3. METHODOLOGY

3.1 Microorganism

*Bacillus amyloliquefaciens* was obtained from culture collection of Clonegen Biotechnology Private Limited, Noida. Bacterial culture was maintained on agar plates.

3.1.1 Culture medium

Generally, LB (Luria and Bertani) is used for growth of bacteria. Composition of LB agar (g/L) is Tryptone 10.0 g, Yeast extract 5.0 g, Sodium chloride 10.0 g and Agar 15.0 g and Distilled water 1000ml. LB Broth requires Tryptone 10.0 g, Yeast extract 5.0 g, Sodium chloride 10.0 g and Distilled water 1000ml.

1. Prepared 50 ml LB agar and Broth in two different 100 ml of conical flasks.
2. Conical flasks were sealed by cotton plugs.
3. Both medium sterilized in autoclave at 121°C for 15 minutes.

3.1.2 Preparation of inoculum

Both medium were prepared for inoculation of bacterial culture.

1. Media were poured in agar plate and tube.
2. Sterilized inoculating loop was used to streak bacteria on LB agar.
3. Used sterilized inoculating loop again for inoculation in LB broth.
4. Agar plate was kept in incubator in inverted position at 37°C for 24 hrs.
5. LB broth was kept in orbital shaker incubator for 24 hrs.

3.2 Strain improvement by UV mutagenesis

The changes in sequence of base or nucleotide of DNA are known as Mutation. Mutation can be induced by UV radiation, chemicals (nicotine and ethyl methane sulphonate), X ray and Gamma rays (Dong et al, 2011). Previous study suggested that enzyme production
can be increased by mutation. During UV radiation mutagenesis, electron of DNA molecule excited and created extra bond between pyrimidine suggested by Michelle Furlong.

3.2.1 UV irradiation

*Bacillus amyloliquefaciens* strain was improved by mutagenesis as described by (Shah et al., 1989; Dong et al., 2011; Ramamurthy et al., 1992; Khattab et al., 2012).

1. Twenty-one LB agar plates were spreaded by 50 µl of culture broth of Bacillus *amyloliquefaciens*.

2. Twenty agar plates exposed to 15 W of UV light from distance of 22 cm.

3. One agar plate was used as control.

4. After UV radiation, the treated cultures were protected from light for two hours.

5. Examined the growth of bacteria after 24 hours to count the number of bacterial colonies and the number of mutated colonies induced by UV light calculated by survival %.

\[
\text{Survival } \% = 100 \times \frac{\text{no. of mutated bacterial colony}}{\text{total no. of bacterial colony}}
\]

6. Selection was done on the basis of enzyme activity and starch hydrolysis test.

3.3 Morphological characteristic

Colonies are described as to such properties size, shape, texture, elevation, pigmentation, effect on growth medium.

3.3.1. Gram Staining

Gram staining method is used to distinguish unknown bacteria bacterial species in gram positive and gram negative. Bacterial cell wall composed of multi layered or thick layer of peptidoglycan, but some bacterial species contained monolayer on thin layer of Pepti-
-doglycan (Gephart et al, 1981; Gram, 1884).

1. Bacterial smear was prepared on glass slide.

2. Added few drops of crystal violet and incubated for 1 minute.

3. Carefully washed slide carefully with water.

4. Now added few drops of iodine and incubated for 1 minute.

5. Carefully washed slide carefully with water.

6. Added few drops of acetone and washed again.

7. Used few drops of safranin and incubated for 1 minute.

8. Carefully washed slide carefully with water.

9. Microscope was used for observation.

3.3.2. Motility

The motility of microbes is determined by this test. It requires motility indole lysine medium (Difco, 1998; Reller et al, 1975). Composition of Motility-indole-lysine medium (g/l): Tryptone 10.0 g, Yeast extract 3.0 g, Ferric ammonium citrate 0.5 g L-lysine hydrochloride 10.0 g, Dextrose 1.0 g, Bromocresol purple 0.02 g, Peptone 10.0 g, Agar 2.0 g and Distilled water 1000ml.

1. Poured medium in tube.

2. Sterilized needle was used to stab medium.

3. Inoculated bacterial culture in same line with sterilized needle.

4. Tube was kept in incubator for 24 hrs.

3.3.3. Growth in air

Bacteria require oxygen for respiration purposes. ATP provides energy to the cellular processes in the bacteria (Prescott et al, 2005). Bacteria that need oxygen for growth are
called as aerobic. Oxygen is toxic to some bacteria and these are called anaerobic.

Composition of Nutrient agar (g/L): Beef extract 3.0g, Peptone 5.0g, Sodium chloride 5.0g, Agar 20.0g and Distilled water 1000ml.

1. Prepared two nutrient agar slants.
2. Streaked the bacteria in it.
3. One tube was cotton plugged and another covered with para film.
4. Tube was incubated for 24 hrs at 37°C.

3.3.4. Growth at different temperature

Mostly bacteria grow on 37°C but some are temperature sensitive. Some temperature range was selected by culturing the inoculums at different temperatures (4, 25, 37 and 50°C) (Mishra and Behera, 2008).

1. Prepared four LB agar plates.
2. Streaked inoculum on it.
3. Incubated according to temperature, 4 °C (in refrigerator), 25 °C (on table), 37 °C (in incubator) and 50 °C (in incubator) for 24 hours.

3.3.5. Salt Tolerance test

In order to determine salt tolerance ability of bacteria, the inoculums incubated at high concentration of NaCl (5% and 10%) in medium (Metin et al, 2010). Composition of 5% NaCl medium (g/L): Yeast extract 5.0g, Tryptone 10.0g, NaCl 5.0g, Agar 15.0g and Distilled water 1000ml. Composition of 10% NaCl medium (g/L): Yeast extract 5.0g, Tryptone 10.0g, NaCl 10.0g, Agar 15.0g and Distilled water 1000ml.

1. Prepared two agar plates containing 5% and 10% NaCl respectively.
2. Streaked inoculum on it.
3. Agar plates were incubated for 24 hrs at 37°C.

3.3.6. Biochemical Test

Biochemical test is group of test included, imvic, oxidase, catalase, gelatin utilization, starch hydrolysis, sugar fermentation etc, used to certain important characteristics of an organism's.

3.3.6.1 IMViC Test

I. Indole Test

This method is used to determine degradation of typtophan amino acid and production of indole.(Harley, 2005; Isenberg, 1958).Composition of Tryptone broth: Tryptone 10.0g, NaCl 5.0g, Distilled water 100ml (Difco, 1998). Composition kovac’s Reagent: N amyl alcohol 150 ml, Conc. HCl 50 ml, p- dimethyl benzaldehyde 10.0g.

1. Prepared 5 ml typtone broth and sterilized in autoclaved.

2. Cooled it to room temperature.


4. Transferred 10 µl of bacterial culture in broth.

5. Incubated tube for 48 hrs at 37°C.

6. After incubation, added few drops of Kovac’s reagent.

7. Observed the changes.

II. Methyl Red and Voges–Proskauer test (MR/VP)

This test consists of two methods. Both methods are performed together due to requirement of same broth. It is used buffered peptone- glucose broth (Barritt 1936; Macfaddin 1980). Composition of Buffered peptone-glucose broth (g/L): Di potassium hydrogen phosphate 5.0g, Peptone 5.0g, Glucose, 5.0g Distilled water 1000ml.
Composition of Methyl red solution: Methyl red 0.1 g and Ethanol (95%) 300 ml.

Solution A: Dissolved 6g of α naphthol in 100 ml of 95% ethyl alcohol.

Solution B: Dissolved 16g KOH in 1000ml of water.

**Methyl Red**

1. Prepared 10 ml buffered peptone- glucose broth in tube and autoclaved.
2. Cooled it to room temperature.
3. Poured 5 ml medium in tube.
4. Transferred 10 µl of bacterial culture in broth.
5. Incubated tube for 48 hrs at 37°C.
6. After incubation, added 4 drops of methyl red solution.
7. Observed the change of color.

**Voges–Proskauer**

1. Transferred remaining 5ml of broth in tube.
2. Transferred 10 µl of bacterial culture in broth.
3. Incubated tube for 48 hrs at 37°C.
4. After incubation, added 1 ml of napthol (solution A) and shaked for mixing.
5. Now added 1 ml of KOH (solution B) and shaked.
6. Incubated for 30 minutes.
7. Observed the change of color.

**III. Simmon’s citrate agar**

This test is used to detect utilization of citrate (carbon source) (Reddy, 2007; Harley, 2005). Composition of Simmons citrate medium(g/L): Magnesium sulfate 0.2 g, Ammonium dihydrogen phosphate 1.0 g, Dipotassium phosphate 1.0 g, Sodium citrate
(dehydrate) 2.0 g, Sodium chloride 5.0 g, Agar 15.0 g, Bromothymol blue 0.08 g and Distilled water 1000 ml.

1. Prepared 5ml citrate agar and autoclaved it.
2. Poured medium in tube and allowed it to solidify in slant form.
3. Streaked bacteria on slant.

3.3.7 Catalase Test (Slide drop method)

It is used to determine catalase enzyme. Most of anaerobic bacteria lack the enzyme (Mahon et al, 2011; McLeod and Gordon, 1923). Catalase enzyme splits hydrogen peroxides into water molecules and oxygen (Clarke and Cowan, 1952; MacFaddin, 2000).

1. Prepared thin smear of bacterial culture on glass slide under (laminar air flow).
2. Added few drops of hydrogen peroxides.
3. Observed the changes.

3.3.8 Oxidase test (Filter Paper Test Method)

This test determines presence of oxidase enzyme (Gaby and Free 1958; MacFaddin 1972; Oser 1965). It produces dark color during reaction (Gaby and Free, 1958; Gerhardt et al, 1981; Lui and Jurtshuk, 1986; Kovac, 1956).

1. Cut squared shape strip from filter paper.
2. Used kovac’s reagent to saturate filter paper strip.
3. Rubbed bacterial colony on filter paper.
4. Observed the changes.
3.3.9 Gelatin Utilization (Nutrient gelatin stab method)

This test is used to detect secretion of extracellular gelatinases enzyme in medium (Leboffe and Pierce, 2010). Composition of medium (g/L): Beef extract 3.0 g, Gelatin 120.0 g, Peptone 5.0 g, and Distilled water 1000 ml.

1. Used a needle to stab the gelatine and to inoculate the media.

2. Incubated, inoculated media for at least 48 hours.

3. Transferred to the refrigerator.

4. The media completely chilled before it was taken out for observation.

3.3.10 Starch Hydrolysis

This test is used to detect production of alpha amylase by bacterial species. Composition of starch agar media (g/L): Beef extract 3.0g, Tyrptone 5.0g, Starch 3.0g, Agar 20.0g and Distilled water 1000 ml.

1. Prepared 100 ml medium and autoclaved it.

2. Transferred in agar plate and allowed it to solidify.

3. Streaked bacterial culture by inoculating loop.

4. Incubated for 24 hrs at 37 °C.

5. After incubation, agar plate was flooded by iodine.

6. Observed the changes.

3.3.11 Carbohydrate Fermentation

This test is used to determine particular carbohydrates (Bartelt, 2000). There were three sources of carbohydrates such as lactose, dextrose, glucose used for fermentation. Prepared different three carbohydrate ferment broth used for fermentation (MacFaddin, 2000; Stanier et al, 1963; Forbes et al, 2007; Mahon et al, 2011). Carbohydrate
fermentation (g/L) Glucose Broth: Beef Extract 3.0g, Peptone 5.0g, Tryptone 5.0g, Glucose 5.0g, Distilled Water 1000ml; Dextrose Broth: Beef Extract 3.0g, Peptone 5.0g, Tryptone 5.0g, and Dextrose 5.0g, Distilled Water 1000ml; Lactose Broth: Beef Extract 3g, Peptone 5.0g, Tryptone 5.0g, Lactose 5.0g, Distilled Water 1000ml.

1. Prepared broth medium and used single carbohydrate for each medium.
2. Added a drop of Bromo cresol purple.
3. Inoculated bacterial species in each tube.
4. Incubated all tubes for 24 – 48 hrs.
5. Observed the changes of color.

3.3.12 Antibiotic Susceptibility Test

This test is used to detect resistance and sensitivity towards bacterial species for particular antibiotic (Bauer et al, 1966). There were four antibiotics used for sensitivity test Chloramphenicol (30 µg/ ml), Nalidixic acid (30 µg/ ml), Polymixin B (30 µg/ ml) and Streptomycin (10 µg/ ml). Composition of medium (g/L); Beef extract 3.0g, Starch 3.0g, Agar 20.0g and Distilled water 1000 ml (Difco, 1984).

1. Prepared 100 ml medium and autoclaved it.
2. Transferred medium in agar plate and kept for solidification.
3. 100-150 µl of bacterial culture spreaded on agar surface.
4. Spreading was done by spreader.
5. After few minutes, kept the antibiotic disc carefully.
6. Incubated for 24 hrs at 37 °C in incubator.
7. Next day, observed zone of inhibition.
8. Used scale to measure zone in millimeter.
9. Marked ‘0’ to no zone of inhibition.

3.3.13 Random amplified polymorphic DNA- PCR

RAPD is one of the most important tools in biological research. The detection of a specific DNA sequence starting from small amounts of DNA requires the design of primers flanking the sequence (Mori et al, 1999). This technique has become very popular due to their ability to easily and rapidly amplification. Random amplification technology is a tool which useful in many areas of genetic research such as gene mapping, individual and strain identification, population genetics and phylogenetics (Micheli et al, 1997; (Kubota et al, 1992, 1995; Peinado et al, 1992; Ionov et al, 1993; Savva, 1996; Shimada and Shima, 1998; Atienzar et al, 1999; Becerril et al, 1999).

RAPD primers can be amplified (3- 20) loci during amplification. It is also used to detect genomic variation and genome mapping RAPD primers are decamer and have 10- 12 nulceotides (Donahue et al, 1994; Nelson et al, 1996). Its preparation is same as PCR in terms of DNA isolation, Quantification of DNA and amplification (White et al, 1990; Welsh et al, 1991; Atienzar et al, 1999).

3.3.13.1 Genomic DNA Isolation by CTAB method

The CTAB (cetyl trimethyl ammonium bromide) method was used for isolation of DNA (Lipp et al, 1999; (Isabel et al, 1995; Via and Falkinham, 1995). Composition of TE buffer : Tris HCL (pH 8.0) 1 ml, EDTA 0.2 ml and Distilled water 100 ml. Composition of 5M NaCl: NaCl 29.22 g and Distilled water 100 ml. Composition of CTAB buffer: NaCl 4.2 g, CTAB 10. 0g and Distilled water 80 ml.

1. Transferred 1.5 ml of culture broth in Eppendorf.

2. Centrifuged at 5000 rpm for 5 mins.
3. Discard the supernatant and added 500 µl TE buffer in pellet.

4. Transferred 20 µl of SDS and 50 µl of proteinase K.

5. Incubated for 15 minutes at 65°C.

6. Added 80 µl CTAB buffer and 100 µl 5M NaCl and vigorously vortexed.

7. Again incubated for 15 minutes at 65°C.

8. Centrifuged at 10,000 rpm for 5 mins.


11. Centrifuged at 10,000 rpm for 5 mins.

12. Added chilled isopropanol and incubated for 60 minutes at -4°C.

13. Centrifuged at 10,000 rpm for 10 mins.

14. Discarded supernatant and pellet was washed with 70% ethanol.

15. Kept it for airdry overnight.


**3.3.13.2 Quantification of DNA**

The concentration of DNA and purity can be detected by Ratio of absorbance at 260/280 and Agarose gel Electrophoresis (Di Pinto et al, 2007; Gupta et al, 2011). One optical density at 260 nm is equal to 50µg/mL DNA (Sambrock et al., 1989). 50-100µg/ml (DNA Standard). Absorbance was taken at 270 nm for DNA Standard. The unknown samples were measured at 260nm and 280nm using UV-VIS Spectrophotometer 119, Systronics.
I. DNA quantification by DPA (Diphenylamine acid) method

A standard method of measuring the amount of DNA in solution is to measure its absorbance of ultraviolet light. For this assay, denatured DNA is reacted with acidified diphenylamine to produce a dark blue colored compound. The reaction depends on the conversion of the pentose to w-hydroxylaevulinc aldehyde which then reacts with diphenylamine to give a blue colored complexed. (Harkulkar 2012).

Composition of (SC) Sodium Citrate Buffer (g/l): NaCl 175.3 g, Tris-sodium citrate 88.2g. Composition of DPA reagent: DPA 0.3 g, Glacial Acetic Acid 30 ml, Conc Sulphuric Acid 750 µl, Distilled water 30 ml.

1. Prepared 1mg/ml of Calf thymus DNA in distilled water and different dilution (100, 200, 300, 400 and 500 µl).

2. Ten test tubes were marked according to DNA standard (S1, S2, S3, S4 and S5) and unknown (W, M1, M2, M3 and M4).

3. Transfered 100, 200, 300, 400 and 500 µl standard DNA in five tubes (S1, S2, S3, S4 and S5) respectively.

4. 10 µl sample DNA added in (W, M1, M2, M3 and M4) five test tubes.

5. Added 1XSC buffer to each tube 400, 300, 200, 100, 0 to DNA standard (S1, S2, S3, S4 and S5) respectively.

6. 490 µl of 1XSC buffer transferred to unknown sample (W, M1, M2, M3 and M4) five tubes.

7. Added 1 ml DPA reagent to each tube.

8. Boiled tubes at 95 °C for 10 minutes in water bath.

9. Cooled at room temperature and observed light blue color.
II. DNA quantification by Absorbance at 260 and 280 nm

The DNA quantification can be done by UV-VIS Spectrophotometer (Systronics, 119). The ratio of absorbance at 260 and 280 nm determines purity of DNA (Moyo et al, 2008).

1. Started Spectrophotometer and set the wavelength.
2. Labeled six test tubes, B, W, M1, M2, M3 and M4.
3. Diluted a portion of the DNA sample of 10 µl in 990 µl distilled water.
4. Make sure that the final volume is 1.0 ml.
5. Recorded optical density $A_{260}$ and $A_{280}$ nm.

III. Qualitative analysis of DNA by Agarose Gel Electrophoresis

The quality of isolated genomic DNA can be detected by Agarose gel Electrophoresis. (Thakuria et al, 2008; Gupta et al, 2011). It requires agarose, TAE buffer and Ethidium Bromide (Etbr) for preparation of gel. Composition of TAE buffer: Tris Base 2.0g, 0.5 M EDTA 100ml, Glacial Acetic Acid1.0 M and Distilled water to make volume 1000ml according to Crandall and Barber.

1. Weighed 0.4 gm of agarose for preparation of 0.8% gel.
2. Dissolved it in 50 ml of 1XTAE buffer.
3. Heated in microwave for 45 seconds.
4. Added 2 µl EtBr after 5 minutes.
5. Assembled the casting tray.
6. Poured gel in tray and placed comb.
7. Kept it for 30 minutes to solidify
8. Removed comb and placed the casting tray in electrophoretic unit and added 1xTAE buffer.
9. DNA ladder loaded in first well.

10. Mixed 1X loading dye (2µl) and 5 µl sample and loaded in wells.

11. Allowed the gel to run at 80 Volts for 35 minutes.

12. After 30 mintues, gel was removed carefully and placed on UV transilluminator.

13. Observed the DNA bands.

3.3.13.3 Amplification by RAPD-PCR

The DNA isolated from an individual is amplified using a PCR reaction and arbitrary oligonucleotide primers, which will hybride the complementary sequences, when these exist. The short primers sequences, random paired (8 – 12 base pairs) are used for DNA RAPD type amplification, usually resulting a presence/absence polymorphism in the gel. A situation like this, which allows the random amplification, in more points, can be realized at the whole genome. The changing of a single base in the genome is sufficient for inhibiting the primer alignment to that place and the fragment amplification, defined by the primer. RAPD analysis can detect the modification of a single base in the genomic DNA, in some conditions (Oroian et al, 2009).PCR (Polymerase Chain Reaction) is used to make billion copies of DNA. There are three main steps included in the process. Denaturation, Annealing and Extension are involved.

Composition of master mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>2.0µl</td>
</tr>
<tr>
<td>Primer (Sigma Genosys)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Taq buffer (Genecea)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Taq polymearase (Bangalore, Genei)</td>
<td>0.50µl</td>
</tr>
<tr>
<td>dNTP (Bangalore, Genei)</td>
<td>2.5 µl</td>
</tr>
</tbody>
</table>
Sterilized distill water 17.0 µl

**Primers**

A single primer with 5’-3’ sequence is needed for RAPD reaction, and we used the following single primers for amplification.

RAPD primers had been used from SIGMA-GENOSYS.

Table 3.1 The decamer sequences of primers used in RAPD reaction

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>$T_m$ (°C)</th>
<th>GC%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VAA 06</td>
<td>GGTCCCTGAC</td>
<td>30.8</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>VAA 16</td>
<td>AGCCAGCGAA</td>
<td>40.5</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>VAA 17</td>
<td>GACCGCTTGT</td>
<td>33.7</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>VAA 18</td>
<td>AGGTGACCCTG</td>
<td>31.6</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>VAA 19</td>
<td>CAAACGTCGG</td>
<td>36.6</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>VAA 20</td>
<td>GTTGCGATCC</td>
<td>34.2</td>
<td>60</td>
</tr>
</tbody>
</table>

**Reaction Conditions**

PCR reaction was carried out for 35 cycles in Thermal cycler (Eppendorf, Germany).

- **Initial denaturation** 94°C 4 minutes
- **Denaturation** 94°C 1 minutes
- **Annealing** 37°C 1 minutes
- **Extension** 72°C 1:30 minutes
- **Final extension** 72°C 10 minutes
- **Hold** 15 minutes
1. Take six 0.5 ml microfuge tubes.

2. Prepared master mix and samples in tubes and final volume was 25 µl.

3. Whole process was carried out on ice.

4. After sample preparation, tubes was kept in thermal cycler.

5. Set the programming for amplification for 35 cycles.

**Visualization of amplified products on 1% Agarose gel**

The amplification products were analyzed by electrophoresis in a 1.0% agarose.

1. Prepared a 1% (i.e. 1.0 grams of agarose per 50 mL 1x TAE buffer).

2. Heat for 35 seconds and after cooling to room temperature, added 2 µL ethidium bromide.

3. Poured in casting tray and allow solidifying.

4. Kept the gel in unit and added sufficient amount of 1x TAE buffer.

5. Loaded DNA standard and PCR product of samples with loading dye.

6. Run the gel at 85 volt for 50 minute.

7. The gel was visualized and photographed under UV Transilluminator (Moyo et al., 2008; Di Pinto et al., 2007).

**3.3.13.4 Data analysis of RAPDs**

These data were used for the calculation of pairwise genetic distances between trees using the Jaccard coefficient similarity matrices. The data sets were analyzed using Dendro UPGMA, a dendrogram construction utility (Garcia-Vallve 1999 DendroUPGMA: Biochemistry and Biotechnology). The Jaccard’s coefficient (Jaccard 1908) was used to prepare similarity matrix. Jaccord suggested formula to create similarity matrix. J represents Jaccord coefficient, $M_{11}$ represents common bands, $M_{10}$ represents band
in first lane and \( M_{01} \) represents band in second lane (Raghunathachari et al., 2000). The unweighted pair group method with arithmetic averages (UPGMA) was used to construct dendrogram (Garcia et al., 1999; Kumar et al., 2010).

### 3.3.14 Fermentation technique by shake flask method

This method is used to produce enzyme from microbes. Fermentation can be carried out by two methods are Submerged Fermentation (SmF) and Solid state Fermentation (SSF). Submerged fermentation is a liquid medium which contains carbon sources, nitrogen sources, nutrients and metallic chlorides, and some times metal ions for growth of microbes. Microbes consume nutrients and release extracellular enzyme in liquid broth suggested (Emma Weir and Colette McSpadden).

Solid state fermentation requires solid substrate. It includes wheat bran, rice husk, coconut oil cake, groundnut oil cake and agricultural by-products.

The composition of the amylase production medium was (g/l) Bacteriological peptone 6.0 g, MgSO\(_4\) 0.5 g, KCl 0.5 g, Starch 1.0 g and 1000 ml distilled water pH 7.

1. Prepared 250 ml fermentation medium and sterilized in autoclave at 121 °C for 15 min.
2. Transferred 50 ml medium in five different 100 ml of conical flasks.
3. Inoculum was prepared by inoculating the loopful of strain of W, M1, M2 and M3 and M4 (Agar plates) into LB broth and it was incubated in shaker for 24 hrs.
4. Transferred 50µl inoculum in amylase production medium and incubated in shaker for 48 hrs (Khan et al, 2011).
5. Incubated in Orbital incubator shaker for 48 hrs.
Culture of parent and four mutant strain of bacillus such as W, M1, M2 and M3 and M4 were prepared for further study (Dey et al, 2002; Syu and Chen, 1997).

3.3.14.1 Preparation of enzyme

After 48 hrs, Culture broth (amylase production medium) was transferred in 1 ml eppendorf. The culture broth was centrifuged (REMI) at 1000rpm for 10 mins. Supernatant was transferred in other eppendorf and used as crude enzyme for estimation of enzyme activity (Tanyilidizi et al. 2005 and 2007).

3.3.14.2 DNS Method for estimation of reducing sugar

Maltose standard curve is used to estimate unknown samples. DNS (3,5- Dinitro salicylic acid) binds to free radical aldehyde and stopped the reaction, which produces red color in solution (Miller, 1959). Composition of DNS Reagent for 100ml: 3,5 DNS acid 0.5g, Sodium PotassiumTartarate 15g, 2N NaOH 10ml and Distilled Water make up to 100 ml. Maltose solution 100 ml: Maltose 180.0g and Distilled water 100ml.

1. Prepare solution of 2N NaOH 10 ml. (Dissolve 0.8g NaOH in 10 ml D/W).
2. Add 0.5 g DNS acid.
3. Make up volume at 100ml.
4. Dissolve thoroughly by magnetic stirrer.
5. After two hrs add small amount of 15 g Sodium Potassium Tartarate at interval of five minutes.

Preparation of Maltose standard

1. Took 11 test tubes wash and clean thoroughly.
2. Labeled tubes with 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0.
3. Transferred appropriate amount of maltose (100 – 1000 µL) to (0.1 – 1.0) labeled test tube respectively.

4. Added distilled water to all test tubes.

5. Transferred 1mL DNS to each tubes.

6. Kept all test tubes in water bath for 10 mins at 95 to 100 °C.

7. Cooled the tubes at room temperature.

8. Pipette 8ml distilled water to test tube and mixed well.

9. Set the blank with 0.5 ml distilled water.

10. Measured the absorbance of each tube at 540 nm.

3.3.15 Optimization of α-amylase

To determine optimum ranges of temperature, pH, substrate concentration and incubation time were determined. Different ranges of temperature were 50, 55, 60, 65, 70, 75, 80 and 85°C. The ranges of pH were 4, 5, 6, 7, 8, and 9. Different times were included for incubation time 24, 36, 48, 60, 72 and 84 hours. Various ranges of substrate concentration were 1, 2, 3, 4, 5 and 6% used for optimization. (Irshad et al, 2012; Gangadharan et al, 2006).

3.3.16 Determination of enzyme activity

Enzyme activity is micromoles of substrate converted per unit time. Soluble starch used as substrate to determine enzyme activity (Berneld, 1955).

\[
\text{Enzyme activity (IU/ml/min) = } \frac{\text{Amount of reducing sugar x 1000}}{\text{Molecular wt of maltose x incubation time}}
\]
3.3.17 Effect of Temperature

The different ranges of temperature were 50, 55, 60, 65, 70, 75, 80 and 85˚ C used to determine optimum temperature for production of alpha amylase (Kumar et al., 2012) from crude enzyme of wild and mutant strains.

1. Transferred 1 ml crude enzyme in five centrifuge tubes marked with W (wild), M1 (mutant 1), M2 (mutant 2), M3 (mutant 3) and M4 (mutant 4).
2. Kept all the tube in water bath at 50 to 85˚C for 10 minutes.
3. Cooled the tubes at room temperature.
4. Pipette 0.5 ml of 1% starch solution in another tube.
5. Added 0.5 ml enzyme and incubate for exactly 3 minutes.
6. Transferred 1 ml DNS reagent to each tube.
7. Kept test tubes in water bath for 10 minutes at 95˚C.
8. Cooled the tubes at room temperature.
9. Added 2ml distilled water to each tube.
10. Set the blank with 0.5 ml distilled water.
11. Measured the absorbance of each tube at 540 nm.

3.3.18 Effect of pH on amylase production

The ranges of pH were 4, 5, 6, 7, 8, and 9 used to determine optimum temperature for production of alpha amylase (Kumar et al., 2012) from crude enzyme of wild and mutant strains (Vidyalakshami et al, 2009).

1. Prepared 50 mL amylase production broth.
2. Checked pH of amylase production broth by pH meter or pH strip.
3. Maintained pH at 4, 5 and 6 by adding few drops of H$_2$SO$_4$ in broth.

4. Applied same method for different pH range.

5. Used NaOH for alkaline medium (pH 8 and 9).

6. Autoclave amylase production broth with different pH.

7. Inoculated 50 µl inoculum of different strains (W, M1, M2, M3 and M4) in broth.

8. Kept each boiling tube with broth in shaker incubator for 48 hrs.

9. Took centrifuge tube and marked with W, M1, M2, M3 and M4.

10. Transferred 1mL broth and centrifuge at 6000 rpm for 20 mins.

11. Took supernatant in another tube.

12. Pipette 0.5 ml of 1% starch solution in another tube.

13. Added 0.5 ml enzyme and incubate for exactly 3 minutes.

14. Transferred 1 ml DNS reagent to each tube.

15. Kept tubes in water bath for 10 minutes at 95˚C

16. Cooled the tubes at room temperature.

17. Added 2ml distilled water to each tube.

18. Set the blank with 0.5 ml distilled water.

19. Measured the absorbance of each tube at 540 nm.

**3.3.19 Effect of incubation time**

The optimum time required for maximum amylase activity, cultures were harvested at different time interval (24, 36, 48, 60, 72 and 84) hours (Ramachandran et al., 2004).

1. Took 1 mL supernatant (enzyme) from amylase production in centrifuge tube marked with W (wild), M1 (mutant 1), M2 (mutant 2), M3 (mutant 3) and M4 (Mutant 4).

2. Transferred 1ml broth and centrifuge at 6000 rpm for 20 mins.
3. Took supernatant in another tube.

4. Pipette 0.5 ml of 1% starch in another tube.

5. Added 0.5 ml enzyme and incubate for exactly 24, 36, 48, 60, 72 and 84 hours respectively.

6.Transferred 1 ml DNS reagent to each tube.

7. Kept tubes in water bath for 10 minutes at 95°C.

8. Cooled the tubes at room temperature.

9. Added 2ml distilled water to each tube.

10. Set the blank with 0.5 ml distilled water.

11. Measured the absorbance of each tube at 540 nm.

### 3.3.20 Effect of substrate concentration

Substrate concentration was carried out at different 1%, 2%, 3%, 4%, 5% and 6% starch concentration in PBS (Phosphate buffer) (Ibrahim et al, 2011).

1. Take 1 mL supernatant (enzyme) from amylase production in centrifuge tube marked with W (wild), M1 (mutant 1), M2 (mutant 2), M3 (mutant 3).

2. Transferred 1mL broth and centrifuge at 6000 rpm for 20 mins.

3. Took supernatant in another tube.

4. Pipette 0.5 ml of 1% starch in another tube.

5. Added 0.5 ml enzyme and incubate for exactly 3 minutes

6. Transferred 1 ml DNS reagent to each tube.

7. Applied same method for 2% to 6% starch (in phosphate buffer saline).

8. Kept tubes in water bath for 10 minutes at 95°C.

9. Cooled the tubes at room temperature.
10. Added 2ml distilled water to each tube.

11. Set the blank with 0.5 ml distilled water.

12. Measured the absorbance of each tube at 540 nm.

### 3.3.21 Effect of Carbon source

Dextrose, lactose, sucrose and maltose carbon source were used for optimization (Lee et al., 2008). Composition of medium (g/L): Bacteriological peptone 6 g, MgSO₄ 0.5g, KCl 0.5g, Starch 1.0g, Carbon source 0.5g and 1000 ml distilled water.

1. Take 1 mL supernatant (enzyme) from amylase production in centrifuge tube marked with W (wild), M1 (mutant 1), M2 (mutant 2), M3 (mutant 3).

2. Transferred 1mL broth and centrifuge at 6000 rpm for 20 mins.

3. Took supernatant in another tube.

4. Pipette 0.5 ml of 1% starch in another tube.

5. Added 0.5 ml enzyme containing (dextrose) and incubate for exactly 3 minutes

6. Transferred 1 ml DNS reagent to each tube.


8. Kept tubes in water bath for 10 minutes at 95°C.

9. Cooled the tubes at room temperature.

10. Added 2mL distilled water to each tube.

11. Set the blank with 0.5 ml distilled water.

12. Measured the absorbance of each tube at 540 nm.

### 3.3.22 Effect of nitrogen source

Nitrogen sources were urea, yeast extract, NH₄Cl and KNO₃ included to determine enzyme activity (Lee et al., 2008). Composition of medium (g/L): Bacteriological peptone
6 g, MgSO$_4$ 0.5g, KCl 0.5g, Starch 1.0g, Nitrogen source 0.5g and 1000 ml distilled water.

1. Take 1 mL supernatant (enzyme) from amylase production in centrifuge tube marked with W (wild), M1 (mutant 1), M2 (mutant 2), M3 (mutant 3).
2. Transferred 1ml broth and centrifuge at 6000 rpm for 20 mins.
3. Took supernatant in another tube.
4. Pipette 0.5 ml of 1% starch in another tube.
5. Added 0.5 ml enzyme containing (urea) and incubate for exactly 3 minutes
6. Transferred 1 ml DNS reagent to each tube.
7. Applied same method for yeast extract, NH$_4$Cl and KNO$_3$.
8. Kept tubes in water bath for 10 minutes at 95˚ C.
9. Cooled the tubes at room temperature.
10. Added 2mL distilled water to each tube.
11. Set the blank with 0.5 ml distilled water.
12. Measured the absorbance of each tube at 540 nm.

3.3.23 Effect of metal ions

The different metal ions were included as CaCl$_2$, MnCl$_2$, FeSO$_4$ and CuSO$_4$ at concentration of (0.5g/l). Composition of medium (g/L): Bacteriological peptone 6 g, MgSO$_4$ 0.5g, KCl 0.5g, Starch 1.0g, Metal ions 0.5g and 1000 ml distilled water. (Muhammad et al, 2012; Mishra et al, 2010; Asgher et al, 2007; Dubey et al, 2011). (Patel et al, 2005) (Li et al, 2005).

1. Transferred 1ml broth and centrifuge at 6000 rpm for 20 mins.
2. Took supernatant in another tube.
3. Pipette 0.5 ml of 1% starch in another tube.

4. Added 0.5 ml enzyme containing (CaCl$_2$) and incubate for exactly 3 minutes

5. Transferred 1 ml DNS reagent to each tube.


7. Kept tubes in water bath for 10 minutes at 95° C.

8. Cooled the tubes at room temperature.

9. Added 2ml distilled water to each tube.

10. Set the blank with 0.5 ml distilled water.

11. Measured the absorbance of each tube at 540 nm.

### 3.3.24 Effect of Polyethylene Glycol (PEG)

Concentration of PEG 600 (Sigma) (1%, 2%, 3%, 4% and 5% were used in medium. (Khan et al, 2011). (Bezerra et al, 2006). (Ulger and Curakoglu 2001). Composition of medium: Bacteriological peptone 6 g, MgSO$_4$ 0.5g, KCl 0.5g, Starch 1.0g, PEG 0.1g and 1000 ml distilled water.

1. Transferred 1ml broth and centrifuge at 6000 rpm for 20 mins.

2. Took supernatant in another tube.

3. Pipette 0.5 ml of 1% starch in another tube.

4. Added 0.5 ml enzyme containing (1% PEG) and incubate for exactly 3 minutes

5. Transferred 1 ml DNS reagent to each tube.

6. Applied same method for 2%, 3%, 4% and 5% PEG.

7. Kept tubes in water bath for 10 minutes at 95° C.

8. Cooled the tubes at room temperature.

9. Added 2ml distilled water to each tube.
10. Set the blank with 0.5 ml distilled water.

11. Measured the absorbance of each tube at 540 nm.

3.3.25 Effect of EDTA and CuO

The effect of EDTA and CuO at 0.5g/L concentration was used to determine effect on enzyme activity (Chanda et al, 2012). Composition of medium : Bacteriological peptone 6 g, MgSO$_4$ 0.5g, KCl 0.5g, Starch 1.0g, EDTA or CuO 0.5 g and 1000 ml distilled water.

1. Transferred 1ml broth and centrifuge at 6000 rpm for 20 mins.

2. Took supernatant in another tube.

3. Pipette 0.5 ml of 1% starch in another tube.

4. Added 0.5 ml enzyme containing (EDTA) and incubate for exactly 3 minutes

5. Transferred 1 ml DNS reagent to each tube.

6. Applied same method for CuO.

7. Kept test tubes in water bath for 10 minutes at 95˚ C.

8. Cooled the tubes at room temperature.

9. Added 2ml distilled water to each tube.

10. Set the blank with 0.5 ml distilled water.

11. Measured the absorbance of each tube at 540 nm.

3.3.26 Effect of low and high concentration of media ingredients

The concentration of different media ingredients were starch, peptone and Magnesium sulphate used to determine effect on enzyme activity. Composition of medium (starch) : Bacteriological peptone 6 g, MgSO$_4$ 0.5g, KCl 0.5g, Starch 0.5 g or 2.0g and 1000 ml distilled water. Composition of medium (Peptone): Bacteriological peptone 6 g, MgSO$_4$
0.5g, KCl 0.5g, Peptone 0.3 g or 12.0g and 1000 ml distilled water. Composition of medium (Magnesium sulphate) : Bacteriological peptone 6 g, MgSO₄ 0.5g, KCl 0.5g, Magnesium sulphate 0.25 or 1.0g and 1000 ml distilled water.

1. Transferred 1ml broth and centrifuge at 6000 rpm for 20 mins.
2. Took supernatant in another tube.
3. Pipette 0.5 ml of 1% starch in another tube.
4. Added 0.5 ml enzyme containing (Starch) and incubate for exactly 3 minutes
5. Transferred 1 ml DNS reagent to each tube.
6. Applied same method for peptone and Magnesium sulphate.
7. Kept test tubes in water bath for 10 minutes at 95˚ C.
8. Cooled the tubes at room temperature.
9. Added 2ml distilled water to each tube.
10. Set the blank with 0.5 ml distilled water.
11. Measured the absorbance of each tube at 540 nm.

3.3.27 Desizing of cotton strip

The removal of starch called as desizing process. Equal size of cotton cloth’s strip was used (2.5 x 2.5 inch)(Chimata et al, 2011; Haq et al, 2010).

1. Weighed the two cotton strip on balance.
2. Noted down weight.
3. Cotton strip dipped in 50 ml of crude enzyme of wild and mutant strain.
4. Incubated it at 60 ° C for 1 hrs.
5. Rinsed cotton strips in running water.
6. Dried in oven.
7. Cotton strip weighed again.

Desizing % can be calculated by divide weight of starch removed by enzyme to starch present on the cotton strip. 0.1N Sulphuric acid is used to determine total starch.

3.3.28 Determination of $K_m$ and $V_{\text{max}}$

The Lineweaver Burk plot is used to determine enzyme kinetics. Lineweaver Burk plot is done by using Graph pad prism 6 (Wanderley et al, 2004; Metin et al, 2010; Avci and Donmez 2012) for estimation of $V_{\text{max}}$ and $K_m$ values. The concentrations of starch solution were 1%, 2%, 3%, 4%, 5% and 6%. The different concentration of starch dissolved separate in 100 ml of Phosphate buffer.

1. Prepared starch solution 1%, 2%, 3%, 4%, 5% and 6% in phosphate buffer.

2. Took six clean and dry test tubes and labeled them 1, 2, 3, 4, 5, and 6.

3. Added 0.5 ml of enzyme (M1) to each tube.

4. Transferred 4 ml of phosphate buffer to each tube.

5. Added 1 ml of starch solution in (1%, 2%, 3%, 4%, 5% and 6%) respected tubes 1, 2, 3, 4, 5, and 6.

6. Incubated for 10 mins.

7. Boiled in water bath for 15 minutes.

8. Measured the absorbance at 540nm.

3.3.29 Partial purification of protein

Partial purification can be done by ammonium sulphate precipitation and acetone precipitation.

3.3.29.1 Protein fractionation (Ammonium Sulfate Precipitation)

Ammonium salts precipitate out protein in solution. It has tendency to breakdown water
molecules and also increases surface tension. It can precipitate 70% of protein in solution (Gbiosciences guide book: Protein fractionation).

1. Transferred 0.5 ml crude enzyme of (W, M1, M2, M3 and M4) in centrifuge tube.
2. Added precipitation buffer in it and mixed well.
3. Centrifuged at 5000 rpm for 5 minutes.
4. Added 100 µl of ammonium sulphate solution and mixed well.
5. Centrifuged at 5000 rpm for 5 minutes.
6. Added 200 µl of ammonium sulphate solution and mixed well.
7. Centrifuged at 5000 rpm for 5 minutes.
8. Added 500 µl of ammonium sulphate solution and mixed well.
9. Centrifuged at 5000 rpm for 5 minutes.
10. Transferred supernatant in fresh tube.

**3.3.29.2 Acetone precipitation of protein**

This method is used to precipitate protein in acetone (Ranish, 2007).

1. Chilled acetone was used for precipitation.
2. Transferred 0.5 ml partial purified protein of W, M1, M2, M3 and M4 in screwed tube.
3. Added 3 ml of acetone in tube.
4. Incubated it overnight at -20°C.
5. Centrifuged at 12,000 rpm for 5 minutes.
6. Discarded supernatant and added 90% acetone to wash the pellet.
8. Centrifuged at 12,000 rpm for 5 minutes.
9. Kept it for air dry and added 50 µl of phosphate buffer.
3.3.30 Estimation of protein by Lowry assay

The concentration of protein is estimated by Lowry assay (Lowry et al, 1951).

1. Prepared protein standard solution by using BSA (Bovine serum albumin).

2. Dissolved 20 mg BSA in 20 ml distilled water.

3. Washed 16 test tubes and labeled them B, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, W, M1, M2, M3 and M4.

4. Transferred 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µl to labeled test tubes (B to 10).

5. Added distilled water to make up volume at 1 ml.

6. Now added 2.0 ml of Lowry solution in each tube.

7. Vortex and incubated for 10 minutes at room temperatures.

8. Then added 0.2 ml of Folin Ciocalteu in each tube.

9. Vortex and incubated for 30 minutes at room temperatures.

11. Now transferred 0.1ml enzyme to W, M1, M2, M3 and M4 marked tubes.

12. Added distilled water to make up volume at 1 ml.

13. Now added 2.0 ml of Lowry solution in each tube.

14. Vortex and incubated for 10 minutes at room temperatures.

15. Then added 0.2 ml of Folin Ciocalteu in each tube.

16. Vortex and incubated for 30 minutes at room temperatures.

17. Measured absorbance at 660 nm.
3.3.30.1 Determination of protein concentration

A standard curve is prepared with bovine serum albumin or other pure protein, and the concentration of unknown protein solutions is determined from the graph. Plot the graph with standard protein concentration and absorbance.

3.3.31 Protein electrophoresis

The separation of protein based on molecular weight as well as charge of protein. Polyacrylamide gel is used for separation because of high resolution (Gbiosciences guide book: Protein electrophoresis).

**Gel Preparation**

Table 3.2 Concentration of gel for SDS- PAGE

<table>
<thead>
<tr>
<th>Gel concentration</th>
<th>10 ml of separating gel</th>
<th>10 ml of stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide gel</td>
<td>3.1 ml</td>
<td>0.55 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>2.5ml (pH 8.8)</td>
<td>0.63 ml (pH 6.8)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.3 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>15 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>10 % APS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

1. Gel plates washed and assembled with spacer.
2. Sealed plates with rubber gasket.
3. Checked leakage by adding distilled water.
4. Dried the plates with filter paper.
5. Prepared separating gel in tube in tube.
6. Transferred gel in casting plates.

7. Added distilled water to remove air bubbles.

8. Removed water carefully.

9. Now prepared stacking gel and transferred it.

10. Put the comb immediately and allowed it to solidify.

11. Kept the plate in electrophoretic unit and remove comb.

12. Loaded marker and samples in wells.

13. Run at 70 V for 2:30 hours.

14. After that, gel was kept in staining solution for overnight.

15. Then placed in destaining solution for 2 hours.

16. Observed the bands.

3.3.31.1 Molecular weight determination

1. Measured the distance of dye front migrated.


3. Calculated Relative Mobility (Rf).

\[ R_f = \frac{\text{distance of protein standards migrated (mm)}}{\text{distance of dye front migrated (mm)}} \]

4. Plot log (Molecular weight) against Rf values.

5. Protein standard were 97, 66, 45 KDa.

6. Calculate unknown Molecular weight with Rf value.

3.3.31.2 Standard Graph

1. Used MS excel and make table of rf and log mol wt.

2. Click on insert.

3. Selected (xy scatter option).
4. Then again selected one chart show fx choose this.

5. Appeared chart tools and selected.

6. Choose layout and clicked on trendline.

7. Selecteted linear trendline.

8. Then selected design.

3.3.32 Response Surface Methodology

Response surface methodology is collection of statistical and mathematical technique for optimization. It is used to optimize effect created by input variables. It requires output and input variables for optimization (Box and Draper, 1987).

Full Factorial Design

CCD (Central composite design) is generally used for optimization. It is consisted with fractional factorial run, center point and axial or star points (Montgomery, 1997). Its objective that interaction between independent variable could be done in one time, which give result in terms of response. CCD has three levels in coded value (-1, 0, +1) and represents low, medium and high respectively (Venter, 1998). CCD has been used to optimize the enzyme activity by using four different variables. It is combined with RSM to investigate the interaction between input and output variables (Vithali, 2000).

CCD is based on ANOVA (Analysis of Variance) to determine significance of data. It also included F – test, T – test, correlation coefficient R, determination coefficient R^2. It generates predicted and experimental values by ANOVA. On the bases of predicted and experimental values represented by contour plots or 3D contour plots. Contour plot is graphical representation of response obtained by predicted and experimental values. It creates ellipse, which can be easily understood. The small ellipse represents maximum
predicted response Tanyildizi et al, 2005).

The elliptical contour shows perfect interaction between input variables. RSM has been applied for optimization of enzyme activity by using four independent variables (Temperature, pH, incubation time and substrate concentration). Design Expert software is used with version of (8.0, Stat-Ease Inc., Minneapolis, USA) to calculate and analyse experiment data.

\[ Y_i = \beta_0 + \sum B_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \]

\( Y_i \) (predicted response); \( X_iX_j \) (input variables); \( \beta_0 \) (offset term); \( \beta_i \) (linear coefficient); \( \beta_{ii} \) (quadratic coefficient) and \( \beta_{ij} \) (interaction coefficient) (Gangadharan et al, 2008).

1. Selected the ranges of independent variables Temperature (50, 60, 70, 80 and 90°C), pH (5, 6, 7, 8 and 9), incubation time (36, 48, 60, 72 and 84 hrs) and substrate concentration (2, 3, 4, 5 and 6).

2. Choosed the fractional \( 2^4 \) factorial design in central composite.

3. The fractional \( 2^4 \) factorial design consist with 30 trials (16 factorial runs, 6 center points and 8 star points).


5. Then selected fit of model for analysis of significance of data.

6. Selected ANOVA to determine correlation coefficient R and determination coefficient \( R^2 \).

7. Significance of coefficient determined by t-values and P-values.

8. Then generate actual and predicted value of enzyme activity (U/ml/min).

9. Selected contour plot option and maximum predicted values were showed by smallest ellipse in 3D contour plots.