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Isolation, Screening of Mangrove Actinomycetes Strain L-3 for the Production of Anti-Bacterial Metabolite and Protease

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Abstract: Marine and mangrove ecosystems are increasingly being investigated as a source of microorganisms with potential to produce novel bioactive compounds and enzymes. Marine and mangrove ecosystems represent a largely untapped source for isolation of new microorganisms. Actinomycetes are of particular interest, since they are traditionally known for their unparalleled capacity to produce biomolecules with diverse biological activities. Actinomycetes were isolated from marine sediments collected off the coast, Goa and samples collected from mangrove wetlands of Krishna estuary, Machilipatnam, Andhra Pradesh. The isolates were evaluated for antibacterial activity and strain L3 was isolated from wetlands of Krishna estuary, Machilipatnam.

The antibacterial and protease activities of the strain were determined and fermentation conditions viz., incubation time, incubation temperature, inoculum age, initial pH, were optimized for maximizing the antibacterial metabolite and enzyme production by strain L3. Further the medium composition with respect to carbon and nitrogen sources was also optimized for increasing the antibacterial metabolite and protease production. With the optimized conditions employed, strain showed antibacterial activity (in terms of inhibition zone diameter) of 38 mm against *Enterobacter aerogenes* and 39.5mm against *Bacillus subtilis* and protease activity of 46.2 U/mL.It was further sent to microbial type culture collection and identification.

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Introduction

Actinomycetes are aerobic, gram-positive bacteria that form branching filaments or hyphae and asexual spores. They have a high guanine (G) plus Cytosine (C) content in their DNA (> 50 mol %). Actinomycetes have considerable practical impact because they play a major role in the mineralization of organic matter in the soil and are the primary source of most naturally synthesized antibiotics (Prescott *et al*., 2008). Marine actinomycetes are an emerging source of novel natural products, particularly novel bioactive compounds. Mangrove forests are inter-tidal ecosystems found in the tropical and sub-tropical regions of the world.

Antibiotics comprise of a large number of metabolites from various microbes. They exhibit the inhibitory or killing response on other microbes, while harming the host as little as possible when used at very low concentrations. Proteases are hydrolytic enzymes found in every organism to undertake important physiological functions. Submerged fermentation, the organism is grown in a liquid medium, i.e., the organism is submerged in a liquid medium.

Materials and methods

Marine sediment samples were collected from 2 different areas. The mangrove wetlands of Krishna estuary, Andhra Pradesh. Mangrove sediment samples were collected from the two sampling sites (1) Machilipatnam, (2) Nizampatnam. About 1g of the each sample was taken into a 250ml conical flask containing 100ml of sterile water and the flasks were kept on a rotary shaker for 15 min. The suspension in each flask was serially diluted up to 10^{-5} level. Isolation was carried on Starch Casein Agar plates supplemented with rifampicin 2.5µg/mL and cycloheximide 75µg/mL to inhibit bacterial and fungal contamination respectively. The plates were seeded with a sediment sample suspension of 1.0ml each and incubated at 28°C for 14 days (Ramesh and Mathivanan, 2009). After 14 days, actinomycete colonies formed on the SCA plates were picked and transferred onto SCA slants and incubated at 28°C for 7 days. Only those isolates that appeared different from one another others were selected.

Results and discussion

The isolates were pooled together and cultures which appeared identical to naked eye in respect of color of aerial mycelium, reverse color, soluble pigment and colony texture were eliminated. About 24 actinomycete isolates were obtained from 3 samples.

Antibacterial Activity Studies

Primary screening by cross-streak method

All the actinomycete isolates were screened for antibacterial activity by cross streak method on agar plates containing starch casein agar (SCA) and nutrient agar in equal proportions. Each plate was streaked with a single isolate at the center along the diameter of the plate and incubated at 28°C for 5 days. After 5 days test organisms were streaked perpendicular to the growth of the actinomycete culture.

The intensity of inhibition produced by each isolate against the test bacteria was noted after 24 h of incubation. Plate with the same medium and without the actinomycete but with the streaking of the test organisms was maintained as a control. About 24 isolates showed antibacterial activity against the test organisms used. The isolates and their antibacterial activity by cross streak method are given in table 1.

Secondary screening by well diffusion method

The 22 isolates which showed antibacterial activity against the test organisms during primary screening were further screened for extracellular antibacterial metabolite production by submerged fermentation using well diffusion method. 4% inoculum prepared from seven day old agar slant cultures of the isolates was transferred into 50 mL of production medium and incubated at 28^0C on a rotary shaker at 180 rpm for 7 days. Then the samples were collected into sterile centrifuge tubes and centrifuged at 10,000 rpm for 20 min, at 8^0C and clear culture filtrate was separated. The clear supernatant was used for antibacterial assay using well diffusion method on nutrient agar plates. Wells were made in the solidified nutrient agar plates using a sterile cork borer and the 50 µL of clear supernatant was added to each well using a micropipette.

The plates were kept in the refrigerator for about 2h for antibiotic diffusion and then incubated at 37°C. After 24 h, the inhibition zones were recorded. The antibacterial activities of the selected isolates during secondary screening done by using well diffusion method are given in table 2.

Isolate L3 has shown good antibacterial activity against *Bacillus subtilis* and *Enterobacter aerogenes* and has been selected for further studies.

Protease Activity Studies

Primary Screening for Protease activity

The actinomycete isolates were streaked on casein agar medium described by Hassan *et al*., (2012) and incubated at 28 ^{0}C for 5 days. After 5 days the plates were observed for clear zones around the actinomycete colonies. L3 has shown a clear zone around the colony, indicating protease activity.

Assay for Protease activity

Isolate L3 was subjected to submerged fermentation in 50 mL of production medium inoculated with 4% inoculum prepared from a 7 day old culture. After 7 days, the fermentation broth was centrifuged at 10,000 rpm for 20 minutes at 8^{0}C and clear culture filtrate obtained after centrifugation was assayed for protease activity using the procedure described by Paranthaman *et al*., (2009). Isolate L3 showed protease activity of 17.56 U/mL.

Optimization studies on antibacterial metabolite production

Effect of incubation time

The effect of incubation time on antibacterial metabolite production, was studied by carrying out the fermentation at different incubation times ranging from 1 day to 7 days. After every 24 hours, the extracts were evaluated for antibacterial activity. The antibacterial activity of strain L3 showed a gradual increase from day 1 to day 3 and further increase in incubation time resulted in a decrease in antibacterial activity (Table 3). The reduced antibiotic activity after day 3 might be due to a reduction in the available nutrients and accumulation of toxic products of metabolism (Zhu *et al.*, 2014).

Effect of incubation temperature

The effect of incubation temperature on antibacterial metabolite production was studied by carrying out fermentation at different temperatures viz., 28^{0}C, 30^{0}C, 32^{0}C, 34^{0}C and 36^{0}C. The extracts of the fermentation broth were evaluated for antibacterial activity after 3 days. The strain L3 showed maximum antibacterial activity at 30 ^{0}C against *Enterobacter aerogenes and Bacillus subtilis* further increase in temperature resulted in a decrease in the antibacterial activity (Table 4). Increase in temperature beyond 30^{0}C might decrease the growth of the organism and the negatively affect the activity of the enzymes involved in antibiotic synthesis (Van der Meij *et al.*, 2017).

Effect of Inoculum age

The effect of inoculum age on antibacterial metabolite production was studied by varying the age of inoculum from 3rd day to 7th day. The production medium was then inoculated with 4% inoculum, incubated at 30 ^{0}C for 3 days and the extracts were then evaluated for antibacterial activity (Table 5). The age of the inoculum influences the growth and product formation by the microorganism. Strain L3 showed maximum antibacterial activity when a 5 day old inoculum was used (Weber *et al.*, 2015).

Effect of initial pH

To study the effect of initial pH on antibacterial metabolite production, pH of the production medium was adjusted to different pH values of 5.0, 6.0, 7.0, 8.0, 9.0 and then fermentation was carried out at 30 ^{0}C for 3 days. After 3 days the extracts were evaluated for antibacterial activity. Initial pH of the medium is an important factor which affects the growth and antibiotic production during submerged fermentation. The results indicate that the antibacterial activity of strain L3 has increased and attained maximum with increase in the initial pH of the medium from 5.0 to 7.0 and then decreased with a further increase in the initial pH (Table 6).

Effect of Carbon source

Different carbon sources such as sucrose, cellulose, maltose, mannitol and glucose were added at a concentration of 1% w/v to the production medium (pH 7) and inoculated with 2 mL of inoculum from a 5 day old culture. The flasks were incubated at 30^{0}C for 3 days and subsequently evaluated for antibacterial activity. The results indicate that strain L3 showed highest antibacterial activity, when starch was used as the carbon source (Table 7). Sucrose has commonly been observed to repress the synthesis of enzymes that are required for antibiotic production. But in this case starch has promoted antibiotic production (Stapley *et al.*, 1972).

Effect of Nitrogen source

Different nitrogen sources such as malt extract, beef extract, peptone, L-Glutamic acid and ammonium nitrate were added at a concentration of 1% w/v to the production medium (pH 7) containing starch as the carbon source instead of sucrose and inoculated with inoculum from a 5 day old culture. The flasks were incubated at 30^{0}C for 3 days and subsequently evaluated for antibacterial activity. Nitrogen source is an important factor in biosynthesis of antibiotics. The results indicate that strain L3 showed highest antibacterial activity, when peptone was used as the nitrogen source (Table 8).

With optimized conditions employed, strain L3 showed inhibition zone diameter of 38mm against *Enterobacter aerogenes* and 39.5mm against *Bacillus subtilis.*

Optimization studies on Protease production

Effect of incubation time

The effect of incubation time on protease production, was studied by carrying out the fermentation at different incubation times ranging from 1 day to 7 days. After every 24 hours, the extracts were evaluated for protease activity. Protease production by strain L3 has increased steadily from day 1 to day 3 and a further increase in incubation time resulted in a decrease in protease production (Table 9 and Figure 4)**.** The decrease in protease production after day 3 might be due to a reduction in the available nutrients and accumulation of toxic products of metabolism (Do *et al.*, 1991).

Effect of incubation temperature

The effect of incubation temperature on protease production was studied by carrying out fermentation at different temperatures viz., 28 ^{0}C, 30 ^{0}C, 32 ^{0}C, 34 ^{0}C and 36 ^{0}C. The extracts of the fermentation broth were evaluated for protease activity after 3 days. The strain L3 has shown maximum protease production at a temperature of 32 ^{0}C (Table 10 and Figure 5). From the result, we can observe that temperature had a profound impact on protease production.

Effect of inoculum age

The effect of inoculum age on protease production was studied by varying the age of inoculum from 2nd day to 7th day. The production medium was then inoculated with 4% inoculum, incubated at 32 ^{0}C for 3 days and the extracts were then evaluated for protease activity. The age of the inoculum influences the growth and product formation by the microorganism. The strain L3 has shown maximum protease production when a 4 day old inoculum was used (Nishikiori *et al.*, (1984) (Table 11 and Figure 6).

Effect of initial pH

To study the effect of initial pH on protease production, pH of the production medium was adjusted to different pH values of 5.0, 6.0, 7.0, 8.0, 9.0 and then fermentation was carried out at 32 ^{0}C for 3 days. After 3 days the extracts were evaluated for protease activity. Initial pH of the medium is an important factor which affects the growth and metabolite production by microorganisms during submerged fermentation. The strain L3 has shown maximum protease production when the initial pH of the medium was 7.0 (Table 12 and Figure 7).

Effect of Carbon source

To determine the effect of carbon source on protease production, different carbon sources such as sucrose, starch, maltose, fructose and glucose were added at a concentration of 1% w/v to the production medium (pH 7) and inoculated with 4% inoculum, incubated at 32 ^{0}C for 3 days and subsequently evaluated for protease activity. The strain L3 has shown maximum protease production when starch was used as the carbon source (Table 13 and Figure 8). Starch has resulted in a significant increase in protease production compared to control where sucrose was used as the carbon source.

Effect of Nitrogen source

To determine the effect of nitrogen source on protease production, different nitrogen sources such as malt extract, beef extract, peptone, L-Glutamic acid and ammonium nitrate were added at a concentration of 1% w/v to the production medium (pH 7) containing starch as the carbon source instead of sucrose and inoculated with inoculum from a 4 day old culture. The flasks were incubated at 32 ^{0}C for 3 days and subsequently evaluated for protease activity. The strain L3 has shown maximum protease production when peptone was used as the nitrogen source (Table 14 and Figure 9). With the optimized conditions employed, strain L3 showed protease activity of 46.2 U/mL.

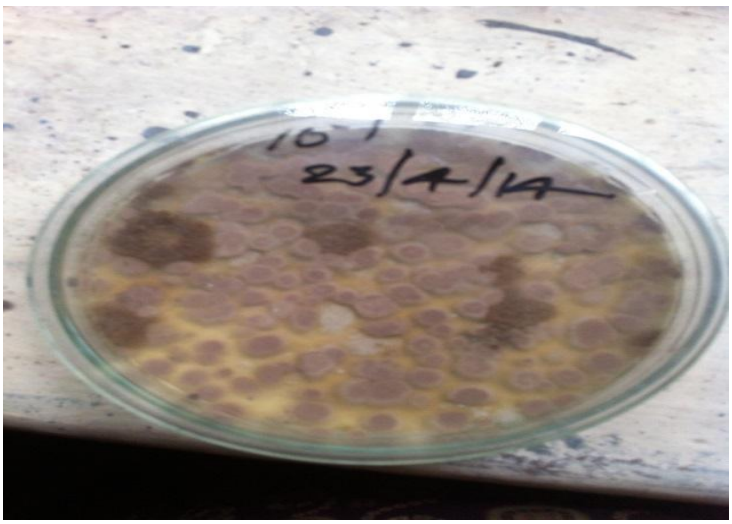

Figure 1. Actinomycetes isolated from mangrove samples using SCA medium

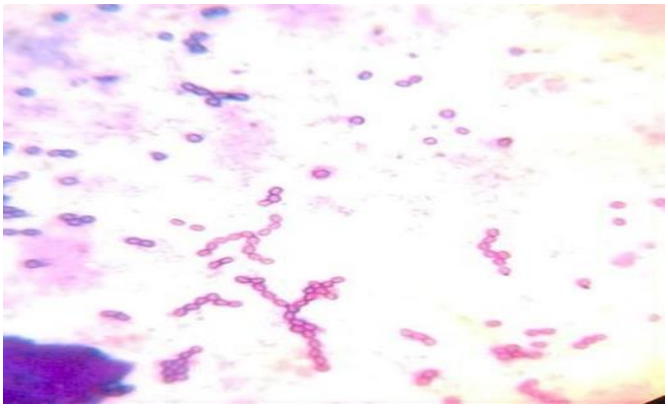

Figure 2. Microscopic view of isolated sample

Figure 3. Isolate L3 showing protease activity

Table 1. Antibacterial activity of actinomycete isolates by cross streak method

Isolate	Gram positive			Gram negative	
	Bacillus cereus	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Enterobacter aerogenes*
C1	+	+	++	-	+
C2	++	+	-	-	+
C3	+	-	-	+	++
C4	+	+	++	-	+
C5	++	++	-	+	+
C6	-	+	++	-	++
L1	+++	++	++	++	+
L2	++	-	++	-	+
L3	**++**	**++**	**+++**	**+**	**+++**
L4	+	-	-	+	-
L5	-	+++	++	-	-
L6	+	++	-	-	++
L7	++	+++	+	+	-
L8	++	-	-	++	++
L9	-	-	-	++	++
L10	+	-	++	++	-
M1	+	+	-	+++	+
M2	+	-	-	++	-
M3	+	-	-	+	-
M4	++	-	-	-	+
M5	++	-	-	++	++
Very good activity (+++); Good activity (++); Moderate activity (+); No activity (-)					

Table 2. Antibacterial activities of the selected actinomycete isolates by well diffusion method

Isolate	Inhibition zone diameter (in mm)				
	Gram positive			Gram negative	
	Bacillus licheniformis	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Enterobacter aerogenes*
C1	17	16	-	16	21
C2	22	14	16	-	-
C5	-	29	20	19	21
L1	13	-	14	-	-
L2	15	-	-	-	-
L3	**17**	**32**	**33**	**26**	**36**
L4	-	-	20	-	20
M1	19	20	22	12	-
M4	22	20	-	35	-
M6	36	-	29	30	19

Table 3. Effect of incubation time on antibacterial metabolite production

Incubation time (days)	Antibacterial activity (inhibition zone in mm)	
	Enterobacter aerogenes	*Bacillus subtilis*
1	-	-
2	3	28
3	**34**	**31**
4	32	29
5	27	27
6	25	26
7	25	25

Table 4. Effect of incubation temperature on antibacterial metabolite production

Temperature (^{0}C)	Antibacterial activity (inhibition zone in mm)	
	Enterobacter aerogenes	*Bacillus subtilis*
28	34	3
30	**36**	**33**
32	34	32
34	3	3
36	33	29

Table 5. Effect of inoculum age on antibacterial metabolite production

Inoculum age (days)	Antibacterial activity (inhibition zone in mm)	
	Enterobacter aerogenes	*Bacillus subtilis*
3	3	32
4	32	34
5	**35**	**36.5**
6	33	32
7	32	31

Table 6. Effect of initial pH on antibacterial metabolite production

pH	Antibacterial activity (inhibition zone in mm)	
	Enterobacter aerogenes	*Bacillus subtilis*
5	31	32
6	33	25
7	**36**	**39**
8	35	36
9	34	32

Table 7. Effect of various carbon sources on antibacterial metabolite production

Carbon source	Antibacterial activity (inhibition zone in mm)	
	Enterobacter aerogenes	*Bacillus subtilis*
Sucrose (control)	29	33
Maltose	32	36
Starch	**37**	**39.5**
Fructose	36	38
Glucose	30	36

Table 8. Effect of various nitrogen sources on antibacterial metabolite production

Nitrogen source	Antibacterial activity (inhibition zone in mm)	
	Enterobacter aerogenes	*Bacillus subtilis*
Malt extract (control)	3	36.5
Beef extract	3	38
Peptone	**38**	**39.5**
L-Glutamic acid	34	37
Ammonium nitrate	26	35

Table 9. Effect of incubation time on protease production

Incubation time (days)	Protease activity (U/mL)
1	5
2	8
3	**19**
4	18
5	16
6	14
7	11

Table 10. Effect of incubation temperature on protease production

Temperature (^{0}C)	Protease activity (U/mL)
28	9.71
30	12.85
32	**19.42**
34	18.57
36	14

Table 11. Effect of inoculum age on protease production

Inoculum age (days)	Protease activity(U/mL)
2	15.6
3	17.31
4	**19.6**
5	18.21
6	17.3
7	15

Table 12. Effect of initial pH on protease production

Initial pH	Protease activity (U/mL)
5	10
6	13.4
7	**19.71**
8	16.57
9	14

Table 13. Effect of carbon source on protease production

Carbon source	Protease activity (U/mL)
Sucrose (control)	12
Fructose	17
Starch	**26**
Glucose	25
Maltose	17.4

Table 14. Effect of nitrogen source on protease production

Nitrogen source	Protease activity (U/mL)
Malt extract (control)	25.6
Beef extract	35.3
Peptone	**46.2**
Ammonium nitrate	32.5
Glutamate	26.8

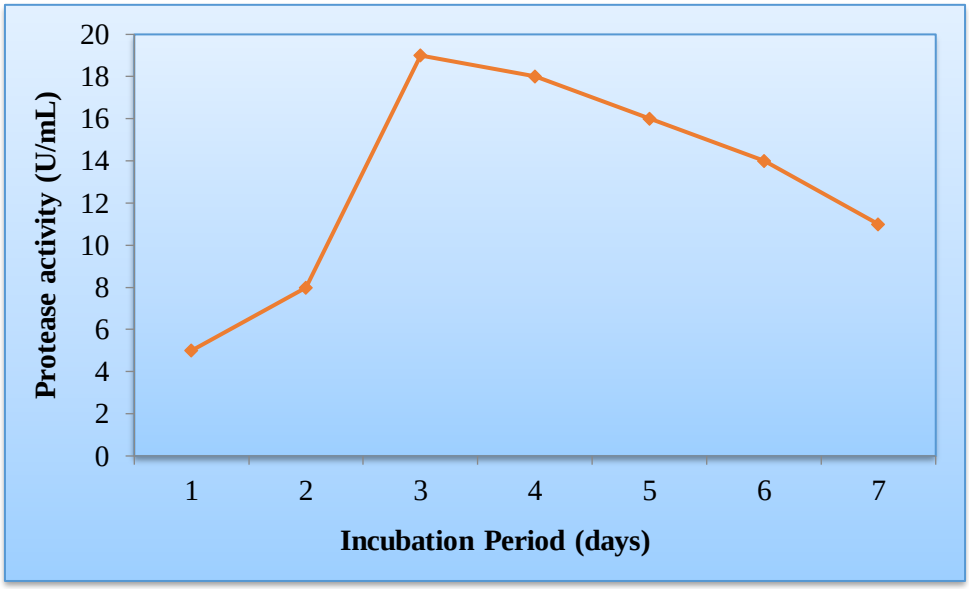

Figure 4. Effect of incubation period on protease production

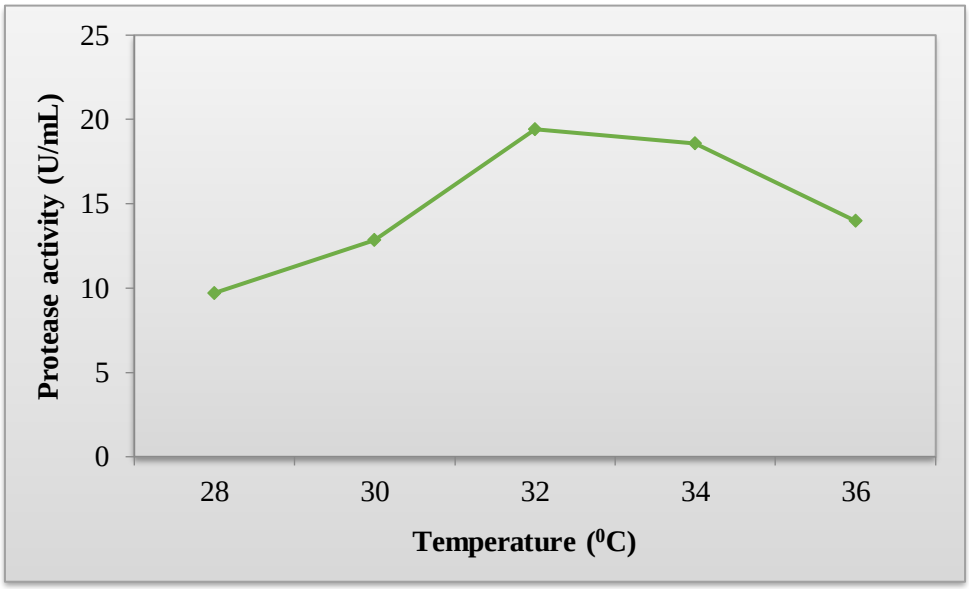

Figure 5. Effect of incubation temperature on protease production

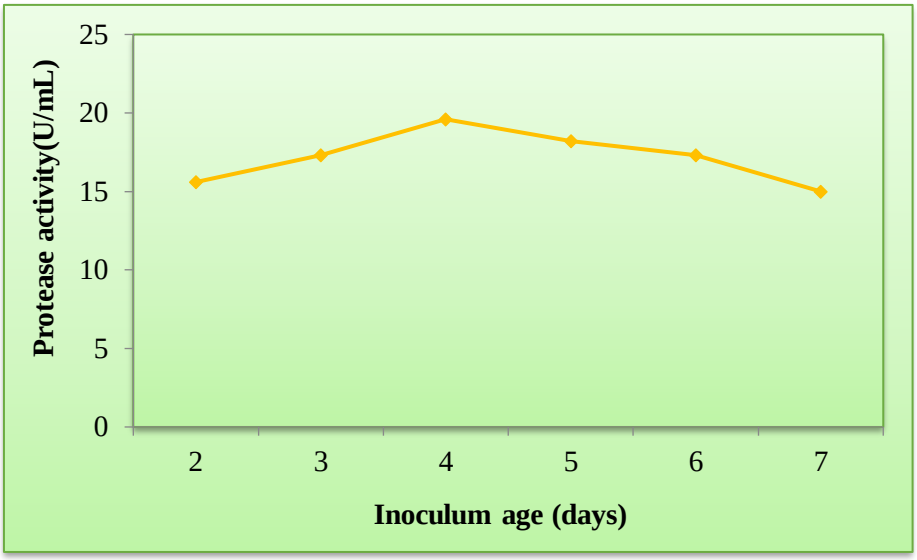

Figure 6. Effect of Inoculum age on protease production

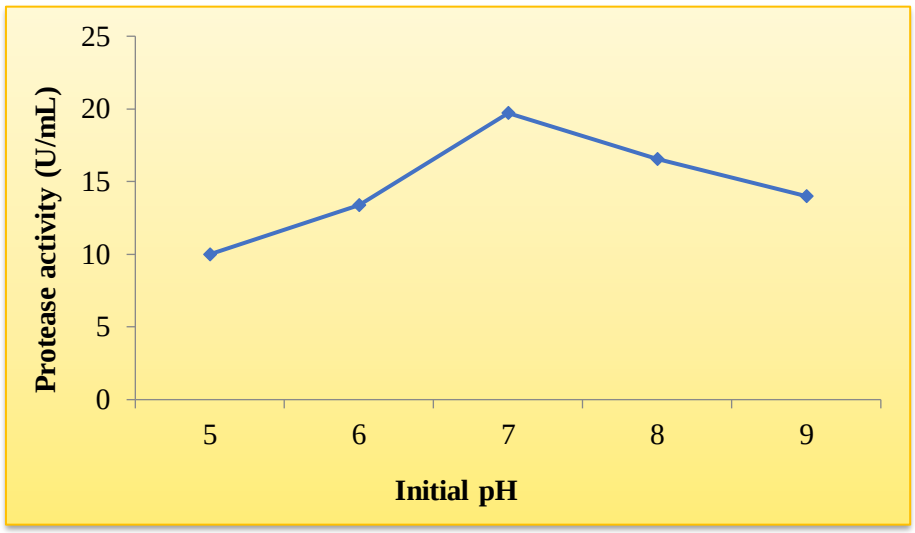

Figure 7. Effect of initial pH on protease production

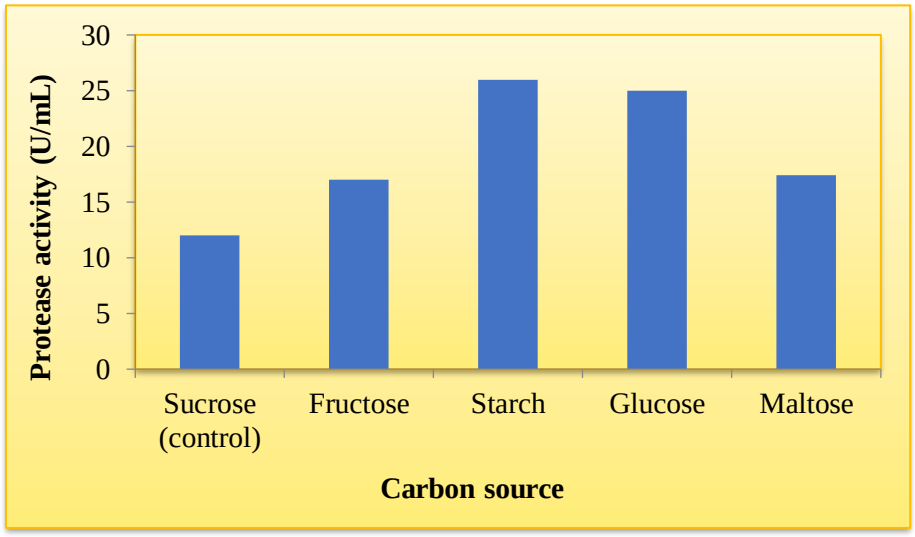

Figure 8. Effect of carbon source on protease production

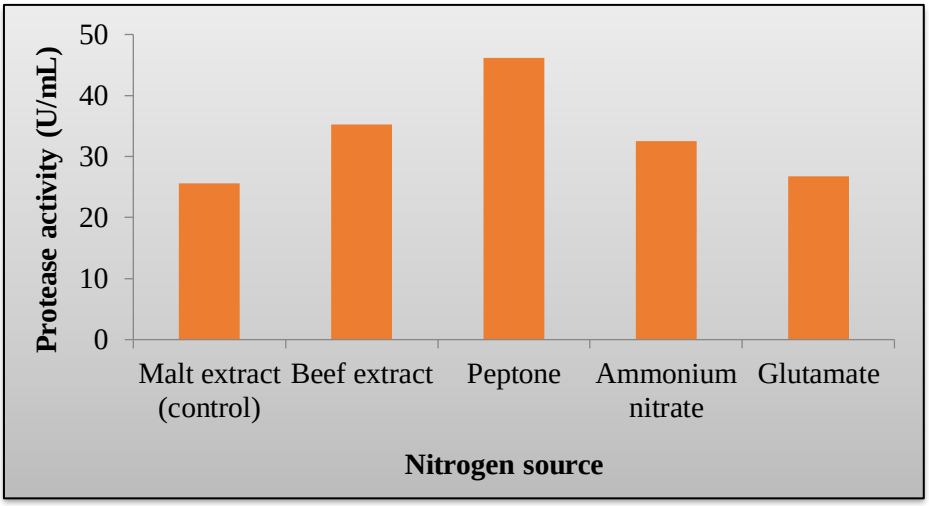

Figure 9. Effect of nitrogen source on protease production

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