45ºC, 5.9 ±0.35mm at 55ºC and 5.3 ±0.27mm at 65ºC which was found to be higher on compared to other isolates at different temperature. 1E showed 5.1±0.17mm zone of clearance at 65ºC and 4.4±0.88mm zone of clearance at 55ºC. 2C showed 3.7±0.09mm at 45ºC, 4.8±0.04mm at 55ºC and 4.8±0.04mm at 65ºC. The zone of clearance of 2D at 45ºC was found to be 1.1±0.34mm, no organism growth was observed at 55ºC and 65ºC. Hence the protease produced by the 1F is found to be stable even at high temperatures. From this study, it is proved that thermostable enzymes can be produced from the microbial mat colonies.

Optimization of conditions for enhanced enzyme production

The enzyme production was optimized under the selected parameters using the bacterial culture 1F isolated from microbial mat-1. The amount of enzyme produced by the strain and its activity under each type of parameters are presented below.

Effect of Carbon supplementation

To determine the effect of each carbon source, casein broth was prepared and inoculated with bacterial culture 1F. After inoculation period, the enzyme was extracted, assayed and determined using UV-V is spectrophotometer at 600nm. In Table-2 Maltose brought the highest enzyme activity about 462.3 (IU). Glucose brought the second highest enzyme activity about 257.5 (IU). Followed by Lactose showed 160.5(IU) and sucrose showed minimum enzyme activity about 39.6 (IU). The present findings also revealed that lactose was the best carbon source to induce the production of protease by *Bacillus subtilis* on production medium, expressing maximum productivity.

Effect of various nitrogen sources/substrates on protease activity

The effect of different nitrogen source on protease activity was determined from the culture inoculated casein broth. After incubation period, the enzyme extracted, assayed and determined using UV-Vis spectrophotometer was presented in below Table. In Table-3 results obtained showed that the yeast extract showed highest enzyme activity of 510.2 (IU).

Peptone showed the second highest enzyme activity of 360.5 (IU) followed by ammonium chloride expressing 156.6 (IU). Ammonium sulphate showed minimum activity of about 89.5 (IU) by the test isolate 1F during the analysis. Our results are in accordance to the findings of Krishna Ash et al., [12], who observed that growth medium containing peptone produced maximum protease. This was followed by yeast extract, tryptone, ammonium sulphate and ammonium chloride.

Effect of various inoculum sizes on protease activity

Inoculum size of the test bacterial isolate was studied to determine the protease activity. Different inoculum size added onto casein broth assayed for enzyme activity was presented in below Table. In Table-4, inoculum size (1%) showed highest enzyme activity of 390.5 (IU). The 2% inoculum size showed the second highest enzyme activity of 155.6 (IU) followed by 3% expressing 75.5 (IU) and minimal activity of 20.2 (IU) in the media inoculated with 4% culture inoculum. Results from the present study showed that the optimum inoculum size of the bacterial isolate for protease production was 1 ml from overnight culture broth. Similar observation has been reported by Gupta et al., [16], where an optimum inoculum size was 1ml from stock suspension for maximum protease.

Effect of various temperatures on protease activity

To determine the effect of temperature all inoculated flasks were incubated at different temperatures such as 28ºC, 37ºC, 45ºC and 55ºC for 24 to 48 hours. After the incubation period, standard protease assay was determined using UV-Vis spectrophotometer at 600nm. In Table-5 results showed that the temperature about 45^{o}C showed highest enzyme activity of about 437.5 (IU). At 37^{o}C, the second highest enzyme activity of 169.2 (IU) by the isolate was evident. At 28^{o}C and 55^{o}C about 385.6 (IU) and 16.8 (IU) of enzyme activity was found respectively.

CONCLUSION

Isolation and production of thermostable enzyme producing bacteria from microbial mats was selected as a novel concept in the present research. As thermostable protease has more commercial value in different industries, the aim of this study was to search for such an enzyme producing bacteria from the microbial mats. Different parameters including cheap carbon and nitrogen substrates, inoculum size and temperature was selected to optimize the enzyme production conditions. Initially five different isolates from two microbial mats collected from different sources were analyzed for its ability to produce thermostable protease after exposing to higher temperature incubation conditions. Test culture tentatively named as 1F from microbial mat-1 was selected as more enzyme producer among the ten isolates. The organism was selected based on the zone of clearance on skim milk agar by the isolate, that indicating more protease production. Under each optimization parameter, each type of carbon (Lactose), and nitrogen (yeast extract) source showed more enzyme production and activity respectively. About 1% inoculum size and a thermostable temperature of 45°C produced significant amount of enzyme and its activity. The obtained results emphasized the need for thermostable protease for different commercial industries in the existing and near future. Also the isolated bacteria (1F) needed to be identified for the bulk production of protease using the selected optimized conditions. This step is considered as the future study of our research work.

CONFLICT OF INTEREST

Authors declare no conflict of research in the present work.

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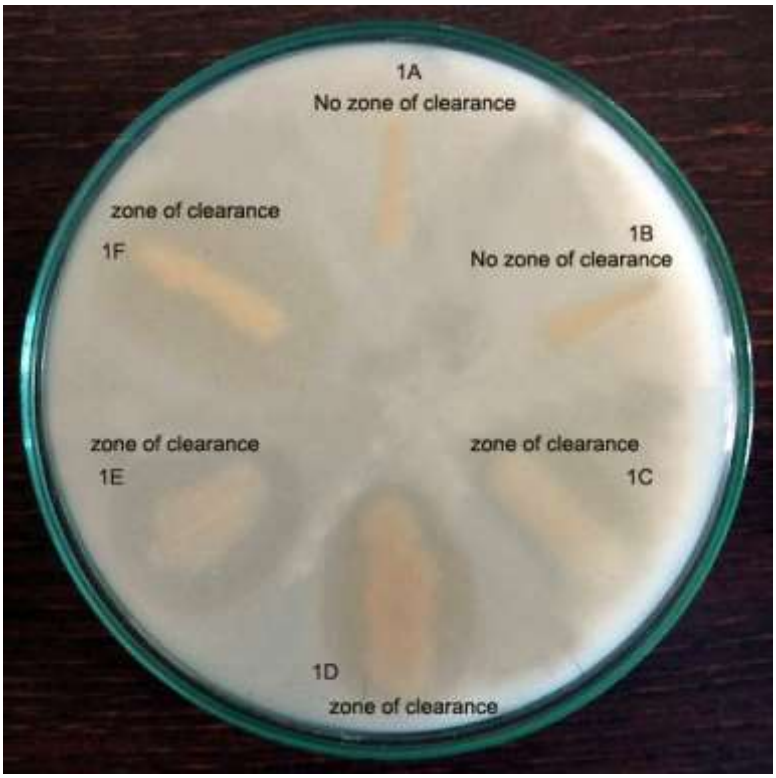
Fig. 1: Isolation of protease producing bacteria from microbial mat source



(Colonies showing maximum zone of clearance are selected – 1F)

Fig. 2: Zone of clearance on Skim Milk Agar media by protease producing bacteria

Zone of clearance around bacterial colony (1F) is evident on Skim milk agar plates

Table-1: *In Vitro* Determination of enzyme activity – Thermostable protease assay

S.No	Bacterial isolates	Zone of Clearance at different temperatures (mm)		
		45ºC	55 ºC	65 ºC
1	1C	3.2±0.15	2.7±0.29	1.3±0.12
2	1D	1.5±0.40	-	-
3	1E	2.1±0.13	4.4±0.88	5.1±0.17
4	1F	2.2±0.26	5.9 ±0.35	5.3±0.27
5	2C	3.7±0.09	4.8±0.04	4.8±0.04
6	2D	1.1±0.34	-	-

Table-2: protease activity at different carbon sources

S. No	Carbon sources	Optical density value	Amount of ptn released (ug)	Enzyme Activity(IU)
1	Glucose	0.64	1210	257.5
2	Sucrose	0.28	460	39.6
3	Maltose	0.81	1590	462.3
4	Lactose	0.52	980	160.5

Table-3: protease activity at different nitrogen sources

S.No	Nitrogen sources	Optical density value	Amount of ptn released (ug)	Enzyme Activity (IU)
1	Peptone	0.79	1250	360.5
2	Yeast extract	0.94	1460	510.2
3	Ammonium sulphate	0.38	620	89.5
4	Ammonium chloride	0.51	910	156.6

Table-4: Protease activity at different inoculum sizes

S.No	Inoculum size	Optical density value	Amount of ptn released (ug)	Enzyme Activity (IU)
1	1%	0.79	1250	390.5
2	2%	0.55	950	155.6
3	3%	0.32	610	75.5
4	4%	0.19	290	20.2

Table-5: Protease activity at different temperatures

S. No	Temperature	Optical density value	Amount of ptn released (ug)	Enzyme Activity (IU)
1	28	0.50	910	145.5
2	37	0.84	1250	385.6
3	45	0.20	390	25.3
4	55	0.16	280	16.8