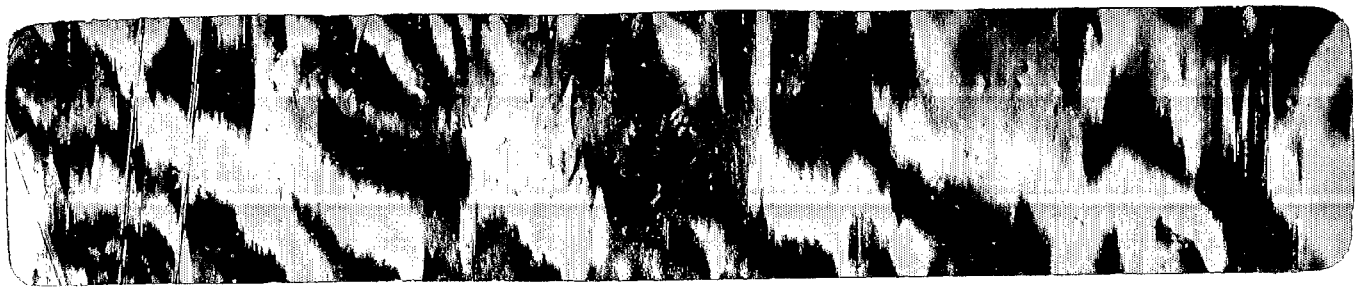
Reprints From :
Journal of Food Science and Technology
1998 Vol. 35, No.2 117 - 121



JWALAMUKHI JOB PRESS
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J. Food Sci. Technol., 1998, Vol 35, No. 2. 117-121

Pre-harvesting Treatments to Recover in a Soluble Form the Cell-bound α-amylase of *Alicyclobacillus acidocaldarius* Grown in Liquid Culture Media Containing Soluble and Granular Starch

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Alicyclobacillus acidocaldarius amylase recovery was investigated, when the strain was grown in liquid medium, containing starch as the sole carbon source. Results indicated that the amylase was non-covalently bound to the cell wall. When *A. acidocaldarius* was grown with soluble as substrate, lysozyme treatment or sonication allowed the amylase release in the liquid phase. When pellets obtained from cultures grown on insolubilized starch, the lysozyme was not effective any more to release the amylase in the liquid. Nevertheless, recovery of the enzyme activity in the supernatant was possible by heating at 70°C for 5 min. It was also possible to separate the amylase directly by SDS-PAGE electrophoresis of cells and starch granule pellets, without any previous treatment. Two bands of 163 and 145 kDa (±5 kDa) respectively, presented amylolytic activity. Hydrolytic activities were found with pullulan, cyclodextrins glycogen, amylopectin and amylose.

Keywords : *Alicyclobacillus acidocaldarius*, α-amylase, Starch, Zymography, Thermoacidophilic enzymes, Electroelution.

Three strains of *Alicyclobacillus acidocaldarius*, that were isolated from acid hot springs (Darland and Brock 1971; Buonocore et al. 1976; Uchino 1982), produced amylases of potential biotechnological interest, because of their acidostability (Boyer et al. 1979). Among them, *A. acidocaldarius* ATCC 27009 (Wisotzkey et al. 1992) produced an unusual glycosylated high molecular weight (160 kDa) amylase, and its structural gene was cloned and sequenced (Koivula et al. 1993; Schwermann et al. 1994). Previous studies suggested that the amylase was cell-bound (Boyer et al. 1979; Koivula et al. 1993). The *A. acidocaldarius* enzyme could not be purified from Darland and Brock (DB) liquid medium, but only from solid DB medium (Darland and Brock 1971; Boyer et al. 1979; Koivula et al. 1993). Nevertheless, Schwermann et al (1994) could purify the enyme from a liquid medium containing maltose. In the present study, investigations were carried out to see whether *A. acidocaldarius* α-amylase could be released from cells cultivated either on soluble or granular starch, by different methods other than reported earlier, thus offering new practical pre-harvesting methods, which could be used for further amylase purification. Combining growth and amylase harvesting on raw starch would avoid the use of pre-hydrolysed starch and would have some effect on the economy of industrial processes.

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Materials and Methods

Chemicals and reagents : Soluble starch was obtained from Merck (Darmstadt, Germany). Other substrates for enzyme assays and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Bacterial strain : The strain used was *Alicyclobacillus acidocaldarius* ATCC 27009.

Culture media and conditions : Stock cultures of *A. acidocaldarius* were maintained at -20°C in M573 medium (Darland and Brock 1971), containing 1% glucose and 40% glycerol. Starter culture was inoculated with cells from 1 ml of glycerol stock and was grown for 48 h with shaking at 55°C, in 250 ml conical flasks containing 100 ml of the M 573 medium, supplemented with 1% of glucose.

Culture in liquid medium: One ml of preculture was used to inoculate 100 ml of medium containing, either 4% insolubilized starch (that was prepared from starch powder sterilized separately and added to the cold medium), or 1% soluble starch, with 10 mM $CaCl_2$. The culture was incubated for 1 to 5 days, with shaking at 55°C. Daily, 1 ml of culture was centrifuged at 8000 rpm and 4°C for 15 min. The pellet and supernatant were subjected to amylase activity assay.

Amylase recovery after different treatments for releasing the pellet associated amylase activity : Culture suspensions were recovered after 5 days

TABLE 1. DESCRIPTION OF THE TREATMENTS USED FOR AMYLASE RECOVERY FROM PELLETS

Treatment	Buffer used	Sample treatment conditions	Reference sample treatment conditions (compared to sample conditions)
Lysozyme (10 mg/ml)	50 mM glucose, 25 mM *Tris*-HCL pH 8	15 min., 37°C	Same buffer, without lysozyme
Sonication	10 mM *Tris*-HCL pH 8	3 times for 20 sec.	Same buffer, without sonication
Alkaline pH	0.2 mM *Tris*-HCl pH 8.8	20 min., 40°C	Same treatment, but in water
0.6 M NaCl	0.6 m NaCl	5 min., 70°C	Same treatment, but in water
Electroelution	Laemmli sample buffer; pellet entrapped in 10% acrylamide	4 h, 30-40 mA	Same buffer, without elution
Heat	0.005 mM acetate buffer, 0.01 mM $CaCl_2$	5 min, various temperatures	Same buffer, without heating
Heat	0.005 mM acetate buffer, 0.01 mM $CaCl_2$	Different times, 70°C	Same buffer, without heating

of incubation and centrifuged at 14,000 rpm. Then, the pellet was washed twice and resuspended in 1 ml of water. One half of a determined volume of resuspended pellet was subjected to various treatments, as described in Table 1 and the other half was used and treated as reference sample. Samples from culture with solubilized starch have been subjected to lysozyme and sonication treatments only. Amylase activity was measured in the supernatant after centrifugation. Each experiment was run twice and each time in triplicate.

Enzyme assay : Pellet and supernatant were used directly for the enzyme assay. The routine α-amylase assay involved measuring the reducing sugars, resulting from the enzymatic hydrolysis of soluble starch. A determined volume of enzyme sample was mixed with 1 ml of 1% soluble starch in 50 mM acetate buffer (pH 4.5) and the mixture was adjusted to a final volume of 1.5 ml with water. After incubation at 55°C for 1h, the amount of reducing sugars was determined by the dinitrosalicylic acid method (Miller 1959). Various substrates were tested under the same conditions to determine the substrate specificity of the amylases. One unit of amylase activity was defined as the amount of enzyme, which liberated 1mg of reducing group per hour under the specified conditions. Each experiment was run 2 times and in triplicate.

Electrophoresis and molecular mass determination : Polyacrylamide gel electrophoresis (PAGE) was performed in 10% (w/v) gels by the method of Laemmli (1970), with or without 0.1% sodium dodecyl sulfate (SDS) or 2.5% β-mercaptoethanol. Samples of washed re-suspended cells were prepared with the Laemmli sample buffer and electrophoresis was performed in a vertical slab unit (model SE 600; Hoefer Scientific Instruments, San Francisco, USA). α-amylase activity in gels was detected after re-naturation of the enzyme, using the technique of Lacks and Springhorn (1980). Protein profiles could be observed in the same gels, by the technique described by Castillo Pompeyro, et al (1993), using silver staining of proteins (Heukeshoven and Dernick 1985).

Characterization of amylase released from cells cultivated in liquid medium with starch granules : Effect of pH and temperature: Amylase activity was determined in presence of different substrates and at several pHs (2.0 to 8.0), with 50 mM phosphate buffer (mono-and di-basic potassium phosphate and phosphoric acid). A determined volume of crude extract was mixed with phosphate buffer and incubated for 1 h at 55°C. The samples were cooled in ice and the residual activities were determined. The thermostability of crude amylase fractions was determined in 50 mM acetate buffer (pH 4.5) in presence of different substrates between 40° and 100°C at various incubation times (up to 2 h.). The effects of metal ions and other chemicals on amylase activity were also determined in presence of 1 mM of metal ions, or 0.5% (w/v) of SDS or substrate. Metals were used as chloride salts, except for Cu^{2+} (sulphate). The activity of the enzyme assayed in 0.05 M phosphate buffer alone, at optimum pH, was taken to be 100%. A standard curve was drawn in the presence of Hg^{2+} in order to eliminate the interference of this metal with the dinitrosalicylic acid method used.

Results and Discussion

Amylase recovery from cells grown in liquid medium containing soluble starch : It has been reported that *A. acidocaldarius* grown in liquid

TABLE 2. EFFECTS OF VARIOUS TREATMENTS ON AMYLASE RECOVERY IN THE LIQUID PHASE OF CULTIVATION MEDIA

Treatments	Amylase activity in the supernatant (U/ml)			
	Cells grown on soluble starch		Cells grown on granular starch	
	Before treatments	After treatments	Before treatments	After treatments
Lysozyme	1.03	7.26	0.95	0.76
Sonication	1.03	10.59	–	–
Electroelution	–	–	1.40	2.40
pH 8.8	–	–	0.95	0.56
0.6 M NaCl	–	–	0.77	0.65
70°C	–	–	0.67	3.35

medium with soluble starch, did not release amylase in the supernatant (Boyer et al. 1979). In the present study, some amylase activity was detected in the liquid phase at the end of the growth of *A. acidocaldarius*. Furthermore, when pellets from centrifuged cultures were either subjected to lysozyme or sonicated, the amylolytic activity increased in the supernatant (Table 2).

Amylase recovery from cells grown in liquid medium containing granular starch : Daily, production of amylase was measured both in the pellet and in the supernatant for 7 days. The maximum production was obtained on the 6th day of cultivation and most of the amylolytic activity was found associated with the cell pellet (Fig. 1).

After 5 days of culture, the pellet containing cells and starch granules were subjected to various treatments (Tables 1 and 2). Lysozyme did not allow

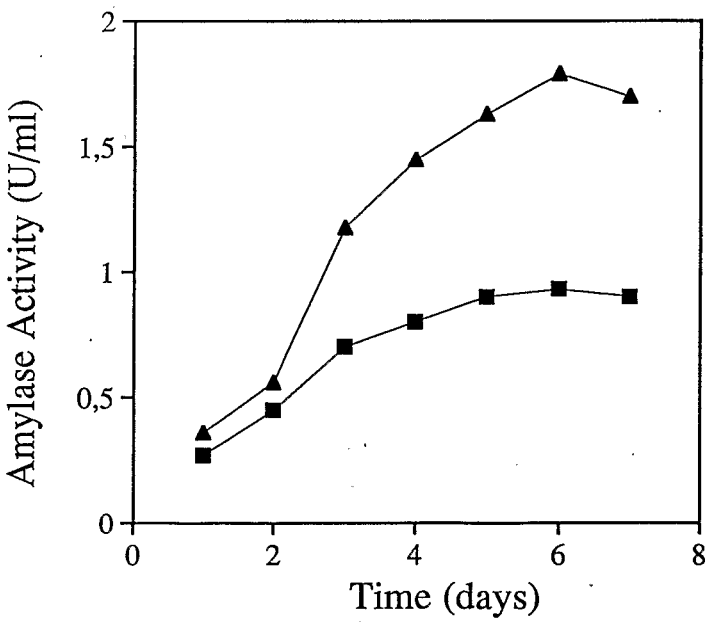

Fig. 1. Kinetics of amylase production during growth of *A. acidocaldarius* with insolubilized starch. ■ : with the supernatant; ▲ : with the cell pellets

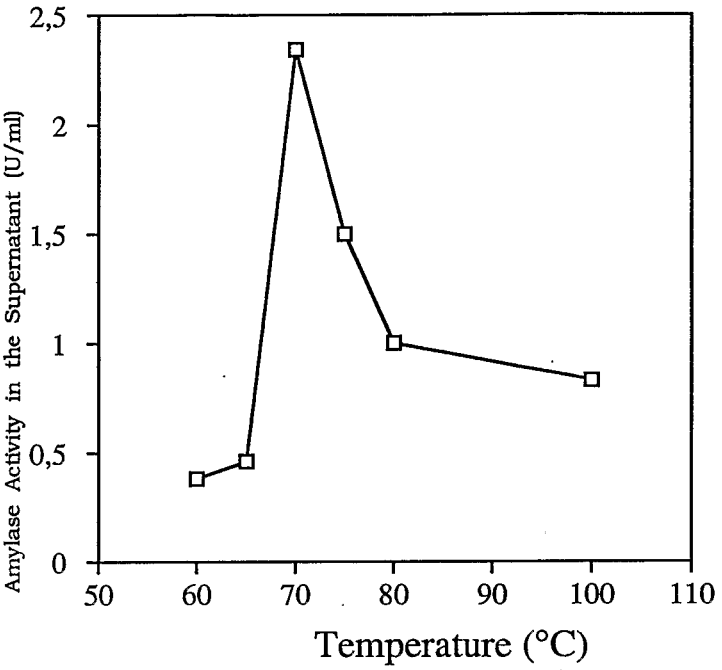

Fig. 2. Effect of temperature on amylase recovery from pellets of centrifuged cultures of cells grown on insolubilized starch granules

the enzyme release, as it did, when cells were grown with insoluble starch (Table 2). Among the various treatments used, electroelution and heat treatment gave positive results. Heat treatment was the most efficient way for enzyme release (Table 2). Various temperatures have been used for releasing the enzyme and heating at 70°C for 5 min was found to be the optimum temperature (Fig. 2). Heating periods at 70°C between 5 and 30 min did not increase the enzyme release. At higher temperature the enzyme was unstable (Fig.4).

Since the pellet-associated amylase could be electroeluted (Table 2), the pellet was directly subjected to SDS-PAGE electrophoresis in parallel to the liquid fraction obtained after heat treatment, in order to check, if the recovered amylases in both, cases were comparable to each other and if they presented the same characteristics other than previously reported. Various proteins were eluted and among them, two bands corresponding to MW of 163 kDa and 145 kDa (±5 kDa), respectively, showed amylase activity (Fig.3). The 163 kDa band was denser than the lower band and corresponded to the amylase MW described by Koivula et al (1993) and Schwermann et al (1994). As reported by the same authors, the 140 kDa band could be a proteolysis product.

A. acidocaldarius - α-amylase localization and excretion were reported to depend on the carbon source used in the culture medium (Koivula et al.

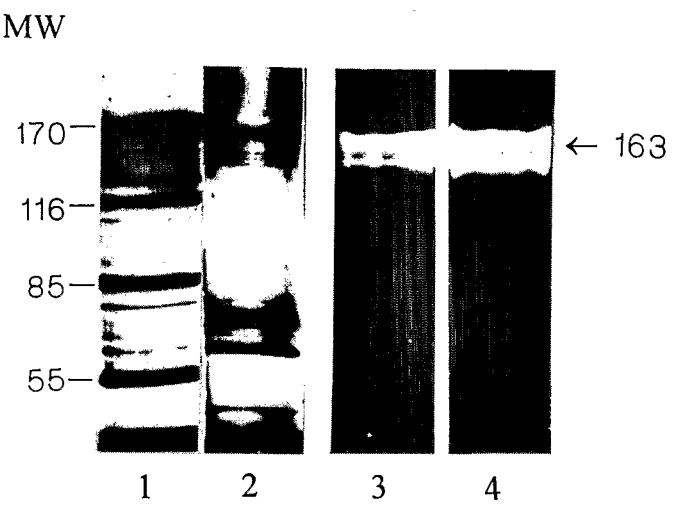

Fig. 3. SDS-PAGE of *Alicyclobacilus acidocaldarius* amylase preparations. Lane 1: molecular-weight (MW) markers whose values (in kDa) are denoted on the left of the panel. Lanes 2 and 3 : *A. acidocaldarius* preparation obtained after heat treatment, under denaturating conditions, with protein silver staining on lane 2 and amylase zymography on lane 3. Lane 4 : amylase zymography obtained from pellet (cells and starch) loaded directly on the gel. The molecular weight of the amylase is indicated by an arrow.

1993; Schwermann et al. 1994). Variability of α-amylase localization has been shown to depend also on growth conditions for *Clostridium thermohydrosulfuricum* (Melasniemi 1988; Antranikian et al. 1987). Further experiments are needed to understand the mechanisms of α-amylase localization and excretion. In the present study, it was shown that significant amylolytic activity could be released from cells grown in a liquid medium containing solubilized starch, by means of lysozyme or physical treatment. Nevertheless, when *A. acidocaldarius* was cultivated with granular starch as substrate, lysozyme treatment was not effective anymore and only physical treatments (either electroelution or heat treatment) released the pellet-associated amylolytic activity. One hypothesis, which could explain these observations, would be that in presence of granular starch, the α-amylase might be bound not covalently to the starch granules. This α–amylase was reported to belong to the group of higher MW amylases (Vihinen and Mantsala 1989). Imam and Harry-O'Kuru (1991) showed the capacity of high MW amylases to adhere and degrade insoluble matrices, such as corn starch and starch plastics. Thus, it should be interesting to see, if the additional region, contained in high MW amylases and not involved in the active site, would play a role in starch-binding, as it has been demonstrated in some glycosylated glucoamylases (Jespersen et al. 1991).

As shown in this study, heat treatment of cell suspensions might be a convenient way to maximize enzyme recovery in a soluble form from *A. acidocaldarius* cultures, by elevating the temperature from 55°C to 70°C for 5 min.

Catalytic properties of the pellet-associated amylolytic activity recovered in the liquid phase by heat treatment : A particular attention was given to substrate specificity and the effect of metal ions, since no data were previously reported on these phenomena.

The substrate specificity of the heat-treated enzyme extract was studied with several polysaccharides (final concentration: 5 mg/ml). The best substrates were soluble starch (100% of relative activity), amylose (81%), glycogen (76%) and γ-cyclodextrin (63%). Amylopectin, α-and β–cyclodextrins, as well as pullulan, were used at lower rates (between 28 and 37% of relative activity). These results are comparable to some extent with those obtained by Schwermann et al (1994), which described the *A. acidocaldarius* purified 160-kDa amylase as an α-amylase of the liquifying type on the basis of the analysis of starch product degradation and with the fact that cyclodextrin-degrading enzymes are mostly α-amylases (Vihinen and Mantsala 1989). The different types of activities displayed by the heat-treated pellet fraction obtained, according to the type of substrate tested, would suggest the possible existence of other polysaccharide-hydrolytic activities, which remain to be characterized.

The effects of metal ions (final concentration 1 mM) and SDS on amylase activity present in the enzyme extract were studied. There were no significant differences in the residual activities with Zn, Ba, Ni, Cu, Ca, Sr and Fe. Hg^{2+}, while SDS had a slight and a strong inhibitory effect, respectively.

Influence of some physico-chemical conditions on the cell pellet-associated amylolytic activity recovered in the liquid phase by heat treatment : Since the enzymatic preparation had high amylolytic activities with glycogen, amylose and starch, effect of pH and thermostability have been studied with each substrate separately, in order to see, if different physico-chemical properties would be obtained according to the substrate used.

The optimum pH was similar to that previously reported by Boyer et al (1979), using amylose as

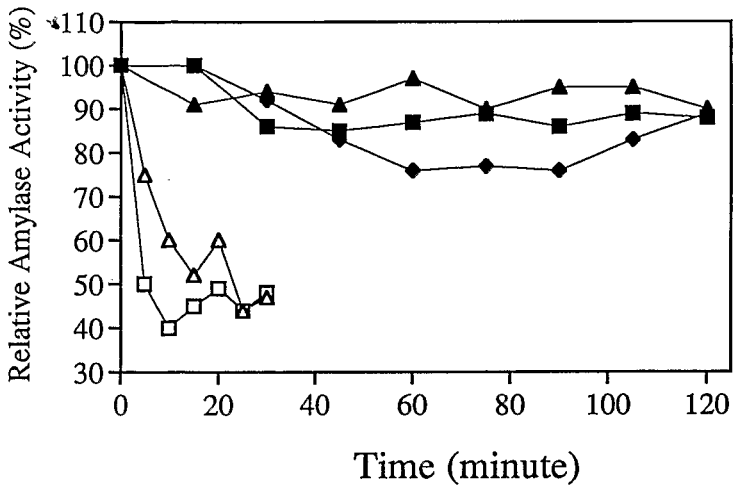

Fig. 4. Thermostability of crude fractions of amylase with starch as substrate. ■ : 50°C; ◆ : 60°C; ▲ : 70°C; Δ : 80°C; □ : 90°C

substrate and was almost identical, whatever the substrate used. Thermostability was identical, when starch (Fig.4), amylose (not shown) and glycogen (not shown) were used as substrates.

Protein electrophoretic profiles of the pellet-associated α-amylase, pH-and thermostability were similar to those previously reported (Koivula et al. 1993; Schwermann et al. 1994). These results confirm that the pre-harvesting heat-treatment allowed the recovery of a crude amylolytic fraction, which presented the main characteristics of the thermoacidophilic *A. acidocaldarius* α-amylase.

Acknowledgement

Authors thank Carlos Castillo Pompeyo for useful discussion.

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Received 2 November 1996; revised 6 April 1997; accepted 10 April 1997