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PRODUCTION AND IMMOBILIZATION OF ALPHA AMYLASE BY USING *BACILLUS SUBTILIS*

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Abstract

The α-amylase producing organisms were isolated from soil sample, such as *Bacillus* sp (2 isolates), *Bacillus cereus* (1 isolates), *Bacillus subtilis (2* isolates) by basal medium and identified by standard biochemical test. The high yielding strain was identified as *Bacillus subtilis* and used for amylase production. Different parameters like temperature (10°C, room temperature (25°-30°C), 37°C & 55°C different pH (6.0, 6.5, 7.0, 7.5, 8.0) and incubation periods (1-5 days) were used for the α-amylase production. The isolate was inoculated to the production medium and incubated, then it was centrifuged and the supernatant containing crude enzyme extract is used for protein estimation and activity. It was found that α-amylase production and activity was high at 37°Cat pH 7.0 in 48 hours. The organism was grown in the optimum conditions mentioned above and the enzyme α-amylase was immobilized by alginate gel entrapment method. From the organism the plasmid and the chromosomal DNA was isolated and detected.

Key Words: *Bacillus*; Immobilization; Plasmid; α-amylase.

Introduction

Enzymes are biological catalysts. They increase the rate of chemical reactions taking place within living cells without themselves suffering any overall change. Amylases are among the most important industrial enzymes and are of great significance in present day biotechnology. Two major classes of amylases have been identified namely α-amylases and glucoamylases in addition to this β-amylases, which is generally plant origin has also been reported a few microbial sources. Amylases are characterized by their ability to hydrolyse glucosidic linkages in polysaccharide. α-amylases (endo 1, 4-α–D–glucan glucohydrolase, EC 3.2.1.1) are extracellular acts on starch components. The α-amylases may be derived from several bacteria, yeasts and fungi. Bacterial amylase, however, is generally preferred over fungal amylase due to several characteristic advantages that it offers. Strains of *Bacillus sp., mainly Bacillus subtills, Bacillus licheniformis, Bacillus amyloliquefaciens and Bacillus megaterium* are employed. Among these *Bacillus subtilis* strains are employed for commercial application (Goyal and Khadeparker, 1979).

Immobilised amylases offer several advantages : (i) can be reused, (ii) involved processes can be operated continuously with better controls (iii) easy separation of the product(s), (iv) simpler handling of materials, (v) alteration in properties, mainly the activity and thermo stability, and (vi) effective reduction in process cost.

Materials and Methods

Isolation of Saccharolytic bacteria

Sample collection

10 Soil Samples were collected from the fertile land. Strains were isolated from different soil samples using a selective medium with the different composition.

For solid medium, agar was used at a concentration of 3%. The soil samples were serially diluted by using sterile distilled water and plated on the selective medium and incubated at 37^{0}C for 24 hours. After incubation, the isolated organisms were streaked over the Starch Medium for the isolation of starch degrading organisms. The plates were incubated at 37^{0}C for 24 hours. After

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incubation, plates were flooded with Iodine Solution. If the organism utilizes starch as a sole source of carbon by synthesizing an enzyme it will form a clear zone while flooded with Iodine solution.

Starch hydrolytic colonies were simultaneously taken and streaked on prepared nutrient agar plates with the same medium used for isolation. The plates were incubated at 37°C for 24 hours.

Identification of Bacteria

The isolated organisms were identified and differentiated by methods like Grams Staining, Motility test and Biochemical tests for the bacterial isolates.

Inoculum preparation for production media

(Ramesh and Lonsane, 1989)

For inoculum preparation, the isolated organism Bacillus subtilis was first grown on the Nutrient Agar slant with 0.3% of soluble starch and incubated at 37°C for 24 hours. A loopful of growth was transferred to Nutrient broth containing 0.3% soluble starch and incubated at 35°C for 18-20 hours

Estimation of total protein from enzyme extract

(Lowry et al., 1951)

Assay of α-amylase activity

Principle

α-Amylase catalyses the hydrolysis of α 1-4 links of starch with the production of reducing sugars. The reducing sugars released were estimated as maltose equivalent by dinitrosalicylic acid method (DNS Method).

Production and activity of α-amylase

The isolated high saccharolytic *Bacillus subtilis* 2 was taken for a-amylase production and activity in the solid state fermentation system involving starch as substract in the basal medium. Three parameters namely pH, incubation period and temperature were taken for the a-amylase production and activity. The α -amylase was estimated by Lowry *et al*., (1951) method and the activity was measured by DNS method (Bernfield, 1955).

Enzyme immobilization protocol

The enzyme immobilization was carried out by enterapment method using alginate gel.

Procedures

Dissolve 30g of sodium alginate in 1 liter to make a 3% solution. Mix approximately 0.015g of enzyme (or an equivalent of concentrated enzyme solution) with 10ml of 3% (wt) sodium alginate solution. The concentration of sodium alginate can be varied between 6-12% depending on the desired hardness. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100ml) of stirred 0.2M $Cacl_2$ solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle guage. A typical hypodermic needle produces beads of 0 5-2 mm in diameter. Other shapes can be obtained by using a mold whose wall is permeable to calcium ions. Leave the beads in the calcium solution to cure for 0 5-3 hours.

Isolation and detection of plasmid (Brinboin and Doly, 1979

Method

Bacterial isolates, which showed antibiotic resistant, was inoculated into prepared LB broth and incubated at 37°C for 24 hrs. After 24 hrs 1.5 ml of sample was taken in the micorcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The pellet was suspended in 300ml of TE Buffer and left in the room temperature for 5 minutes. The 300ml of NaOH was added and allowed to stand for 5 minutes. Then 300ml of 3M potassium acetate was added and kept in the ice for 15 minutes and then centrifuged at 10,000 rpm for 10 minutes. Supernatant was removed and 50ml of 90% ethanol was added to the pellet and gently mined. Then centrifuged at 10,000 rpm for 10 minutes and the Supernatant were discarded. Keep the tube in air dry for 10 minutes and dry the pellets. The DNA was stored at 20°C. The gel was stained with ethidium bromide and the plate was prepared and kept in the electrophoretic tank containing electrophoresis buffer. The prepared plasmid and chromosomal DNA samples were mixed with loading dye and loaded on to the slots of the submerged gel using micorpipette. Then the agarose gel was run at 100 Volts for 1 hour. The presence of plasmid and chromosomal DNA with their molecular weights were confirmed by restriction fragmentation using EcoRI and Hind III as by restriction fragmentation. Then the gels were visualized by UV Transilluminator. Then plasmid and chromosomal DNA in each track were recorded.

Results and Discussion

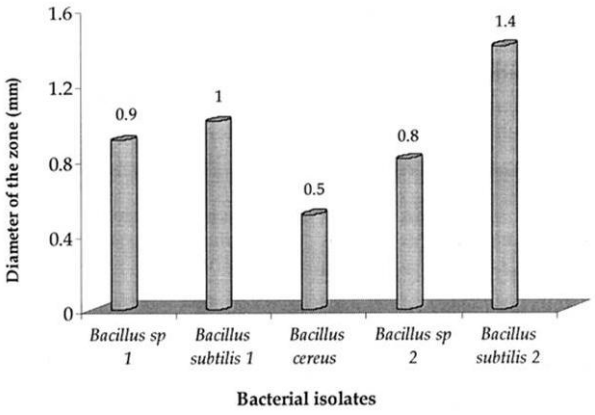
Table 1. Screening the saccharolytic activity of bacterial isolates on starch medium

Sl. No	Bacterial Isolates	Diameter of the zone (mm)
1.	*Bacillus sp 1*	0.9
2.	*Bacillus subtilis 1*	1.0
3.	*Bacillus cereus*	0.5
4.	*Bacillus sp 2*	0.8
5.	*Bacillus subtilis 2*	1.4

In the present study a total of 10 different soil samples were collected for isolating saccharolytic (α-amylase producing) bacteria. Among those, 5 isolates

were identified as a-amylase producing strains on starch medium. These isolates were identified as *Bacillus* sp (2 isolates), *Bacillus cereus* (1 isolates) and *Bacillus subtilis* (2 isolates) by standard biochemical test and culture characteristics (Table 2).

FIG. I. SCREENIG THE SACCHAROLYTIC ACTIVITY OF BACTERIAL ISOLATES ON STARCH MEDIUM



Bacillus subtilis is identified as the most favorable species for α- amylase production. The results were tabulated in (Table 1, Figure I).

Table 2. Characterization of the screened saccharolytic isolates from the soil

Name of the Isolates	Gram	Mot	Cat	Oxi	Glu	Suc	Man	Ind	MR	VP	Cit	Ure
Bacillus sp	+	Motile	+	-	A	A	A	-	-	-	+	-
Bacillus subtilis	+	Motile	+	-	A	A	A	-	-	+	+	-
Bacillus cereus	+	Motile	+	-	A/G	A	A	-	-	+	-	-
Bacillus sp	+	Motile	+	-	A	A	A	-	-	-	+	-
Bacillus subtilis	+	Motile	+	-	A	A	A	-	-	+	+	-

Gram	-	Gram straining	Man	-	Manitol
Mot	-	Motility	Ind	-	Indole
Cat	-	Catalsase	MR	-	Methyl Red
Oxi	-	Oxidase	VP	-	Voges Proskauer
Glu	-	Glucose	Cit	-	Citrate utilization
Suc	-	Sucrose	Ure	-	Urease
A	-	Acid	A/G	-	Acid/ Gas
'+'	-	Positive result	'-'	-	Negative result

Effect of pH and incubation period on α-amylase production at 10°C.

As per the table 4, the production of α-amylase in 24 hours of incubation was 62 µg/ml at pH 6.5. On comparison with other pH and incubation time, it was high. This is shown in Figure II.

Effect of pH and incubation period at room temperature (25 °-30°C) on α-amylase production.

It was found that the production of α-amylase in 48 hours of incubation period was high at pH 7.0 (80 (µg/ml), when compared with other pH and incubation time (Figure III). The results were tabulated in table 5.

Effect of pH and incubation period at 37°C **on α-amylase production.**

From the table 6, it was found that at pH 7.0 in 48 hours, 85 µg/ml of the α-amylase was produced. This is found to be the highest production at 37°C. This is shown in figure IV.

Table 3. Standards for protein estimatlon

S.No	Concentration of Bovine serum albumin(ug/ml)	Absorbance (OD value) at 660 n m
1	20	0.046
2	40	0.092
3	60	0.125
4	80	0.181
5	100	0.215

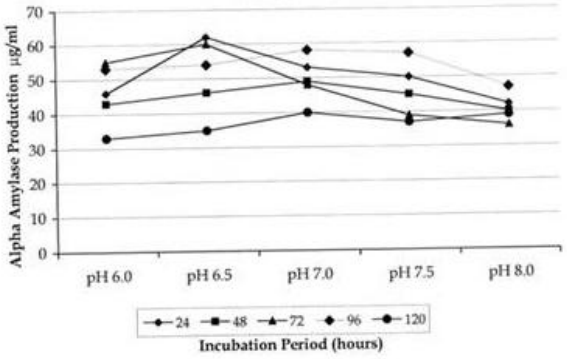
Table 4. Effect of pH at 10°c on α- amylase production

S. No	Incubation Period(hrs)	α-Amylase production (µg/ml)				
		pH6.0	pH6.5	pH7.0	pH7.5	pH.8.0
1	24	46	62	53	50	42
2	48	43	46	49	45	40
3	72	55	60	48	39	36
4	96	53	54	58	57	47
5	120	33	35	40	37	39

Effect of pH and incubation period at 55°C **on α-amylase production.**

From the figure V and table 7, at 55°C the production was low compared to other temperatures selected. Moreover the high pr6duction at 55°C was in 24 hours at pH 6.5 found to be 60 µg/ml.

FIG. NO. II EFFECT OF pH AT 10 ° C ON ALPHA AMYLASE PRODUCTION



For the assay of α-amylase activity, the estimation of total reducing sugars produced by DNS method was used. The maltose was produced in major amount by the enzyme action on substrate starch and maltose standard graph was plotted from which the activity of enzyme was noted in umoles per minute. The standard maltose readings were tabulated in table 8.

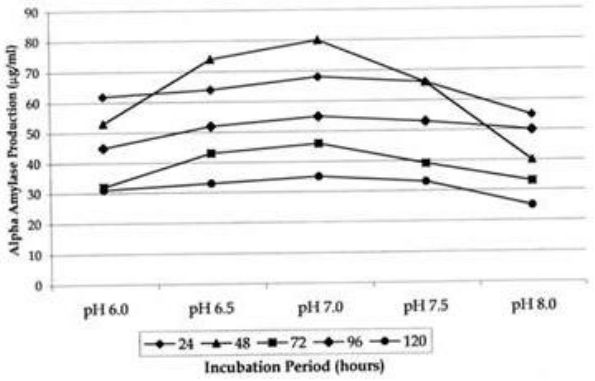
The results for the assay of α-amylase activity at different pH (6.0, 6.5, 7.0, 7.5 and 8.0) and for different

incubation periods (1-5 day) were tabulated in tables 9,10,11,12 and in figures VI, VII, VIII, and IX. From that it was inferred that the enzyme activity was high at 37°C at pH 7 and in 48 hours incubation.

Table 5. Effect of pH at room temperature (25-30°c) on α-amylase production

S. No	Incubation Period(hrs)	α-Amylase production (µg/ml)				
		pH6.0	pH6.5	pH7.0	pH7.5	pH.8.0
1	24	62	66	68	66	55
2	48	53	74	80	66	40
3	72	32	43	46	39	33
4	96	45	52	55	53	50
5	120	31	33	35	33	30

FIG. NO.III EFFECT OF pH AT ROOM TEMPERATURE (25 °- 30° C) ON ALPHA AMYLASE PRODUCTION



In that same condition the production of enzyme (protein) was also high.

Thus from the organism isolated, it was inferred that if it was grown for 48 hours at pH 7.0 at 37°C, it will produce α-amylase in increased amount with high activity.

Table 6. Effect of pH at 37°c on α-amylase production

s. No	Incubation Period(hrs)	α -Amylase production (µ/ml)				
		pH6.0	pH6.5	pH7.0	pH7.5	pH.8.0
1	24	50	65	74	66	64
2	48	56	67	85	70	51
3	72	49	56	76	50	48
4	96	36	47	44	38	33
5	120	33	37	27	28	21

It was reported that (Sivanandane *et al* 1983**)** the most important microbial sources of α-amylase are *Bacillus subtilis* and *Bacillus licheniformis* which is similar to another report . The amylase production in *Bacillus subtilis* isolated from Indian soil (Sivanandane *et al.* 1983) is seems to similar to this result at pH 7.0 at 37°C.

The progress of amylase production and activity in *Bacillus subtilis* were observed for 5 days in selective medium, which is used for isolation procedure with starch as the carbon source. The formation of amylase started from the beginning of the growth and lasted until the end of log phase where the amylase reached its peak value.

The strains of *Bacillus subtilis* (Coleman and Elliot, 1962) and in *Bacillus stearothermophilus* it has been shown that the formation of amylase takes place during the log phase in parallel with cell mass. The present findings are in agreement with these findings. The cell lysis was noticed after the log phase in *Bacillus subtilis* with concomitant decrease in the amylase level.

FIG. NO. IV EFFECT OF pH AT 37 °C ON ALPHA AMYLASE PRODUCTION

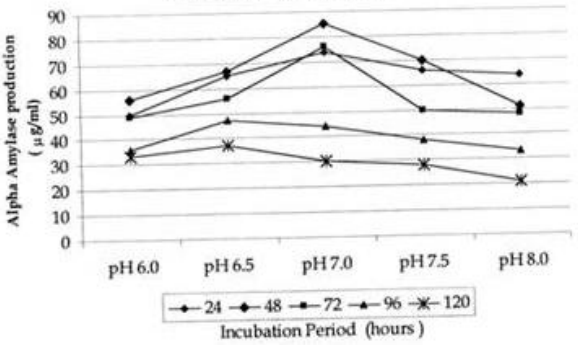
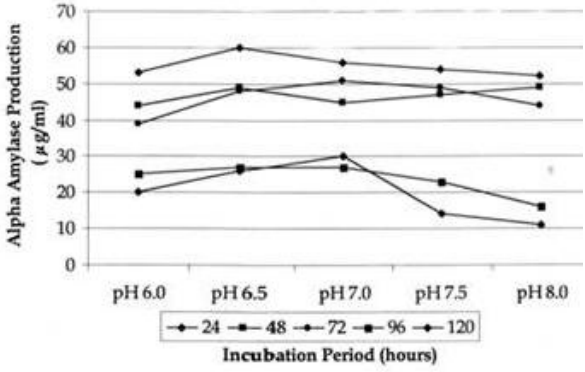


Table 7. Effect of pH at 55°c on α-amylase production

S. No	Incubation Period(hrs)	α -Amylase production (µ/ml)				
		pH6.0	pH6.5	pH7.0	pH7.5	pH.8.0
1	24	53	60	59	54	52
2	48	44	49	45	47	48
3	72	43	48	51	49	44
4	96	25	27	27	23	16
5	120	20	26	30	14	10

Similar to the immobilization of α-amylase by alginate gel Sardar and Gupta (1998) immobilized the enzyme using alginate beads. Moreover Patel *et al* (1996) used similar method in presence of glutaraldehyde. Because of the mild conditions needed for gelatin, calcium alginate is widely used for cell immobilation procedures. The immobilized enzyme retained 77% activity and could be stored for 28 days without any significant loss of activity.

FIG. NO. V EFFECT OF pH AT 55 °C ON ALPHA AMYLASE PRODUCTION



The plasmid and chromosomal DNA isolated was detected by agarose gel electrophoresis. The result is similar to the reports of Titok M A *et al* (2003) who

collected 55 *Bacillus subtilis* strains isolated from various natural sources of the territory of Belarus. Twenty percent of the strains contained one or two plasmids of either. 6-8 or approximately 90 kb. *Bacillus subtilis* is the best-characterized member of the gram-positive bacteria. Its genome of 4,214,810 base pairs comprises 4,100 protein coding gens. According to them many genes were involved in the synthesis of secondary metabolites, including antibiotic, that were more typically associated with streptomyces species.

Table 8. Standards for maltose for α-amylase activity

S. No.	Concentration of Maltose (mg/ml)	Absorbance (OD value) at 540nm
1	1	0.12
2	2	0.24
3	3	0.35
4	4	0.45
5	5	0.65

Table 9. Effect of pH at 10°c on α- amylase activity

S. No	Incubation Period(hrs)	α-Amylase production (10^{-4} µg/ml)				
		pH6.0	pH6.5	pH7.0	pH7.5	pH.8.0
1	24	1.2	2.3	2.5	2.6	1.4
2	48	2.1	2.4	2.3	2.0	1.9
3	72	1.3	2.5	3.1	2.9	1.7
4	96	1.9	2.3	2.3	2.1	2.0
5	120	1.5	1.9	2.1	1.4	1.1

FIG NO. VI EFFECT OF pH AT 10 O C ON ALPHA AMYLASE ACTIVITY

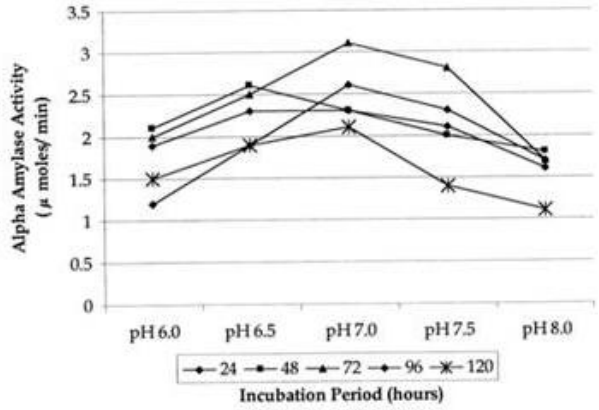


Table 10. Effect of pH at room temperature (27° - 30°c) on α- amylase activity

s. No	Incubation Period(hrs)	α-Amylase production (10^{-4} µg/ml)				
		pH6.0	pH6.5	pH7.0	pH7.5	pH.8.0
1	24	1.6	1.9	2.4	2.0	1.5
2	48	1.2	2.0	4.5	3.1	2.5
3	72	2.9	3.1	6.5	2.3	3.4
4	96	1.9	2.3	2.5	1.8	1.5
5	120	1.8	1.5	1.9	1.6	1.4

It was inferred that one of the gene in plasmid DNA will code for α-Amylase production which may be characterized. It may be cloned into DNA of amylase-negative cells for increased α-amylase production. The chromosomal DNA isolated and digested with restriction enzymes EcoRI & *Rind* III may be used for further research to use this *Bacillus subtilis* as host for increased synthesis of α -Amylase.

FIG. NO. VII EFFECT OF pH AT ROOM TEMPERATURE (25 ^{O} - 30^{O} C) ON ALPHA AMYLASE ACTIVITY

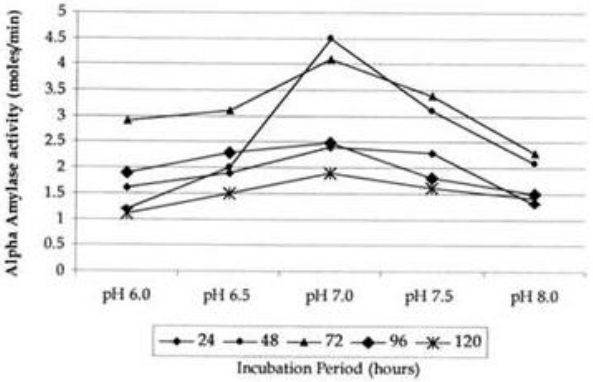


Table 11. Effect of pH at 37°c on α- amylase activity

s. No	Incubation Period(hrs)	α-Amylase production (10^{-4} µg/ml)				
		pH6.0	pH 6.5	pH 7.0	pH 7.5	pH. 8.0
1	24	1.7	2.5	3.7	2.4	1.5
2	48	2.8	3.3	8.7	4.0	3.2
3	72	3.5	4.9	5.6	5.0	2.9
4	96	3.2	4.1	5.0	4.3	2.1
5	120	2.5	3.8	4.5	2.8	1.5

FIG NO. VIII EFFECT OF pH AT 37 ^{O} C ON ALPHA AMYLASE ACTIVITY

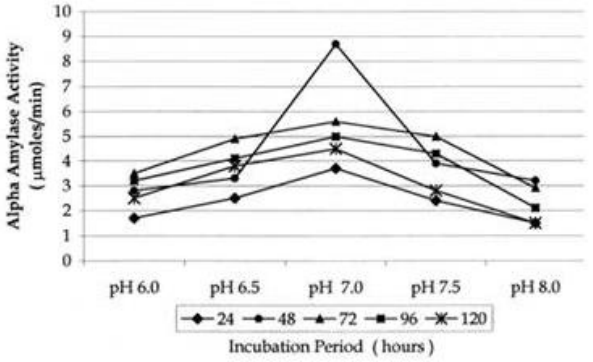


Table 12. Effect of pH at 55°c on α- amylase activity

s. No	Incubation Period(hrs)	α-Amylase production (10^{-4} µg/ml)				
		pH6.0	pH 6.5	pH 7.0	pH 7.5	pH. 8.0
1	24	1.2	2.0	2.3	1.7	1.3
2	48	1.5	2.4	2.9	2.3	1.0
3	72	1.9	2.0	2.1	1.0	0.5
4	96	1.3	2.3	2.5	2.0	1.5
5	120	1.8	2.5	2.9	1.5	1.0

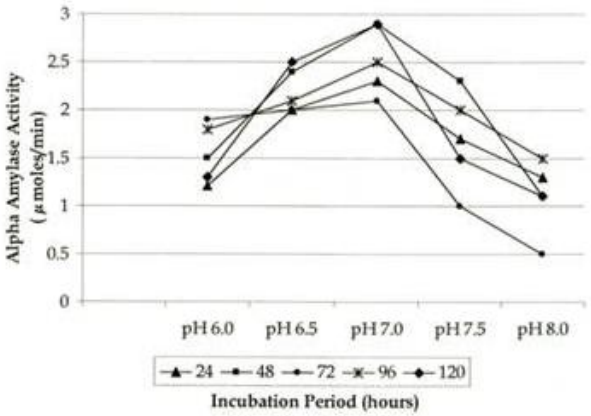
Isolation of Plasmid and Chromosomal DNA

The plasmid and chromosomal DNA was isolated and detected in agarose gel electrophoresis.

In the electrophoretic separation; the track 1 is the separation of DNA marker (Lambda DNA) Eco RI + Hind

III double digest). The marker Lambda DNA, was separated with DNA fragments of following base pairs 21226, 5148, 4973, 4268, 2027, 1904, 1584, 1375, 947, 831. The track 2 has shown the plasmid *of Bacillus subtillus* with 3150 base pairs. In track 3 the chromosomal DNA of *Bacillus subtillus* was visualized with 21224 base pairs. The track 4 picture out the Hind III digest of chromosomal DNA with 2 DNA segments of 22552 & 21224 base pairs. The track 6 shows the EcoRI digest of chromosomal DNA this reaction enzyme digest the chromosomal DNA with DNA fragments of 21226 & 2026 base pairs.

FIG. NO. IX EFFECT OF pH AT 55 ^{O} C ON ALPHA AMYLASE ACTIVITY



Based on the results discussed above it may be conducted that if the organism is grown with the conditions mentioned above, the enzyme production and activity may be increased.

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