

Use of Agro Waste Biomass for α -Amylase Production by *Anoxybacillus amylolyticus*: Purification and Properties

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Abstract

Knowledge accumulated from fundamental and applied studies on *Anoxybacillus* suggests that this genus can serve as a good alternative in many applications related to starch and lignocellulosic biomasses, waste treatment, enzyme technology, and bioenergy production.

We investigated the purification, biochemical characterization and immobilization of a thermostable α -amylase from the thermophilic *Anoxybacillus amylolyticus*, strain MR3CT, isolated in Antarctica and its production on vegetable wastes. In particular, the rhizome from *Arundo donax*, waste biomass of *Cynara cardunculus* and potato peels were tested either in Submerged Fermentation (SmF) and Solid State Fermentation (SSF) conditions.

The amylase from *A. amylolyticus*, with a molecular weight of about 60 kDa, displayed an optimum enzyme activity at 60°C and pH 5.6. Moreover, by retaining up to 70% of total activity after 48 h at 60°C, it showed high thermostability in the presence of 2 mM calcium ion. The immobilized enzyme maintained the 48% of its initial activity after the sixth reuse. The optimal conditions for its production in SmF were achieved at 60°C for 24 h with 1% of rhizome from *Arundo donax*, which was about 2126 U/gds. SSF cultures reached maximum α -amylase yield (102 U/gds) when grown on waste biomass of *Cynara cardunculus* as substrate, with a substrate-water ratio of 1:1 (w/v), and incubation at 60°C for 4 days. In this study, rhizome of *A. donax* resulted to be a good substrate for amylase production in SmF thus allowing a cheaper alternative to obtain amylolytic enzymes. Indeed by using rhizomes from *A. donax* as growth substrate it was possible to recovery an amylase activity level higher than that obtained by synthetic complex medium. Amylase production was also investigated under SSF conditions by using the above listed wastes as sole carbon source for *A. amylolyticus* growth. Under these conditions, *C. cardunculus* gave a higher enzyme yield per reactor volume.

Keywords: *Anoxybacillus amylolyticus*; Vegetable wastes; Amylases; SSF; SmF; Immobilization

Introduction

α -Amylase (E.C 3.2.1.1) catalyses the hydrolysis of α -D-(1,4) glycosidic linkages in starch components and related carbohydrates. Amylases are among the most important enzymes in global market that have potential application in a number of industrial processes such as food, pharmaceuticals, cosmetics, fermentation, textile and paper industries. Although they can be derived from different sources, including microorganisms, plants and animals, microbial enzymes generally meet industrial demands. The performance, economics and feasibility of each α -amylase applications is influenced by important enzyme characteristics, including specificity, stability, optimum temperature and pH performance [1]. Therefore, selection of the suitable microbial strains which produce enzymes that show good activity and stability at industrially desirable conditions will be valuable.

Although, amylase has been produced by Submerged Fermentation (SmF), Solid State Fermentation (SSF) has remarkable potential for the production of industrial enzymes in view of its economic and engineering advantages. It can be of particular significance in those processes where a crude product may be used as an enzyme source [2]. Agroindustrial residues are generally considered the best substrates for the SSF processes and enzyme production. In fact, the use of SSF for the production of enzymes and other products has many advantages over SmF [3,4] and these have been widely discussed in the literature [5]. Such systems have proved more efficient in the production of several enzymes and metabolites by microbes, as they more closely mimic the microbes' natural habitat. This cultivation technique has acquired a

special relevance in the field of biotechnology because it gives higher productivity per reactor volume, has lower operating costs, produces less wastewater, less demands in terms of energy and space, requires simpler equipment, gives higher product concentration, avoids foaming and lowers the risks of contamination [6,7].

In the light of these, our extremophiles data bank collection has been screened to identify species able to produce amylolytic enzymes. This report describes purification, biochemical characterization and immobilization of a thermostable α -amylase by the extremophile *Anoxybacillus amylolyticus*, strain MR3CT [8], a thermophilic species isolated in Antarctica and its production, either in Submerged Fermentation (SmF) and Solid State Fermentation (SSF) conditions. The enzyme production were investigated in relation to different kinds of raw vegetable waste biomass, i.e. potato peeling residues, rhizomes of *Arundo donax* and biomass from *Cynara cardunculus* as growth medium.

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Received June 16, 2014; Accepted July 23, 2014; Published July 30, 2014

Citation: Finore I, Donato PD, Poli A, Kirdar B, Kasavi C, et al. (2014) Use of Agro Waste Biomass for α -Amylase Production by *Anoxybacillus amylolyticus*: Purification and Properties. J Microb Biochem Technol 6: 320-326. doi:10.4172/1948-5948.1000162

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Materials and Methods

Microorganism

Anoxybacillus amylolyticus, strain MR3CT (ATCC BAA-872T=DSM 15939T=CIP 108338T), was cultivated at 60°C on YN complex medium containing (w/v): 0.6% yeast extract, 0.6% NaCl at pH 5.6 (standard growth conditions) [8]. Growth was followed for 24 h by measuring the optical densities at 540 nm. For amylase purification, the bacterial culture was grown on SS complex medium containing (w/v): 0.6% yeast extract, 0.6% NaCl, 0.2% soluble starch in the above pH, temperature and time course conditions.

α -Amylase assay and protein determination

The amylase activity was assayed as previously described [9], incubating at 60°C for 10 min 0.1 ml of appropriately diluted enzyme solution, 0.4 ml solution of 0.25% starch (w/v) in 50 mM acetate buffer pH 5.6. One unit of amylase activity was defined as the amount of the enzyme causing 10% reduction in starch-iodine color in 10 min in the standard assay conditions. Enzyme activity was expressed in terms of units per gram dry substrate (U gds⁻¹). The total protein content was determined according to Bradford method [10]. All values given are averages of three assays

Biomasses

Potato peel wastes were obtained from local market. The biomass was first washed with tap water and then with distilled water in order to remove surface dust particles. After washing, a blanching operation was carried out by immersing them into hot water at 75–80°C for 20 min, followed by a drying step in an oven at 45°C [11]. Rhizomes of *Arundo donax* and biomass of *Cynara cardunculus*, were kindly provided by the Agraria Department of “Federico II” University, Naples. Rhizomes of *Arundo donax* and biomass of *Cynara cardunculus*, were provided by the Agraria Department of “Federico II” University, Naples and were obtained from an experimental plantation located in Bellizzi (SA). The three biomasses were grinded at 4 mm. Successively, in order to obtain a homogenous fine powder (particle size of about 1 mm) the material was further grinded by means of a Waring Blender mixer and stored at room temperature until used.

Optimization of fermentation conditions

Amylase production was investigated both in SmF (1) and in SSF (2) condition growing *A. amylolyticus* on different carbon sources including vegetable wastes.

(1) The effect of different carbon sources on amylase production was observed in the media containing (% w/v) 0.6 NaCl and one of the following substrates (% w/v): 0.6 yeast extract (YN); 0.6 yeast extract +0.2 soluble starch (SS); one of the following vegetable waste sources (% w/v): 1.0 potato peel wastes (PP); 1.0 rhizomes of *Arundo donax* (AD); 1.0 biomass of *Cynara cardunculus* (CC). The pH was adjusted at 5.6. All experiments were carried out in triplicate in 1 L-flasks filled with 100 mL of broth culture and incubated at 60°C for 24 h. Cells were removed in late exponential growth phase, and the supernatants obtained by centrifugation at 10000 rpm for 30 min were used for protein content and amylase activity determinations.

(2) The production of α -amylase under SSF was studied utilizing the following vegetable wastes as substrate: potato peel wastes (PP), rhizomes of *Arundo donax* (AD) and biomass of *Cynara cardunculus* (CC). The strategy was to optimize each parameter independently and employ optimal conditions subsequently in all experiments for every

substrate. 1 gr of each crude substrate was taken in 100 ml Erlenmeyer flask and to this distilled water was added according to 1:1 w/v ratio and sterilized at 121°C for 20 min. After cooling, they were inoculated with 2 mL of 24 h old culture in YN medium and observing the effects after 72 h of incubation at 60°C, unless otherwise stated.

The parameters, which were optimized for maximal enzyme production, were: the ratio (w/v) of the solid substrate to the moisturizing agent (1:1, 1:1.5, 1:2); the inoculum size: 1, 2 and 3 mL of culture, in order to obtain an average optical density at 540 nm of ~ 0.700; substrate size: 1 and 4 mm; incubation time: 1, 2, 3, 4, 5 and 6 days. In separate experiments, instead of water, moisturizing agents were made up by aqueous solution containing one of the following sources (NH₄)₂SO₄, NaNO₃, yeast extract, peptone, (NH₄)Cl, (NH₄)NO₃ at 1% (w/w), or vitamin solution and metal solution (DSM 141) at 0.1% (v/w), with the respect the weight of solid substrate used.

To assess bacterial biomass production, fermented substrates were suspended in 12.5 ml of 50 mM acetate buffer pH 5.6, and centrifuged at 10000 rpm for 30 min; the supernatants were used for protein content and amylase activity determinations. All optimization experiments were run in triplicate.

Statistical analysis

Statistical significance of results for growth analysis and enzyme assay in SmF was assessed by t test by means of SYSTAT 7.0 software. For both parameters the analysis comparison was made for all tested media with respect to YN complex medium and each one respect to the other media.

Amylase purification

500 ml of MR3CT culture broth on SS complex medium were centrifuged at 10000 rpm for 30 min and the cell-free supernatant was precipitated with 80% ammonium sulphate. The crude enzyme was then loaded onto a Q-Sepharose Fast Flow (1.6×27 cm, Pharmacia) column pre-equilibrated in 50 mM sodium acetate, pH 5.6. Bound proteins were eluted at a flow rate of 3 ml min⁻¹ by applying a linear NaCl gradient (0-1 M NaCl in 50 mM sodium acetate, pH 5.6). The collected fractions with alpha-amylase activity were pooled, concentrated by ultrafiltration (Amicon, YM 10) and then dialyzed against the same buffer. The amylase sample recovered from the anion exchange chromatography, was purified by Pharmacia FPLC (ÄKTA, Amersham, Biosciences) system equipped with a pre-packed 16/60 Sephacryl S-100 column (Pharmacia), pre-equilibrated in 50 mM sodium acetate buffer, pH 5.6 containing 150 mM NaCl. Elution was performed with the same buffer at a flow rate of 0.5 ml min⁻¹ and active fractions were pooled, concentrated by ultrafiltration (Amicon, YM 10) and dialyzed against the same buffer.

Electrophoresis analysis and zymogram

Protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 15% gels according to the method of Laemmli [12] and zymographic analysis [13]. The molecular mass was determined by using a low molecular weight protein marker (Pharmacia). The relative molecular mass of the enzyme was determined by gel filtration chromatography on a Sephacryl S-100 column (Pharmacia), using a standard gel filtration calibration kit (Pharmacia).

Biochemical characterization of amylase

To study the pH effect, the enzyme was incubated at 60°C at

different pH values ranging from 3.0 to pH 10.0 by means of the following buffers: 50 mM glycine-HCl (pH 3.0); 50 mM acetate (pH 3.5, 4.0, 5.0, 5.6); 50 mM phosphate (pH 6.0, 6.5, 7.0, 7.6, 8.0); 50 mM glycine-NaOH (pH 9.0, 10.0). To study the temperature effect, activity assay was performed at pH 5.6 by varying the incubation temperatures from 30°C to 90°C.

The effect of Ca^{2+} on the amylase thermostability was evaluated by treating the purified enzyme at 60°C in the presence of 2 mM CaCl_2 for various time intervals, until max 96 h, then the residual enzyme activity was determined under standard assay conditions.

To study the effect of metals, additives and enzyme inhibitors on amylase activity purified amylase was pre-incubated, before enzyme assay, for 30 min at room temperature in the presence of one of the follow ions: 2 mM Hg^{2+} , Fe^{2+} , Co^{2+} , Ba^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} , (in chloride form), Zn^{2+} and Cu^{2+} (in sulphate form). Different additives and inhibitors were tested including: EDTA (5 mM, 10 mM), Urea (2 M, 4 M), N-Bromosuccinimide (NBS) (10 mM), β -mercaptoethanol (10 mM, 20 mM), SDS (0.1% w/v, 0.2% w/v) and Triton X-100 (0.1% w/v, 0.2% w/v). The amylase assay was carried out under standard conditions; the activity measured in the absence of metal ions was taken as the 100% reference value.

Analysis of Hydrolysis Products

α -Amylase was incubated at 60°C in 50 mM acetate buffer pH 5.6 in the presence of (1% w/v) soluble starch. Incubation was carried out for 20 min, 2, 6 and 24 hrs by stopping the reactions in ice-cold bath. The hydrolysis products were analysed by TLC chromatography (eluant n-butanol/ acetic acid/ water 6:2:2, v/v/v), the bands were revealed by the alpha-naphthol-sugars reactions, followed by heating at 150°C for 5 min.

Entrapment of amylase and its reuse

Entrapment of amylase was carried out as described previously [14]. 0.5 mL of crude enzyme solution and 3.5 mL of 2% (w/v) sodium alginate (medium viscosity) were mixed and extruded dropwise through a syringe (0.8 mm diameter) into a 200 mL of a gently stirred 100 mM CaCl_2 solution for 30 min at 4°C. The calcium alginate beads containing the enzyme were separated from the CaCl_2 solution by filtration. They were washed on a filter, three times with 10 mM CaCl_2 in 50 mM sodium acetate buffer, pH 5.6. The bead diameter ranged from 2 to 3 mm.

The immobilized crude enzyme was assayed as described before.

In order to test the reuse of entrapped α -amylase, the activity in the beads was assayed several times for the hydrolysis of soluble starch. After each amylase activity assay of the beads, they were removed, washed thoroughly with distilled water and stored at 4°C. Then, the beads were reassayed for α -amylase activity and the same process was repeated till the sixth use. The decrease in activity for each cycle was determined assuming activity of beads in the first cycle as 100%.

Sequencing of α -amylase

The gene coding for the α -amylase from *Anoxybacillus amylolyticus* was amplified according as previously [9]. The sequence obtained was deposit in DNA Data Bank of Japan.

Results and Discussions

Growth and enzymatic production on SmF

The effect of the carbon sources on growth and enzymatic

production on SmF was studied by growing the microorganism in a medium containing different carbon sources including agriculture wastes. In these conditions, Soluble Starch (SS) gave highest biomass production (26.3 ± 0.6 mg of total protein) (Table 1).

In terms of amylase activity, AD resulted as the best inducer source (2126 ± 26 U gds⁻¹), which was 2,2-fold higher than the activity in standard growth medium YN (965 ± 9 U gds⁻¹), and almost comparable to that recovery by SS media; CC resulted poor inducer giving the lowest yield in terms of growth and amylase activity (8.7 ± 0.1 mg of total protein and 575 ± 11 U gds⁻¹).

Production of enzyme under SSF

The use of Solid State Fermentation (SSF) represents an alternative and low cost production method. SSF holds tremendous potential for the production of enzyme and it can be of special interest in those processes where crude fermented product may be used as enzyme source [3-5]. For amylase production on SSF each carbon source was studied independently by varying various physicochemical and cultural conditions.

During the time course study, enzyme activity was detected in the culture supernatant from 24 h to 144 h, and the maximum α -amylase production was found for all substrates tested after 96 h. After that, a significant decrease was observed which could be due to denaturation of enzyme as already observed in other studies [15] or to the presence of secondary metabolites produced at the end of the stationary phase and toxic for the microorganism, thus resulting in a lower yield of enzyme. This value was comparable to the amylase production reached in SSF conditions by other species of *Bacillus* [16,17]. Therefore, 96 hours was the incubation time used in later studies.

The inoculum size was evaluated because it is also an important factor for production of α -amylase [18]. The results showed that there was a significant increase in α -amylase production with an increase in inoculum size up to an optimum level after which growth and enzyme yield decreased. Indeed maximum enzyme production was obtained with an inoculum size of 2 mL of preculture for all substrates tested. The decrease seen with larger inoculum sizes could be due to an early competition for nutrients among the cell population, as observed in *Bacillus firmus*-I-4071 [19] and *T. harzianum* [20].

Another crucial factor in SSF is the substrate moisture and its importance for enzyme production has been well established [21]. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities, and the moisture level of the substrate is therefore essential [22]. The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles [18]. Low and high moisture levels of the substrate affect the growth of the microorganism

Growth media	Growth (mg of total protein)	Activity (U/gds)
YN	16.6 ± 0.3	965 ± 9
SS	26.3 ± 0.6	1904 ± 33
PP	10.75 ± 0.3	1000 ± 26
AD	9.4 ± 0.2	2126 ± 24
CC	8.7 ± 0.1	575 ± 11

Table 1: Effect of different carbon sources (YN: yeast extract; SS: soluble starch; PP: potato peels; AD: rhizomes of *Arundo donax*; CC biomass of *Cynara cardunculus*) on *A. amylolyticus* growth and amylase production after 24 h of incubation at 60°C in SmF conditions. The values are the average of two assays. Significance was determined by t test for all media versus YN complex medium and for each medium respect to the other media. P resulted <0.01 for all comparison media.

resulting in lower enzyme production [23]. The ratio 1:1 (w/v) resulted the best for amylase production in presence of CC; while for PP and AD 1:1.5 (w/v) was the most suitable moistening ratio. Therefore, the moistening ratio described above for each substrate was used to investigate the effect of substrate particle size (mm) on α -amylase production in further studies.

In the SSF processes, particle size is the most significant for microbial growth and activity [24]. The maximum enzyme production for every substrates was found to be at 4 mm particle size. With smaller particles (1 mm), the surface area for growth is greater, and thus it should be considered as a desirable factor. However, too small particles may result in substrate agglomeration in most cases, which interfere with aeration and may thus result in poor growth. At the same time, larger particles provide better aeration efficiency, but provide limited surface for microbial attack [25]. Hence, it is stated that determination of particle size corresponding to optimum growth and enzyme production is necessary [23]. Thus, 4 mm particle size of substrate was used to select the most suitable moistening agent.

Among the various agents tested in this study, distilled water was found to be the best agent to moisturize AD while vitamin solution was found to be the most favorable for CC and $(\text{NH}_4)_2\text{SO}_4$ is the moistening agent that was the most effective for the production of α -amylase for PP.

Definitively, maximum amylase yield (102 U gds^{-1}) was obtained with CC, by using vitamin solution as moistening agent with ratio 1:1 (w/v) (Table 2).

PP was the second best amylase inducer, about one third of enzyme recovered by CC, with $(\text{NH}_4)_2\text{SO}_4$ solution as moistening agent in the ratio 1:1.5 (w/v). The lowest amylase recovery was observed with AD as substrate with 1:1.5 (w/v) moistening ratio; distilled water gave the higher amylase production with this waste source.

Comparison between SmF and SSF

Although a direct comparison between SmF and SSF modes is difficult to make because the two processes are different, it results evident that one of the most ecological (environment-protective) advantage of SSF is due to the fact that it is conducted in the absence of a free aqueous phase. This result in minimum water consumption and thus a low production of effluent water by the process, minimizing the contamination risk and therefore the possibility to perform the fermentation under semi-sterile conditions. This means that, energy- and instrumentation-demanding sterilization processes can be reduced or eliminated [26]. Figure 1 shows the comparison of amylase production from *Anoxybacillus amylolyticus* in SmF and SSF in function of water volume employed during the growth. From this point of view the biomass from *Cynara cardunculus* resulted as best substrate in SSF with a highest enzyme yield.

In addition, all the waste biomass afforded suitable microbial biomass production and moreover they positively affected also production of enzymes by the studied extremophilic *Anoxybacillus*

Growth media	Growth (mg of total protein)	Activity (U/gds)
PP	0.55 ± 0.01	32 ± 0.9
AD	0.87 ± 0.02	7 ± 0.01
CC	1.4 ± 0.03	102 ± 1.9

Table 2: Effect of different carbon sources (PP: potato peels; AD: rhizomes of *Arundo donax*; CC biomass of *Cynara cardunculus*) on *A. amylolyticus* growth and amylase production after 96 hours of incubation at 60°C in optimal SSF conditions for each substrate. The values are the average of two assays.

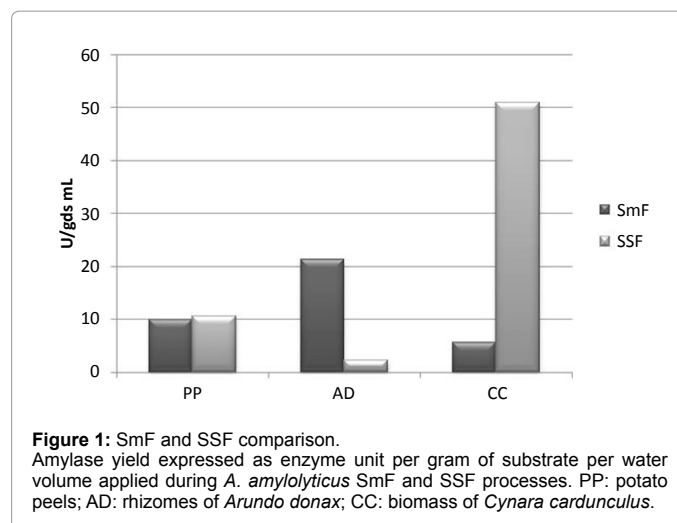


Figure 1: SmF and SSF comparison. Amylase yield expressed as enzyme unit per gram of substrate per water volume applied during *A. amylolyticus* SmF and SSF processes. PP: potato peels; AD: rhizomes of *Arundo donax*; CC: biomass of *Cynara cardunculus*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
$(\text{NH}_4)_2\text{SO}_4$	118	7533	63	1	100
Q-Sepharose F.F.	5	4578	915.6	14.5	61
Sephacryl S-100	0.024	1722	71750	1138	23

Table 3: Purification of amylase from *A. Amylolyticus*.

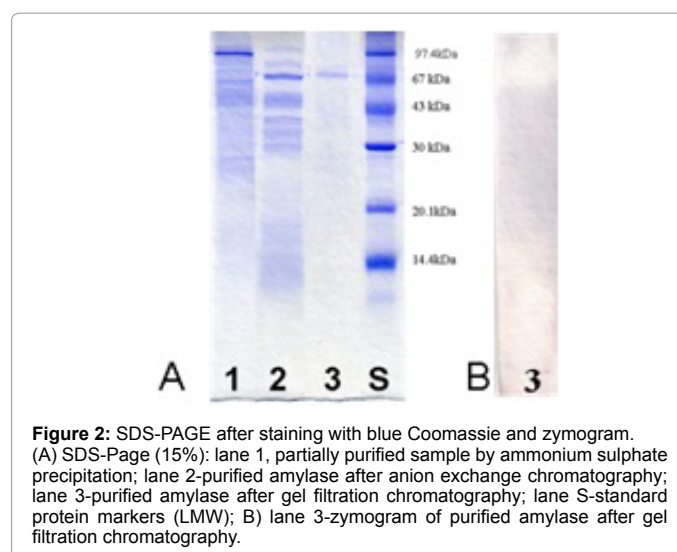


Figure 2: SDS-PAGE after staining with blue Coomassie and zymogram. (A) SDS-PAGE (15%): lane 1, partially purified sample by ammonium sulphate precipitation; lane 2-purified amylase after anion exchange chromatography; lane 3-purified amylase after gel filtration chromatography; lane S-standard protein markers (LMW); B) lane 3-zymogram of purified amylase after gel filtration chromatography.

amylolyticus. The results obtained encourage exploiting such wastes as zero cost fermentation media for biotechnologically useful α -amylase, thus also providing an alternative and less environmental impacting method for vegetable wastes management.

Purification of extracellular α -amylase

Amylase was purified, from the growth of *A. amylolyticus* on SS complex medium, via three chromatographic steps and data regarding each step are summarized in Table 3. The applied protocol yielded a purified fraction with a specific activity of 71750 U/mg of proteins, a purification factor of 1138 and a yield of 23%. Gel filtration chromatography showed that amylase has a molecular weight of about 60 kDa as also confirmed by SDS-PAGE of the purified fractions, which additionally established a single polypeptide chain structure

for this protein (Figure 2A). The purified enzyme was also subjected to zymographic analysis (Figure 2B): the clear halo, due to starch digestion, was observed at about 60 kDa thus confirming the presence of amylase activity.

Biochemical characterization of amylase

The enzyme was active in the acidic to neutral pH range, showing the optimum value at pH 5.6 and retaining about 50% of its original activity at pH 4.5 and 7.5. The optimum activity of the enzyme at acidic pH values makes this enzyme suitable for industrial applications [27,28] since it would also avoid the required pH adjustment step when going from the liquefaction to the saccharification step, thus resulting in an overall simplification of the process [29].

The influence of temperature on enzyme activity was observed by incubating the enzyme in standard conditions at different temperatures ranging from 30°C to 90°C. The maximal activity was found at 60°C. In addition, the enzyme was active even at 80°C, retaining at that temperature about the 60% of total activity.

The thermal stability of the enzyme was studied by assaying the activity in standard conditions, after sample pre-incubation for different intervals of time, at 60°C with and without calcium. Like other amylases, the isolated enzyme was more thermostable in the presence of Ca^{2+} than without it [1]. Indeed, the half-life was reached after 65 h of incubation at 60°C, while in the presence of 2 mM calcium ions it was 130 h.

The influence of different divalent metal cations was investigated. The amylase activity was almost completely inhibited in the presence of Hg^{2+} as already observed for other microbial α -amylases [23,30] and this effect should suggest a possible involvement of indole amino acid residues in enzyme function [31]. Specific activity was also significantly lowered by the presence of 2 mM Cu^{2+} ; a similar effect was observed for *Lactobacillus manihottivorans*, for *Bacillus circulans* [32] and *Bacillus coagulans* enzymes [33]. Except for Zn^{2+} , which was responsible of a 10% decrease of the activity, all the other tested metals and in particular Ca^{2+} , Fe^{2+} and Mn^{2+} slightly stimulated the amylase activity (Table 4).

The effect of metal chelators, denaturing agents and detergents on specific activity was also investigated (Table 5).

The addition of 5 mM EDTA resulted in about 70% activity inhibition thus confirming that metal play a fundamental role in the enzyme function. The amylase was insensitive to β -mercaptoethanol, which might suggest that disulfide bonds are not involved in preserving the enzymatic structure; it was totally inactivated by 10 mM N-bromosuccinimide, indicating the existence of essential tryptophan residues in the active site. Triton as well as SDS did not affect the activity and such resistance to detergents could make the MR3CT amylase a

Metal concentration (2 mM)	Relative enzyme activity (%)
None	100
Hg^{2+}	1.3
Fe^{2+}	122.4
Co^{2+}	120
Cu^{2+}	14.2
Ba^{2+}	116.4
Mg^{2+}	103
Mn^{2+}	123.8
Zn^{2+}	90.8
Ca^{2+}	129

Table 4: Effect of various metal salts on the activity of α -amylase.

Additives and Inhibitors	Concentration	Relative enzyme activity (%)
Control*	0	100
EDTA	5 mM	35.68
	10 mM	33.92
UREA	2 M	67.28
	4 M	37.68
N-bromosuccinimide	10 mM	0
β -mercaptoethanol	10 mM	104.88
	20 mM	108.72
SDS	0.001	99.52
	0.002	111.76
Triton X-100	0.001	99.84
	0.002	109.52

*Control: enzyme activity measured in the absence of additives and inhibitors, assumed as 100%

Table 5: Effect of various additives and inhibitors on the activity of α -amylase.

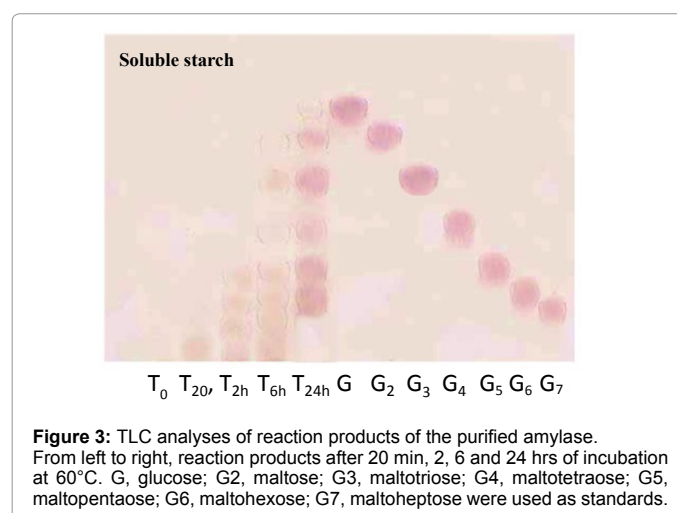


Figure 3: TLC analyses of reaction products of the purified amylase. From left to right, reaction products after 20 min, 2, 6 and 24 hrs of incubation at 60°C. G, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexose; G7, maltoheptose were used as standards.

good candidate for applications in detergent industries. Finally, this enzyme was also sufficiently stable to the action of 2 mM urea that caused only 30% loss of its activity.

Study of hydrolysis products

The Thin-Layer Chromatography (TLC) technique was used for analyzing of hydrolytic products of the enzyme action on soluble starch (Figure 3). When the enzyme was incubated with soluble starch, large malto oligosaccharides were produced during the early stage of reaction, as the reaction proceeded (24 h), the main hydrolysis products were maltoesose, maltopentaose, maltotriose. Maltopentaose (G5), maltose (G2) and maltotriose (G3) are the end products of starch hydrolysis by the α -amylase of *B. licheniformis* NH1 [34].

Reuse of the entrapped amylase

The immobilized crude enzyme showed the same behavior respect to the free enzyme. The entrapped amylase in alginate beads was evaluated by reusing beads for enzyme assay (after washing with distilled water) for several cycles. The α -amylase used in this study exhibited successful reuse. It maintained 48% of its initial activity after 6-time uses. It was found that its repeated reuse and starch hydrolysis ability were suitable for starchy processes.

Sequencing of α -amylase

The amyA gene sequence (1,724 bp) belonging to *Anoxybacillus amylolyticus* was deposited in Data Bank of Japan [DDBJ:AB908318].

Conclusions

Optimization of amylase production was tested in SmF and SSF conditions by using potato peel wastes, rhizome of *Arundo donax* and biomass from *Cynara cardunculus*. The cost of enzyme production in submerged fermentation conditions is high due to the need to use the very expensive synthetic media and from the ecological point of view for the higher water request. Therefore, the need to reduce the enzyme's production cost by alternative methods has prompted the search of cheaper alternative such as the exploitation of agro-wastes. In this study, rhizome of *Arundo donax* resulted to be a good substrate for amylase production in SmF thus allowing a cheaper alternative to obtain amylolytic enzymes. Indeed by using rhizomes from *Arundo donax* as growth substrate it was possible to recovery an amylase activity level higher than that obtained by synthetic complex medium. Amylase production was also investigated under SSF conditions by using the above listed wastes as sole carbon source for *A. amylolyticus* growth. Under these conditions, *Cynara cardunculus* gave a higher enzyme yield per reactor volume, encouraging for further studies on the optimization of process parameters under SSF conditions.

Acknowledgement

The authors are grateful to Massimo Fagnano and to Mauro Mori of the Agraria Department of "Federico II" University of Naples that kindly supplied *A. donax* and *C. cardunculus* waste biomass. This work has been implemented in the frame of the project PON01_01966 "Integrated agro-industrial chains with high energy efficiency for the development of eco-compatible processes of energy and biochemicals production from renewable sources and for the land valorization" funded by MIUR.

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