Advances in Biochemistry and Biotechnology
Mehdi A, et al. Adv Biochem Biotechnol 8: 10121.
www.doi.org/10.29011/2574-7258.010121
www.gavinpublishers.com

Research Article

Production of A Stable Recombinant α-amylase Enzyme from Thermoanaerobacterium: Thermoanaerobacterium in Pilot Scale Fermenter

Mehdi A[1], Javaid Z[2], Aftab MN[1*]

[1]Dr. Ikram-ul-Institute of Industrial Biotechnology, Government College University Lahore, Pakistan

[2]Lahore Grammar School, 1-A-1 Gulberg Lahore, Pakistan

[*]**Corresponding author:** Aftab MN, Dr. Ikram-ul-Institute of Industrial Biotechnology, Government College University Lahore, Pakistan.

Citation: Mehdi A, Javaid Z, Aftab MN (2024) Production of A Stable Recombinant α-amylase Enzyme from Thermoanaerobacterium: Thermoanaerobacterium in Pilot Scale Fermenter. Adv Biochem Biotechnol 8: 10121. DOI: https://doi.org/10.29011/2574-7258.010121

Received Date: 21 June, 2024; **Accepted Date:** 27 June, 2024; **Published Date:** 01 July, 2024

Abstract

This study investigated the production of stable α-amylase from thermopile bacteria *Thermoanaerobacterium thermoanaerobacterium*. The gene encoding this enzyme was cloned and expressed in *E. coli* using IPTG as the standard inducer of T7 promoter. For large-scale production optimization, several parameters were adjusted. High recombinant enzyme yield was achieved u*nde*r conditions where the medium volume comprised 60% of the fermenter's total volume, aeration rate was set at 2.5 vvm, dissolved oxygen was maintained at 15%, and agitation speed of 150 rpm was used. Using the optimized conditions, highest enzyme production was reached to 6.17 ± 0.32 U/ml/min with total proteins of 4.73 ± 0.04 mg/ml and viable cells 5.82 $\times 10^7$ cells/ml. When the *E. coli* cells were induced with IPTG and lactose, maximum enzyme production (16.8 U/ml/min) was obtained when induction was done with 0.5 mM lactose. Maximum protein produced was 9.62 ± 0.24 mg/ml.

Key words: Recombinant; α-amylase; *Thermoanaerobacterium thermoanaerobacterium*; pilot scale.

Introduction

Amylases are essential enzymes that hydrolyze the α-1-4 glycosidic linkages in starch, breaking it down into glucose polymers [1]. They are among the most significant industrial enzymes, accounting for about 25% of the global enzyme market, and hold great importance in biotechnology [2]. Amylases are found in animals, plants, and microorganisms, with microbial amylases being the most suitable for industrial needs because of their versatility and stability [3]. These enzymes are used extensively across various industries, including food, paper, starch saccharification, fermentation, textile desizing, detergents and pharmaceuticals [4]. Thermostability is a crucial feature for industrial applications of amylases, as starch becomes soluble at temperatures above 100°C. Therefore, most industrial processes involving α-amylases require them to function efficiently at temperatures more than 100°C [5].

To meet industrial demands, continuous research is necessary on thermostable amylases to investigate and highlight their unique properties [6]. Growing thermophilic organisms at an industrial scale is challenging due to their stringent requirements. This issue is often resolved by employing recombinant DNA technology to clone genes into mesophilic hosts [7]. Achieving high levels of gene expression from these cloned genes necessitates developing innovative processes. One such process involves the highly effective T7-expression system in the *Escherichia coli* (strain BL21), which is widely used to produce heterologous recombinant proteins [8]. T7 promoter centered pET-vectors, as initially reported by Studier et al., are the most commonly employed vectors for this purpose [9]. Typically, IPTG is used to induce gene expression u*Nde*r the influence of a promoter. However, IPTG is costly and toxic to cells, highlighting the need for an alternative [10]. Lactose, the natural inducer of the lac operon, is a cheaper and non-toxic substitute for IPTG. Additionally, lactose serves as a carbon and energy source for cells, making it a more efficient and economical option for

regulating recombinant gene expression [11].

The main objective of the current research was to optimize culture conditions and fermentation media to enhance cost-efficiency and maximize enzyme production. Various fermentation methods, such as submerged and solid-state fermentation, were employed. Submerged fermentation is favored in many industrial applications because it offers excellent process control, efficient bioreactor design, and facilitates fermentation modeling [12]. Furthermore, submerged fermentation presents distinct advantages in downstream processing, particularly in reducing costs associated with medium components [13].

Recent advancements in fermentation techniques have heightened interest in scaling up α-amylase production. Within biotechnology, maximizing enzyme output has become pivotal [14]. Improving culture conditions, including adjusting inoculum size, optimizing dissolved oxygen levels, refining agitation rates, and employing advanced techniques, plays a critical role in enhancing enzyme production significantly [15]. The objective of this study was to optimize critical parameters for developing a fermentation approach to produce cloned α-amylase from *Thermoanaerobacterium thermoanaerobacterium* in *E. coli* on a large scale.

Methodology

Bacterial Strain and Plasmid

Genomic DNA from *Thermoanaerobacterium thermoanaerobacterium* was sourced from the DSMZ collection in Germany for the amplification of the amylase gene. The amplified amylase gene was then expressed in *Escherichia coli* BL21 using the pET-21b (+) vector.

The amylase gene of *Thermoanaerobacterium thermoanaerobacterium* was amplified using genomic DNA, with primers designed using Primer 3 software. The forward primer included an *Nde*I site at its 5' end, and the reverse primer had *EcoR*I site at its 5' end. Primer sequences were: Forward 5'-GCCATATGATAGGTGATTTGCGTGGGA-3', Reverse 5'-GCTCACTCCGTCATTCGCCACATCGTA-3'. The 1544 bp amylase amplifies gene was run and examined on agarose gel (1%), purified utilizing Qiagen purification kit, then double digested with *Nde*I and *EcoR*I. After ligation into pET-21a (+), the plasmid-PCR product was transformed into competent cells of *E. coli* BL21 [16]. Positive clones were confirmed via restriction analysis with *EcoR*I and *Nde*I of the recombinant pET-21a (+) ligated with α-amylase gene.

Escherichia coli BL21 cells expressing recombinant a-amylase gene

For recombinant enzyme expression, *E. coli* BL21 cells were cultured in 0.1 L Luria–Bertani medium augmented with 100 mg/ml ampicillin at 37°C until reaching an optical density of 0.5–0.7 at 600 nm. Induction was achieved with 0.5 mM IPTG, followed by a 4-hour incubation at 37°C. Cells were harvested by centrifugation (6000 rpm, 10 min, 4°C), lysed via 10 min sonication in 5 ml 50 mM Tris–HCl (pH 7.5–8), and centrifuged (12,000 rpm, 10 min). Total proteins were quantified using Bradford assay [17]. α-amylase expression was assessed in both cell fractions using SDS-PAGE (12%) stained using Coomassie blue [18].

Fermentation Techniques

Fermentation was carried out for 4 hours at 37°C in a 2 L stirred fermenter (glass). LB medium was added in the glass vessel and sterilized at 121°C and 15 psi for 30 minutes. After autoclave, the assembly was fitted. Inoculum transfer was performed under aseptic conditions and incubated at 25°C. Aeration and agitation were set at 200 (rpm) and 2.0 vvm, respectively, until O.D. reached 0.5. Air sterilization was done using a 0.3 mm membrane filter. Silicone oil (10%) was used to control frothing. Upon reaching an OD of 0.5, cells were induced with 0.5 mM IPTG and incubated at 25°C for an additional 8 hours. The broth was centrifuged at 5000 g at 4°C for 10 minutes. Supernatant was kept at 4°C, and cells of *Escherichia coli* cells were sonicated. Intracellular and extracellular enzyme estimations were performed using clear cell lysate and supernatant, respectively.

Effects of Various Parameters:

Agitation

Agitation speeds from 100 to 300 rpm were tested with 3% inoculum, 20% dissolved oxygen, 60% fermentation medium volume, and 3 vvm aeration.

Inoculum

Different inoculum sizes (1-6%) were tested with 200 rpm agitation, 20% dissolved oxygen, 60% fermentation medium volume, and 3 vvm aeration.

Dissolved oxygen

The 10-30% of D.O. were examined using 3% inoculum, 150 rpm agitation, 2.5 vvm aeration, and 60% fermentation medium volume.

Fermentation medium volume

Volumes from 40-90% were tested with 3% inoculum, 150 rpm agitation, 20% dissolved oxygen, and 2.5 vvm aeration.

Aeration rate

Aeration rates from 0.5-3.0 vvm were studied with 3% inoculum, 150 rpm agitation, 20% dissolved oxygen, and 60% fermentation medium volume.

Enzyme assay

Enzyme activity was determined using the DNS method, where reducing sugars were measured [19]. Maltose acted as the control, and the released reducing sugars were quantified at 550 nm using a spectrophotometer. Enzyme action was defined as the amount of enzyme needed to liberate one mole of reducing sugar from substrate using specified conditions.

Protein assay

Enzyme activity was assessed via the DNS method, quantifying reducing sugars with maltose as a standard and measuring absorbance at 550 nm. This method defines enzyme activity as the enzyme quantity needed to release one mole of reducing sugar under defined conditions.

Results and discussion

Amplification of *T. thermoanaerobacterium* α-amylase gene was achieved using specific primers, yielding a 1544 bp PCR product (Fig. 1). After double digestion with *Nde*I and *EcoR*I, the gene was ligated into pET-21b (+) using T4 DNA ligase. Transformation into *E. coli* BL21 (DE3) confirmed successful cloning through *EcoR*I and *Nde*I digestion and gene sequencing. A 6754 bp band post-*EcoR*I digestion validated amylase gene ligation into pET-21b (+) (Figure 1).

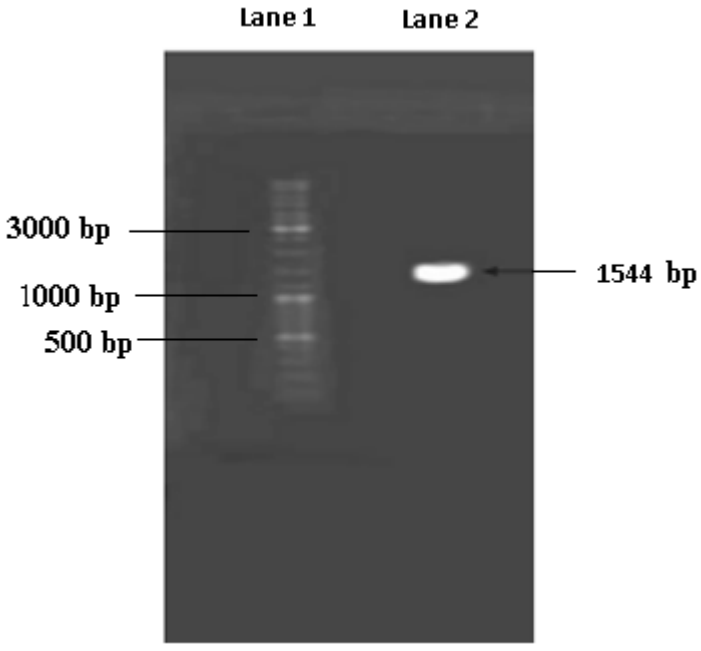

Figure 1: Agarose gel showing amplified α-amylase gene. Lane 1: DNA ladder (Fermentas#0341), Lane 2: amplified α-amylase gene (1544 bp).

Cloning of α-amylase gene in pET-21b (+) Vector

The cloning of the α-amylase gene from *T. thermoanaerobacterium* into the pET-21b (+) expression vector was meticulously executed to facilitate its expression in *Escherichia coli* BL21 (DE3). Initially, specific primers were designed and employed to amplify a target DNA fragment of 1544 base pairs (bp), corresponding to the α-amylase gene. This amplified product was then purified and subjected to double digestion using *Nde*I and *EcoR*I restriction enzymes, ensuring the creation of compatible cohesive ends required for subsequent ligation steps. Following digestion, the purified α-amylase gene fragment was ligated into the pET-21b (+) vector using T4 DNA ligase. This vector, renowned for its robust expression capabilities in *E. coli*, was chosen to facilitate high-level production of the recombinant α-amylase. The resulting construct, designated as pET-21b (+)/α-amylase, was then introduced into competent *E. coli* BL21 (DE3) cells via transformation. To confirm successful cloning, the recombinant plasmid was analyzed through double digestion with *EcoR*I and *Nde*I enzymes, revealing a distinct band at 6885 bp on agarose gel electrophoresis. This band corresponds to the combined size of the pET-21b (+) vector and the inserted α-amylase gene fragment, confirming the accurate insertion of the gene into the vector. In literature one study was reported in which thermostable α-amylase gene from *Pyrococcus woesei* in Escherichia coli was cloned [20].

Expression of the cloned α-amylase gene

After successful cloning, the expression of the α-amylase gene in *E. coli* BL21 (DE3) was assessed both intracellularly and extracellularly. Cultivated bacterial cells harboring the pET-21b (+)/α-amylase construct were subjected to sonication to lyse the cells and release their contents, facilitating the extraction of intracellular proteins. Intracellular extracts exhibited significant α-amylase activity, quantified at 2.5 U/ml/min, indicating robust expression within the bacterial cells. Conversely, extracellular samples showed minimal α-amylase activity, suggesting that the majority of enzyme production occurred intracellularly. To further validate α-amylase expression, SDS-PAGE analysis was conducted on both intracellular and extracellular fractions. In the intracellular fraction, a prominent band approximately 70 kDa in size was observed, corresponding to the molecular weight of the α-amylase enzyme from *T. thermoanaerobacterium*. This band was absent in negative controls (wild-type *E. coli* BL21 (DE3)) and showed only faint presence in the extracellular fraction, affirming successful intracellular expression of the α-amylase gene. In recent study, it has been shown that amylase possess size of 56 kDa [21].

Optimization of parameters for α-amylase production

To enhance α-amylase production, various fermentation parameters were optimized using a 2 L fermenter equipped with a 1 L round-bottom flask.

Agitation Rate

Agitation rate plays a crucial role in oxygen supply and mixing efficiency within the fermentation medium, directly impacting enzyme production [22]. The study evaluated agitation rates ranging from 100 to 250 rpm using 3% inoculum, 20% D.O level, 60% medium volume, and 2.5 vvm aeration rate. Optimal enzyme activity of 4.35 ± 0.07 U/ml/min was achieved at 150 rpm, accompanied by a total protein amount of 3.27 ± 0.11 mg/ml. Higher agitation intensities beyond 200 rpm led to reduced enzyme synthesis because of oxidative stress and excessive foaming, whereas lower rates hindered oxygen supply and mixing efficiency, limiting enzyme synthesis [23]. Simair and Mangrio demonstrated that maximum amylase production was achieved at 200 rpm [24] Amylase production at 100, 200 and 250 rpm was analyzed as 2.31 ± 0.05 U/ml/min, 3.72 ± 0.06 U/ml/min and 2.74 ± 0.07 U/ml/min and total proteins were calculated to e 1.85 ± 0.07 mg/ml, 2.86 ± 0.09 mg/ml and 2.27 ± 0.07 mg/ml respectively. Total viable cells were 2.13 x 10^7/ml, 3.26 x 10^7/ml, 2.41 x 10^7/ml and 1.92 x 10^7/ml and total protein was 2.18 mg/ml, 2.86 mg/ml and 1.53 mg/ml at 100, 150, 200 and 250 rpm, respectively (Figure 2).

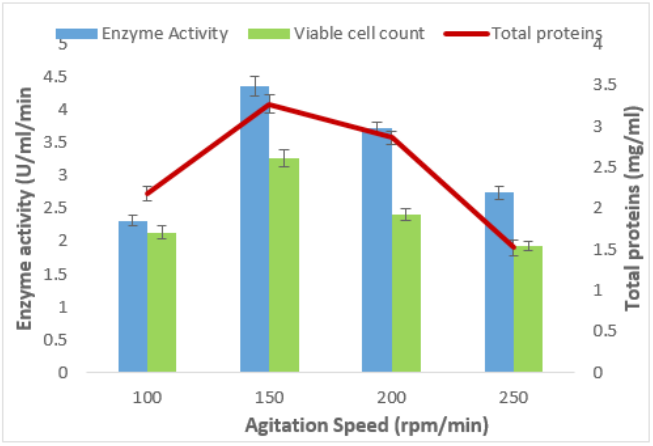

Figure 2: Effect of aeration rate on α-amylase production.

Inoculum Size

The size of the inoculum significantly influences microbial growth and subsequent enzyme production in fermentation. Testing inoculum sizes ranging from 1% to 5%, optimal α-amylase activity of 4.48 ± 0.12 U/ml/min was attained with a 3% inoculum, agitation rate, 60%, volume of the medium, 20% dissolved oxygen level and 2.5 vvm aeration rate yielding a total protein content of 3.41 ± 0.16 mg/ml and a viable cell count of 3.08 × 10^7/ml (Fig 3). Less inoculum sizes resulted in inadequate microbial growth and decreased enzyme activity, while higher sizes led to nutrient depletion and metabolic inefficiencies, thereby reducing enzyme synthesis [25]. The activity of α-amylase was noted to be 2.83 ± 0.09 U/ml/min and 3.04 ± 0.10 U/ml/min having total protein content of 1.73 ± 0.09 mg/ml and 3.31 ± 0.07 mg/ml, 3.67 ± 0.11 mg/ml and 1.97 ± 0.08 mg/ml with viable cell count of 1.78 x 10^7 / ml and 2.43 x 10^7/ml, 3.08 x 10^7/ml and 1.26 x 10^7/ml once 1% and 2%, 4% and 5% inoculum was utilized, respectively (Fig. 3). One study has shown that maximum enzyme production from Bacillus was observed when 5% inoculum was utilized [26].

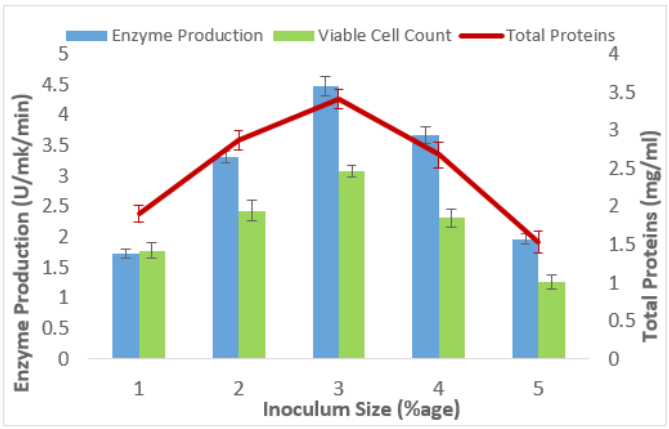

Figure 3: Effect of inoculum size on α-amylase production.

Dissolved Oxygen Level

Maintaining appropriate dissolved oxygen levels is critical for aerobic fermentation and enzyme production. Studies explored dissolved oxygen concentrations ranging from 10% to 25%, using inoculum (3%) at agitation speed (150 rpm) and aeration rate (2.5 vvm) and adding medium (60%), revealing peak α-amylase activity of 4.58 ± 0.21 U/ml/min at 15% dissolved oxygen. The viable cell count and total proteins were 3.29 ± 0.16 mg/ml and 2.54 x 10^7/ml respectively (Fig 4). Lower levels impeded microbial growth, while higher concentrations induced oxidative stress and toxicity, both detrimental to enzyme synthesis [27]. At high concentrations, oxygen can exceed the saturation level, resulting in the production of lethal compounds such as hydrogen peroxide and superoxide in the fermentation media [28, 29]. At 10%, 20%, and 25% dissolved oxygen, reduced enzyme activity was observed at 1.74 ± 0.12 U/ml/min, 3.25 ± 0.17 U/ml/min, 2.67 ± 0.13 U/ml/min and low level total proteins was obtained (2.48 ± 0.11 mg/ml, 2.69 ± 0.16 mg/ml and 2.37 ± 0.14 mg/ml) and viable cell count was 1.87 x 10^7 /ml, 1.47 x 10^7 /ml and 1.79 x 10^7/ml were calculated (Fig. 4). According to Elmansy et al. [30], amylase production from thermos halophilic bacteria was increased in a fermenter using dissolved oxygen (15%).

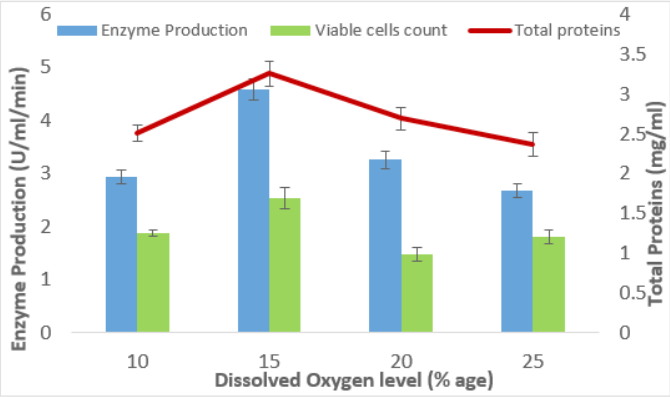

Figure 4: Effect of dissolved oxygen on α-amylase production.

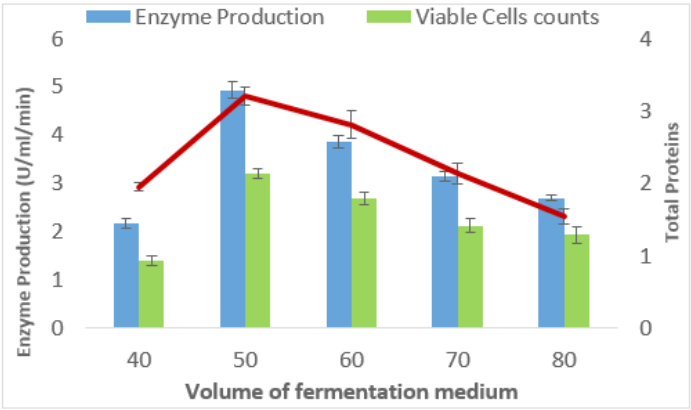

Figure 5: Effect of volume of fermentation on α-amylase production.

Volume of Fermentation Medium

The fermentation medium volume showed a vital role in the enzymes production as in Bacillus sp. utilizing submerged fermentation. In the realm of industrial microbiology, optimizing fermentation conditions is crucial for maximizing enzyme production [31]. This is exemplified in studies focusing on *Bacillus* sp. where submerged fermentation is utilized. To investigate the impact of fermentation medium volume on enzyme production, experiments were conducted using different volumes ranging from 40% to 80% of the total fermenter capacity. The experiments were carried out in a fermenter under controlled conditions: inoculum size (3%), agitation speed (150 rpm), dissolved oxygen (15%), and 2.5 vvm (volume of air per volume of liquid per minute) aeration rate. The results indicated a clear dependency of enzyme production on the volume of the fermentation medium. Optimal enzyme production, measured at 4.93 U/ml/min, was achieved when the medium volume was set at 60%. This volume facilitated the highest enzyme activity due to favorable conditions for microbial growth, adequate nutrient availability, and optimal oxygen supply [32]. The corresponding total protein content was recorded at 3.29 ± 0.14 mg/ml/min, with a viable cell count of 3.2 × 10^7 cells/ml (Fig 5). In contrast, deviations from this optimal volume resulted in diminished enzyme yields. For instance, at lower volumes (40%), enzyme activity dropped significantly to 2.16 ± 0.06 U/ml/min, with a lower total protein content of 1.41± 0.08 mg/ml and a viable cells of 1.39 × 10^7 cells/ml. Similarly, at higher volumes (80%), enzyme activity decreased to 2.69 ± 0.11 U/ml/min, accompanied by a total protein content of 1.54 ± 0.10 mg/ml and a viable cells of 1.93 × 10^7 cells/ml (Fig 5). These observations underscored the critical balance needed in medium volume to sustain optimal conditions for microbial growth and enzyme production. Similar finding was reported by Ye et al who worked on cellulase of *Bacillus amyloliquefaciens* [33].

Aeration Rate

The rate of aeration also played a pivotal role in optimizing enzyme production [34]. Different aeration levels ranging from 0.5 to 3.0 vvm were tested while maintaining consistent parameters of inoculum size (3%), agitation speed (150 rpm), dissolved oxygen (20%) and volume of the medium (60%). The highest enzyme activity of 6.17 ± 0.32 U/ml/min was observed at an aeration rate of 2.0 vvm. This level of aeration provided optimal conditions for microbial respiration and metabolic activity, resulting in the highest enzyme yield. The corresponding total protein content was 4.73 ± 0.04 mg/ml, with a viable cell count of 5.82 ×10^7 cells/ml (Fig 6). In recent study, it has been shown that maximum α-amylase production by *Aspergillus flavus* was carried out at 0.5 vvm [35]. Deviations from this optimal aeration rate led to varied enzyme activities: at lower aeration rates (0.5 to 1.5 vvm), enzyme production decreased due to inadequate oxygen supply, impacting microbial growth and metabolic efficiency. Conversely, higher aeration rates (2.5 to 3.0 vvm) showed a decline in enzyme activity, potentially due to oxidative stress on the microbial cells, despite sufficient oxygen availability. These findings highlight the delicate balance required in aeration levels to sustain optimal enzyme production (Fig 6). Moderate levels of aeration (around 2.0 vvm) proved to be most favorable, providing adequate oxygen for microbial growth and metabolic activity without inducing oxidative stress. One study has demonstrated that optimal production of thermostale α-amylase from *Thermotoga petrophila* can be produced at 3.0 vvm [36].

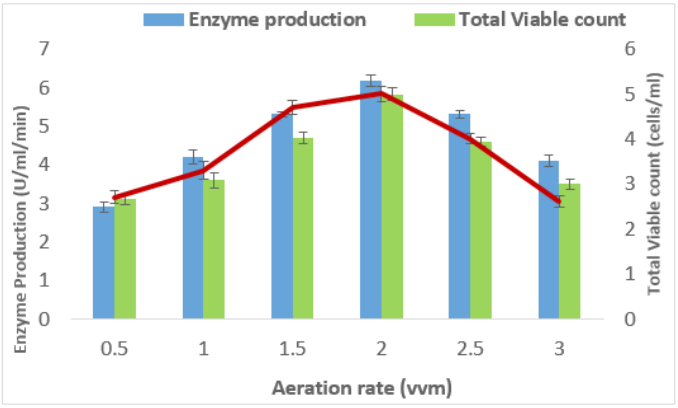

Figure 6: Effect of aeration rate on α-amylase production.

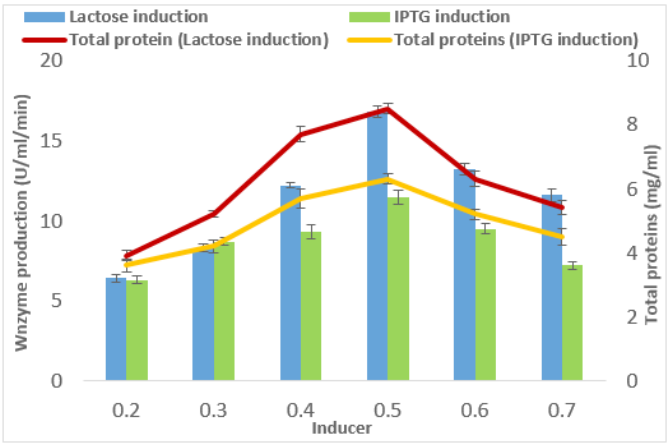

Figure 7: Effect of inducers on α-amylase production.

Inducers (IPTG and Lactose)

In the context of recombinant enzyme production, inducer optimization is crucial for maximizing protein expression. Using the T7-regulated pET 21a (+) vector, two inducers, IPTG and lactose, were evaluated for their efficiency in inducing α-amylase production. These experiments were conducted with different concentrations (0.2 to 0.7 mM) of IPTG and lactose, each with the constant conditions of 3% inoculum size, 150 rpm agitation speed, 2 vvm aeration rate, 20% dissolved oxygen, and 60% medium volume. The results demonstrated significant variations in α-amylase production based on the inducer used. Lactose, as a natural inducer of the lac operon, exhibited superior performance, yielding a maximum enzyme activity of 16.8 ± 0.32 U/ml/min and a total proteins of 9.62 ± 0.24 mg/ml when used at concentration of 0.05 mM. One research group has worked on the stimulation of lac operon with lactose and got high yield of metabolites in *E. coli* [37]. In comparison, IPTG induction with same concentration resulted in a lower enzyme yield of 11.47 ± 0.37 U/ml/min and a total proteins of 7.67 ± 0.23 mg/ml (Fig 7). The enhanced performance of lactose as an inducer highlighted its non-toxicity to microbial cells and its effective utilization as a carbon source, contributing to higher enzyme yields across different media formulations.

Conclusion:

The recombinant αamylase produced at large scale with high yield in pilot scale fermenter. The produced amylase could be used in various industries like food, fermentation, textile desizing, paper, detergents and pharmaceuticals.

Acknowledgments: Thanks to Higher Education Commission (HEC) Pakistan for providing funds for this project.

Ethical Considerations: Not Applicable.

Conflict of Interest: There is no conflict of interest.

References

1. Souza PM, Magalhaes PO (2010) Application of microbial α-amylase in industry – A review. Braz J Microbiol 41:850-61.
2. Rajagopalan G, Krishnan C (2008) Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresour Technol 99:3044-50.
3. Far BE, Ahmadi Y, Khosroshahi AY, Dilmaghani A (2020) Microbial alpha-amylase production: Progress, challenges and perspectives. Adv Pharm Bull 10:350-358.
4. Lim SJ, Oslan SN (2021) Native to designed: microbial -amylases for industrial applications. PeerJ 18:9:e11315.

5. Akhavan T, Ebrahimi, M, Hekmat A (2020) Structural evidence for kinetic and thermal stability changes of α-amylase due to exposure to [emim][lactate] ionic liquid. Turkish Journal of Biochemistry Volume 45:785-791.

6. Soy S, Nigam VK, Sharma SR (2021) Enhanced production and biochemical characterization of a thermostable amylase from thermophilic bacterium *Geobacillus icigianus* BITSNS038. JOURNAL OF TAIBAH UNIVERSITY FOR SCIENCE Vol 15:730 – 745.

7. Fakruddin M, Mazumdar RM, Bin Mannan KS, Chowdhury A, Hossain MN, et al. (2012) Critical factors affecting the success of cloning, expression, and mass production of enzymes by recombinant *E. coli*. ISRN Biotechnol 2013:590587.

8. Pouresmaeil M, Azizi-Dargahlou S (2023) Factors involved in heterologous expression of proteins in E. coli host. Arch Microbiol 205:212.

9. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 185:60-89.

10. Briand L, Marcion G, Kriznik A, Heydel JM, Artur Y, et al. (2016) A self-inducible heterologous protein expression system in *Escherichia coli*. Sci Rep 6:33037.

11. Khani MH, Bagheri M (2020) Skimmed milk as an alternative for IPTG in induction of recombinant protein expression. Volume 170:105593.

12. Ashok A, Doriya K, Mohan Rao DR, Kumar DS (2016) Design of solid state bioreactor for industrial applications: an overview to conventional bioreactors. Biocatalysis and Agricultural Biotechnology Volume 9:11-18.

13. Straathof A (2011) The proportion of downstream costs in fermentative production processes. Comprehensive Biotechnology 2:811-814.

14. Aneesa Fasim, Veena S More, Sunil S More (2021) Large-scale production of enzymes for biotechnology uses. Current Opinion in Biotechnology Volume 69:68-76.

15. Melchor-Moncada JJ, Garcia-Barco A, Zuluaga-Velez A, Veloza LA, Sepulveda-Arias JC, et al. (2024) Scale-Up of the fermentation process for the production and purification of serratiopeptidase using silkworm pupae as a substrate. Methods Protoc 7:19.

16. Cohen SN, Chang AC, Hsu L (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proc Natl Acad Sci U S A 69:2110-4.

17. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-54.

18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5.

19. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Journal of Analytical Chemistry 31:426-428.

20. Grzybowska B, Szweda P, Synowiecki J (2004) Cloning of the thermostable alpha-amylase gene from *Pyrococcus woesei* in *Escherichia coli*: isolation and some properties of the enzyme. Mol Biotechnol 26:101-10.

21. Chen Y, Armstrong Z, Artola M, Florea B I, Kuo CL, et al. (2021) Activity-based protein profiling of retaining α-amylases in complex biological samples. Journal of the American Chemical Society 143:2423-2432.

22. Zhou Y, Han LR, He HW, Sang B, Yu DL, et al. (2018) Effects of agitation, aeration and temperature on production of a novel glycoprotein gp-1 by *Streptomyces kanasenisi* ZX01 and Scale-Up Based on Volumetric Oxygen Transfer Coefficient. Molecules 23:125.

23. Enas MM, Elsoud MA, Salem MS, Tabakh EI, Sidkey NM, et al. (2021) Optimization of aeration and agitation for α-amylase production by *Aspergillus flavus* from water hyacinth. International Journal of Industry and Sustainable Development Volume 2:25-35.

24. Simair A, Mangrio GS, Qureshi AS, Khushk I, Ali CH, et al. (2017) Production and partial characterization of α-amylase enzyme from Bacillus sp. BCC 01-50 and potential applications. Biomed Res Int 2017:9173040.

25. Simair AA, Qureshi AS, Qureshi AS, Khushk I, Ali CH, et al. (2017) Production and partial characterization of *α*-amylase enzyme from *bacillus* sp. bcc 01-50 and potential applications. Biomedical Research International 2017:9173040.

26. Singh J, Kaur P (2012) Optimization of process parameters for cellulase production from Bacillus sp. JS14 in solid substrate fermentation using response surface methodology. Brazilian Archieve for Biological Technology 55:505-512.

27. Fasnacht M, Polaceck N (2021) Oxidative stress in bacteria and the central dogma of Molecular Biology. Front Mol Biosci 10:8:671037.

28. Korshunov S, Imlay JA (2006) Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. J Bacteriol 188:6326-34.

29. Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem 77:755–776.

30. Elmansy EA, Asker MS, El-Kady EM, Hussnain S M (2018) Production and optimization of α-amylase from thermo-halophilic bacteria isolated from different local marine environments. Bulletin of National Research Center 42:31.

31. Nigam PS (2013) Microbial enzymes with special characteristics for biotechnological applications. Biomolecules 3:597-611.

32. Raveendran S, Parameswaran B, Ummalyma SB, Abraham A, Mathew AK, et al. (2018) Applications of microbial enzymes in food industry. Food Technol Biotechnol 56:16-30.

33. Ye M, Sun L, Yang R, Wang Z, Qi K (2017) The optimization of fermentation conditions for producing cellulase of *Bacillus amyloliquefaciens* and its application to goose feed. R Soc Open Sci 4:171012.

34. Techapun C, Poosaran N, Watanabe M, Ken Sasaki K (2003) Optimization of aeration and agitation rates to improve cellulase-free xylanase production by thermotolerant Streptomyces sp. Ab106 and repeated fed-batch cultivation using agricultural waste. J Biosci Bioeng 95:298-301.

35. Enas MM, Abo Elsoud MM, Salem MS, El-Tabakh EI, Sidkey NM (2021) Optimization of Aeration and agitation for α-amylase production by *Aspergillus flavus* from water hyacinth. International Journal of Industry and Sustainable Development. 2:25-35.

36. Zafar A, Aftab MN, Iqbal I, Din ZU, Saleem MA (2019) Pilot-scale production of a highly thermostable α-amylase enzyme from *Thermotoga petrophila* cloned into *E. coli* and its application as a desizer in textile industry. RSC Advances 9:984-992.

37. Tan JS, Ramanan RN, Ling TC, Ramanan N, Lin TC, et al. (2012) The role of *lac* operon and *lac* repressor in the induction using lactose for the expression of periplasmic human interferon-α2b by *Escherichia coli* . Annals of Microbiology 62:1427-1435.