, R. H. R. Sajedi , Mehdi. and J. Vahab. Characterization of an α amylase with broad temperature activity from an acid-neutralizing *Bacillus cereus* strain. *Iran. J. Biotech. 8* (2) , (2010)103-1110.
[11] R. Iraj, D. A A . Shakiba , B. Hojjat and A. B. Kamal. A Thermostable α Amylase Producing Natural Variant of *Bacillus s*pp. Isolated from soil in Iran**.** *Amer. J. Agri. Biol. Sci.*3 (3) ,(2008) 591-596.
[12] A. A. Saad . Isolation**,** Phylogeny and Characterization of New α- amylase Producing Thermophilic *Bacillus* sp. From the Jazan Region, Saudi *Arabia Inter. J. Biotech. Biochem.* 6 (4), (2010) 537–547.
[13] D. Claus and P.C.W. Berkeley. Genus Bacillus In : " Bergey's Manual of Systematic Bacteriology". Vol.2 : Sneath, P. H. A., Bair, N. S. Sharpe, M. E. and Holt, J. G. (eds.) (1986) pp. 1115 – 1123. Williams and Wikins, Baltimore.
[14] G. l. Miller .Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, (1959) 426 – 428.
[15] M.M. Bradford . A rapid and sensitive method for the quantification of microgram quantities of protein – dye binding. *Anal. Biochem.* 72 , (1976) 248 254.
[16] J. M. Khire, and A. Pant . Thermo stable salt tolerant amylase from *Bacillus spp.* 64. World *J. Microbiol. Biotech.* 8, (1992) 167 – 170.
[17] H. S . Kwan , K. H. So. K.Y. Chan, and S. C. Cheng . Production of thermo tolerant β- amylase by *Bacillus circulans*. *World J. Microbiol Biotechnol.* 9 , (1993) 50 –52.
[18] S. Patel N. Jain, and D. Madamwar . Production of α- amylase from *Halebacterium halobium*. *World J. Microbiol. Biotech.* 9 , (1993). 25 – 28.
[19] R . Aliya, S . Qadar, A . Anwar, A. Iqba and S. Bano. Production and Characterization of thermostable α-amylase from a newly isolated strain of *Bacillus subtilis* KIBGE-HAR . *Int. J. Microbiol.* 6 (1) , (2009) 51-54.
[20] Z. Konsula, and M. M. liakopoulou-Kyriakides. Hydrolysis of starches by the action of α -amylase from *Bacillus subtilis* *Process Biochem.* 39 , (2004) 1745-1749.
[21] K. Kathiresan and S. Manivannan. α-Amylase production by *Penicillum fellutanum* isolated from mangrove rhizosphere soil. *Afri. J. Biotechnol.* 5 , (2006) 829 -832.
[22] M.A . Amoozegar, F. Malekzadeh and K.A. Malik. Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. *J Microbiol. Methods* 52 , (2003) 353-359.
[23] K. Horikoshi . Production of alkaline enzymes by alkalophili microorganisms. II. Alkaline amylase produced by *Bacillus* No. A- 40 (2). *Agri. Biol. Chem.* 35 , (1971) 1783-1791.
[24] M. Asgher, M. A. Javaid, S.U. Rahman and R.L. Legge . A thermostable a-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J. Food Engin.* 79 , (2007). 950–955.
[25] W. M. Fogarty and C. T. Kelly . Recent advances microbial amylases In: "Microbial Enzymes an Biotechnology." Fogarty, W. M. and Kelly, C. T. (eds.) 2nd ed. (1990) pp 71-132. Elsevier Applied Science Publishers.
[26] C.J. Hewitt and G.L. Solomons . The production of –amylase (E.C.3.2.1.1) by *Bacillus amyloliqifaciens*, in a complex and a totally defined synthetic culture medium, *Indian J. Microbiol.* **17** , (1996) 96–99.
[27] P. Hillier, D. Wase , A. J. Emery, and G. L. Solomons.Instability of a- amylase production and morphological variation in continuous culture of *Bacillus amyloliquefaciens* is associated with plasmid loss. *Pro. Biochem.* 32 , (1997) 51–59.
[28] C.E.D. Teodoro, and M.L.L ., Martins . Culture condition for the production of thermostable amylase by *Bacillus* sp., *Brazil J. Microbiol.* 31, (2000) 298–302.
[29] P .V. Aiyer . Amylase and their applications. *Afri. J. Biotech.* 4 (13), (2005) 1525-1529.
[30] C.O. Justina , O. N . Christian, N. N . Rose, E. Christopher, N. Nwanyanwu; S. Chinwe and N. E**.** Ebuta . Production and Properties of α- amylase from *Citrobacter* species. *J. Appl. Sci*. 4 (1) , **(**2009) 45-57.

Thanks to Our Valuable Reviewers

Dr. Ashish Uzgare
Wilson College
Mumbai, INDIA

Dr. Ben Chiba Amar
Department of Biotechnology
Faculty of Sciences
University of Sciences and Technology
Oran-Algeria