Short Communication

Effect of UV Radiations on Enzyme Kinetics of Extracellular Amylases Isolated from *Bacillus subtilis*

Amit Pandey[1*] and Love Gupta[2]

1. R&D Division, MRD LifeSciences, Lucknow 226010, India.
2. Institutes of Biosciences & Biotechnology, CSJM University, Kanpur 802024, India

Abstract

Bacillus subtilis is one of the potent species of bacteria which were found to synthesize α-amylase in a very large quantity by fermentation process. In this study it was found that a UV exposure of 254 nm from a height of near about 30 inches for 4 and 8 min increased the enzyme activity up to 2.08%. This value get further increased up to 16% by optimizing the media and enzymatic reaction conditions. Peptone (0.5%) as a nitrogen, Beef extract (0.3%) with starch (1%) as a carbon source and calcium ion (1%) as an elicitor were used as a media component. Media pH was optimized near about neutral. During enzymatic reaction, the reaction time for enzyme substrate reaction was 3 min at room temperature with substrate of neutral pH.

Keywords: Fermentation; UV exposure; Optimization; Enzymatic reaction

Introduction

Microbial enzymes find increasing industrial application and among them amylases occupy a large share of the commodity enzyme market. Amylase is a class of hydrolases enzymes which are use for the breakdown of starch. Starch is a polysaccharide made up of two types of glucose polymer, amylose and amylopectin. Amylose consists of long, unbranched chains of D-glucose residue connected by (α1-4) linkages. Amylase works on (α1-4) linkage and hydrolyzes it. Most of the amylases are metalloenzymes which require calcium ion for their activity (Burhan *et al*, 2003). A seed containing starch as a reserve food, also has this enzyme, and is secreted by many fungi. In the starch liquefaction process (Bessler *et al.,* 2003) it is worthwhile to select a potent strain of microorganism for enzyme production. *Bacillus* species such as *B. amyloliquefaciens, B. subtilis, B. licheniformis B. stearothermophilus* are known as potent producers (Rasiah & Rehm, 2009; Gangadharan *et al.,* 2006). The microbial alpha amylase production by bacteria is depends on various factors such as, the type of strain, media composition, cultivation methods, cell growth, requirement of nutrients, metal ions, pH, temperature, incubation time and thermostability (Pandey *et al.,* 2000). Optimization means from the set of variables which influences the growth of bacterial colonies and synthesis of α-amylase, changing one independent variable keeping the other factors constant and demonstrating the effect of it on bacterial growth and enzyme production. Optimization of various parameters and manipulation of media are one of the most important techniques used for the overproduction of enzymes in large quantities to meet industrial demands (Kar and Ray, 2008). The present study is carried out by effect of UV radiation on extracellular amylases and optimization of culture conditions.

Materials and Methods

The pure bacterial culture of *B. subtilis* was used in present study. This bacterial sample was obtained from IMTECH, Chandigarh and maintained by MRD LifeSciences, Lucknow, with the help of sub culturing method. It was found that in nutrient agar/broth with 1% starch, the growth of *B. subtilis* was best (Ajayi and Fagade, 2003). *B. subtilis* culture was maintained on nutrient agar medium and nutrient broth along with 1% starch. Treatment with physical mutagen was done for increasing the production of alpha amylase, as physical mutagen ultra violet exposure was used. UV light of 254 nm was given from a height of about 30 inches. 6 plates of nutrient agar + 1% starch were prepared and after solidification 50 µl of pure cultures on each plate were spread,1st plate took as control and next 5 plates were treated with 4, 8, 12, 16 and 20 min UV, plates were placed in the incubator at 37°C for 24 hrs (Haq *et al*, 2010). For standard graph preparation, enzyme assay by DNS method is done by taking maltose as standard reducing sugar (Miller, 1959). DNS is 3,5-dinitrosalicylic acid, which mainly reacts with reducing sugar and itself converted in to 3-amino, 5-nitrosalicylic acid, to stop the reaction. Standard reducing sugar maltose stock concentration was 500 µg/ml was taken; this was give orange colour solution. The optical density (OD) of reaction mixture was determined at 540 nm. This was used for the extracellular alpha amylase assay; here the bacterial culture was centrifuged at 5,000 rpm for 5 min. The supernatant was taken as enzyme source as the amylase is an extracellular enzyme, the substrate use in the reaction is the 1% starch in phosphate buffer saline (PBS), for preparation of PBS- take 20 ml of 0.02 M disodium hydrogen phosphate (Na_2HPO_4) and add 20 ml of 0.02 M sodium dihydrogen phosphate (NaH_2PO_4). Adjust the pH 6.9 with these two reagents. Na_2HPO_4 increases the pH and NaH_2PO_4 decreases the pH. After adjusting add 0.006 M NaCl. The mixture of enzyme and substrate was incubated for 15 min and the reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid, and then followed by incubating in water bath at 100°C for 10 min, diluted it with 5 ml distilled water and the reducing sugar released was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that releases 1 mol of reducing sugar as glucose per min, under assay conditions and expressed as U/g of dry substrate. Enzyme activity in units can be measure by using the formula

*Corresponding Author
E mail : amit@mrdlifesciences.com

below:

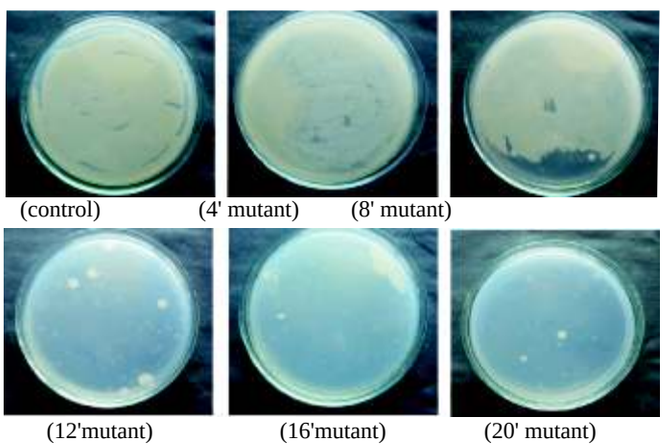
$$\text{Enzyme activity} = \frac{\text{mg/ml maltose released x } 0.36}{\text{Volume of enzyme taken} \times \text{incubation time}}$$

Optimization of media was done for increasing the growth as well as production of α-amylase (Zambare, 2011). It was done by taking five types of media i.e. Nutrient Broth (1.3%), Nutrient Broth (1.3%) + Starch (1%), Nutrient Broth (1.3%) + Glycine (1%), Beef Extract (1%) and Peptone (1%). The above media were inoculated with bacterial culture and put in the incubator shaker for 24 hrs. Bacterial growth was observed by using colorimeter at 600 nm. On the basis of growth, the best media was taken for further alpha amylase assay. pH of media was adjusted to 5, 6, 7, 8, 9 and was autoclaved further inoculated with bacterial culture and put in the incubator shaker for 24 hrs. The growth and α-amylase production was observed at different pH (Pandey *et al.*, 2000). Effect of different metals i.e. calcium, magnesium, zinc and iron on media was observed. 1% metal ion was added in media. Inoculated with bacterial culture and put in the incubator shaker for 24 hrs. The used parameters (a, b, c and d) contain the variables which influences the enzyme activity and was analysed by α-amylase assay for determining the optimum conditions for α- amylase production (Kar and Ray, 2008). The used cultures were observed for incubation time of 3', 6', 9', 12', 15' and 18 min. Cultures were kept for effect of different temperature i.e. room temperature, 0°C, 37°C and 50°C during incubation period. Cultures were kept for effect of different pH of PBS i.e.5, 6, 7 and 8. Cultures were kept for effect of different concentration of starch i.e., 0.5%, 1% and 2%.

Results

50 µl of the pure culture of *B. subtilis* was spread on Nutrient agar + 1% starch plates and UV treatment was given.After 24 hrs incubation following results were obtained

Figure 1. Effect of UV at different time intervals. The Figure shows that



(control) (4' mutant) (8' mutant)

(12'mutant) (16'mutant) (20' mutant)

when UV exposure increases, number of colonies decreases i.e., in control plate number of colonies was maximum while in case of 20' UV mutant, there were minimum colonies present.

Estimation of enzyme activity by DNS method was done by taking OD at 540 nm and compared this value on standard curve to evaluate the amount of maltose released by enzyme action.

From the above graph it was clearly shown that 4' and 8' mutants release

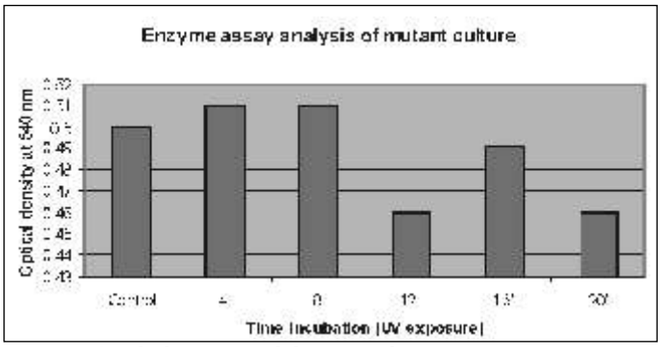


Figure 2. Graph shows optical densities of UV mutants.

α-amylase in the medium greater than other cultures. So for further experiments only control, 4', and 8' UV mutants were taken.

Media Optimization was done to increase the growth of *Bacillus subtilis* and to enhance the alpha amylase production, following results were observed:

This was done to find out the best media for the growth of bacterial species and for the maximum production of α-amylase. The growth was measured in form of optical density at 600 nm.

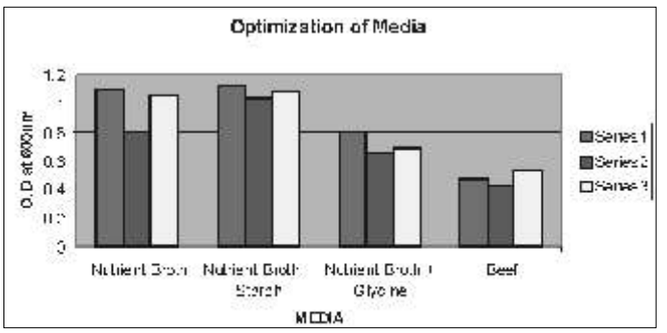


Figure 3.Optimization of media [*Note:* Series 1= control, Series 2 =4 min, Series 3 =8 min]

The above bar diagram shows that Nutrient broth + 1% starch give best

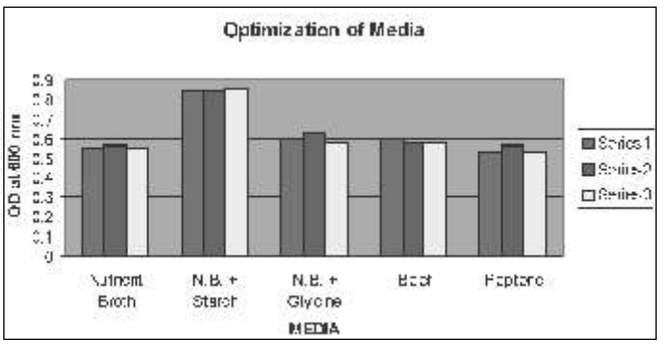


Figure 4. Optical densities of cultures plotted against different media *[Note:* Series 1= control, Series 2 =4 min, Series 3 =8 min]

result for alpha amylase production as compare to others.

The above bar diagram shows that Nutrient broth + 1% starch give best result for alpha amylase production as compare to others.

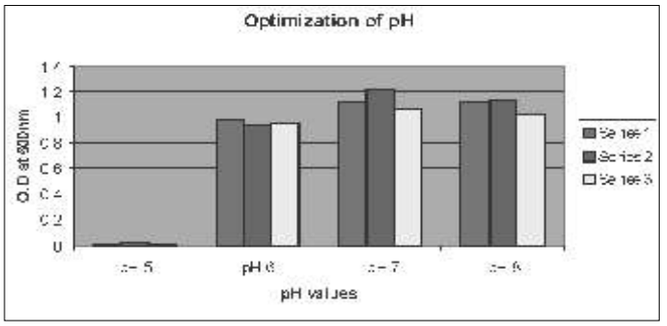


Figure 5. Growth of bacteria in media of different pH

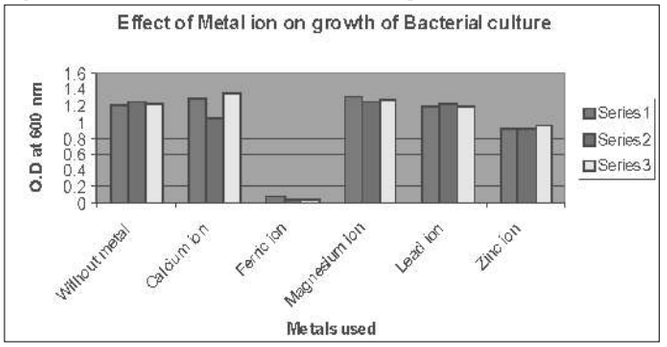


Figure 6. Effect of metal ion [*Note*: Series 1= control, Series 2 =4 min, Series 3 =8 min. The above graph shows that in the presence of Ca ++ and Mg ++, cultures were showing maximum growth].

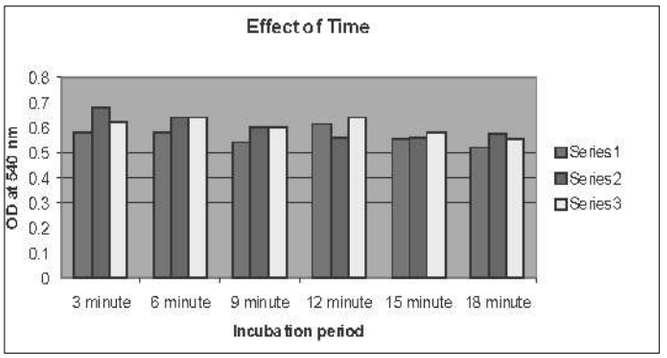


Figure 7.Activity of enzyme in different incubation periods [*Note*: Series 1= control, Series 2 =4 min, Series 3 =8 min. The above graph shows that 3 min incubation time was showing best result for enzyme activity].

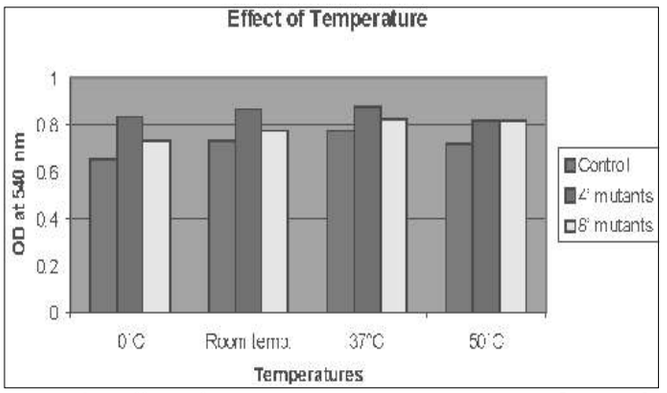


Figure 8. Effect of Temperature on Enzyme activity *[Note*: The above graph shows that maximum activity was observed at 37^{0}C].

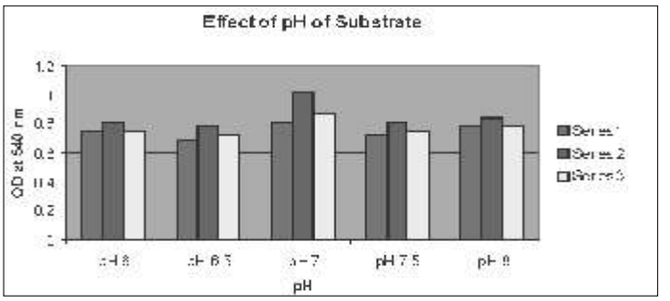


Figure 9. Effect of pH of Substrate [Note: Series 1= control, Series 2=4 min, Series 3 =8 min.The above graph shows that maximum enzyme activity was obtained at the pH 7 of substrate].

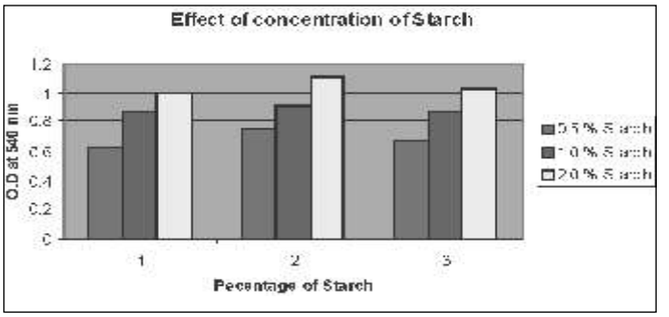


Figure 10. Effect of starch concentration on α-amylase activity [Note: The above graph shows that maximum activity was observed at 2% starch conc].

Culture	OD at 540 (nm)	Enzyme activity
Control	0.50	0.035
4'	0.51	0.036
8'	0.51	0.036
12'	0.46	0.033
16'	0.49	0.035
20'	0.46	0.033

Table 1. Comparative analysis of Mutant cultures (UV exposure) *[Note:* It shows that compare to control plate, 4 min and 8 min UV treated plates were having maximum OD which shows the maximum enzyme activity].

Media Used	Control (O.D at 600 nm)	4' mutant (O.D at 600 nm)	8' mutant (O.D at 600 nm)
Nutrient Broth	1.10	0.80	1.05
Nutrient Broth + Starch	1.12	1.02	1.08
Nutrient Broth + Glycine	0.80	0.65	0.68
Beef	0.47	0.42	0.53
Peptone	0.90	0.93	1.06

Table 2. Optimization of media for the growth of *Bacillus subtilis [Note:* It shows that compare to all media, NB+ Starch was suitable for growth of cultures].

Media used	Control (O.D at 600 nm)	4' mutant (O.D at 600 nm)	8' mutant (O.D at 600 nm)
Nutrient Broth	0.55	0.56	0.54
N.B. + Starch	0.84	0.84	0.85
N.B. + Glycine	0.60	0.62	0.58
Beef	0.59	0.58	0.58
Peptone	0.53	0.56	0.53

Table 3. Effect of media on enzyme production

pH of media	Control (O.D at 600 nm)	4' mutant (O.D at 600 nm)	8' mutant (O.D at 600 nm)
5	0.01	0.02	0.01
6	0.97	0.94	0.95
7	1.12	1.21	1.06
8	1.12	1.13	1.02

Table 4. Effect of media pH on bacterial growth [Note: It shows that at pH 7 cultures were showing best growth. Different pH of PBS i.e. 5, 6, 7 and 8 were analysed].

Metal used	Control (O.D at 600 nm)	4' mutant (O.D at 600 nm)	8' mutant (O.D at 600 nm)
Without metal	1.2	1.25	1.22
Calcium ion	1.28	1.03	1.34
Ferric ion	0.07	0.03	0.03
Magnesium ion	1.31	1.25	1.26
Lead ion	1.18	1.21	1.18
Zinc ion	0.92	0.90	0.95

Table 5. Effect of metal ions on growth of cultures *[Note*: 1% concentration of metal ions was used. The above Table shows that in the presence of Ca ++and Mg ++, cultures were showing maximum growth].

Incubation period (min)	Control (O.D at 540 nm)	4' mutant (O.D at 540 nm)	8' mutant (O.D at 540 nm)
3	0.58	0.68	0.62
6	0.58	0.64	0.64
9	0.54	0.60	0.60
12	0.61	0.56	0.64
15	0.55	0.56	0.58
18	0.52	0.57	0.55

Table 6. Effect of incubation time during enzyme assay. *[Note*: The above Table shows that incubation time was 3 min for maximum enzyme activity].

Temperature (°C)	Control (O.D at 540 nm)	4' mutants (O.D at 540 nm)	8' mutants (O.D at 540 nm)
0	0.65	0.83	0.73
Room temp.	0.73	0.86	0.77
37	0.77	0.87	0.82
50	0.71	0.81	0.81

Table 7. Effect of temperature on á-amylase activity.[Note: The above table shows that maximum activity was observed at 37^0C].

pH of substrate	Control (O.D at 540 nm)	4' mutant (O.D at 540 nm)	8' mutant (O.D at 540 nm)
6	0.75	0.80	0.74
6.5	0.69	0.77	0.71
7	0.81	1.01	0.86
7.5	0.73	0.81	0.75
8	0.77	0.84	0.78

Table 8. Effect of different pH of substrate on α-Amylase activity. The above table shows that maximum enzyme activity was obtained at the pH 7 of substrate].

Conc. Of starch (%)	Control (O.D at 540 nm)	4' mutant (O.D at 540 nm)	4' mutant (O.D at 540 nm)
0.5	0.62	0.75	0.75
1.0	0.87	0.91	0.91
2.0	1.00	1.11	1.11

Table 9. Effect of concentration of starch on enzyme activity. The above table shows that maximum activity was observed at 2% starch conc].

Incubation period is the time for the enzyme to react on substrate molecule and convert it into product molecule.

Discussion

This study concludes that by using physical mutagen, production of alpha amylase can be increased which was shows by U.V irradiation for 4–8 min. The results of the present study proved that a bacterial culture of *B. subtilis* can be successfully used for the production of alpha amylase that has lots of applications in industry and Biotechnology, from the previous studies it was found that N.B.+ 1% Starch is the suitable media for the growth of *B. subtilis* (Ajayi and Fagade, 2003) and also for the synthesis of α-amylase so at the starting of this experiment N.B + 1% Starch was taken as an initial media for the growth of *B. subtilis* when media plates spreaded with bacteria were exposed in the UV treatment, no morphological changes were observed except a fall in number of bacterial colonies. Mutants produced by the 4 min and 8 min UV exposure synthesized α-amylase in higher amount than control, for further studies only normal (control), 4' and 8' mutants were taken. Media optimization for these three cultures were done and found that cultures shows best growth and also produced higher enzyme in N.B.+ starch (1%), at pH 7, in the presence of calcium ion (Agrawal, 2005) whereas beef media was unsuitable for the growth and α-amylase production. Peptone media also gave nearly similar results as beef, during pH optimization it was found that at pH 5 the growth of bacteria was inhibited, but at pH 7-8 bacterial growth was optimum. In case of

optimization for metal ions it was found that calcium ion enhances the growth (Srivastava and Baruah, 1986) whereas in 1% ferric ion media, the growth was inhibited. In optimization conditions, to enhance the activity of enzymes it was found that 3 min incubation period at 50°C with higher substrate concentration of pH 7 was most suitable for the enzyme activity.

Conclusion

The result obtained in the present study indicate that effect of UV radiation can increased the alpha amylase production rate obtained by *B. subtilis* and also the optimization condition for *B. subtilis* provided the suitable growth at Peptone (0.5%) as a nitrogen, Beef extract (0.3%) with starch (1%) as a carbon source and calcium ion (1%) as an elicitor were used as a media component. Media pH was optimized near about neutral pH (7.0). During enzymatic reaction the reaction time for enzyme substrate reaction was 3 min at room temperature with substrate of neutral pH (7.0).

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