ISSN : 0974 - 7427 Volume 5 Issue 1

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 5(1), 2011 [40-43]

Production of alkaline isoamylase by isolated *Micrococcus varians*

Iman A.Ghazy[1]*, Reda A.Bayoumi[2], Magdy A.Gad Allah[1], Samier S.El-Louboudy[2], Mostafa M.Abo El-Soud[1]

[1]Microbial Biotechnology Dept., NRC, (EGYPT)

[2]Botany and Microbiology Dept., Faculty of Science, El-Azhar Univ., (EGYPT)

Received: 30th August, 2010 ; Accepted: 9th September, 2010

ABSTRACT

Micrococcus varians isolated from soil produced extracellular isoamylase. The optimized conditions for isoamylase production was found when liquid shaking culture was incubated 72h at 30c with initial pH 9.2. The optimum inoculum size on the production of isoamylase was 1000 ml/ 50 ml medium. Soluble starch at 1.5% was the best inducer for enzyme production as a carbon source, and ammonium sulfate at 1%was better than the other nitrogen sources for production of enzyme.

© 2011 Trade Science Inc. - INDIA

KEYWORDS

Micrococcus;
Extracellular enzyme;
Alkaline;
Isoamylase;
Industry.

INTRODUCTION

Isoamylase activity at alkaline pH was first detected purified and characterized in the culture medium of an alkalophilic strain of *Bacillus sp.* by Ara et al.[2]. Isoamylase is useful not only for the structural analysis of polysaccharides and derived oligosaccharides[6], but also for the starch industry in producing glucose, maltose, and higher oligosaccharides from starch with the action of exo-type hydrolases[8,12]. Isoamylase.also can be used in conjunction with CGTase to enhance the production of cyclodextrins from starch[15] and to improve their solubility and hemolytic product[9] through the reversed action of enzyme. Also, isoamylase can be used as an effective additive in dishwashing and laundry detergents[2].

MATERIALS AND METHODS

Strain isolation

The organism was isolated from soil samples were taken from various places including crop fields in Egypt in clean plastic bags and stored at 4°C. 0.2 ml of 10 % soil suspension were added to 50 ml of the media in 250 ml flasks which prepared according to Ara et al.[3], containing g/L: tryptone, 2; yeast extract, 1; $(NH_4)_2SO_4$, 1; KH2PO4, 3; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.2; Na_2CO_3, 5 and glycogen, 10. For solid medium: agar, 20. Medium was autoclaved at 121°C for 20 min., $CaCl_2$. 2H2O and Na_2CO_3 were autoclaved, separately, and final pH was adjusted at 9.2. Culture was incubated at 30°C under shaking conditions at 120 rpm for 24 h. Each isolate was done by agar streaking technique, and incubated at 30°C for 4 days. Finally, one iolate was selected according to the largest zone for further investigation.

Characterization of organism

The morphological and taxonomic characteristics were examined according to the method of Mac Faddin[4,5], and Sneath[10]. The organism was identified

Regular Paper

TABLE 1 : Characterization of alkali tolerant bacterial isolates

Character	Organism	Bergey's 1986	Character	Organism	Bergey's 1986
1- Morphological and microscopic character			**4- Growth at different Temperatures (ºC)**		
Colony color	Yellow	Yellow	5	-	ND
Cell form	Coccobacilli	Cocci	10	+	ND
Motility	Non motile	-	20	+	ND
Gram's stain	+		30	+	ND
Spore formation	-		40	+	+
Nitrate reduction to nitrite	+	+	50	+	ND
Gas from Nitrate	-		55	-	ND
2- Enzyme production			65	-	ND
Amylase	+	D	**5- Growth at different pH values**		
Catalase	+	ND	3.6	ND	ND
Caseinase	-	ND	4.6	-	ND
DNase	+	ND	5.6	+	ND
Gelatinase	-	+	6.6	+	ND
Oxidase	-	-	7.6	+	ND
Urease	-	+	8.6	+	ND
Indole production	-	ND	9.6	+	ND
H_2S production	+	ND	10.6	+	ND
L-Lycine decarboxylase:	-	ND	**6- Carbohydrates fermentation**		
L-Lycine deaminase:	-	ND	D (-) Arabinose:	-	ND
Oxidation / Fermentation	Fermentative	ND	Aesculin utilization:	ND	-
Ornithine decarboxylase:	-	ND	Cellobiose:	-	ND
V.P test:	+	ND	D (+) Galactose	-	-
Blood hemolysis:	γ-hemolysis	-	D-Glucose:	+	+
Growth on MacConkey agar:	-	ND	α-Lactose:	+	-
Citrate utilization:	-	+	Mannitol:	-	ND
Methyl Red Test:	+	ND	D (+) Mannose:	+	-
Growth under anaerobic conditions	+	ND	D-(+)-Xylose:	-	ND
3- Tolerance of high NaCl concentration (%)			Gas form glucose	-	ND
2	+	ND			
5	+	ND			
7	-	+			
10	-	-			

NB: D, different reactions in different taxa; d, 11-89% of strains are positive; ND, No data available

as *Micrococcus varians*.

Enzyme production

A series of 250 ml Erlenmeyer flasks each containing 50ml of the liquid medium were inoculated with 1 ml of 18 h broth culture of the organism and incubated for 3 days at 30 C under shaking condition. Crude enzyme preparation was obtained from the supernatant

TABLE 2 : The effect of incubation period on *Micrococcus varians* isoamylase production

Incubation period (Hours)	Bacterial growth O.D at 660 (µm)	Isoamylase activity (U/ ml)	Total protein (µg/ml)
6	0.37	0	38.45
12	1.32	0	47.42
24	2.95	0	50.10
48	5.14	10.67	53.07
72	5.66	54.67	57.03
96	5.42	33.67	64.72
120	5.34	16.67	77.60
144	5.14	11	75.27

***The test was carried out at pH 9.2, temp. 30°C and under shaking conditions. * Starch was added to the medium as carbon source (1.5 %), $(NH_4)_2SO_4$ as nitrogen source and inocula size (1000 µl/50 ml medium)**

TABLE 3 : The effect of different temperatures on *Micrococcus varians* isoamylase production

Temperature (?C)	Isoamylase activity (U/ ml)	Total protein (µg/ml)
20	0	26.39
25	27.33	31.57
30	55.67	58.66
37	12.67	60.58

***The test was carried out at pH 9.2 under shaking conditions for 3 days. *Starch was added to the medium as carbon source (1.5 %), $(NH_4)_2SO_4$ as nitrogen source and inocula size (1000 µl/50 ml medium)**

TABLE 4 : Effect of different pH values on *Micrococcus varians* isoamylase production using different carbon sources

KE	Starch		Glycogen		Amylopectin	
	Isoamylase activity (U/ml)	Total Protein (µg/ml)	Isoamylase activity (U/ml)	Total Protein (µg/ml)	Isoamylase activity (U/ml)	Total Protein (µg/ml)
Control	34.67	50.97	22.67	20.33	28.00	66.41
6.6	3.67	97.46	24.67	99.50	0.00	99.56
7.6	7.00	105.91	7.00	110.51	10.33	114.47
8.6	11.00	120.53	5.00	144.06	5.33	131.36
9	4.00	129.44	8.33	108.12	3.00	151.64
9.6	0.00	177.62	4.00	107.60	2.33	105.21
10.6	0.00	109.23	2.00	102.70	0.00	104.39

*** The medium pH was adjusted using buffers; citrate-phosphate buffer (6.6), phosphate buffer (7.6) and glycine-NaOH buffer (8.6 - 10.6). * Control: indicates medium with pH 9.2 adjusted using Na2CO3**

Regular Paper

TABLE 5 : Effect of different inocula sizes on *Micrococcus varians* isoamylase production

Inoculum size (µl/ 50 ml medium)	Isoamylase activity (U/ ml)	Total protein (µg/ml)
25	0	64.20
50	10.00	60.7
100	30.67	55.81
125	32.67	50.27
150	42.33	55.52
200	55.67	57.26
500	60	59.65
1000	61	60.41
2000	46.67	60.76
3000	39.33	62.1
4000	38	63.5
5000	28.67	67.98

*** The test was carried out at pH 9.2, temp. 30°C and under shaking conditions for 72 (hrs). * Starch was added to the medium as carbon source (1.5 %) and $(NH_4)_2SO_4$ as nitrogen source**

after centrifugation of the broth culture at 5000 g in sigma cooling centrifuge for 20 min.

Assay of isoamylase

Isoamylase activity was assayed according to modified method from Maruo and Kobayshi[7]. The reaction mixture contained 0.5 ml amylopectin 2% (w/v) as substrate in 0.2M glycine / NaOH buffer at ph 9.0 and 0.5 ml enzyme solution. After incubation at 40 C for 1hr, 0.2 ml of the reaction mixture was with drown and mixed with 2.0 ml of iodine solution (0.005% I_2 and 0.015% KI), water was then added to a total volume of 8ml. Activity was monitored by measuring absorbance at 620nm in a spectrophometer (UV-Visible 240 IPC shimadzu, Kyoto, Japan).

Enzyme unit of isoamylase activity (U) was defined as an increment in absorbency at 620 nm of 0.01 in 60 min at 40°C under assay conditions.

Protein determination

Protein was determined by Bradford method[3] using borine serum albumin as standard.

Effect of incubation period, temperature and pH on Enzyme production

The effect of incubation period, temperature and pH on isoamylase production were investigated by cultivating the organism at: different incubation time (6-144) hrs, and different temperatures (20-37°C) for obtaining the optimum time and at pH (6.6-10.6). The organism was incubated, the isoamylase activity and protein were determined in supernatant.

TABLE 6 : The effect of different starch concentrations on *Micrococcus varians* isoamylase production

Starch concentration (%)	Isoamylase activity (U/ ml)	Total protein (µg/ ml)
0.00	0	34.66
0.50	8	75.61
1.00	33.33	53.24
1.50	42	58.14
2.00	22.33	66.18
2.50	12.33	72.41

*** The medium pH was adjusted at 9.2 and starch was used as a carbon source at different concentrations and (NH4)2SO4 as a nitrogen source. *After inoculation, flasks were incubated at 30°C for 72 hrs under shaking conditions**

Inoculum size, carbon and nitrogen sources

Various inoclum sizes (25-5000) µl per 50ml medium broth 24 hr old were inoculated into the media,and incubated at 30°C for 72h. Three major of carbon sources, starch,amylopectin and glycogen were tested at 1% (w/v) concentration. Nitrogen sources: peptone, urea, $(NH_4)SO_4$, NH_4Cl, and ammonium molybdate at concentration 1%.

RESULTS AND DISCUSSION

Characterization of the bacterial isolate

TABLE 1 and figure 1 represent the characteristics and gram staining of the isolated organism, respectively.

Factors affecting isoamylase production by *Micrococcus varians*

The time course from 6 to 144 hrs was followed in liquid shaking culture medium at 30°C and initial pH 9.2. Results in TABLE 2 show that there was a gradual increase in isoamylase activity up to 72h.of incubation at which maximum enzyme activity was observed. These results agree with those of Ara, et al.[2].

TABLE 3 indicated that the maximum production of isoamylase enzyme in the temperature at 30°C. These data agree with the data previously published by Ueda & Nanri[13] and Wu, et al.[14].

Micrococcus varians failed to grow at pH 5.6 un-

Regular Paper

TABLE 7 : The effect of different nitrogen sources on *Micrococcus varians* isoamylase production

Nitrogen source		Bacterial growth O.D at 660 (nm)	Isoamylase productivity(U/ml)	Total protein (µg/ ml)
Control *	0	5.95	24	45.96
Peptone	0.143	6.66	0	53.77
Ammonium molybdate	0.312	8.23	10	58.84
Ammonium citrate	0.196	9.00	0	67.17
Urea	0.045	6.18	0	63.44
$(NH_4)_2SO_4$	0.100	8.76	34.33	51.5
$(NH_4)H_2PO_4$	0.174	8.38	0	75.5
$(NH_4)_2HPO_4$	0.100	9.83	3.33	63.32
NH_4Cl	0.081	8.94	0	66.99
NH_4NO_3	0.061	7.74	0	64.14
$NaNO_3$	0.129	7.37	0	75.38

*** Control: is a medium without (NH4)2SO4. * The test was carried out at pH 9.2, Temp. 30°C and under shaking conditions for 72 hrs. * Strach was added as carbon source.Nitrogen Sources were added in equal nitrogen content**

der shaking medium, growth was increased between pH 6.6-10.6. Data recorded in TABLE 4 indicated that the optimum pH for isoamylase production at 9.2. This data agree with data previously published by Ara et al.[2].

The effect of inoculum size on the production of isoamylase was shown in TABLE 5 indicated that the inoculum size at 500 ML/50ml medium and 1000 ml/ 50ml affected the enzyme production.Even the important of inoculum size as aparameter controlling of the production of enzyme[1] most workers on isoamylase production did not take it in consideration.

Only Three carbon sources (starch amylopectin and glycogon)were used in this wark at 1% (W/V)in production medium,starch at the concentration 1% proved to be the best inducer for isoamylase production (32.7V/ml) compared with amylopectin and glycogen (27.7 and 24.0) U/ml respectively. Data showed in TABLE 6 indicated that the 1.5% w/v is the optimum soluble starch concentration for isoamylase production (42.0u/ml). Soluble starch at 1 and 2% (w/v), the isoamylase production decrease by 20.6% and 46.8% respectively compared with 1.5% (w/v). Spencer-Murtins[11] used soluble starch for isoamylase production by yeast.

Effect of nitrogen source

Data recorded in TABLE 7 indicated that the ammonium.

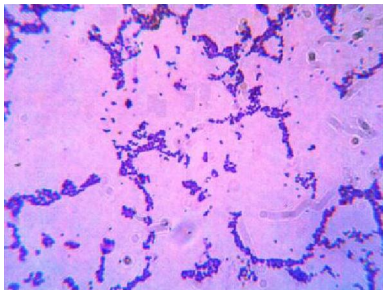

Figure 1 : A photomicrograph of gram's stain of *Micrococcus varians* KE

REFERENCES

[1] M.A.Abd El-Rahman; 'Production of Thermostable Microbial Enzymes for Application in Bio-detergent Technology', M.Sc., Thesis., Al-Azhar Univ.Fac.Of Science (boys), Bot.and Microbiol.Dept.Cairo, Egypt, **(2006)**.

[2] K.Ara, K.Saeki, S.Ito; J.of general Microbiology, **139**, 781-786 **(1993)**.

[3] D.M.Bollag, S.J.Edelstein; ,Protein Methods', John Wiley & Sons, Inc. New York, **(1991)**.

[4] J.F.Mac Faddin; 'Biochemical Tests for Identification of Medical Bacteria', Williams & Wilkins Co., Baltimore, MD, **(1976)**.

[5] J.F.Mac Faddin; 'Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria', Baltimore, MD., Williams & Wilkins, **1**, **(1985)**.

[6] J.M.Martinez, P.Schoenmakers, W.Kok; Journal of Chromatography A, **1053**, 227-234 **(2004)**.

[7] B.Maruo, T.Kobayashi; Nature, London, **167**, 606-607 **(1951)**.

[8] B.E.Norman; Starch \ Starke, **34**, 340-346 **(1982)**.

[9] Y.Okada, Y.Kubota, K.Koizumi, S.Hizukuri, T.Ohfuji, K.Ogata; Chem.Pharm.Bull., **36**, 2176-2185 **(1988)**.

[10] P.H.Sneath, (Ed.); 'Bergey's Manual of Systematic Bacteriology', William and Wilkins press (Baltimore, London, Los-Angeles, Sydney), **2**, **(1986)**.

[11] I.Spencer-Martins; Applied and Environmental Microbiology, **44**, 1253-1257 **(1982)**.

[12] L.Stominska, M.Maczeynski; Starch/Starke, **37**, 386-390 **(1985)**.

[13] S.Ueda, N.Nanri; Applied Microbiology, **15(3)**, 492-496 **(1967)**.

[14] D.Wu, C.Wen, W.Chu, L.Lin, W.Hsu; Biotechnol.Lett., **15(9)**, 883-888 **(1993)**.

[15] O.B.Zofia; 'Isoamylase from Pseudomonas Amyloderamosa', Chemical and Technical Assessment, Ph.D., (CTA), **(2007)**.