Full Paper

Strain improvement studies on *Microbacterium foliorum* GA2 for production of α-amylase in solid state fermentation: Biochemical characteristics and wash performance analysis at low temperatures

(Received February 7, 2017; Accepted February 17, 2017; J-STAGE Advance publication date: November 15, 2017)

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Microbacterium foliorum GA2, an alkali-tolerant bacterium, was randomly mutated using UV radiation and sodium azide to obtain a mutant with a higher cold-active extracellular amylolytic activity. A mutant, designated as MFSD20, was selected owing to its higher amylase activity at 20°C. Under optimized conditions, amylase production was achieved best with raw banana peels (5000 units) in solid-state fermentation (SSF). The enzyme was purified by salt precipitation and chromatographic methods and afterwards characterized biochemically. The purified enzyme showed maximal activity at temperatures between 15–25°C and at pH 8.0. Interestingly, this mutant biocatalyst (MFSD20) displays higher catalytic activity under conditions of low temperature (4°C) and high pH (10.0), in the presence of SDS (0.1 and 1%), and exhibited 85% and 50% requirement of divalent metallic ions Ca²⁺ and Mg²⁺, respectively. This mutant enzyme extract in combination with “Wheel detergent” was highly effective in the removal of tomato sauce and chocolate stains from white cotton fabric was demonstrated by ~50% additional reflectance compared with detergent alone, in a wash performance analysis at 20 ± 2°C. The features shown by mutant *M. foliorum* GA2 make it a promising candidate for industrial applications involving starch degradation at low temperatures.

Key Words: alkali-tolerant; cold-active amylase; *M. foliorum*; mutagenesis; solid state fermentation

Introduction

Amylase-producing microbes, especially those isolated from extreme environments such as the deep sea, polar regions, deserts and volcanic vents, etc., have been reported with their particular interest on carbohydrate modification and other industrial applications (Debashish et al., 2005; Feller and Gerday, 2003; Siezen and Wilson, 2009; Simonato et al., 2006). However, various molecular analyses have confirmed that >99% of microorganisms cannot be cultured by conventional methods (Streit and Schmitz, 2004). This unexplored microbial diversity represents an untapped source of potentially novel and unique enzymatic activities and metabolic pathways. Unfortunately, for industrial purposes, high temperatures and an alkaline pH result in the formation of side reactions generating undesirable by-products (together with metal ions) that need to be removed at the end of the process, thereby increasing the production costs (Lee et al., 2005; Rhimi et al., 2007). Microorganisms growing in low-temperature regions are important for their enzymes with potential industrial applications. Such cold-adapted microorganisms offer a wide biotechnological potential over the use of organisms and their enzymes that operate at higher temperatures (Kuddus and Ramteke, 2009). Protein engineering has also been used to generate enzymes acting at low temperatures (Lee et al., 2005; Prabhu et al., 2010; Rhimi et al., 2009). Mutation induction and/or selection techniques, along with cloning and protein engineering strategies, have been used extensively to develop enzymes with a higher activity (Kitamura and Kamei, 2003). Physical mutagenesis using different irradiation methodologies has also been adopted to mutate bacteria. Ultraviolet radia-
tion as a physical mutagen is one of the most well-known and commonly used mutagens, which is generally used to induce genetically improved strains. Microbial enzymes have been known to be used for improving the cleaning efficiency of detergents. Detergent enzymes account for about 30% of the total worldwide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology (Hasan et al., 2006). The performance of enzymes in detergents depends on a number of factors, including the wash temperature. Bearing in mind the diverse potential of microbial enzymes, the aim of this study was to provide an economical method to enhance the cold-active α-amylase activity of M. foliorum GA2 in SSF by mutation. This study also included the purification and characterization of cold-active amyrase along with its wash performance analysis at low temperatures.

Materials and Methods

**Microorganism and culture conditions.** A cold-tolerant bacterium, M. foliorum GA2 (GenBank accession No. HQ832574), obtained from the Gangotri glacier, Western Himalaya, India, was reported as an excellent producer of cold-adapted α-amylase (Roohi et al., 2011). The culture was maintained on amyrase-producing basal media agar slants (Abe et al., 1988). The slants were incubated at 20°C for 5 days, stored at 4°C, and sub-cultured periodically.

**Alpha-amylase assay.** Amylolytic activity with starch as a substrate was assayed by the modified method of Swain et al. (2006). This amylase assay was based on the reduction in blue color intensity resulting from the enzyme hydrolysis of starch. One unit of enzyme activity is defined as the quantity of enzyme that causes a 0.01% reduction of absorbance at 690 nm of starch-iodine solution at 20°C in one min per ml.

**Strain improvement studies.** Random mutagenesis by the treatment of cells with UV rays: Physical mutagenesis: A single colony of the wild-type strain M. foliorum GA2 was inoculated into 3 ml of basal medium and incubated for 48 h at 20 ± 1°C with shaking at 100–120 rpm. This culture (0.5 ml) was diluted in 50 ml of fresh basal medium and incubated at 20 ± 1°C until an optical density (OD660) of 0.5 was reached after 6 h of incubation. Subsequently, mutagenesis with short wavelength UV radiation (240 nm) was carried out using a Philips tube (15 W). The cell suspension was distributed in 90 mm petri plates (2–3 ml in each plate) on a magnetic stirrer and exposed to UV radiation for variable time periods from 10 s–10 min by keeping the distance of the UV source fixed at 40 cm (Vahed et al., 2013). After exposure, the sample was incubated for 30 min in the dark. The cultures were then spread onto starch-agar plates and incubated overnight at 20 ± 1°C. Each colony was then evaluated for its ability to produce α-amylase by using a standard assay method. The alpha-amylase activity of mutants was compared with the wild type. An attempt was also made to improve the yield of amyrase production from M. foliorum GA2 by UV mutation when grown under submerged fermentation conditions at a distance of 40 cm from a UV-lamp (240 nm) for variable time periods (2, 4, 6, 8, 10 min) with shaking condition at 20 ± 1°C. The cultures were tested for enzyme production as mentioned above.

Random mutagenesis by the treatment of cells with sodium azide: Chemical mutagenesis: A forty-eight hours old cell suspension of M. foliorum GA2 grown in a basal media was used for this mutation, in a stage which involved treatment with sodium azide (5–100 μg/ml). Different concentrations of fresh sodium azide solution consisting of 5.0–100 μg/ml were added separately to 100 ml of culture (final pH maintained at 7.0). The solution was then incubated at 20 ± 1°C with shaking at 100–120 rpm for 3 h. Immediate centrifugation was carried out at 10,000 g for 10 min at 4°C to stop the reaction (Vahed et al., 2013). Alpha-amylase assay was performed with this 0.2 ml crude enzyme (isolated from the supernatant) by the modified method of Swain et al. (2006) and all activities were calculated in 100 ml of culture supernatant and expressed in terms of “units”. Finally, a mutant with the highest α-amylase activity was selected and compared with the wild type and also with the UV-mutants.

**Culture conditions for the production of cold-adapted wild and mutant α-amylase.** SSF was conducted for the production of cold-adapted mutant α-amylase with 5.0 g of each agricultural residue (raw banana peel, sugarcane-bagasse, saw-dust, potato peel, wheat bran, rice bran, cassava peel, orange peel), with a particle size of 6–8 mm, as the sole carbon source and 1% of soybean meal as the nitrogen source. Soybean meal (7.94% N as determined by the Kjeldahl method) was purchased locally and used in all experiments. The solid medium was moistened with sterile distilled water in the ratio 1:1.5 (w/w) and harvested with 10 ml of basal medium (gm/L: peptone, 0.6; KCl, 0.05; MgSO₄·7H₂O, 0.05; starch, 0.1). The SSF culture was incubated at 20–22°C for 7 days under static conditions. For harvesting, 50 ml of 0.1 M phosphate buffer (pH 6.0) was used on a rotary shaker at 250 rpm for 30 min (Anto et al., 2006). The cells and residual substrates were removed by centrifugation at 10,000 g for 10 min, twice. The supernatant was used for the enzyme activity assay. All the activity measurements were made in triplicate and experiments were repeated twice.

**Partial purification of cold-adapted wild and mutant α-amylase.** Supernatants of wild and mutant cold-adapted α-amylase were filtered separately by a dialysis membrane (molecular weight cut off between 12000 to 14000, pore size 2.4 nanometers) and concentrated by ammonium sulfate precipitation. The amount of ammonium sulfate required for percentage saturation was calculated by the method of Deutscher (1990). The precipitate was resuspended in 0.1 M phosphate buffer, (pH 6.0), dialyzed against the same buffer overnight at 4°C, freeze-dried, and again resuspended in 0.1 M phosphate buffer. Wild and mutant enzymes were further purified by ion-exchange chromatography using a DEAE-cellulose column. Proteins were eluted at a flow rate of 3 ml/min, by sequential application of 50 ml of 0.1 M phosphate buffer, (pH 6.0) containing NaCl at a concentration of 0 to 1 M, increased stepwise in 0.2 M increments. Active fractions were...
pooled, freeze-dried and re-suspended in a small volume of the same buffer. The molecular weight of purified amylase was determined by SDS-PAGE. Electrophoresis was performed by the method of Laemmli (1971). The gel was stained with Coomassie Brilliant Blue R-250 and the relative molecular mass of the protein was calculated using standard protein markers (Sigma), run simultaneously. An SDS-PAGE zymogram was performed for identity confirmation by the proposed method of Lin et al. (1998).

Estimation of protein. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Properties of partially purified cold-adapted wild and mutant α-amylase. Partially purified enzymes were incubated with buffers of different pH for 1 h at 20°C, viz. 0.1 M acetate buffer (pH 5), 0.1 M phosphate buffer (pH 6–8), 0.025 M borax-NaOH buffer (pH 9–10) or 0.05 M sodium hydrogen phosphate buffer (pH 11–12). To determine the thermal stability, wild and mutant α-amylases were incubated in 0.1 M phosphate buffer (pH 6) for 1 h at different temperatures (4, 15, 25, 35 and 45°C) without the addition of a substrate. The remaining enzyme activity was measured by the standard procedure. The effects of metal ions and inhibitors were also examined. Wild and mutant α-amylases were incubated for 30 min at 20°C in 0.1 M phosphate buffer (pH 6.0) containing either 5 mM metal ions (Ca²⁺, Zn²⁺, Hg²⁺, Co²⁺, Fe²⁺, Mg²⁺, Cu²⁺ and Ba²⁺) or a variable concentration of inhibitors such as EDTA and H₂O₂ (1 mM and 10 mM), SDS and CuSO₄ (0.1% and 1%) and Urea (1 M and 10 M). Remaining activities were measured as per standard protocol. Kinetic parameters (Vmax and km) were determined on the basis of Lineweaver-Burk plots. Assays were carried out in 0.1 M phosphate buffer (pH 6.0) with 0.2 to 1.4 mg/ml soluble starch, at 20 ± 1°C. The relative activity at each exposure was measured as per standard method.

Evaluation of enzyme for detergent formulation (compatibility and wash performance analysis). Compatibility and stability of wild and mutant α-amylase with commercially available laundry detergents was studied by the method of Roohi et al. (2013). The used detergents were Surf Excel and Wheel (Hindustan Lever Ltd., India), Ariel and Tide (Procter and Gamble, India) and Ghari (Rohit Surfactants Pvt. Ltd., India). Partially purified enzymes were mixed with a compatible detergent at a proportion of 5 units of enzymes per gram of detergent. Four different sets were prepared each having the same concentration of enzyme : detergent, which was 1:1. The respective sets were: detergent only with no enzyme additive, detergent with enzyme, only enzyme in water, and a control of only water without any detergent. These were tested for stain (tomato sauce and chocolate) removal. These starchy stains were selected as they are very hard to remove from cloth; moreover, chocolate and tomato-sauce contain 8.3 and 2.9 gm starch, respectively, per 100 gm of food as per food additives legislation, Guidance notes, 1995 (www.fao.org/production/faqstat). White cotton cloth pieces (8 × 8 cm) were stained with tomato sauce and chocolate and dipped in the above formulations followed by incubation for 1 h at 20°C. After incubation, cloth pieces were taken out, rinsed with cold tap water and dried. The wash performance was analyzed using a reflectance meter (Digital Reflectance Meter, Aimil Ltd., New Delhi India). The relative reflectance of all pieces was examined to test the efficiency of the enzymes to remove the stains. Untreated cloth pieces stained with tomato sauce and chocolate were taken as control.

Results and Discussion

Effect of UV irradiation on amylolytic activity of cold-adapted M. foliorum GA2

The amylolytic M. foliorum GA2 was subjected to random mutagenesis by first exposing the colonies to UV irradiation. The results (Table 1) showed that no significant increment in α-amylase production was observed when UV mutation (40, 50, 60 and 120 s) was carried out in a solid plate at 40 cm distance from a UV-lamp (240 nm) at 20 ± 1°C. UV irradiation mainly caused a decrease in activity. Some colonies produced a wider halo, but this was negligible. However, other mutants showed either the same or a decreased hydrolysis zone, when compared with the wild type (Table 1). An attempt was also made to improve the yield of amylase from M. foliorum GA2 by UV mutation (2, 4, 6, 8, 10 min) when grown under submerged fermentation conditions. A colony survived after a 4-min UV exposure produced 116% activity (9130 units) com-

| S. No. | Time for UV exposure | Diameter of colony growth (mm) (a) | Diameter of hydrolysis zone + Colony growth (mm) (b) | Difference (b)−(a) |
|--------|----------------------|-----------------------------------|--------------------------------------------------|-------------------|
| Control | —                    | 12                                | 21                                               | 9                 |
| 1      | 10 sec               | 12.5                              | 22                                               | 9.5               |
| 2      | 20 sec               | 15                                | 24.5                                             | 9.5               |
| 3      | 30 sec               | 9                                 | 17.5                                             | 8.5               |
| 4      | 40 sec               | 11                                | 21                                               | 10                |
| 5      | 50 sec               | 15                                | 25                                               | 10                |
| 6      | 60 sec               | 13                                | 23                                               | 10                |
| 7      | 2 min                | 10                                | 20                                               | 10                |
| 8      | 4 min                | 11                                | 19                                               | 8                 |
| 9      | 6 min                | 12                                | 21                                               | 9                 |
| 10     | 8 min                | 11                                | 19                                               | 8                 |
| 11     | 10 min               | 11                                | 19                                               | 8                 |
pared with the original strain (7827 units). A shorter or longer UV-exposure gave a lower, or no, enzyme production. This result suggests that the high dose of UV-radiation proved to be lethal for this bacterium whereby 100% initial cells were killed. The mutant of M. foliorum GA2, named as MFUV4, with a higher amylolytic activity was isolated. This finding also demonstrates that for improvement of amylolytic activity by random UV-mutagenesis, submerged culturing is much better than a solid plate method.

**Effect of sodium azide treatment on amylolytic activity of cold-adapted M. foliorum GA2**

The wild strain of M. foliorum GA2 was subjected to strain improvement by the treatment of cells with sodium azide (5–100 \( \mu \)g/ml), a highly potent chemical mutagen as well as a carcinogen for bacteria. The results indicated that 20 \( \mu \)g/ml of sodium azide was better for maximum \( \alpha \)-amylase production (5854 units) at 20 ± 1°C compared with the wild strain. This best screened mutant was tentatively named as MFSZ20. A higher dose of sodium azide proved to be lethal for the growth of bacteria as enzyme activity continuously decreased and, after 40 \( \mu \)g/ml of sodium azide, almost all cells were likely to be killed. A mutant colony, MFSZ20, with the highest \( \alpha \)-amylase activity relative to that of the wild type, as well as the UV-mutant (MFUV4), was selected for further studies. In other words, the above-mentioned chemically mutagenized strain consumed starch at a higher rate, and produced a high yield of \( \alpha \)-amylase. Consequently, this investigation has provided a different and simple approach regarding the improvement of enzyme yield and characteristics. Apart from the overall efficacy, microbial modifications using various types of chemical mutagenesis methods require the broader attention of investigators in the field.

The results of this study demonstrate the clear advantage in using chemical mutagens over the most commonly used UV-radiation for random mutagenesis that improves enzyme activity. During these stages of mutagenesis, lethal doses and the frequency of selected mutants were estimated.

**Production of cold-active \( \alpha \)-amylase from wild and mutant strain (MFSZ20) in SSF**

Among the eight tested agro-substrates, maximum amylase production was observed with raw banana peels (5000 units) at 20°C by the MFSZ20 strain, followed by sugarcane bagasse (4550 units), while enzyme production was not significant with sawdust (950 units), cassava peel (10120 units) and orange peel (1250 units). In previous published reports of Abou-Elela et al. (2009) and Gangadharan et al. (2006), luffa pulp for immobilized Nocardiopsis aegyptia cells and wheat bran for Bacillus amyloliquefaciens ATCC 23842, respectively, was found best for the production of cold-active \( \alpha \)-amylase under low temperature conditions. This is the first report of the production of cold-active \( \alpha \)-amylase from mutant cells of M. foliorum GA2 (MFSZ20) in SSF using raw banana peels.

### Purification of \( \alpha \)-amylase from wild and mutant (MFSZ20) M. foliorum GA2

Cold-active \( \alpha \)-amylase was purified to homogeneity by precipitating with ammonium sulphate (60% for the wild type and 80% for the mutant type) and using single-step ion exchange chromatography on a DEAE-cellulose. Enzyme was eluted from the column as unbound fractions with 0.7 M NaCl gradient. The lyophilized \( \alpha \)-amylase from wild type M. foliorum GA2 exhibited a specific activity of 595.5 U/mg, corresponding to a purification factor of 14.2-fold and a total yield of 30.4% (Table 2). Whereas lyophilized alpha-amylase from mutant type M. foliorum GA2 showed a 31.3% yield with 19.6-fold purification corresponding to 700 U/mg of specific activity (Table 2). The molecular mass of amylase obtained from M. foliorum GA2 was determined from its mobility relative to the protein standards on SDS-PAGE by using a gel documentation system. By interpolation, the molecular mass of the amylase was estimated to be 66 kDa, confirming that the enzyme is composed of a single polypeptide chain. Also, one single sharp band on a dark background observed in the zymogram, confirmed the presence of amylase activity (Fig. 1).

### Characterization of purified \( \alpha \)-amylase from wild and mutant (MFSZ20) M. foliorum GA2

**pH-optima and pH-stability profile.** The partially purified wild \( \alpha \)-amylase showed good activity over a broad pH range (pH 7–10) and the optimum pH for enzyme activity was found to be pH 8 (804.88 units/mg). The specific activity was 512.27, 599.65, 773.29 and 757.5 units/mg at pH 5, 6, 7 and 9, respectively (Fig. 2). Specific activity was still 694.43 units (86%) at pH 10. But beyond pH 10, enzymatic activity drastically reduced and only 536.9 units/mg specific activity was observed at pH 11 which further fell to 157.72 units/mg at pH 12. Similar type of results was also reported for the amylase from Wangia sp. C52 by Liu et al. (2011) and Bacillus sp. by Ashwini et al. (2011), where the optimum pH was 7.18 at 20°C and pH 7 at 40°C, respectively. But \( \alpha \)-amylase from the MFSZ20 strain showed entirely different pH-optima

| Purification steps                  | Total activity (Units) | Total protein (mg) | Specific activity (U mg\(^{-1}\)) | Purification fold | Yield (%) |
|------------------------------------|------------------------|--------------------|----------------------------------|-------------------|-----------|
|                                    | wild       | mutant   | wild       | mutant   | wild      | mutant   | wild       | mutant   |
| Crude enzyme                       | 8827       | 7827     | 210        | 220      | —         | —        | 100        | 100      |
| (NH\(_4\))\(_2\)SO\(_4\) precipitation (80%) | 5444 | 5061 | 50        | 46       | 108.8     | 110      | 2.6        | 3        | 61.6      | 64.6     |
| Dialysis                           | 4674       | 4644     | 13         | 10       | 359.5     | 464.4    | 8.6        | 13       | 57.9      | 59.4     |
| DEAE cellulose pool (lyophilized)  | 2680       | 2450     | 4.5        | 3.5      | 595.5     | 700      | 14.2       | 19.6     | 30.4      | 31.3     |
from the wild type enzyme (Fig. 2). Here, a maximum specific activity (1234.44 units/mg) was obtained at pH 10 which is the optimum for this mutant enzyme. This result suggests that mutation probably affects the change of amino acid at the active site which finally changes its conformation and makes it more suitable for substrate binding at the more alkaline side. Specific activity was 1176.66 and 906.52 units/mg at pH 9 and 11, respectively. The results obtained from the present study recommend that the α-amylase produced by MFSZ20 strain is a typical alkaline amylase. These alkaliphiles producing alkaline enzymes are unique microorganisms, with a great potential for microbiology and biotechnological exploitation. Alkaline enzymes have received great attention in recent years since they are valuable for several industries, such as chill-haze removal in brewing, biological wood pulping and the production of sophisticated enzyme detergents (Horikoshi, 1999).

The stability of the partially purified wild type α-amylase was determined by the pre-incubation of the enzyme in various buffers at different pHs for one hour without the substrate. The enzyme was less stable at pH 5 and 6 (~50%) but was relatively stable (above 70%) over a pH range of 7 to 11. The enzyme retained 96.2 and 91.1% of the original enzyme activity at pH 8 and 10 with 100% at pH 9 followed by 77% activity at pH 11 (Fig. 2). Whereas partially purified mutant type α-amylase showed more stability towards the alkaline range. This enzyme was relatively stable (above 70%) over a pH range of 8 to 12. The mutant enzyme retained its 90.6% activity at pH 9 with 100% at pH 10, and 81.2 and 80.2% activity were retained at pH 8 and 11, respectively. The enzyme was even quite stable at pH 12 where 70.6% activity still remained (Fig. 2). A similar result is the stability of cold-adapted α-amylase from P. arctica GS230 which was between pH 7.0–8.5 as reported by Lu et al. (2010) at 30°C for 1 h. Zhang and Zeng (2008) also reported that cold-adapted α-amylase produced by Nocardiosis sp. 7326 isolated from Prydz Bay, Antarctica, was stable between pH ranges of 5 to 10 and exhibited a maximal activity at pH 8.

**Temperature optima and thermal stability profile.** The activity of partially purified cold-active α-amylase was determined at different temperatures ranging from 4–45°C (Fig. 3). The optimum temperature for enzyme activity was noted at 15°C (678.63 units/mg). The enzyme showed the same specific activity (568.18 units/mg) both at 4°C and 25°C. Even at 35°C, the enzyme was active and showed 422.95 units/mg specific activity, but, beyond this temperature, activity drastically reduced and went to just 31.47 units/mg at 45°C. On the other hand, the temperature-optima of mutagenized α-amylase from the MFSZ20 strain shifted to 4°C (1022.36 units/mg), that is at the low temperature side. The specific activity slightly decreased as the temperature increased from 25°C to 35°C (945.13 and 906.52 units/mg, respectively) but it drastically went down to 173.61 units/mg at 45°C (Fig. 3). On the contrary, Xu et al. (2014) reported that the optimum temperature for the best amylase activity of purified AmyPL was 50°C. Thus, it can be assumed that mutation might improve the activity by altering the dynamic properties of residues in the active site of the enzyme. Thus, this mutation keeps this strain and its enzyme in a true psychrophilic category and thereby can be exploited for various biotechnological applications which can be successfully carried out at low temperatures.

In the case of thermal stability, both the wild and mutant type enzyme was relatively stable (more than 90% activity) over a temperature range of 4°C to 25°C when incubated for 1 h without the substrate. Wild α-amylase retained approximately 97.8% and 93.6% of the original enzyme activity at 15°C and 25°C with 100% at 4°C followed by 70.2 and just 6.3% activity at 35°C and 45°C, respectively (Fig. 3). Similarly, mutant α-amylase from the MFSZ20 strain retained its 98% and 96% activity at 15 and 25°C, respectively with 100% at 4°C (Fig. 3) and only 19% activity remained at 45°C. Thermal inactivation studies of Cotarlet et al. (2009) showed that cold-adapted α-amylase isolated from Streptomyces retained almost 90% and 80% of its activity at the optimum temperature (30°C) during the interval of 20–60 minutes of
incubation, but after 20 minutes of incubation at low temperature (20°C) α-amylase activity decreased. According to literature reports based on molecular adaptation studies shown by X-rays, the catalytic cavity seems to be larger and more accessible to ligands in psychrophilic enzymes than in mesophilic enzymes (Aghajari, 2003; Feller and Gerday, 2003). This is achieved by the deletion of bulky residues or side chains bordering the active site, allowing better accommodation of the substrate at a low energy cost and also to facilitate the release of the reaction products. The same reason might apply to the mutation studies carried out here to catalyze the hydrolysis of starch at low temperatures, psychrophilic α-amylase (from MFSZ20) improve the flexibility of the structural elements that are involved in the catalytic cycle, thereby resulting in more activity and stability as compared with normal amylase at low temperatures. A more relevant and exact reason for the high activity and stability of the mutant at 4°C will be available after a detailed study using a molecular biotechnology tool.

Effect of metal ions and inhibitors. The effect of various metal ions and inhibitors on the activity of the purified enzyme was evaluated. Of the tested metal ions, Fe²⁺ (5 mM) was shown to be a strongest inhibitor for both the wild and mutant α-amylase enzyme as the activity fell to zero. An inhibitory effect was also observed in the presence of Zn²⁺, Cu²⁺, Hg²⁺ and Co²⁺, where only 46, 38, 27 and 53% of enzyme activity remained, respectively. However, enzyme activity was enhanced up to 85% in the presence of Ca²⁺ but no significant change was observed with Mg²⁺ in the mutant case. The results suggest that probably the Fe²⁺ and Cu²⁺ metal ions apparently destabilize the active conformation of the enzyme and prevents it from breaking down the starch, thereby decreasing the activity. In previous reports, similar results were also reported where Fe²⁺ and Cu²⁺ inhibited amylase activity (Alexsandro et al., 2011; Kikani and Singh, 2011; Li, S. et al., 2011; Lu et al., 2010). Ca²⁺ independent α-amylase have also been reported by several authors (Kikani and Singh, 2011; Li, F. et al., 2011; Sindhu et al., 2011; Swain et al., 2006). All the tested inhibitors (EDTA, H₂O₂, CuSO₄, Urea and SDS) in their highest concentrations were proved to be potent inhibitors for amylase except SDS. Both the wild and mutant amylase showed 70–80% more stability towards SDS compared with the control. The chelating agent EDTA inhibited enzyme activity, indicating that the enzyme has a divalent ion required for its structural integrity. A similar result was also observed by Shafiei et al. (2011), Zhang and Zeng (2008) and Sindhu et al.
as shown in Table 3. A similar result was shown by Dahiya et al. (2011) where the chelating agent EDTA inhibited amylase activity.

Effect of substrate concentration on enzyme activity for the determination of kinetic parameters. When the activity of the purified enzyme was measured with different concentrations of substrate at previously optimized temperature and pH values (15°C, pH 8 and 4°C, pH 10 for the wild and mutant, respectively), it was observed that the amylolytic activity of wild α-amylase increased with substrate concentration linearly and reached a maximum at 1 mg/ml, before becoming constant favoring an ideal enzymatic behavior. Whereas, in the case of mutant α-amylase isolated from the MFSZ20 strain, the best substrate concentration was found to be 0.8 mg/ml which is less than in the case of the wild type. A Lineweaver-Burk plot (Fig. 4) indicates that apparent $K_m$ and $V_{max}$ values for the hydrolysis of soluble starch are 4 mg/ml and 1111 units/mg and 2 mg/ml and 844.5 units/mg, for the wild and mutant enzyme, respectively. The result of $K_m$ (2.68 mg/ml) was in accordance with the results of Bano et al. (2011) for α-amylase purified from Bacillus subtilis. The $K_m$ and $V_{max}$ values of the α-amylase for soluble starch were 7.28 mg/ml and 13.07 mg/ml min, respectively, for cold-adapted α-amylase isolated from P. arctica GS230 as reported by Lu et al. (2010). Similarly Femi-Ola and Olowe (2011) determined apparent $K_m$ and $V_{max}$ values of 16.67 mg/ml and 3.82 units, respectively, for α-amylase purified from Bacillus subtilis.

Feller and Gerday (2003) reported that the $K_m$ value of the cold-active chitobiase is 10 times lower than that of a mesophilic homologue at the respective environmental temperatures, and, interestingly, both enzymes display minimal (and therefore optimal) $K_m$ values at these temperatures. The same is also observed by this mutant enzyme whose $K_m$ value is just half of the wild amylase. The X-ray crystal structure of the mesophilic chitobiase reveals that hydrophobic interactions are involved in substrate binding. According to Feller and Gerday (2003), hydrophobic interactions are strengthened as the temperature increases, with an increase in the $K_m$ value at elevated temperatures. By contrast, in the psychrophilic chitobiase these interactions are replaced by electrostatic interactions that are stabilized at low temperatures, therefore resulting in optimal $K_m$ values at low temperatures. So, there may be a probability that, also in this research work, discrete mutations in the active site of the mutant enzyme allow fine kinetic optimization of the cold-active enzyme by modifying the thermodynamic properties of the weak interactions that are involved in substrate binding.

Compatibility and wash performance analysis

The α-amylase from the mutant M. foliorum GA2 showed excellent stability and compatibility with tested commercial detergents (1% w/v) such as Ariel, Ghari, Surf-excel, Tide and Wheel, at 20 ± 2°C as compared with wild enzyme. Both showed the highest compatibility with “Wheel” detergent, but the mutant enzyme retained more activity in comparison with the wild enzyme (Table 3). After the Wheel detergent the enzymes were more compatible with Tide followed by Ariel, Surf-excel and Ghari as shown in Table 3. A similar result was shown by Dahiya et al. (2010) where the compatibility of Bacillus amyloliquefaciens amylase was shown to be good, as the enzyme retained 96.4, 94 and 86% of its activity after 1 h of incubation but at 40°C in the presence of the detergent brands Surf Excel Blue, Surf Excel and Fena Bar, respectively. Wash performance analysis is carried out to determine the efficacy of the enzyme as a detergent additive for the removal of starchy stains from white cotton fabric. Alkaline, SDS-stable mutant α-amylase exhibit increase in reflectance from 46% to 88% when washed with a mixture of detergent + enzyme in comparison with detergent only, at 20 ± 2°C (Fig. 5). However, only a 46% to 78% increase in reflectance was reported in the case of the wild enzyme. These results suggest that cold-active α-amylase from mutant M. foliorum GA2 can be profitably used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods.

Conclusion

The results revealed the advantage of random mutagenesis with sodium azide proving to be a more effective mutagenic agent than UV-light for strain improvement of M. foliorum GA2 for enhanced cold-active α-amylase productivity. This investigation has attempted to establish a cost-effective and simple approach, in order to concomitantly increase the rate of amylase activity and improve product characteristics in a proficient manner. Based on various biochemical properties, this SDS-stable, alkaline, and waste waters in cold regions.

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