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Research Article

Enhanced Bacterial α-Amylase Production Using Mutant Strains Through Submerged Fermentation

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Authors' Contributions
MAF and AH conceived the idea and conducted the experiments. SA, AH and MS designed the methodology and wrote manuscript. AL and TT collected the data. SA, AH and MS, RS and HS analyzed the data. SA supervised the study. MAF and MAK wrote and edited manuscript.

Keywords
α-amylase; *Bacillus subtilis; Bacillus licheniformis*, Mutant strains, Fermentation

Abstract | The imperative enzyme alpha-amylase is used in various commercial and research applications. Due to the high market demand for α-amylase, the production of this enzyme was increased in the current study employing a mutation in wild strains. To reduce costs, less expensive agro-wastes were used, such as soybean meal, wheat bran, apple peels, rice husk, and cucumber peels. The mutant strains of *Bacillus subtilis* (BSAA-5 to BSAA-40) and *Bacillus licheniformis* (BLAA-5 to BLAA-40) were prepared by exposure to UV radiation for 5 to 40 min to synthesize α-amylase via submerged fermentation. Then the crude α-amylase synthesized by these mutant strains was optimized and partially characterized. In contrast to wild and all other mutant strain BSAA-25 and BLAA-25 strains showed the optimum production of α-amylase 331.4±6.9 U/mL and 310.8±11.3 U/mL, respectively, at 37±0.5°C and pH 7.0±0.2 for 48 h on wheat bran-based broth. BSAA-25 demonstrated maximum biosynthesis of α-amylase as compared to BLAA-25. Optimum α-amylase activity was measured at 40±0.5°C, pH 7.0±0.2 and 1% starch solution by BSAA-25 (338.6±11.0 U/mL) and BLAA-25 (326.8±6.4 U/mL). A considerable increase was seen in the biosynthesis of α-amylase from mutant strains of *B. subtilis* and *B. licheniformis* using agro-waste as substrate.

Novelty Statement | This scientific study presents an innovative strategy for increasing α-amylase production through the mutation and use of non-toxic agro-wastes such as soybean meal, wheat bran, apple peels, rice husk, and cucumber peels. The potential application of this approach contributes to the advancements in the fields of industrial biotechnology, and bioengineering.

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Introduction

Amylase plays a crucial role in industrial biotechnology. Industries always prefer microbial α-amylases because they are simple and inexpensive to produce (Gopinath *et al.*, 2017). Amylases are well-known enzymes due to their large number of applications in food, paper, textile, distilling, brewing and pharmaceutical industries (Chimata *et al.*, 2010). Amylases account for approximately 25% of the global enzyme market (Abedi *et al.*, 2022).

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The enzyme commission number of α-amylase is EC 3.2.1.1 and α-1, 4-glucan-4-glucanohydrolase known as a scientific name of the α-amylase. It is naturally produced in higher animals, microorganisms and plants (Kandra, 2003). It is a metalloenzyme, use calcium as co-factor (Farooq *et al*., 2021). It is an endo-amylase, cleaves α-D-(1, 4) glycosidic linkage and hydrolyzes the starch into glucose monomer and/or maltose. It is unable to hydrolyze α-1, 6-linkages and terminal glucose (Salim, 2021).

Starch is the main substrate of amylase which is one of the essential ingredients of human diet. Chemically and enzymatically processed starch is consumed to produce various products like glucose and fructose syrups, cyclodextrins or maltodextrin derivatives and starch hydrolysates (Agrawal *et al*., 2005; Patil *et al*., 2021).

According to the modern notion of fermentation, microorganisms like yeast, fungi and bacteria are used for the synthesis of beneficial products such as enzymes, antibiotics, metabolites and recombinant products. Fermentation is used at industrial scale to produce a wide range of products that benefit humanity (Paulová *et al*., 2013; Paul and Joshi, 2022). There are two categories of fermentation, solid substrate fermentation and submerged fermentation.

In SmF, substrates and nutrients are used in liquid form called broth and the microbial products formed in the presence of substrate/nutrients are directly secreted in this broth. The consumption of substrates is rapid, so the continuous supply of substrates is required (Couto and Sanromán, 2006; Salim, 2021). Genetically engineered microbes perform efficiently in SmF. The sterilization and purification processes can also be done with great ease (Paulová *et al*., 2013). Due to these advantages, SmF is preferred by several researchers to synthesize α-amylase by using bacterial strains (Hashemi *et al*., 2011; Abd-Elhalem *et al*., 2015; Farooq *et al*., 2021; Rodrigo *et al*., 2022).

The α-amylase production from bacterial strains can be enhanced by varying the physiological and genetic properties of the bacterial strains (Suribabu *et al*., 2014; Jujjavarapu and Dhagat, 2019; Zhang *et al*., 2021). Several researchers have reported that mutations in wild strains have resulted in enhancement of enzyme production. Exposure to ultraviolet radiation has been identified to be adequately mutagenic for improving α-amylase synthesize by *Bacillus* sp. The mutant strain of *Bacillus* sp. produced amylase at a higher rate (Abdullah *et al*., 2013; Suribabu *et al*., 2014).

Along with increasing the enzyme activity, it is necessary to keep production cost as low as possible to make it more affordable and commercially feasible. To achieve the target of producing low-cost enzymes, the medium used for its production must be economical. The use of agricultural waste as substrates helps to develop economical fermentation medium. Pakistan, being an agricultural country, produces large amounts of agricultural wastes, most of which finds no application other than contributing towards the pollution. The presence of high starch content depicts potential of agricultural wastes to serve as substrate to produce low cost α-amylase (Egbune *et al*., 2022).

Several researchers have used various agricultural wastes such as soybean meal, wheat bran and rice hull to produce α-amylase using various microorganisms (Negi and Banerjee, 2010; Nwagu and Okolo, 2011; de Castro and Sato, 2013; Pathak and Rekadwad, 2013). These agro-wastes are the source of nutrient for the bacteria. These wastes contain many anti-nutritional agents like such as phytic acid, trypsin inhibitors, saponins and some amino acids. Fermentation reduces this hindrance and promotes their nutritional values (Khan *et al*., 2021; Wang *et al*., 2021; Mittal *et al*., 2022).

The highest level of α-amylase production through the fermentation depends on the nutrient values of the substrates. Amongst the substrates that are used in current studies, wheat bran is an excellent substrate for fermentation. Due to the loose binding of wheat bran particles, air can easily circulate among them (Yan *et al*., 2019). Wheat bran also contains high carbon and nitrogen for microbial growth because it contains xylan and proteins (Tran *et al*., 2021).

Other researchers have used different substrates for synthesis of α-amylase, such as fruit and vegetable peels (Jadhav *et al*., 2013; Khawla *et al*., 2014; Uygut and Tanyildizi, 2018; Paul and Joshi, 2022).

The present study aims to screen different mutant strains of *Bacillus* sp. *(Bacillus subtilis* and *Bacillus licheniformis)* to yield highest α-amylase production using different agricultural waste based economical fermentation media. Furthermore, optimize the α-amylase activity with different physico-chemical parameters such as temperature, pH and starch contents.

Materials and Methods

Culturing of microorganisms

B. subtilis (FCBP-SB-0324) and *B. licheniformis* (FCBP-SB0019) were found from the Government College University's Institute of Industrial Biotechnology in Lahore. For the culturing of these bacterial strains, the nutrient agar (yeast extract, peptone, NaCl and agar) was used with pH 7.0±0.2. The microorganisms were streaked on these slant and plates and kept them in incubator for 24 h at 37±0.5°C. The under observed plates and slants were

preserved at 4°C for future use and weekly refreshed these cultures.

Production of mutant strains

Eight mutant strains of *B. subtilis* (BSAA-5, BSAA-10, BSAA-15, BSAA-20, BSAA-25, BSAA-30, BSAA-35 and BSAA-40) and *B. licheniformis* (BLAA-5, BLAA-10, BLAA-15, BLAA-20, BLAA-25, BLAA-30, BLAA-35 and BLAA-40) were prepared by exposing wild strains to UV radiation (254 nm at distance 6 cm by removing the lids of agar plates) for 5, 10, 15, 20, 25, 30, 35 and 40 min, respectively.

Inoculum preparation

The nutrient broth was used to prepare the inoculum. Twenty-five milliliters of the broth were taken in the 100 ml Erlenmeyer flasks. Autoclaved the flasks and allowed them to cool. When the temperature fell below the 40°C then a full loop of bacterial strain was inoculated into each flask. After the inoculation, these flasks were left for incubation at 37±0.5°C for 24 h in shaking incubator at 170 rpm and these cells were used as inoculum source.

Preparation of fermentation media using different agro-wastes

Five different fermentation media were prepared using five different agro-wastes. Each medium contained starch (0.5%), calcium carbonate (0.4%), and citric acid (0.1%) with 5% one of the agro-waste such as wheat bran, rice hull, soybean meal, cucumber peels and apple peels which labeled from M1 to M5, respectively.

Screening of bacterial strains for α-amylase production

Freshly prepared inoculum (10 ml) of bacterial strains *B. subtilis* (BSAA-5 to BSAA-40) and *B. licheniformis* (BLAA-5 to BLAA-40) were aseptically added in the 100 ml of media M1 to M5, respectively and incubated for 48 h at 37±0.5°C. These media were vortexed and centrifuged at 6000 rpm for about 20 min time duration after the fermentation. The supernatant was obtained which was employed in the enzyme assay.

Influence of temperature on α-amylase biosynthesis

For the determination of the influence of temperature on the biosynthesis of α-amylase, the media were incubated at 33, 37, 41 and 45°C and pH 7.0±0.2 for duration of 48 h and estimated the production of α-amylase.

Influence of pH on α-amylase biosynthesis

To determine the influence of pH, fermentation media were prepared with the pH values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Then fermented with inoculum at 37±0.5°C and 200 rpm for 48 h and observed the productivity of α-amylase.

Enzyme assay

The DNS method was used to determine the amount of -Amylase produced (Gusakov *et al*., 2011).

Enzyme characterization

Influence of temperature on the α-amylase activities

The influence of various temperatures (31, 34, 37, 40 and 43°C) at pH 7±0.2 was determined on the activity of crude α-amylase by using DNS method.

Influence of pH on the α-amylase activity

The activity of crude α-amylase was measured with starch solution at different values of pH (4.0, 5.0, 6.0…….10.0) under optimized temperature by using DNS method.

Influence of starch concentration on the α-amylase activity

For this purpose, different concentrations of starch solutions (0.5, 1.0, 1.5 and 2%) were prepared. Then these solutions were used to measure the activity at optimum pH and temperature according to the DNS method.

Statistical analysis

The statistical analysis of the result data was carried out using Graph Pad Prism (version 5.03) for Windows. One-way analysis of variances and Bonferroni's test were used to estimate the effect of various parameters on production of α-amylase. As a minimum criterion of significance, the probability level for these tests was 5%. Data are presented, Mean ±SEM.

Results

Screening of mutants for α-amylase production

All bacterial strains (wild and mutated strains) of *B. subtilis and B. licheniformis* were screened for the production of α-amylase through medium M1 (soybean meal). BSAA-25 and BLAA-25 produced higher yield of α-amylase as compared to the wild strains (untreated strains).

The yields of α-amylase produced by BSAA-25 and BLAA-25 were 245.1±7.6 U/mL and 199.7±9.1 U/mL, respectively, that were significantly higher than all other strains and commercial α-amylase (170.92 U/mL) ($F_{8,18}$ = 43.45; P< 0.001 and $F_{8,18}$ = 58.29; P< 0.001, respectively) (Figure 1A, B).

Production of α-amylase from BSAA-25 and BLAA-25 strains by utilizing different substrates

The mutant strains, *B. subtilis BSAA25-* and *B. licheniformis* BLAA-25, provided maximum production of alpha-amylase up to 331.4±6.9 and 310.8±11.3, respectively with medium M2 containing wheat bran as substrate than other media containing other agro-wastes and also greater than commercial α-amylase (170.68 U/mL) (Figure 2A, B) ($F_{4,10}$=93.5; P< 0.001 and $F_{4,10}$=75.5; P< 0.001, respectively). So, for further analysis, these two strains were only used with medium (M2).

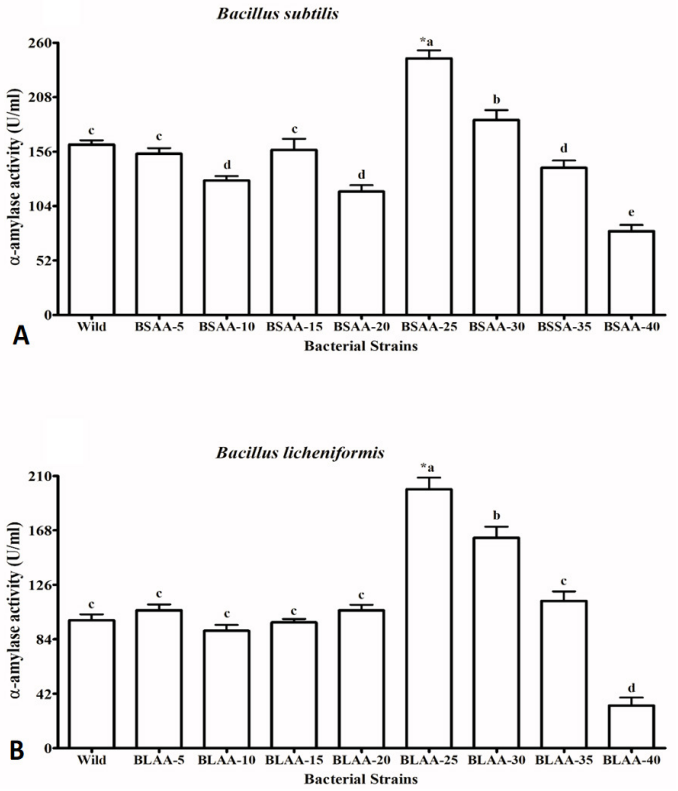

Figure 1: Production of α-amylase (U/ml) obtained by wild and different mutant strains of (A) *Bacillus subtilis* and (B) *Bacillus licheniformis* is compared, using medium M1 containing Soya bean meal as agro-waste. Each bar represents SEM (standard error of the mean) for the mean of three replicates. Different alphabets on different columns represent that these are statistically different (p< 0.001).

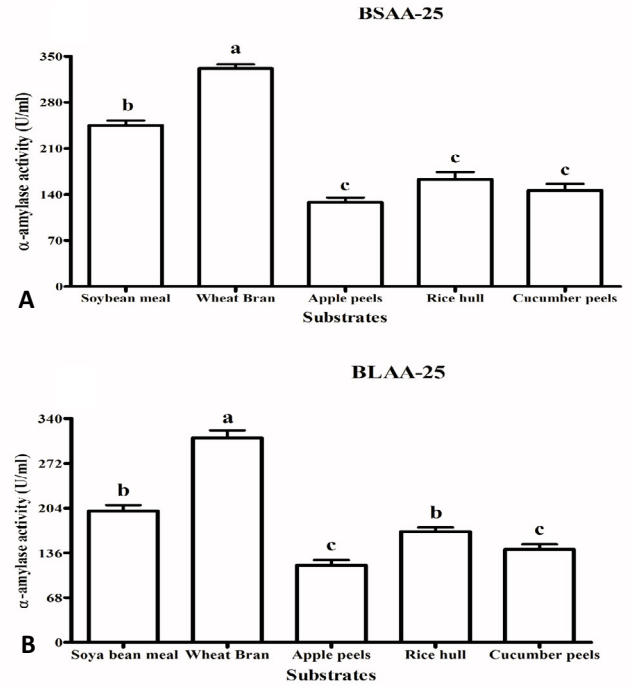

Figure 2: Biosynthesis of α-amylase (U/ml) obtained using different media containing different agro-wastes like soybean meal M1, wheat bran M2, apple peels M3, rice hull M4, cucumber peels M5 by (A) BSAA-25 and (B) BLAA-25 strains. Each bar represents SEM for the mean of three replicates. Different alphabets on different columns represent that these are statistically different (p< 0.001).

Influence of temperature on the biosynthesis of α-amylase

The mutant strains, *B. subtilis* BSAA-25 and *B. licheniformis* BLAA-25, were used to produce α-amylase at different temperatures by using wheat bran as a substrate. Different temperatures (33, 37, 41 and 45°C) were provided to the media during fermentation and pH was maintained at 7.0±0.2 for each temperature. At 37±0.5°C and pH 7.0±0.2, the highest biosynthesis of α-amylase was shown by both these strains that were 331.4±6.8 U/mL by BSAA-25 and 310.8±11.3 U/mL by BLAA-25 ($F_{3,8}$=13.0; p=0.001 and $F_{3,8}$= 28.4; P< 0.001, respectively). When the temperature was more than or less than 37°C, there was decreased in α-amylase production (Figure 3A, B).

Influence of pH on the biosynthesis of α-amylase

B. subtilis BSAA-25 and *B. licheniformis* BLAA-25 have an optimum pH 7.0±0.2 for the biosynthesis of α-amylase at 37±0.5°C. To optimize the pH, media with different pH (5.0, 6.0, 7.0, 8.0 and 9.0) were used for α-amylase production but temperature of each pH media was maintained at 37±0.5°C during fermentation. The maximum productivity of α-amylase enzyme by the strains *B. subtilis* BSAA-25 (331.4±6.8 U/mL) and *B. licheniformis* BLAA-25 (310.8±11.3 U/mL) was observed

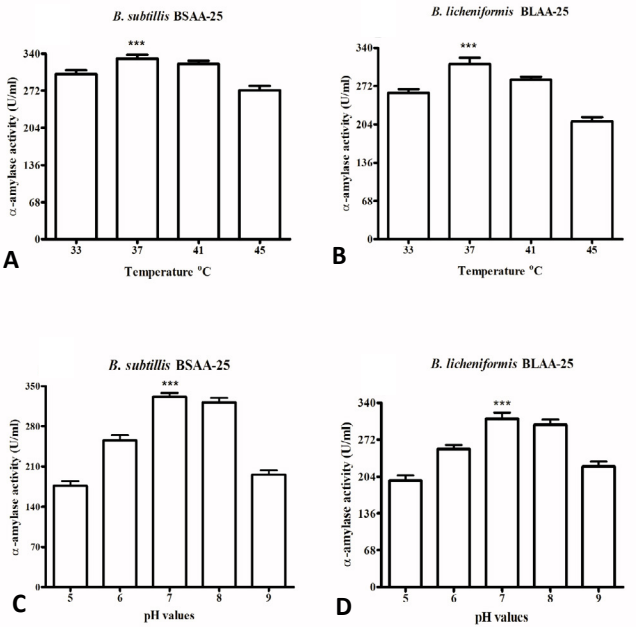

Figure 3: Influence of different temperatures and pH values on the biosynthesis of α-amylase produced by BSAA-25 and BLAA-25. Influence of temperatures on the biosynthesis of α-amylase by (A) BSAA-25 and (B) BLAA-25 strains. Influence of pH values on the biosynthesis of α-amylase produced by (C) BSAA-25 and (D) BLAA-25 strains, using the medium M2 containing agro-waste (wheat bran). Each bar represents SEM for the mean of three replicates (p< 0.001).

at pH 7.0±0.2 and 37±0.5°C ($F_{4,10}$=85.3; P< 0.001 and $F_{4,10}$=27.4; P< 0.001, respectively). At high acidic and basic pH, biosynthesis of α-amylase was abated (Figure 3C, D).

The production of α-amylase was optimized at pH7.0±0.2 and 37±0.5°C for both strains. Other than the optimal temperature and pH value, BSAA-25 and BLAA-25 showed a clear difference in α-amylase production (Figure 3).

Enzyme characterization

Influence of temperature on the activity of α-amylase

The activities of supernatants obtained from the cultures of the mutant strains, *B. subtilis* BSAA-25 and *B. licheniformis* BLAA-25 by using wheat bran as substrate were measured at varied ranges of temperatures (31, 34, 37, 40 and 43°C). Increasing the temperature from 31 to 40°C increased the amylase's activity. The highest activities of *B. subtilis* BSAA-25 (338.6±11.0 U/mL) and *B. licheniformis* BLAA-25 (326.8±6.4 U/mL) with $F_{4,10}$=19.9; P< 0.001 and $F_{4,10}$=30.9; P< 0.001, respectively were obtained at 40±0.5°C (Figure 4A, B).

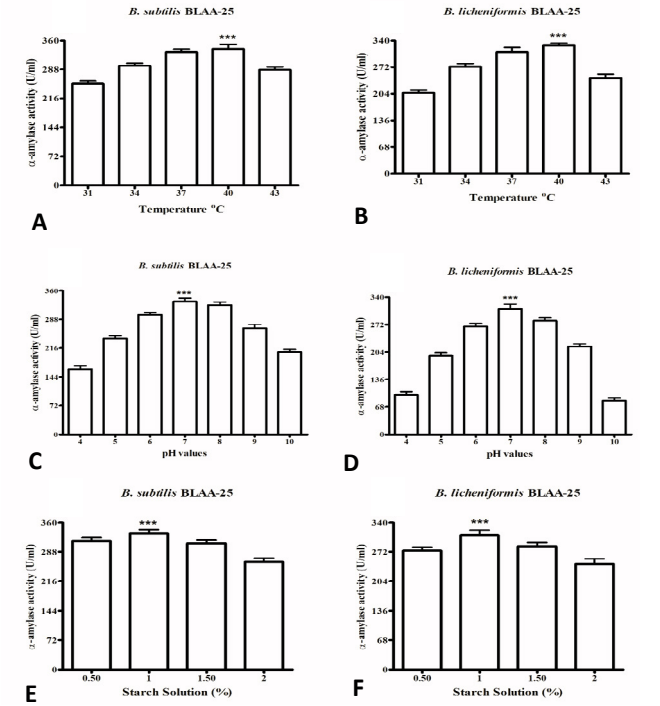

Figure 4: Influence of different temperatures, pH values and concentrations of starch solution on the activity of α-amylase (U/ml) synthesized by BSAA-25 and BLAA-25. Influence of different temperatures on the activity of α-amylase (U/ml) synthesized by (A) BSAA-25 and (B) BLAA-25 strains. Influence of pH values on the activity of α-amylase (U/ml) synthesized by (C) BSAA-25 and (D) BLAA-25 strains. Influence of the concentrations of starch solution on the activity of α-amylase (U/ml) synthesized by (E) BSAA-25 and (F) BLAA-25 strains, utilizing the medium M2 containing agro-waste (wheat bran). Each bar represents SEM for the mean of three replicates (p< 0.001).

Influence of pH on the activity of α-amylase

The supernatant obtained from the cultures of the mutant strains, *B. subtilis* BSAA-25 and *B. licheniformis* BLAA-25, using wheat bran as substrate was utilized to find the activity of α-amylase at varied pH values such as (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and10.0). The maximum activity up to 333.0±8.2 U/mL by *B. subtilis* BSAA-25 and 310.8±11.3U/mL by *B. licheniformis* BLAA-25 were recorded at pH 7.0 ±0.2. The activity decreased above and below pH 7.0±0.2 (Figure 4C, D) ($F_{6,14}$=63.71; P< 0.001 and $F_{6,14}$=125.6; P< 0.001, respectively).

Influence of the concentrations of the starch solution on the activity of α-amylase

Various concentrations of starch solution (0.50, 1.0, 1.50, and 2.0%) were used to measure the activity of α-amylase of the mutant strains *B. subtilis* BSAA-25 and *B. licheniformis* BLAA-25. The highest activity of α-amylase enzyme by *B. subtilis* BSAA-25 and *B. licheniformis* BLAA-25 was obtained with a 1% starch solution. Other concentrations presented less α-amylase activity in comparison with 1% starch solution (Figure 4E, F) ($F_{3,8}$=12.1; p=0.002 and $F_{3,8}$=7.24; p=0.01, respectively).

Discussion

In the current study, mutant strains of *Bacillus* (*B. subtilis* and *B. licheniformis)* prepared by exposure to ultraviolet (UV) radiation showed a higher potential for biosynthesis of α-amylase than wild strains. Two mutant strains BSAA-25 and BLAA-25 showed maximum α-amylase production. Ultraviolet (UV) radiation enhances the α-amylase synthesis by producing mutant strain of *B. subtilis* (Demirkan, 2011). α-amylase production was increased by producing the random mutagens of *Aspergillus oryzae* through the exposure to UV radiation (Abdullah *et al.*, 2013). The production of mutation in *Brevibacillus borstelensis* through UV radiation also enhanced the α-amylase production (Suribabu *et al.*, 2014).

In the current study, five different media having different substrates like M1 (soybean meal), M2 (wheat bran), M3 (apple peels), M4 (rice hull) and M5 (cucumber peels) were used. Amongst these media, the maximum biosynthesis of α-amylase was observed by BSAA-25 and BLAA-25 mutant strains with medium M2 embracing wheat bran as substrate. The second highest biosynthesis was shown by the medium M1, containing soybean meal, with same strains. The similar results were followed in the previous studies (Negi and Banerjee, 2010; de Castro and Sato, 2013). Other media M3, M4, and M5 showed less production as compared to media M1 and M2. Different agricultural residues were used as substrates and among those substrates, wheat bran showed the maximum synthesis of α-amylase by using *Bacillus megatherium* in SSF. *Clostridium thermosulforegenes* provided the highest

synthesis of α-amylase by utilizing wheat bran (Mrudula *et al.*, 2011). Due to the loose binding of wheat bran particles, air can easily circulate among them. Therefore, wheat bran is an excellent substrate for fermentation (Yan *et al.*, 2019). Wheat bran also contains high carbon and nitrogen for microbial growth because it contains xylan and proteins (Tran *et al.*, 2021). The *Aspergillus oryzae* was used to produce α-amylase by using wheat bran, soybean meal, and other substrates (de Castro and Sato, 2013). Wheat bran had the greatest α-amylase activity, which was higher than the activity shown by soybean meal and the commercially available α-amylase. The biosynthesis of enzymes by *Bacillus* species was greatly affected by the temperature of incubation (Horak *et al.*, 2019). Different bacteria produce amylases at a large range of temperatures. Submerged fermentation with *Bacillus* species produced the most α-amylase at 37°C (Rajagopalan and Krishnan, 2008; Viswanathan *et al.*, 2014). The maximum α-amylase production by *Bacillus subtilis* was reported at 45°C (Al-Johani *et al.*, 2017). In current studies, mutant strains of both species BSAA-25 and BLAA-25 also showed maximum α-amylase production at 37±0.5°C.

The pH of the media influences both organism growth and enzyme synthesis, when the pH was increased from 5.0 to 7.0, the synthesis of the enzyme also increased (Asgher *et al.*, 2007). In present study, both mutant strains, BSAA-25 and BLAA-25, showed greatest production at pH 7.0±0.2. The highest α-amylase production was determined at pH 7.0 using *Bacillus* species (Viswanathan *et al.*, 2014).

In present study, both the mutant strains BSAA-25 and BLAA-25 showed the greatest increased in α-amylase activity at 40±0.5°C. For *Bacillus* species, the optimum temperature range for maximum α-amylase activity differed from 35 to 50°C (Liu and Xu, 2008; Simair *et al.*, 2017). Crude α -amylase activity was highest at 50°C and lowest at 65°C (Dash *et al.*, 2015).

Denaturation is not only the property of high temperature but a high pH also denatures the enzyme. In current study, BSAA-25 and BLAA-25 strains showed optimal activity at neutral pH *i.e.,* 7.0±0.2. At pH 8.0, these enzymes showed activity near that of pH 7.0, but further increased in pH suddenly abated the activity due to denaturation. The bacterial strain *Bacillus* sp. produced the most crude α-amylase activity at pH 9.0 (Simair *et al.*, 2017). *Bacillus subtilis* BI19 and *Bacillus licheniformis* B4-423 had the highest crude-amylase enzyme activity at pH 6.0 and pH 5.0, respectively (Dash *et al.*, 2015; Wu *et al.*, 2018). The optimum pH of α-amylase was reported 8.5 which was produced from *B. subtilis* by using wheat bran as substrate in submerged fermentation (Irfan *et al.*, 2016). The optimal activity of α-amylase produced by *B. subtilis* was also observed at pH 6.0 (Özdemir *et al.*, 2011).

The concentration of the substrate has a significant impact on enzyme activity. The amylase synthesized by BSAA-25 and BLAA-25 strains demonstrated the highest activity with 1% solution. Further increases in starch concentration had no effect on enzyme activity. Even as the concentration of the substrate increased, the enzyme activity remained constant. The reduction in enzyme activity with increase in starch concentration might be associated with complete occupation of all active sites of enzymes with substrate (Wang *et al.*, 2022).

Conclusions and Recommendations

The biosynthesis of α-amylase might be improved by submerged fermentation with inexpensive nutrient-rich agricultural residue (substrate). The highest production of α-amylase was recorded with wheat bran after that soybean meal. The UV radiation based mutated strains of *B. subtilis* and *B. licheniformis* produce maximum α-amylase production than wild strains. The production as well as the activity of α-amylase obtained by BSAA-25 and BLAA-25 mutagenic strains can be optimized at the temperature (37°C and 40°C, respectively) and pH at 7.0. The activity of α-amylase produced via microbial mutant strains could be higher than the commercially available α-amylases. The genome sequencing of mutant strains and enzyme purification are future goals of the present study.

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Availability of data and materials

Data is available on request.

Consent for publication

All authors are agreed to publish the manuscript in its current form.

Ethics approval consent to participate

Not applicable.

Conflicts of interest

The authors have declared no conflict of interest.

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