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# SOLUBLE EXPRESSION OF *BACILLUS LICHENIFORMIS* ATCC 27811 α-AMYLASE AND CHARACHTERIZATION OF PURIFIED RECOMBINANT ENZYME

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## ABSTRACT

Alpha amylase (α-amylase) from *Bacillus licheniformis* shows great role in starch hydrolysis for industrial application due to its thermostability, long half-life and specificity. However, it is difficult to produce this enzyme in large quantities because of formation of inclusion bodies, but lowering the temperature to 18 °C leads to a soluble expression of protein. This also highlighted the efficacy of *Escherichia coli* as one of the best host for secretion of recombinant proteins. A further study to find an approach for a soluble expression of α-amylase was conducted here, using pET-22b(+) as an expression vector and temperature optimization. Purified monomeric enzyme displayed a specific activity of 584.61 U/mg at 37 °C and a molecular mass of 52 *kDa*. Purification fold of recombinant α-amylase was 2.04 times in comparison to crude amylase extract. Relative activity of enzyme was remarkably enhanced by $Ca^{+2}$ but strongly impeded by $Cu^{2+}$ and $Na^{+1}$, highlighted the significance of $Ca^{+2}$ as a cofactor for optimal amylase activity.
**Key words:** Hydrolysis, Soluble, Thermostable, Industrial.

## INTRODUCTION

Alpha amylases (α-amylases) (α-1,4-glucanohydrolase; E.C 3.2.1.1), are endohydrolases that catalyze hydrolysis of starch at α-1,4-glycosidic linkages and yield monomers of glucose, maltose and oligosaccharides (Omemum *et al*., 2005; Bhanja *et al*., 2007: Leman *et al*., 2009). They were well-thought-out to be an extracellular endoglycan hydrolases but intracellular α-amylases have also been documented to have an important role in cell growth (Dong *et al*., 1997). Knockout of intracellular α-amylase coding gene in *Streptococcus bovis* has shown 15-20% of cell growth with respect to wild type, highlighted its role in cell growth (Brooker and McCarthy, 1997). In contrast, inactivation of intracellular α-amylase expression in *Streptococcus mutans* neither reduced cell growth nor effected its role in carbohydrate fermentation (Simpson and Russell, 1998).

α-amylases has been extracted from varied sources that includes; plants, animals, fungi and bacteria. They have extensive applications in detergent industry, paper industry, food manufacturing and textile industry for hydrolysis of starch (Pandey *et al*., 2000). At industrial scale microbes (Table 1) are a major source of amylases production (Lonasane and Ramesh, 1990). An important feature of amylase is its stability in harsh conditions (acid, base, temperature, UV and mutagens). 86 acid stabilizing alpha amylases has been purified from different strains of *Aspergillus* (Tokhadze *et al*., 1975).

As far as structure of alpha amylases are concerned, these metalloenzymes exhibit great structural resemblance with each other. All contained three domains; A, B and C. A barrel of eight α helixes, eight β sheets and a chloride ion present in the N-terminus of enzyme exhibits domain A. The key component of domain B is an aspartate residue that holds a calcium ion (Qian *et al*., 1993), although this region is quiet inconstant in term of size among various amylases (Janecek, 1997), but extremely crucial for proper activity of enzyme (e.g pH stability) and it has been hypothesized that active site, substrate binding site and $Ca^{+2}$ binding site are positioned somewhere between domain A and B. Domain C comprehends a cluster of β sheets in a Greek Key motif with no pronounced role, but still required by enzyme to execute its normal function. Few fungal amylases incorporate additional calcium ions at their active site, one such example is *Aspergillus niger* (Boel *et al*., 1990).

Asp195, Glu223 and Asp300 are dynamic constituents of active site, located at C-terminus of domain A in close vicinity to β-barrels with side chains are submerged somewhere in a cleft of domain A and B. Asp195 and Glu223 has pronounced role as nucleophile but purpose of Asp300 is not known. There might be a possibility that Asp300 is crucial for protonation and stabilization of an intermediate state (Uitdehaag *et al*., 1999; McCarter and Withers 1994).

Genetic engineering has reinforced the production of thermostable α-amylase to meet the growing ultimatum of brewing, textile, detergent and sugar industry. *Bacillus licheniformis* is previously known for its aptitude to increase the yield of significant industrial enzymes that include proteases, mannases, keratinases, amylases and β-lactamases among many

other. *Escherichia coli* is the frequently manipulated host system to achieve this purpose via different expression vectors, among them, pET-expression vectors are valuable because of T7 promoter that give better control of inserted genes along with vector self-ability to overcome host metabolic stresses (Janknecht *et al*., 1991).

This study was deliberated for production and purification of α-amylase from the *Bacillus licheniformis* by manipulating pET22b(+) expression vector in *Escherichia coli* (*E. coli*) and characterization of genetically modified recombinant enzyme.

## MATERIALS AND METHODS

**Chemicals, Bacterial strains, Plasmid and Media:** Chemicals; Kanamycin (Catalog number: 11815024) Thermo Fisher Scientific, Thermo Scientific GeneJET plasmid miniprep kit, Gene ruler (SM0311 1Kb) Fermentas Inc, Thermo Scientific GeneJET Gel Extraction Kit. T4 DNA ligase and Restriction endonucleases were acquired through Thermo Fisher Scientific (USA), were utilized according to manufacturer recommendations.

Bacterial strains; *Bacillus licheniformis* ATCC 27811-*α-amylase*, *E. coli*; DH5α cells for cloning and BL21-CodonPlus (DE3)-RIPL for expression of *α-amylase* gene.

Media; (Luria-Bertani) LB medium containing yeast extract, tryptone and NaCl was used for proliferation of all three bacterial strains at 37 °C. Plasmid; pET-22b(+)- Novagen | 69744 - EMD Millipore was used as expression vector.

**Cloning of *Bacillus licheniformis* ATCC 27811 *α-amylase* gene:** *α-amylase* gene was recuperated from recombinant *B.licheniformis* strain ATCC 27811 (Rashid *et al*., 2009; Malik *et al*., 2013) by double digestion of pET-21a(+)-*N-amy* using *EcoR*1 and *Hind*III restriction endonucleases and gene (1419 bp) was purified by Thermo scientific Gel Extraction kit. Purified gene was ligated in pET-22b(+) at site between *EcoR*1 and *Hind*III. pET-22b(+)-*amylase* was transformed to *E. coli* strain DH5α following the standard protocol (Sambrook and Russell, 2001). Positive clones were screened (restriction analysis) by isolation of recombinant plasmid following (Thermo scientific Miniprep Kit) manufacturer recommendations and results were visualized on 1% agarose gel. Isolated pET-22b(+)-*amylase* plasmid was then transformed into calcium chloride mediated competent cells of BL21 for comprehensive analysis of gene expression.

**Expression of *α-amylase* gene:** Confirmed BL21-Codon Plus (DE3-RIPL) clones were preceded for expressional analysis in LB culturing medium containing 30 µg/ml of ampicillin (antibiotic) at 37 °C for overnight. Next day secondary inoculum was placed and continued to induction with a total concentration of 0.1, 0.5 and 1 mM IPTG along with control (without IPTG). When the $OD^{600}$ of the bacterial culture reached 0.4. control and samples were harvested (5000 rpm for 15 minutes at 4 °C) to make a final suspensions in Tris-Cl pH 8.0 buffer. The protein expression was checked side by side on 15% Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

**Purification of α-amylase:** Expression mixture (5 mL) was subjected to ammonium sulphate precipitation (2.795 g/ml) to precipitate out some extra proteins by salting out method followed by dialysis. Protein mixture (5 ml) was filled in an activated dialysis tubing at 4 °C in 500 ml of dialysis buffer (20 mMTris-Cl pH 8.0) for 6-8 hours and old buffer was substituted with fresh buffer solution at regular intervals (6 hours). Protein solution was removed from dialysis tubing after 18 hours of continuous stirring and stored at -20 °C.

Dialyzed protein was further purified by means of Sephacryl S-200 HR column (1.5 cm × 40 cm) equilibrated by 20 mM Tris-Cl (pH 8.0) for gel filtration chromatography. Recombinant α-amylase was eluted by collecting 1.5 ml fractions (0.5 ml/min). Eluted protein was lyophilized and examined by 15% SDS-PAGE. Total amount of purified recombinant protein was assessed by Bradford assay (Bradford, 1976).

Partially purified sample was loaded on DEAE-sephadex (ion exchange chromatography) column (1.5 cm × 20 cm) and equilibrated by 20 mM Tris-Cl buffer (pH 8.0). NaCl Gradient (0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M) was used for enzyme elution with a flow rate of 0.7 ml/minute. After purification, sample was lyophilized and accessed by 15% SDS-PAGE. Bradford assay was done for estimation of total concentration of purified enzyme.

**Amylase activity assay:** This assay is suitable for the colorimetric detection of amylases activity in cells and tissues. In this assay purified enzyme (50 µl) and 1% starch solution (90 µl) was incubated at 80 ºC for 15 minutes. After incubation sample was ice chilled for 10 minutes and centrifuged at 12000 rpm for 5 minutes. Centrifuged sample along with 100 µl of 0.1 N NaOH and 2 ml of iodine solution (10 gm KI, 5 gm Iodine, 100 ml distilled water) was added in a separate microfuge tube. Control contains all the reaction components except enzyme. Absorbance of control and sample was taken at 660 nm. Total enzyme units were estimated as:

Total enzyme units= $Abs^{660}$ x D.F x Total amount of enzyme ÷ amount of enzyme in reaction.

**Starch-agar plate assay:** Soluble starch (1 gm) and agar (2 gm) was mixed in 100 ml of distilled water and poured on a disposable plate. Mixture was allowed to solidify at room temperature and fine holes were dug on the surface

of solid agar by slightly pressing a yellow tip. Purified enzyme (10 µl) was poured into the wholes and incubated at 70 ºC for 1 hour. Iodine solution (10 gm KI, 5 gm Iodine, and 100 ml distilled water) was added after incubation to perform the colorimetric analysis.

**Effect of temperature, pH and metal ion on enzyme activity:** Purified α-amylase was incubated at 8 different temperature ranges varies from 20 °C to 90 °C with an interval of 10 °C between each incubation, in Tris-Cl buffer (pH 8.0) with starch as mediator for 1 hour to determine the optimum temperature. Similarly, optimum pH was estimated by incubating enzyme with starch in Tris-Cl buffer at 5 different pH ranges from 5.0 to 9.0 at 37 ºC for 1 hour. Effect of metal ion was estimated in presence of 0.5 mM $K^{+1}$, $Na^{+2}$, $Mg^{+2}$, $Cu^{+2}$and $Ca^{+2}$.

## RESULTS

**Cloning of Bacillus licheniformis ATCC 27811 α-amylase gene:** α-amylase was known to have an insoluble expression before, but in previous study, we reported a soluble expression of α-amylase in *E. coli* at low temperature using pET-21a(+) expression system to minimize the problem associated with solubilization of insoluble inclusion bodies (Malik *et al*., 2013). In the present study α-amylase (*Bacillus licheniformis* ATCC 27811) gene provided in pET-21a(+) expression vector was double digested with *EcoR*1 and *Hind*III. The isolated gene fragment of 1419 bp was ligated in pET-22b(+) to check whether vector pelB coding sequence (signal sequence for periplasmic localization) will improve the expression and stability of α-amylase. Recombinant vector was transformed to *E.coli* strains (DH5α and BL21) and positive clones were preceded for plasmid isolation (Figure 1) followed by restriction analysis to confirm successful ligation.

**Expression of α-amylase gene:** Positive BL21-Codon Plus (DE3-RIPL) clones were headed for expression analysis of α-amylase gene at 0.1 mM, 0.5 mM and 1 mM IPTG concentration at 37 ºC for 16 hours. Maximum enzyme activity of 1.265 U was perceived in case of soluble (1 mM IPTG) induction sample as shown in figure 2.

The yield of active amylase was confirmed at 1 mM IPTG concentration in supernatant and this culture medium served as primordial stage of amylase purification following a series of purification techniques. Ammonium sulphate precipitates out a number of different proteins by salting out method. Dialyzed sample was preceded to gel filtration chromatography and eluted fraction were quantitatively and qualitatively analyzed (Figure 3). Multiple bands were seen on 15% SDS-PAGE as shown in figure 4A. On the basis of ambiguous results, we re-planned to cultured protein in bulk for Ion exchange chromatographic analysis and results were examined on 15% SDS-PAGE (Figure 4B). A single band of 52 *kDa* confirmed the successful purification of α-amylase enzyme. Purification scheme of α-amylase from *Bacillus licheniformis* is shown in table 2.

**Characterization of α-amylase:** Iodine-starch assay was used for analysis of purified enzyme and total activity came out to be 0.06 U. Result of starch-agar plate are shown in figure 5, where enzyme at each successive step of purification was analyzed, a prominent white zone of starch degradation was marked in region 2 of agar plate. Enzyme showed maximum activity between 65 °C to 72 °C with an optimum temperature of 70 °C (Figure 6A) and at pH 7-9 with an optimum pH of 8.0 (Figure 6B). Estimated $V_{max}$ and *Km* of the active enzyme was 584.61 U/mg and 5.4 mg/ml respectively (Table 3). $Cu^{+2}$ and $Na^{+1}$ have maximum inhibitory effect on α-amylase activity in contrast to $K^{+1}$and $Mg^{+2}$ which showed mild inhibitory effect. α-amylase showed maximum activity in presence of $Ca^{+2}$ with a total relative activity of 78% (Figure7).

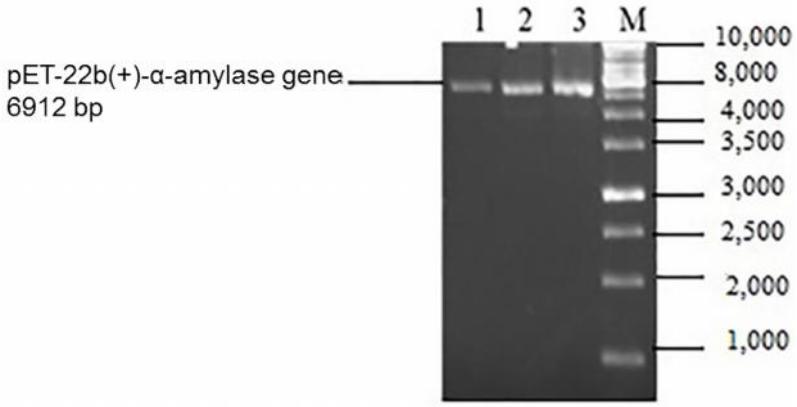


**Figure 1. Agarose gel analysis (1%) of plasmid DNA pET22b*(+)-α-amylase*; Lane M: shows 1kb DNA ladder, lane 1: Positive DH5α clone, Lane2,3: Positive BL21-Codon Plus (DE3-RIPL) clones.**

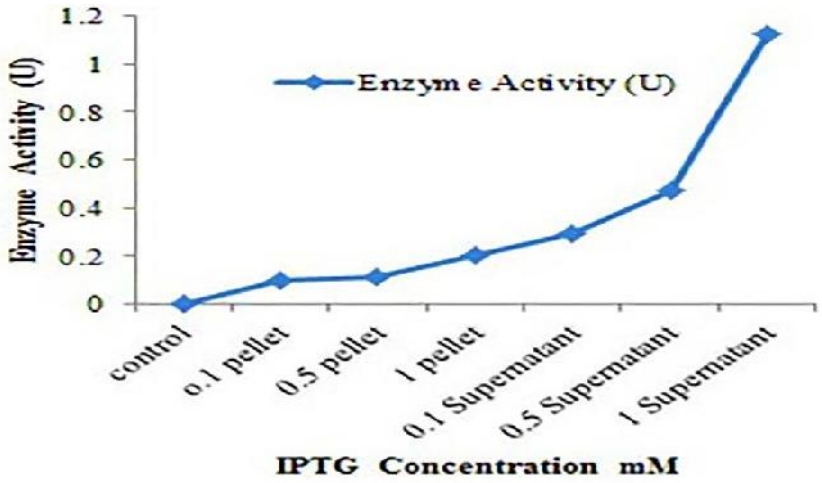


**Figure 2. Recombinant α-amylase activity (U) at different IPTG concentrations.**

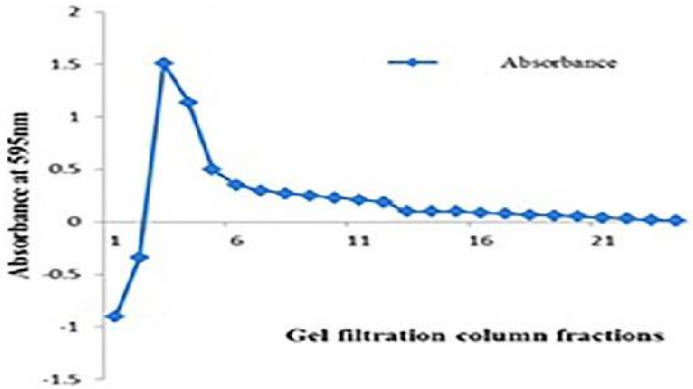


**Figure 3. The elution profile for recombinant α-amylase enzyme by Gel filtration Sephacryl S-200 HR column.**

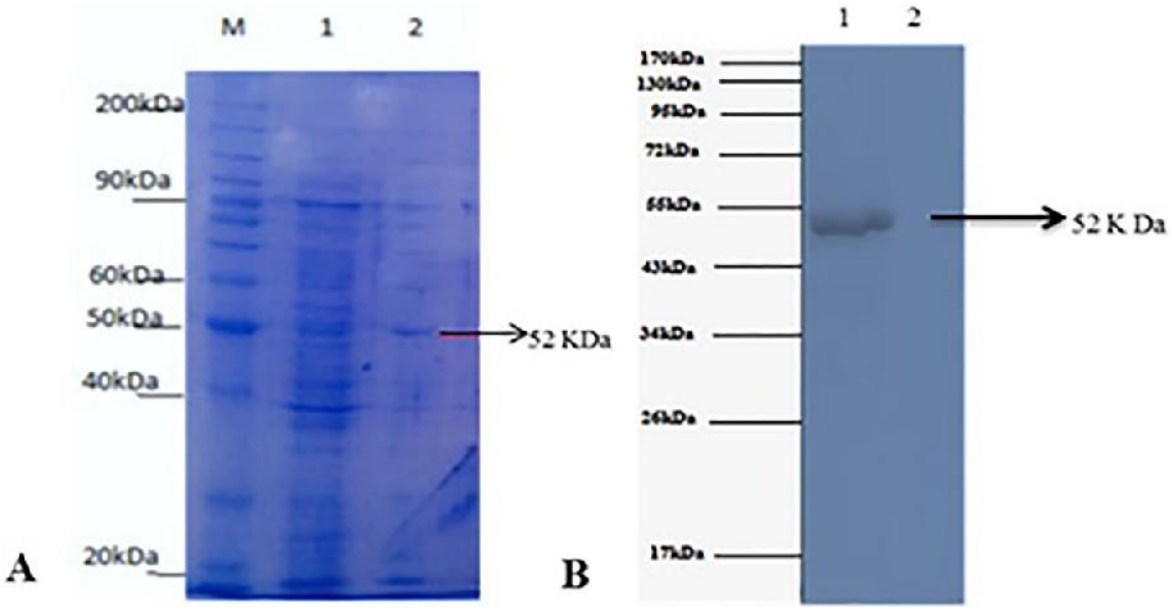


**Figure 4.** ***SDS-PAGE analysis (15%) of recombinant purified alpha amylase enzyme,*** **(A) Gel filtration chromatography. Lane M: Protein marker (Fermentas cat# Sm 0661); Lane 1; sample of alpha-amylase after dialysis, Lane 2: lyophilized fraction 2. (B) Ion exchange chromatography. Lane 1-2; lyophilized fraction 6 and 7.**

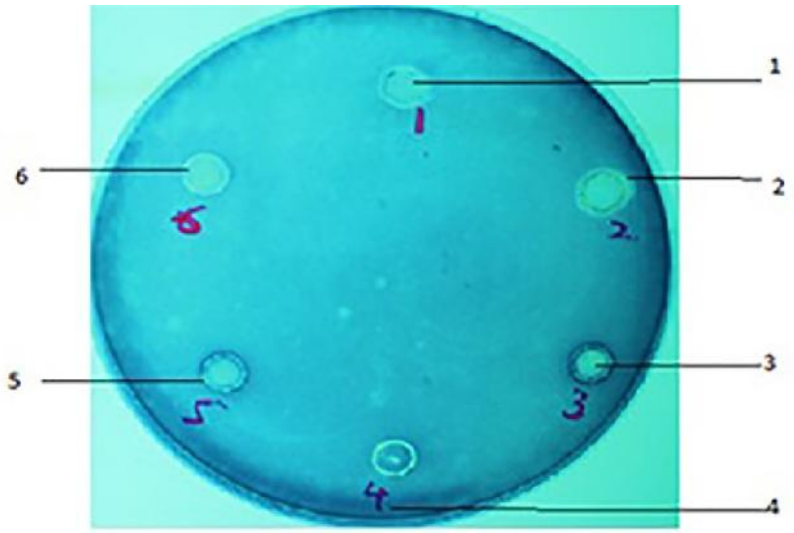


**Figure 5. Starch-agar plate (1%), 1; control, 2; Sample after ion exchange chromatography, 3; Sample after gel filtration chromatography, 4;Ammonium sulphate precipitated sample, 5-6; Dialysis sample.**

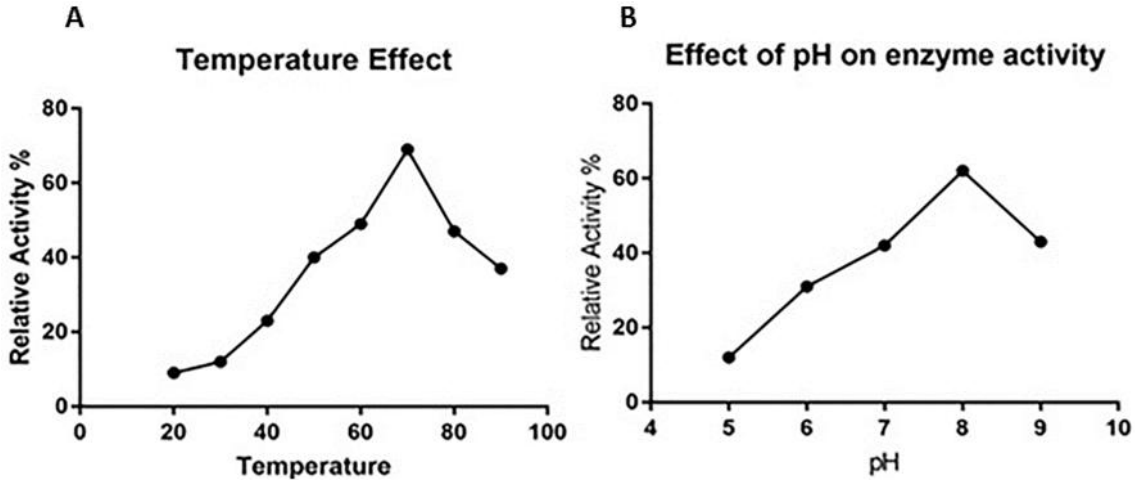


**Figure 6. *Optimum conditions for Bacillus licheniformis α-amylase* (A) Optimum temperature for optimum activity of enzyme. (B) Optimum pH for enzyme activity.**

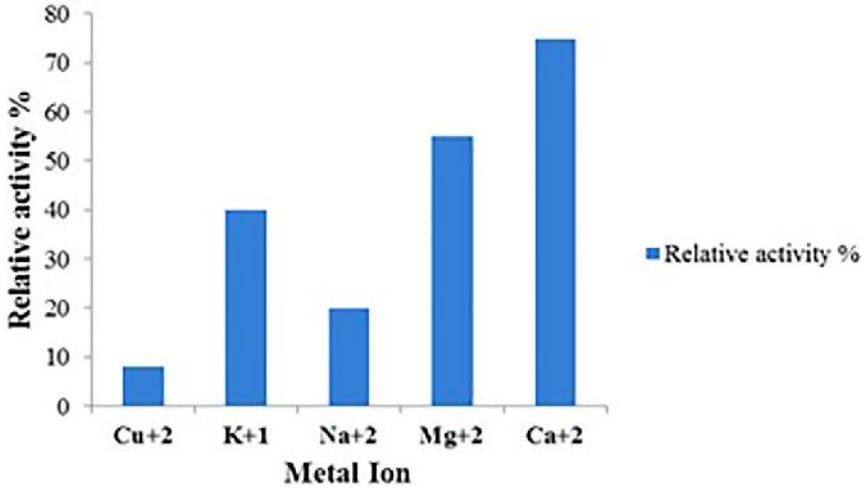


**Figure 7. Effect of Metal ions on *Bacillus licheniformis* α-amylase activity.**

**Table 1. Commonly reported sources for purification and characterization of α-amylases from literature.**

| Source of α-amylases | Reference |
|---|---|
| *Aspegillus flavus* | El-Safey *et al*., 2002 |
| *Aspergillus fumigatus* | Planchot and Colonna, 2008 |
| *Aspergillus oryzae* | Yabuki *et al*., 1977 |
| *Aspergillus niger* NRRL-337 | Mahmoud *et al*., 1978 |
| *Aspergillus niger* ATCC 16404 | Djekrif-Dakhmouche *et al*., 2006 |
| *Aspergillus niger* NRRL 3112 | Mariani *et al*., 2000 |
| *Aspergillus ficuum* | Hayashida and Teramoto, 1986 |
| *Aspergillus kawachii* | Omori *et al*., 1994 |
| *Aspergillus nidulans* | Agger *et al*., 2001 |
| *Anoxybacillus species* | Chai *et al*., 2012 |
| *Bacillus stearothermophilus* | Demirkan *et al*., 2005 |
| *Bacillus coagulans* | Hawary, 1991 |
| *Bacillus subtilis* | Huang *et al*., 2003 |
| *Bacillus licheniformis* | Haq *et al*., 2005 |
| *Bacillus amyloliquefaciens* | Tayeb *et al.,* 2007 |
| *Bacillus thermooleovorans* | Ngernyuang *et al.,* 2011 |
| *Bombyx mori L* | Ngernyuang *et al.,* 2011 |
| *Clostridium acetobutylicum P262* | Madihah *et al.,* 2000 |
| *Escherichia coli* | Rashid *et al*., 2009 |
| *Geobacillus thermoleovorans* | Mehta and Satyanarayana, 2013 |
| *Humicola lanuginose* | Singh *et al*., 2009 |
| *Lactobacillus manihotivorans* | Ray, 2001 |
| *Penicillium sp.* | Ray, 2001 |
| *Pyrococcus furiosus* | Jorgensen *et al.,* 1997 |
| *Pichia pastoris* | Rydberg *et al.,* 1999 |
| *Streptococcus bovis* | Satoh *et al*., 1993 |
| *Thermomyces lanuginosus* | Petrova *et al*., 2000 |
| Oryza sativa | Karrer *et al*., 1991 |

**Table 2. Scheme for purification of recombinant α-amylase from *Bacillus licheniformis*.**

| Purification step | Total enzyme activity (U/ml) | Total protein concentration (mg/ml) | Specific activity of enzyme (U/mg) | Purification fold Of enzyme | Yield of enzyme (%) |
|---|---|---|---|---|---|
| Culture Supernatant | 89 | 0.9 | 98.88 | 1 | 100 |
| Ammonium-Sulphate precipitation | 80 | 0.62 | 129.03 | 1.30 | 89.88 |
| Gel-Filtration chromatography | 63 | 0.22 | 286.36 | 2.21 | 70.78 |
| Ion-Exchange chromatography | 76 | 0.13 | 584.61 | 2.04 | 85.39 |

**Table 3. Kinetics study of purified α-amylase enzyme from *Bacillus licheniformis*.**

| Substrate | *Km* (mg/ml) | *Vmax* (U/mg) | Optimal pH | Optimal Temperature (°C) | Molecular Weight (kDa) |
|---|---|---|---|---|---|
| Starch | 5.4 | 584.61 | 8.0 | 70 °C | 52 |

* Data is given as Mean of triplicate experimental value

## DISCUSSION

Enzymes are extensively used in all industries that deals with organic compounds. They are used variably, for example, as an ingredients of detergents, reagents for analysis of drugs and blood components, food additives, in the paper industry, and for purification purposes. Enzymes that are involved in starch hydrolysis occupied 30% of total industrial catalysts. A hallmark of these enzymes is to endure high temperature, acid and

basic conditions that are customary requirements in order to attain desired products in industrial processes. Recombinant DNA technology has facilitated the production of α-amylases with projected specificities from bacterial, fungal and algal extracts in order to cope up with severe reaction conditions to attain maximum yield.

In this study α-amylase from *Bacillus licheniformis* ATCC27811 was purified and characterized by manipulating pET-22b(+) expression system. *α-amylase* gene was ligated between *EcoR*1 and *Hind*III site (Figure 1) and expressed in BL21 strain of *E. coli*. Enzyme activity of 1.265 U was estimated in soluble sample with 1 mM IPTG concentration at 37 °C after 16 hours (Figure 2). These results are supported by another study conducted by Pervaiz *et al*., where soluble expression of *B.licheniformis* α-amylase-pET-28a(+) was reported at 37 °C after 4 hours with 1 mM IPTG concentration (Pervaiz *et al*., 2017) but contrary to Rashid *et al*., as they documented insoluble expression of *B.licheniformis* ATCC27811 *α-amylase* gene by exploiting pET-21a(+)expression vector (Rashid *et al*., 2009).

A purification scheme containing three discrete steps was employed for purification of α-amylase with a specific activity of 584.61 U/mg and 85.39% yield (Table 2). Purification efficiency of purified enzyme was 2.04 times in comparison to crude amylase extract and molecular weight was 52 *kDa* (Figure 4) in comparison to 56 *kDa* documented before (Pervaiz *et al*., 2017). Purified enzyme was analyzed by iodine-starch analysis (0.06 U) and starch-agar analysis as shown in figure 5.

α-amylase from *B.licheniformis* remained stable at 65 °C to 72 °C with a relative activity at 77% at 70 °C (Figure 6A). From literature the stated temperature at which enzyme showed a good range of activity is 40-100 ºC, but the optimum temperature for α-amylase is 90 ºC (Rashid *et al*., 2009; Morgan and Priest, 1981). These enzymes maintain 79% of its activity at 80 ºC and 92% activity at 100 ºC (Hmidet *et al*., 2008).

A number of studies have declared the optimum pH of α-amylase from *Bacillus licheniformis* was 6.5 and a maximum activity falls within range of 5-10. At pH 5 it shows 89% activity, at pH 9 it shows 96.6% activity and at pH 10 it shows 90% activity (Hmidet *et al*., 2008; Bose and Das, 1996). Sometimes it depicts maximum activity at pH 6.0 but drastically lost 80% of its activity at pH 9.0 (Lee *et al*., 2006). In our study, optimal pH range for enzyme activity was 7-9 with relative activity of 62% was observed at pH 8.0 (Figure 6B).

Kinetics of α-amylase have been calculated using Michealis and Menten equation by incubating enzyme at different concentrations of its substrate (1% soluble starch). $K_m$ and $V_{max}$ of enzymes showed a great degree of variability because of their dependence on a number of factors like experimental conditions and substrate employed. (Eisenthal *et al*. 2007). In our study, estimated $V_{max}$ and $K_m$ of recombinant α-amylase form *Bacillus licheniformis* was 584.61 U/mg and 5.4 mg/ml respectively (Table 3). Decrease in *Km* referred to increase affinity of enzyme towards its substrate. Our data is conferring the previously reported results where $V_{max}$ and $K_m$ values for *Bacillus amyloliquefaciens* and *Lactobacillus manihotivorans* were 3.076 mg; 4.11 mg/min (Gangadharan *et al*., 2008) and 0.45 mg; 3.44 mg/ml respectively (Goyal *et al*., 2005).

Amylases are sensitive to chelating agents which make them an impeccable metallo-enzyme. Different ions have different impact on amylase activity; $Zn^{+2}$ and $Hg^{+2}$ inhibits while $Ca^{+2}$, $Ba^{+2}$ and $Fe^{+2}$ enhance amylase activity, however, $Mg^{+2}$ has no pronounce effect. These findings coincide with our results where $Cu^{+2}$ and $Na^{+1}$ impeded α-amylase whereas $Ca^{+2}$ strongly enhanced its activity (Figure 7).

**Conclusion:** This research contributes to soluble production of α-amylase in order to minimize the complications associated with insoluble expression of recombinant enzyme. pET-22b(+) expression system provides an insight for better yield of soluble, active and thermostable α-amylase enzyme in *Escherichia coli,* that could be appropriate for hydrolysis of starch at elevated temperatures. Therefore, recombinant alpha amylase could be a good alternative to use at industrial scale with some modifications to withstand adverse reaction conditions. Present study provides a base for further exploration of thermodynamics and industrial application of above mentioned recombinant enzyme.

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**Conflict of interest:** No conflict of interest.

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