IMPROVEMENT OF ALPHA-AMYLASE ACTIVITY FROM *BACILLUS LICHENIFORMIS* USING UV RADIATION AND MODIFIED MEDIA COMPOSITION

Bioresearch Communications
Volume 9, Issue 2, July 2023

DOI:
doi.org/10.3329/brc.v9i2.67092

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ABSTRACT

Alpha-amylases are glycosidic bond hydrolyzing enzymes produced in many microorganisms. In the modern era, the spectrum of amylase applications has expanded into various industries, including medical fields. Among other approaches, strain improvement by mutation and culture optimization are attractive methods for increasing the productivity of alpha-amylase in bacterial strains. The present study aimed at enhancing the yield of the enzyme alpha-amylase from the host strain *Bacillus licheniformis*. The UV irradiation has been used to induce mutations in the bacterial strain with different time durations. Afterwards, the mutant strains were cultured in different modified growth media for enzyme production. Among the four time periods,the highest enzyme activity (705.23 U/ml) was observed after 24 hours which is more than two-fold higher compared to the enzyme activity of the host stain (345.31 U/ml). In addition, enzyme yield varied significantly in modified culture media (i.e, MP1 and MP2, MP3 and MP4) with different carbon and nitrogen compositions. The mutant strain cultured in MP3 medium had the highest amylase activity which was 1461.2 U/ml at 24 hours. More interestingly, this medium also had the highest enzyme activity (1080.5 U/ml) at 48 hours. The trend of enzyme activity showed a decreasing tendency when the strains cultured in MP1, MP2, and MP3 media, but exceptionally, a sharp decreasing trend was seen in MP4 media from 622.1 U/ml at 24 hours to 428.4 U/ml at 48 hours. From the findings, it can be concluded that *B. licheniformis* is a good candidate to employ for alpha-amylase production in a modified MP3 culture condition for a short time. However, other limiting factors need to be considered for increasing the yield.

KEYWORDS: Alpha-amylase, Enzyme, Mutation, UV irradiation, *Bacillus licheniformis*

RECEIVED: 11 February 2023, ACCEPTED: 17 May 2023

TYPE: Original Research

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Introduction

Enzymes are biocatalysts that are important components of biochemical reactions. Among others, alpha-amylases are crucial enzymes that have been the most useful in industrial biotechnology (Periasamy Anbu and Lakshmipriya, 2017). Amylase can be obtained from several fungi, yeast, bacteria an d actinomycetes (Dike Precious Elechi, 2022). Enzymes that are collected from bacterial sources have a wide range of applications in industrial sectors. Bacterial sources for the production of amylases are more preferred than any other sources because of their plasticity and vast availability and ability to produce in bulk and ease with which they can be manipulated for desired products (Arora et al., 2020). These contribute a major class of industrial enzymes constituting 25% of the enzyme market (Hiteshi et al., 2016). The global market for industrial enzymes estimated at $2 billion in 2004 and expected to rise at an average annual growth rate of 3.3% (Periasamy Anbu and Lakshmipriya, 2017, He et al., 2023).

Bacillus licheniformis is one of the most characterized gram-positive bacteria with unique genetic background and safety characteristics. It has the important biologic applications in the food industry, including, the biosynthesis of high value-added bioproducts, probiotic functions, biological treatment of wastes derived from food production (He et al., 2023). *B. licheniformis* is a well-known amylase producer, and improvement of its amylase production is of great interest. The biodegradable polymers like starch are used to control the drug release rate in controlled delivery systems. The release rate is also dependent on α-amylase activity contained in the dissolution media. The supplementation of α-amylase to cross-linked amylose (CLA) tablets can amend the kinetics of those drugs that are in a drug vehicle (Surendra Sing, 2011).

In many previous studies, the production of the amylase was carried out by the screening of *Bacillus species* in soil, e.g. purification, immobilization along with variations of enzymatic parameters like pH, temperature, salt tolerance, substrate concentration, enzyme activity was checked (Dash et al., 2015, Aida and Hanan, 2016, Mohammad et al., 2017). Unfortunately, very few research on α-amylase production was reported from Bangladesh, such as by *Trichoderma viride* (Rahman, 2008) or mixture of various extracellular enzymes from *Bacillus aryabhattai* (Khandaker Md. Khalid-Bin-Ferdaus

and Ahmed Sajib, 2018). Nevertheless, traditional mutation and selection techniques have been exploited for the improvement of bacterial phenotypic expression (Hormoznejad et al., 2022). Moreover, the optimal growth of bacterial culture and subsequent α-amylase production is greatly affected by the cultivation method and supplementation of essential nutrients (S. Sankaralingam, 2012). Amylase production was enhanced by codon optimization through recombinant technology in *B. licheniformis* (Wang et al., 2015) and peptide optimization (Yao et al., 2019). On this note, the optimization of fermentation conditions for α-amylase production by a chemically treated mutant strain is needed. In another study, a bacterial strain of *B. licheniformis* GCB-30UCM was improved for α-amylase production using ethyl methane sulfonate (EMS) as an inducing mutagen (Ikram-Ul-Haq et al., 2009). Screening and selection of a better enzyme producer was performed by optimization of cultural conditions such as temperature, pH, volume of medium, incubation period and size of inoculum for enzyme production by the mutant were optimized to gain an insight of the batch fermentation process (Niu et al., 2009, Al-Johani et al., 2017).

B. licheniformis has been used in the fermentation industry for over a decade for production of proteases, amylases, antibiotics, or chemicals. This species is easily differentiated from other members of the genus that are pathogenic to humans and animals. Amylases from *B. licheniformis* are deployed for the hydrolysis of starch, desizing of textiles and sizing of paper (He et al., 2023). In Bangladesh, α -amylase is being used in food and pharmaceuticals industries for production of high glucose and maltose syrups. Moreover, garments and textile industries also have enormous demand of amylases for various purposes. However, there is no industry for amylase production in this country. So, amylases are imported from other countries which is very costly. Moreover, production and characterization of α-amylase activity from *B. licheniformis* have not yet been reported in Bangladesh. The objectives of the present study were to develop a mutant strain of the host strain *B.licheniformis*, to optimize different growth media of the strains and enhance enzyme production by increasing enzyme activity. The findings of this study will help to develop new dimensions for the exploration of microbial biotechnology that will enrich the economy of Bangladesh.

Materials and Methods

Bacterial culture media

The host strain of *B.licheniformis* was collected from a company named Active Fine Chemicals (AFC) Ltd, Dhaka in agar slant test tubes. A loopful of bacteria was then introculated in liquid Luria Bertani (LB) media for reviving and then cultured overnight on seeding medium (Table 2) for further experiment.

Inducing mutation by UV irradiation and Determination of enzyme activity

In order to induce mutations in the overnight culture of host B. *licheniformis*, bacterial strain was centrifuged at 6500 rpm for 15 minutes and the bacterial pellet was resuspended in 10 ml of 0.85% NaCl solution. Bacterial cell density was adjusted to an OD_{560} of 2.0 for UV radiation. The UV radiation was applied inside a metallic black box. The exposure durations were 2 minutes, 4 minutes, 6 minutes and 8 minutes. The control was the parental strain, which was not exposed to any radiation. The petridishes containing the mutated strains were covered with aluminum foil. Resulting strains were named as indicated below.

Table 1. *B. Licheniformis* strains used in this study

Name	UV treatments
LR-0	Exposed for 0 minutes (Host Strain)
LR-2	Exposed for 2 minutes
LR-4	Exposed for 4 minutes
LR-6	Exposed for 6 minutes
LR-8	Exposed for 8 minutes

After incubating for 45 minutes, the UV treated strains were plated in LB medium and incubated overnight at 37˚C. Firstly, 1 ml of an overnight *Bacillus licheniformis* culture was inoculated in liquid culture media: Starch 10g/L, peptone 14 g/L, yeast extract 6g/L, $CaCl_2$ 0.25 g/L, KCl 1.0 g/L $MnSO_4$ 0.001g/L, $FeSO_4$ 0.0005 g/L at 37˚C, and 150 rpm for enzyme production. The experiment was carried out in triplicate.

Production of α-amylase from B. licheniformis mutant in modified media

The production of α-amylase was carried out using four different enzyme production media namely MP1, MP2, MP3 and MP4. The media compositions are as follows:

Table 2. MP1 media composition

Components	Amount (g/L)
Starch	10
Peptone	14
Yeast Extract	6
NaCl	1
$CaCl_2$	0.2
$MgSO_4$	0.2
KCl	0.2

Table 3. MP2 media composition

Components	Amount (g/L)
Lactose	10
Peptone	14
Tryptone	6
NaCl	1
$CaCl_2$	0.2
$MgSO_4$	0.2
KCl	0.2

Table 4. MP3 media composition

Components	Amount (g/L)
Starch	10
Peptone	7
Yeast Extract	3
NaCl	1
$CaCl_2$	0.2
$MgSO_4$	0.2
KCl	0.2

Table 5. MP4 media composition

Components	Amount (g/L)
Glucose	10
Peptone	14
Yeast Extract	6
NaCl	1
$CaCl_2$	0.2
$MgSO_4$	0.2
KCl	0.2

1 ml of the *B. licheniformis* from the liquid seeding culture of the strain was added using sterile pipette tips. The flasks were incubated at 37˚C and 500 rpm for culturing. The activity of amylase produced for the different media were determined from the culture at 24 hours and 48 hours after inoculation, respectively. The experiment was carried out in triplicate.

Preparation of 3,5-Dinitrosalicyclic reagent

The reagent used to color the solution is a 3,5-dinitrosalicyclic Acid **(**DNSA) reagent. In order to prepare the reagent, the chemicals are listed in Table 6. Rochelle salt was then added to DNSA solution. 2M NaOH solution was then pipette into the solution and the final volume made up to 100 ml with distilled water. The solution was heated using a magnetic stirrer in order to dissolve the constituents. Once preparation of the solution has been completed, the beaker was wrapped up completely in aluminum foil, and stored in a cool dark cabinet for one week to use.

Table 6. Components of DNSA

Components	Amount
3,5-dinitrosalicyclic acid	0.438 gm
Rochelle salt	12 gm
2M NaOH	8 ml
Distilled water	Up to100 ml volume

Preparation of standard curve

The standard curve was prepared using the reducing sugar maltose monohydrate. In order to produce the curve, samples containing specific concentrations of the reducing sugar were incubated with 3,5-dinitrosalicyclic acid reagent. Using a water bath, the solutions were boiled for 15 minutes. The temperature of the water was allowed to reach above 90˚C, until the water inside had started boiling. Afterwards, the test tubes were placed in flaked ice for complete cooling. The Humalyzer 3000 machine was then used to obtain a standard curve. One unit of activity (unit, U) of the enzyme alpha-amylase, is the quantity of the enzyme required for the production of 1 µmole of maltose in 1 min, when the enzyme is incubated along with the substrate at 37 °C.

Determination of α-amylase activity using DNSA

For determination of enzyme activity, crude extracts of α-amylase were added to a substrate (paper) and incubated. The amount of reducing sugar produced was then used to quantify the enzyme activity. Then, 1.5 ml of samples was collected from the bacteria cultures. The samples were then centrifuged at 6500 rpm for 30 minutes. After centrifugation, the supernatant was poured into small test tubes, containing the substrate. The contents of the test tubes were mixed by mechanical agitation using a pipette tip. The open end of the test tube was sealed with aluminum foil, and it was incubated at 37˚C for 30 minutes. After that, 1ml of DNSA added to the tubes. Following the addition of DNSA, the test tubes were resealed with aluminum foil, and boiled for 15 minutes in water bath and then cooled using ice. The absorbance of the solution was determined using the Humalyzer 3000 at 545nm to obtain the concentration of sugar in the solution. This reading was then used to calculate the enzyme activity, using the following formula (Miller, 1959).

$$EnzymeActivity = \frac{molar\ conc.\ of\ reducing\ sugar\ released \times total\ vol.\ in\ cuvette}{final\ concentraion\ of\ enzyme \times volume\ of\ enzyme} \quad \ldots\ldots (1)$$

The amount of reducing sugar produced was compared to a standard curve, which was used to obtain the amount of reducing sugar from the absorbance of the samples.

Results and Discussions

Classical strain improvement by UV irradiation has significant advantages over chemical mutation. Since the mutagenesis might produce novel criteria or random recombination, there is enormous potential to enhance metabolic phenomena. The objective of our work was to improve the yield of α-amylase production from *B.licheniformis* using UV mutation and modified media composition. In order to do mutagenesis, the bacteria were irradiated with UV radiation forvarious durations. Variation in the composition of the liquid media in which the bacteria were grown yielded a great change in results.

Determination of α-amylase yield from strains after UV mutation

Induction of mutagenesis using UV radiation resulted in a variation in the production of α-amylase by the bacteria. The variable selected for inducing mutagenesis was the exposure time, with a control being used for comparison. Upon employing the mutated bacteria the activity of the enzyme was seen to have followed a general trend related to the exposure time.

Figure1 shows the changes in activity for the enzyme produced by different strains. In the first 24 hours, the enzyme activity was highest (705.2 U/ml) for LR -6, followed by LR-4. The LR-8 strain the lowest enzyme activity (480.63 U/ml) among the four mutant and host strain.

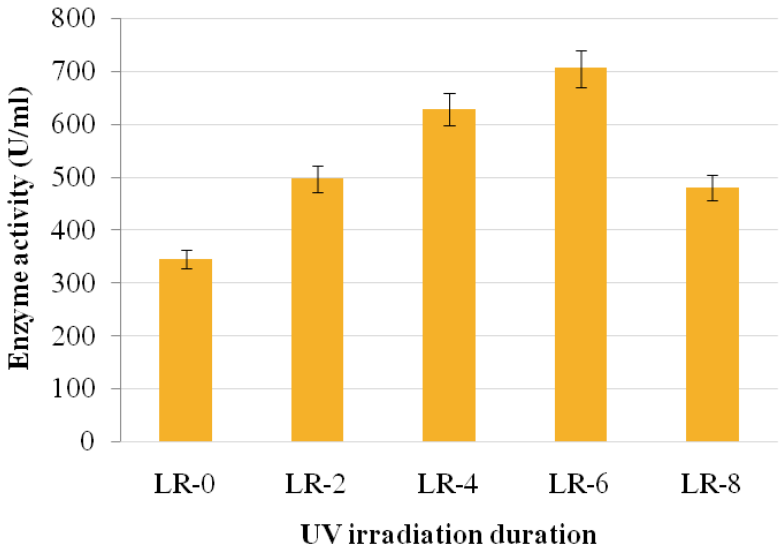

Figure 1. Enzyme activity of host and mutant strains of *B.licheniformis* at 24 h

Moreover, host strain showed the lowest activity (345.3 U/ml). The activity generally increased for UV exposure of up to 6 minutes, and then declined for increasing time period. At 48 hours, the enzyme activity showed a decline trend for all the strains. It is clearly depicted that mutant LR-6 has the highest potentiality to produce highest enzyme among others after 24 h. Afterwards, LR-6 was selected for further experiment using modified media to check its enhanced amylase production capacity compared to the host strain LR-0.

Determination of α-Amylase yield in modified culture media

The media that were selected for amylase enzyme production displayed great variation in the enzyme yield. As seen in Figure 2, the mutant strain LR-6 cultured in liquid MP1, MP2, MP3 and MP4 media for amylase production. Interestingly, the highest amylase activity was archived by the strain cultured in MP3 media which was 1461.2 U/ml at 24 hrs. The enzyme activity produced by the strain cultured in MP3 medium was approximately two-fold higher than cultured in MP1, MP2 and MP4 media. As shown in Table 4, MP3 media contained less protein composition (half of others) compared to other media and rest of constitutes remained similar.

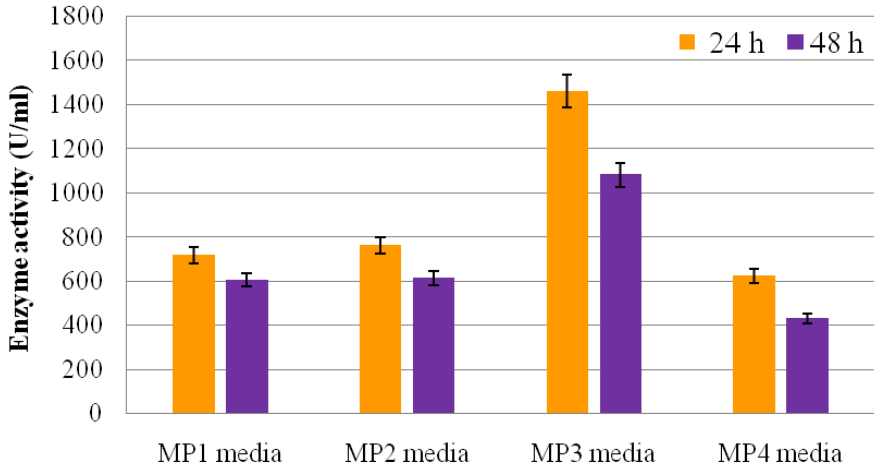

Figure 2. Enzyme activity at 24 hours and 48 hours for different culture media

More interestingly, this medium also had the highest enzyme activity at 48 hours (1080.2 U/ml).The trend of enzyme activity with time showed a decrease for MP1, MP2, and MP3 media, but a sharp decrease in enzyme activity was seen for strains cultured in MP4 media. The promising enzyme activity in MP3 might be due to the medium composition that contained a reduced amount of nitrogen source (C:N ratio) though the molecular mechanism is unknown. It was expected that the α-amylase production by the bacteria would be enhanced by growing the organisms on MP4 medium with the addition of glucose. However, the synthesis of α-amylase by the mutant LR-6 was seriously concealed when the bacteria were grown on glucose containing medium MP4 (Figure 2) though the growth of the bacteria in glucose containing media was found to be very good. The inhibitory effect of glucose on α-amylase synthesis was reported in a previous study (Campbell, 1963).

Conclusions

The study offers the establishment of a promising alternative strategy to develop bacterial cell factories for improved production of high value industrial products like amylase. The enzyme activity obtained by the host strain was 345.3 U/ml at 24 hours. After UV mutation, LR-6 was selected for growing in modified culture media since it showed the highest enzyme activity (705.2 U/ml). However, the enzyme activity was increased for UV exposure of up to 6 minutes, and then it showed declining trend with time extension. Nevertheless, we can see the strain LR-6 used starch and lactose easily as carbon source than glucose which has a reverse impact on enzyme activity. Here, the mutant LR-6 showed the highest enzyme activity (1461.2 U/ml) in MP3 media which was optimized with lower nitrogen content and starch as the carbon source (Figure 2). The amylase activity could be elevated due to change in transcription level of alpha amylase gene in the mutant *B. licheniformis*. Therefore, transcription level could be characterized for further study. A medium composed of 1:1 ratio of concentration of carbon and nitrogen sources showed an increase in trend of enzyme activity with time. Further observation on the activity of alpha-amylase from this medium for an extended duration of culture time can be used to establish the overall productivity of the medium.

Acknowledgment

All the financial support for the research work was provided by the Ministry of defense and the research cell of Military Institute of Science and Technology (MIST), Dhaka. The authors expressed their gratitude and thanks for the grant. We would like to thank Dr. Asrafuzzaman for his cordial help in contact with Active Fine Chemicals (AFC) Ltd. Dhaka.

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