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Full Length Research Paper

Bacillus sp. R2 α-amylase production optimization: Pasta cooking water as medium of amylase production

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α-Amylases (EC 3.2.1.1; 1,4-α-D-glucanglucanohydrolase) are enzymes that are widely studied and used in industries. Their major source is microorganisms. This study aimed to prepare a readily available medium for industrial amylase production. An optimization of amylase production conditions by *Bacillus sp.* R2 and medium composition were done. Pasta cooking water was selected as basal medium due to its richness in starch. Maximum α-amylase production was achieved after 72 h and at pH 6 and 40°C. Glucose and sodium nitrite were the best secondary carbon and nitrogen sources, respectively. The optimum substrate concentration, sea-water concentration, inoculum size and NaCl concentration were 50, 75, 4 and 15%, respectively. These outcomes show that pasta cooking-water may be a promising medium for industrial amylase production due to its availability.

Key words: *Bacillus* sp. R2, α-amylase, pasta cooking-water, production, optimization.

INTRODUCTION

Starch is the major plant polysaccharide reserve and it is considered as the third biomass source on earth after lignocelluloses and chitins. α-Amylase (EC 3.2.1.1; 1,4-α-D-glucanglucano hydrolase) hydrolyzes this biopolymer into smaller polymers like dixtrins and gradually smaller oligomers to finally obtain glucose units.

Microorganisms are top α-amylase producers organisms due to their availability and readiness that meet with industrial demand. Indeed, amylases are reported to occur in archae (Canganella et al., 1994; Di lernia et al., 1998; Kwak et al., 1998; Horvathova et al., 2006), actinomyces (Upton and Fogarty, 1977; Kar and Ray, 2008; Stamford et al., 2001; Uguru et al., 1997), bacteria (Bozic et al., 2011; Cheba and Zaghloul, 2008; Raju and Divakar, 2013; Fincan et al., 2014; Najafi et al., 2005) and fungi (Hernandez et al., 2006; Sahnoun et al., 2012; Singh and Gupta, 2014; Michelin et al., 2010; Kunamneni et al., 2005; Kusuda et al., 2003).

A large number of industrial amylases are derived from bacteria belonging to the very wide and diversified genus *Bacillus*. These bacteria are widely found in nature and can live in very different habitats and undergo rough conditions. The production enhancement of chitinase by the marine bacteria *Bacillus* sp. R2 has been previously

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described (Cheba et al., 2011). In this study, its α-amylase production and optimization using submerged fermentation (SmF) was considered. This fermentation technique is of utmost importance due to its economic and environmental advantages; it is best suited for microorganisms such as bacteria and permits an easy purification of products (Subramaniyam and Vimala, 2012). Pasta cooking water was chosen as substrate for amylase production because of its starch abundance. Indeed, pasta is chemically composed of starch, proteins (gluten), non-starch polysaccharides and other minor components. During cooking, under the effect of heat; the protein matrix becomes too loose to retain gelatinized starch granules from leaching (Sissons, 2008).

Also, with 13.5 million tons produced and more than 10 million tons consumed around the world in 2013 (International Pasta Organization), pasta cooking water can be considered as a potential substrate for amylase production at large scale especially in regions with large consumption; however, a strategy of collection should be considered beforehand. In this work, the effect of many physico-chemical parameters needed for an optimal amylase production from *Bacillus* sp. R2 was monitored.

MATERIALS AND METHODS

All chemicals used in this study were of technical grade and obtained from commercial sources. Seawater was collected from Western Algerian Costal Region (Oran). The seawater was initially filtered through bolting cloth to eliminate the dirt and then sterilized by autoclaving.

Bacterial strain and culture conditions

The strain *Bacillus* sp. R2 was isolated from Red Sea water (Hurghada-Egypt) and was identified by 16s rRNA sequencing method and submitted to the GenBank database, with the accession number (DQ923161). The strain was cultured on nutrient broth and preserved in glycerol (50%, v/v) at -21°C.

Amylase production optimization

The factors that affect amylase production such as: pasta cooking water concentration, seawater concentration, secondary carbon sources, nitrogen sources, NaCl concentration, temperature, pH, inoculum size and incubation period were optimized. The optimization was performed using the one-variable-at-a-time (OVAT) method.

Amylase production

Factory-made pasta (spaghetti) was cooked in boiling water for 30 min. Recovered cooking water was allowed to cool, then mixed with seawater (v/v) and adjusted to pH 7. This mixture was used for optimization of parameters that influence amylase production.

The overnight bacterial culture 5% (v/v) was used to inoculate Erlenmeyer flasks containing production media. The culture was incubated at 37°C with 150 rpm shaking for 24 h. It was then centrifuged at 10,000 rpm for 10 min at a temperature of 4°C. The supernatant was finally recovered and used for amylase assays.

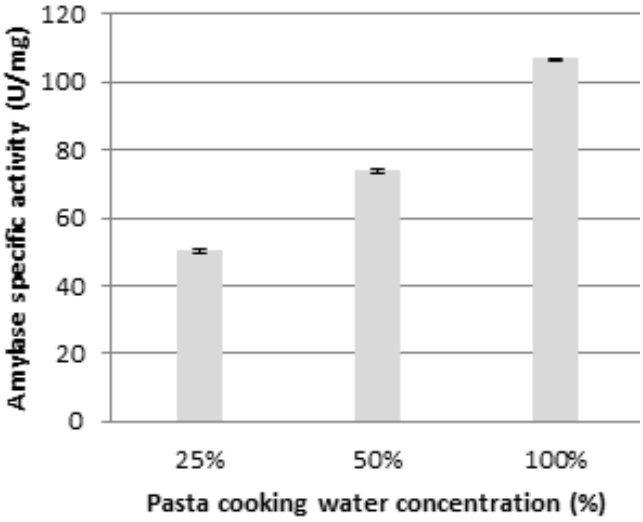

Figure 1. Effect of pasta cooking water concentration on amylase production by *Bacillus* sp. R2. Each value is an average of three parallel replicate. Error bars show the standard deviation.

The effect of physico-chemical parameters on amylase production

The experiments were conducted after autoclaving the production medium. The flasks were then cooled and inoculated with the culture seed and maintained under the following operational conditions: a) Starch concentration: 25, 50 and 100% ; b) Secondary carbon sources: Glucose, cellobiose, arabinose, saccharose, xylose, galactose (0,5%) and cellulose, pectin, tween 20, tween 80, glycerol (0, 2%); c) Sea water concentration: 50, 75 and 100% ; d) NaCl concentration: 2.5, 5, 10, 15 and 20% ; e) pH: 5, 6, 7, 8, 9 and 10 ; f) Temperature: 25, 37, 40 and 50°C ; g) Inoculum size: 2, 3, 4, 5, 6 and 7% ; h) Incubation period: 24, 48, 72, 98 and 147 h.

Amylase assay and total protein assay

Amylase activity and total protein content were measured to calculate the amylase specific activity. Amylase activity was analyzed by estimating the released reducing ends of sugar according to the method of Miller (1959). One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of reducing sugar as maltose per minute under the assay conditions. Soluble proteins were determined as described by Bradford (1976).

RESULTS AND DISCUSSION

Effect of starch concentration on amylase activity

The results showed in Figure 1 indicate that pasta cooking water at 100% gave the best amylase activity (107.1 U/mg). It is difficult to find data to compare these results knowing that this is the first investigation using starch-rich cooking water as substrate for amylase production. Most studies on amylase production are done using commercial starch or starchy residues.

Table 1. Effect of secondarycarbon sources on amylase production by *Bacillus*sp. R2. ± indicates standard deviation.

Carbon source	Carbon source	Amylase specific activity (U/mg)	Standard deviation
Monosaccharides	Glucose	452.89	±0.89
	Galactose	315.9	±0.410
	Xylose	82	±0.287
	Arabinose	336	±0.436
Disaccharides	Cellobiose	249.1	±0.550
	Saccharose	82	±0.574
Polysaccharides	Cellulose	122	±0.141
	Pectin	136	±0.563
Micellaneous	Glycerol	82.1	±0.590
	Tween 20	74.3	±0.368
	Tween 80	80.9	±0.4

Effect of secondary carbon sources on amylase activity

Results indicate that glucose was the most efficient supplementary carbon source for amylase production (452.89 U/mg), followed by arabinose and galactose (Table 1). In contrast, disaccharides and polysaccharides supported a lower enzyme levels. Narang and Satyanarayana (2001) reported that glucose was among the best carbon sources for amylase production by *B. thermooleovorans,* although several studies report that glucose at some levels triggers an inhibition of amylase production (Dey et al., 2003; Reddy and Abouzied, 1986). Paradoxically, Mørkeberg et al. (1995) indicated that at low glucose concentrations, derepression results in an increased amylase production rate in the fungi *Aspergillus oryzae*. Also, Lachmund et al. (1993) showed that glucose led to a low amylase activity always with *A. oryzae*.

Effect of nitrogen source on amylase production

Among nitrogen sources tested, sodium nitrite ($NaNO_2$) gave the best amylase production (181.76 U/mg). Concerning organic nitrogen sources, peptone was the most efficient (90.1 U/mg) (Figure 2). In a general way, these results prove that inorganic nitrogen sources are clearly more suitable for optimal amylase production by *Bacillus* sp. R2. This could be due to the fact that *Bacillus* sp. R2 is a marine strain isolated from a region of red-sea of an intense maritime traffic that causes nitrogen pollution of the aquatic ecosystem. Avdiiuk and Varbanets (2008) reported that the best nitrogen source (0.2%) for α-amylase production by *Bacillus subtilis* 147 was sodium nitrate$NaNO_3$. Similarly, Zar et al. (2013) reported a production of α-amylase, which was slightly superior using *Bacillus amyloliquefaciens* IIB-14 when NH_4NO_3 was added, compared to organic carbon sources tested. In contrast, many other studies stated that organic nitrogen sources like peptone (Aiyer, 2004) and casein (Akcan, 2011) are better for amylase production than *Bacillus*.

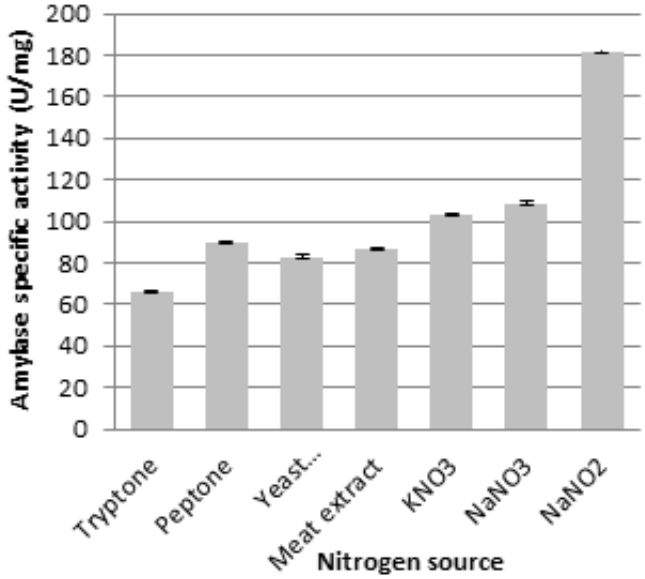

Figure 2. Effect of nitrogen source on amylase production by *Bacillus* sp. R2. Each value is an average of three parallel replicate. Error bars show the standard deviation.

Effect of seawater concentration on amylase production

Given that *Bacillus* sp. R2 is a marine bacteria isolated

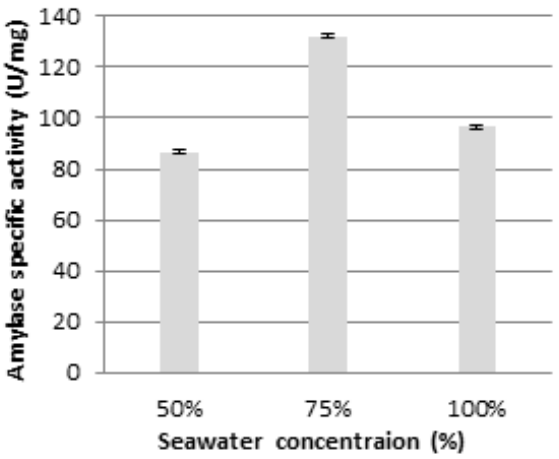

Figure 3. Effect of seawater concentration on amylase production by *Bacillus* sp. R2. Each value is an average of three parallel replicate. Error bars show the standard deviation.

from sea water, it was obvious that it will be environmentally suitable for its growth. The results show therefore that the best amylase activity (132.25 U/mg) was obtained when cultivated with seawater diluted at 75% (Figure 3). The growth of marine bacteria and production of extracellular amylase using seawater-based medium would further reduce the costs of enzyme production at large scale.

Komives et al. (2005) showed that *Bacillus methanolicus* PB1 was able to grow on methanol in semi-defined medium with 100% seawater with good growth yields. Similarly, Haifeng et al. (2007) reported an optimal amylase production by the bacteria *Pseudoalteromonas* sp. and the yeast *Aureobasidium pullulans* N13d, respectively using seawater based medium.

Effect of NaCl concentration on amylase production

Salt concentration is known to be a crucial feature in microbial life. Yet, it influences other environmental features. In fact, Valera et al. (1985) reported that increased salt concentrations resulted in oxygen concentration and pH decrease; in contrast, total biomass and diurnal temperatures increase. Given that *Bacillus* sp. R2 was isolated from a marine environment, it was expected that it will show a tolerance to salinity like most marine micro-organisms. Indeed, the results shown in Table 2 indicate that 15% was the best NaCl concentration that yields the best amylase activity (126.48 U/mg). Accordingly, Mahmoudinia et al. (2013) found that 15% was the best salinity for maximum amylase production from amylase producing halothermophilic bacteria. Other bacteria had been reported to yield maximum amylase production at high salt concentrations. Sarethy et al. (2012) indicated that addition of 10% NaCl in

Table 2. Effect of NaCl concentration on amylase production by *Bacillus sp*. R2.± indicates standard deviation.

NaCl concentration (%)	Amylase specific activity (U/mg)
2.50	114.59 ± 0.525
5	108.86 ± 0.148
10	112.26 ± 0.75
15	126.48 ± 0.48
20	104.56 ± 0.583

Table 3. Effect of pH on amylase production by *Bacillus sp*. R2.± indicates standard deviation.

pH	Amylase specific activity (U/mg)
5	81.51 ± 0.16
6	123.74 ± 0.375
7	91.75 ± 0.25
8	89.39 ± 0.357
9	81.67 ± 0.156
10	68.52 ± 0.188

medium enhanced *Bacillus* sp. SI-136 amylase activity. Likewise, maximum α-amylase production was achieved in the presence of 20% NaCl from moderately halophilic *Halomonas* sp. AAD21 (Uzyol et al., 2012).

Effect of pH on amylase production

Most enzymes are active only over a narrow pH range and have specific pH at which the activity is at its maximum. An increase or decrease in pH also causes denaturation in enzymes, thereby affecting their activity. In this work, the maximal amylase activity was noticed at pH 6 (Table 3). Most of the *Bacillus* strains used commercially for the production of bacterial α-amylases by submerged fermentation SmF have an optimum pH between 6 and 7 for growth and enzyme production (Gupta et al., 2013).

Effect of temperature on amylase activity

The data exhibited in Table 4 indicate that the temperature suitable for amylase production by *Bacillus* sp. R2 is 40°C (149.09 U/mg). This result is consistent with data about *Bacillus* species which is known to give optimal amylase production at temperatures above 40°C. Many other similar investigations concerning *Bacillus* sp.; *Bacillus cereus* and *B. subtilis* confirm these outcomes(Raj and Hemashenpagam, 2012; Singh et al., 2010; Swain et al., 2006). Also, Charoenlap et al. (2004) reported an optimal cyclodextringly cosyltransferase production; which is an enzyme belonging to the same family of α-amylase, *Bacillus circulans* in a range of 55-60°C.

Table 4. Effect of temperature on amylase production by *Bacillus sp*. R2.± indicates standard deviation.

Temperature (°C)	Amylase specific activity (U/mg)
25	93.31 ± 0.3
37	134.15 ± 0191
40	149.09 ± 0.11
50	147.46 ± 0151

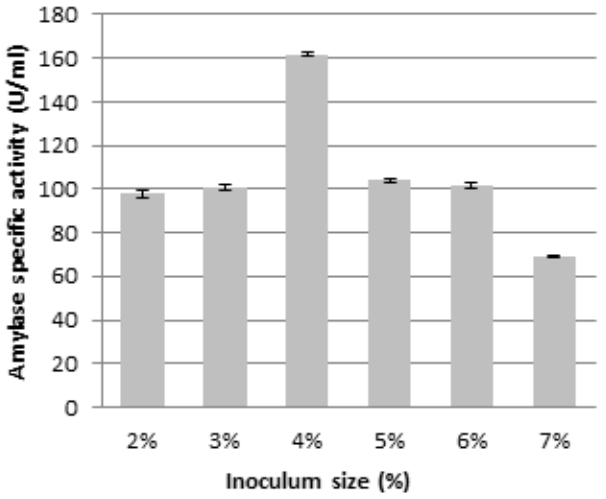

Figure 4. Effect on inoculum size on amylase production by *Bacillus* sp. R2. Each value is an average of three parallel replicate. Error bars show the standard deviation.

Effect of inoculum size on amylase activity

The effect of inoculum size is of utmost importance in microbial enzymes processes. In this study, it was found that there is an increase in amylase activity, from inoculum size of 1 to 4% where the activity reaches its highest level (161.71 U/mg) (Figure 4).

It is noteworthy that there is no precise bacterial inoculum volume suitable for amylase production. It can vary from 0.5% for *B. amyloliquefaciens* (Haq et al., 2010) to 2.95% (Zambare, 2011) for *Bacillus* sp. and 8% for *B. cereus* (Sivakumar et al., 2012).

Effect of incubation period on amylase production

The changes in amylase activity and protein content were monitored each 24 h during six days. Figure 5 shows that the maximum amylase specific activity (203.73 U/mg) was obtained after 72 h and declined gradually thereafter. This result is consistent with other similar studies that found that amylase production by different *Bacillus* strains reached its maximum on the third day (Divakaran et al., 2011; Bozic et al., 2011; Nanganuru et al., 2012). This cultivation period is slightly above the average, given that bacterial amylases are produced highly within short periods; although, good amylase production was noticed after 24 and 48 h as well.

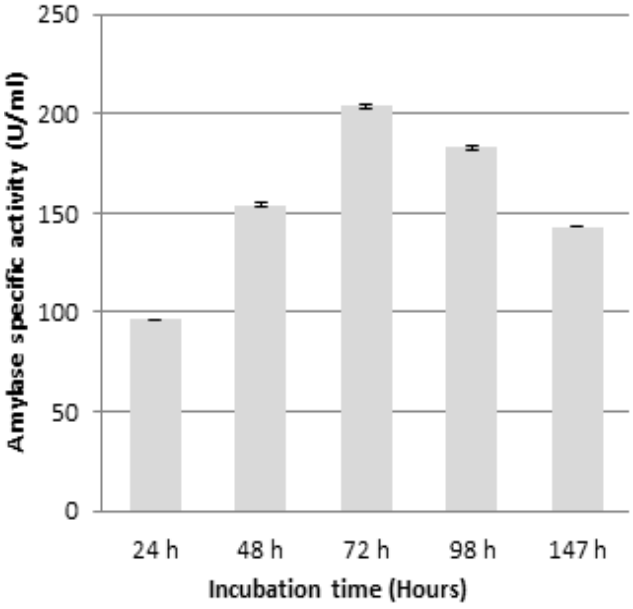

Figure 5. Effect of incubation period on amylase production by *Bacillus sp R2..* Each value is an average of three parallel replicate. Error bars show the standard deviation.

Conclusion

The use of pasta cooking-water based medium for α-amylase production by *Bacillus* sp. R2 using SmF is a simple and economical process regarding the readiness and availability of this material. This medium was efficient and yielded high amylase activity. Therefore, it could be very interesting to use it in amylase production at a large scale regarding its wide production and consumption around the world. The optimization of physico-chemical parameters of amylase production revealed good features of the strain. In fact, the using of seawater based medium can be an economical asset at industrial enzyme production scale.

Conflict of interests

The author(s) did not declare any conflict of interest.

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