Bulletin of Faculty of Science, Zagazig University (BFSZU)
e-ISSN: 1110-1555
Volume-2024, Issue-2, pp-1-14
https://bfszu.journals.ekb.eg/journal
Research Paper DOI: **10.21608/bfszu.2023.180467.1222**

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Environmental culture optimization for maximizing alkaline protease production by local Bacillus subtillis isolated from vegetables water waste

Ghaly, M. F.; Elsaid Ali Elsaid; Elkafass, Eetmad. Nasser

Botany & Microbiology Department, Faculty of Science, Zagazig University, Egypt.

***ABSTRACT*: Alkaline protease have tremendous applications in different industries. Therefor in this study, we reported the production of thermostable alkaline protease from thermotolerant alkaliphilic bacterial isolates. This bacteria was isolated from vegetable water waste, screened proteolytically showing the maximum zone of inhibition and identified as *Bacillus subtilies* based on 16S rDNA sequencing. Maximum level of protease production was founded at 40 ^{0}c of fermentation in alkaline skimmed milk nutrient broth media. The optimum incubation period, size of inoculum and initial pH of the media for maximum production of alkaline protease were 48h, 2ml% and 8 respectively at shaking conditions of 150 rpm. Where the enzyme activity was reported as following: 0.470 U/ml for optimum pH, 0.612 U/ml for optimum incubation period, 0.504 U/ml for optimum incubation temperature, 0.351 U/ml for best inoculum size and 0.452 U/ml for shaking conditions. Thus, such additions can influence alkaline protease production and improve their use in various applications of industry.**

KEYWORDS: Alkaline protease, Bacillus subtilies , Temperature.

Date of Submission: 13-12-2022 Date of acceptance: 20-07-2023

I. INTRODUCTION

Enzymes are proteins that produced by plants, animals and microorganisms Namasivayam et al.,(2011). Enzymes are important to all forms of life Aaisha et al.,(2016). Enzymes could take place chemical bio-catalysts in industrial processes. Proteases are one of the most important groups of enzymes of industrial interest. By optimizing parameters, Protease do functions most efficiently and deliver the desired. The various environmental parameters that can be optimized are pH, incubation temperature, Incubation period, etc. On account of their tremendous applications, protease used in detergent, tannery, food, pharmaceutical,medical, leather industries as well as in environmental bio-remediation Culp et al., (2017) & Ammasi et al., (2020). The bacterial genus, Bacillus has been the best for laboratory and commercial production of proteases. Species like B. licheniformis, B.cereus, B. subtilis, B. safensis, etc. have carved commercial niches for themselves Singh et al., (2017).

1.1.Optimization of culture conditions for production of alkaline protease

For maximum production of enzyme, fermentation media could be properly optimized. Different factors like pH, incubation period, temperature and size of inoculum have great affect on enzyme production.

1.1.1.Effect of pH

pH is an important parameter which needs to be controlled for maximum cell growth and protease enzyme production. Alkaliphilic microorganisms require optimum pH of around 10 for their growth. Generally, Optimum pH for alkaline protease production range from 8 to 10.5 Ji et al., (2012). This includes different fungal and bacterial species that produce alkaline protease like Pseudoalteromonas sp. 129–1 (pH 8) Wu et al., (2015)., Aspergillus oryzae (pH 8) Yepuru et al., (2018)., Streptomyces fungicidicus MML1614 (pH 9) Ramesh et al., (2009)., Yarrowia lipolytica YlTun15 (pH 9) Bessadok et al., (2017)., Alcaligenes faecalis APCMST-MKW6 (pH 9) Maruthiah et al., (2016)., Psiloteredo healdi (pH 9) Griffin et al., (1992)., Aureobasidium pullulans 10 (pH 9) Ma et al., (2007)., Bacillus sp. SD11 (pH 10) Cui et al., (2015)., Bacillus alveayuensis CAS 5 (pH 9) Annamalai et al., (2014)., Bacillus sp. APCMST-CS4 (pH 8) Maruthiah, et al., (2017)., Pseudomonas aeruginosa (pH 9) Satheeskumar et al., (2013)., Bucillus subtilis NS (pH 9) Nisha et al., (2014)., Pseudoalteromonas arctica PAMC 21717 (pH 9) Park et al., (2018)., Actinomycete MA1–1 (pH 9) Hameş-Kocabaş et al., (2007)., Bacillus altitudinis HHT597 (pH 9) Yuan et al., (2019)., and Vibrio sp. (pH 9) Subhashini et al., (2012). However, there are some exceptions reported by Kirk et al., (2002). & Hameş-Kocabaş et al., (2007)., like Bacillus sp., and Bacillus vallismortis which showed optimum pH from 6.5 to 7.0 respectively.

1.1.2. Effect of temperature

Alkaliphilic microorganisms are strongly dependent upon Temperature for maximum cell growth and protease enzyme production. Alkaline protease shows optimum activity at temperatures ranging from 40 to 70 °C. This includes different alkaline protease producing marine bacterial and fungal species like Bucillus subtilis NS (40 °C) Nisha et al., (2014)., Vibrio sp. (55 °C) Subhashini et al., (2012)., Psiloteredo healdi (42 °C) Griffin et al., (1992)., Pseudoalteromonas arctica PAMC 21717 (40 °C) Park et al., (2018)., Streptomyces fungicidicus MML1614 (40 °C) Ramesh et al., (2009)., Bacillus spp. (45 °C) Karikalan et al., (2018)., Aureobasidium pullulans 10 (45 °C) Ma et al., (2007)., Yarrowia lipolytica YlTun15 (45 °C) Bessadok et al., (2017)., Pseudoalteromonas sp. 129–1 (50 °C) Wu et al., (2015)., Bacillus alveayuensis CAS 5 (50 °C) Annamalai et al., (2014)., Pseudomonas aeruginosa (50 °C) Satheeskumar et al., (2013)., Actinomycete MA1–1 (50 °C) Hameş-Kocabaş et al., (2007)., Bacillus vallismortis (54 °C) Cheng et al., (2015)., Bacillus sp. APCMST-CS4 (60 °C) Maruthiah et al., (2017)., Bacillus sp. SD11 (60 °C) Cui et al., (2015)., Alcaligenes faecalis APCMST-MKW6 (60 °C) Maruthiah et al., (2016)., Bacillus pumilus TMS55 (60 °C) Ibrahim et al., (2011)., and Bacillus sp. (70 °C) Padmapriya et al., (2012). Despite there are some exceptions out of the given temperature range, like Aspergillus oryzae (30 °C) Yepuru et al., (2018).

1.1.3. Effect of incubation period

The time duration of alkaline protease production varied from 1 day to 7 days. The production of enzyme in liquid media takes less time than the production time required for solid state fermentation where the incubation period for protease synthesis in solid state fermentation was 96 h Ammasi et al., (2020). & Ranjithkumar et al., (2017). to a maximum of 7 days Zekeya et al., (2019). as maximum, but in liquid medium the incubation period for protease synthesis was 24 h at least Fitriyanto et al., (2021)., with 72 h as maximum Moonnee et al., (2021) & Nyakundi et al., (2021).

The aim of the present study was interested to isolate and purify the highest alkaline protease producing bacteria and optimize the enviromental conditions for maximum production of alkaline protease.

2. Materials and Methods

2.1. Quantitative screening of thermo-alkaline protease-positive isolates

The pure isolates that give positive results on the qualitative screening media by giving larger inhibition zone were selected and tested quantitatively by culturing them on alkaline protease screening media excluding agar (alkaline skimmed milk broth medium). The pH of the medium was adjusted to 8.0 by 0.1N NaOH before sterilization and then autoclaved at 1210c for 30 min. A volume of 25 ml of alkaline protease screening media in 100 ml flasks were inoculated by selected bacterial strains then, the cultures were grown for 72 h in a static incubator at 40°C . At the end of each fermentation period, the broth was filtrated and centrifuged at 5,000 rpm at 4°C for 20 min. and the clear supernatant was used as a crude enzyme.

2.2. Alkaline protease assay :

alkaline protease activity in the cell-free supernatant was determined according to the method of Takami et al. (1989) . By using 1% casein as substrate dissolved in 50 mM Glycine-NaOH buffer(pH 9) . The assay was carried out routinely in a mixture containing 2.5 ml casein solution added to 0.5mL of the suitable diluted enzyme. After incubation at 30 0C for 60min, the reaction time ended by adding 2.5 ml of 0.44M trichloroacetic acid(TCA) .After 10min, The reaction mixture was centrifuged for 10 min at 8000 rpm. Then 0.5ml of supernatant was mixed with 2.5ml of 0.5M Na2CO3 and 0.5ml of folin-ciocalteu,s phenol solution and kept for 30min at room temperature. The optical densities of the solutions were determined with respect to sample blank at 660nm.

One unit of protease activity was defined as the amount of enzyme resulting in release 1 µgm of tyrosine per minute under the defined assay conditions.

2.3. Total soluble protein determination (Lowry et al., 1951) :

This method of protein determination by Lowry et al., (1951) to estimate soluble proteins in the biological samples. In this method, protein reacts with Folin phenol reagent to produce a blue color. The absorbance of this blue color solution can be measured at 750nm. The intensity of the color is in direct relation to the protein concentration. Bovine serum albumin is used to prepare a standard curve.

2.4. Dry weight determination

The dry weight of the bacterial cells were measured by weighting the cells after washing them twice with distilled water and centrifuged each time and putting them in a drier at 105°C for 48h until the weight become stable.

2.5. Identification of isolated bacteria:

Morphological and biochemical tests were done to identify the bacterial isolates according to (Bergey's Manual of Determinative Bacteriology 1994).

2.5.1. Gram staining technique:

Smear of about 200mm in diameter on grease free slide which was also fixed above a burning flame. A crystal violet dye was putted to cover the smear for 30 seconds , then washed with distilled water. Secondly lugol's iodine was putted to the surface for 30 seconds. Ethyl alcohol was used to decolorize the stain and finally, the safranin dye solution was applied to the surface of slide for one minute then washed with distilled water and remains slide to dry at room temperature. Then the stains have been scanned under microscope with oil immersion and Gram- negative bacteria is observed as red color and Gram- positive bacteria is observed as violet color Cheesbrough, (2000).

2.5.2. Biochemical identification of isolated bacteria:

2.5.2.1. Citrate agar :

To identification and differentiate the members of Enterobacteriaceae on the basis of citrate utilization from clinical and non-clinical samples Eaton et al.,(2005).

Composition: (g/l)

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Bromothymol blue	0.08
Sodium chloride	5
Sodium citrate	2
Agar	15
Final pH (at 25°C)	6.8± 0.2

Procedure:

Dissolve 24.28 grams in 1000 ml pure distilled water, heat and boiling to complete dissolve the medium, mix well and distribution tubes, sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes, finally before using water, ensure pH of water is 6.5 to 7.0.Initial color of the to medium may different from expected color, if the pH is ignored.

2.5.2.2. Indole test:

This test is performed to identify the E .coli, Shigellae and Salmonellae from tested colonies. A sterile loop was used to inoculate overnight growth cultures into a test tube of 5ml peptone water then incubated at 35 - 37 ºC for 24 hours then add 5 drops of Kovac'sindole reagent and shaken gently. Appearance of red color on the top layer. E. coli and Shigella were indole positive while Salmonella was indole negative (Cheesbrough, 2000).

2.5.2.3. Catalase test:

Rapid air bubbling or effervescence indicates positive result by adding few drops of hydrogen peroxide to colony stand on clean slide by platinum loop. The lack of bubbles indicates the absence of catalase (Forbes et al., 1998b).

2.6. Fermentation process for alkaline protease production

2.6.1. preparation of inoculum

After the quantitative screening of protease producing bacteria, Bacillus subtilis no 28 was selected among the isolated bacterial isolates for the studying the factors affecting alkaline protease production. The spore suspension was prepared by adding 5 ml sterile distillated water to slant culture 18-30 h old scratched with the aid of sterile needle.

2.6.2. Factors affecting alkaline protease production by Bacillus subtilies

2.6.2.1. Environmental factors:

5.2.1.1Cultivation

For the cultivation of Bacillus subtilies , 100 ml Erlenmeyer conical flasks containing 25 ml of alkaline skimmed milk broth medium were used. Each flask was plugged with cotton plug and sterilized at 1210c for 20 min at 1.5 atmospheric pressure. Each flask was inoculated with 0.5 ml inoculum after cooling under sterile conditions. Then the flasks were incubated at 400c at static incubator for the required time depending on the

purpose of the experiment. At the end of each incubation periods, the culture broth filtrated and centrifuged at 5.000 rpm for 20 min using cooling centrifuge. then the filtrate was used directly for enzyme activity determination, protein, specific activity and final pH. Duplicate flasks were used for each condition.

5.2.1.2. Initial pH of the fermentation medium

Effect of different initial pH value of the fermentation medium was studied. The pH of the fermentation broth media were adjusted before sterilization. Different pH values of medium ranging from 5 - 11 were prepared using 1.0N NaOH and 0.1N HCl. The flasks were sterilized, cooled, inoculated and incubated at 400c for 72 h. at the end of each incubation period, the usual parameters were determined.

5.2.1.3. Effect of different incubation periods on alkaline protease production

Bacillus subtilis was cultured on two types of media : free and skimmed nutrient broth media. The culture broth were incubated at 400c for 5 days. Samples of two broth media were collected every day for determination of different parameters.

*free skimmed nutrient broth media is the same of skimmed nutrient broth media excluding addition of skimmed milk.

5.2.1.4. Effect of different incubation temperature on alkaline protease production

The flasks containing culture broth were incubated for 48 h at different temperature 10, 20, 30, 40, 50, 600c. At the end of incubation period, the enzyme activity, protein, specific activity and final pH determine.

5.2.1.5. Effect of different inoculum size.

Different volume of freshly actively 18-30 h cultivated slanta were used as inocula for experimental culture flasks containing fermentation media. The effect of inoculum size was studied.

5.2.1.6. Effect of static and shaking conditions

The flasks containing culture broth were incubated at 400c at static and shaking incubator (150 rpm/min) for 48 h. At the end of the incubation period, the usual parameters were determined.

3. Results

1. Quantitative screening for the best alkaline protease isolates with highest qualitative detection:The bacterial isolates that giving the highest inhibition zone diameter in the qualitative screening were selected for quantitative screening using alkaline skimmed milk nutrient broth media. The results in tables(1, 2 & 3) explained the final pH, enzyme activity, protein , specific activity and biomass. It has been founded that the chosen isolates showed protease activity. The activity of all isolates were measured at 400c and pH 8.0 for 3 periods (24,48,72 hr) . Isolates number 28 was selected for further studies as it showed the best alkaline protease productivity and specific activity after 48h.

Table(1) Quantitative screening of alkaline protease production by alkaliphilic selected bacterial isolates after 24 hr.

Sample No.	Final pH	Enzyme activity(U/ml)	Protein detection(mg/ml)	Specific activity(U/mg protein)	Dry wt. (g/100ml)
6	7.82	0.309	1.008	0.30	0.04
14	8.1	0.513	0.924	0.55	0.12
19	7.92	0.519	1.026	0.50	0.08
25	7.84	0.372	0.918	0.40	0.08
28	7.87	0.368	1.086	0.34	0.04
29	7.93	0.467	0.918	0.50	0.12
33	7.82	0.339	1.218	0.28	0.04
34	8.36	0.349	1.014	0. 34	0.04
41	8.13	0.417	0.993	0.42	0.04
42	7.75	0.272	0.993	0.27	0.08
45	7.90	0.289	0.969	0.30	0.12
52	7.92	0.514	1.02	0.50	0.04
55	7.64	0.238	1.14	0.21	0.04

Incubation for the best bacteria producing alkaline protease after 24 hr at pH 8.0 and temperature 400c.

Table(2) Quantitative screening of alkaline protease production by alkaliphilic selected bacterial isolates after 48 hr.

Sample No.	Final pH	Enzyme Activity(U/ml)	Protein detection(mg/ml)	Specific activity(U/mg protein)	Dry wt.(g/100ml)
6	8.65	0.384	0.957	0.40	0.08
14	8.68	0.446	0.939	0.47	0.04
19	8.16	0.346	0.615	0.56	0.04
25	8.73	0.436	0.780	0.56	0.08
28	8.79	0.614	0.879	0.69	0.08
29	9.25	0.447	1.002	0.45	0.12
33	8.48	0.604	0.930	0.65	0.04
34	8.69	0.456	0.894	0.51	0.04
41	8.96	0.421	1.140	0.37	0.04
42	8.53	0.456	0.927	0.49	0.12
45	8.48	0.417	0.831	0.50	0.08
52	8.26	0.383	0.942	0.41	0.04
55	8.24	0.369	0.849	0.43	0.08

Incubation for the best bacteria producing alkaline protease after 48 at pH 8.0 hr and temperature 400c.

Table(3) Quantitative screening of alkaline protease production by alkaliphilic selected bacterial isolates after 72 hr.

Sample No.	Final pH	Enzyme activity(U/ml)	Protein detection(mg/ml)	Specific activity(U/mg protein)	Dry wt. /25ml(g/100ml)
6	8.40	0.482	0.897	0.54	0.08
14	8.74	0.466	0.966	0.48	0.08
19	8.16	0.357	0.921	0.39	0.08
25	8.75	0.477	0.885	0.54	0.16
28	8.05	0.421	0.966	0.43	0.04
29	8.61	0.495	0.966	0.51	0.12
33	8.83	0.488	1.038	0.47	0.08
34	8.66	0.330	0.945	0.35	0.08
41	9.09	0.453	1.041	0.43	0.08
42	8.58	0.462	1.032	0.45	0.08
45	8.78	0.494	0.879	0.56	0.36
52	8.12	0.480	0.906	0.53	0.08
55	8.63	0.449	0.807	0.55	0.08

Incubation for the best bacteria producing alkaline protease after 72 hr at pH 8.0 and temperature 400c.

2. Identification of potent alkaline protease producing isolates (28)

Morphological & biochemical identification of isolated bacteria:

About 58 isolates were isolated and screened for alkaline protease production. Bacterial isolate no. 28 strain exhibiting highest enzyme production was selected for further studies. Morphological and biochemical characteristics of the isolate revealed that it is bacilli shaped, motile Gram +ve, catalase positive, citrate positive and indole negative (Table 4).

Table (4) Morphological and biochemical identification of isolated bacteria

Gram staining	Gram positive
Citrate agar	Positive +ve
Indole test	Negative -ve
Catalase test	Positive +ve
Shape	Bacilli
Motility	Motile

3. Optimization of fermentation enviromental factors for maximum production of alkaline protease by local Bacillus subtiles isolates

3.1. Effect of different initial pH of culture media on the production of alkaline protease enzyme

The pH of the fermentation media were initially adjusted by 0.1N HCl or 0.1N NaOH to adjust pH value ranging from 5.0 - 11.0. All the pH values were adjusted before sterilization of the media. 100ml of flasks were inoculated with 2.0ml spore suspension freshly active cultivated and incubated at 400c for 3 successive days (24,48&72hr). Samples were collected every day by filtration and centrifugation for determination of extracellular protein, enzyme activity, specific activity and final pH. The data obtained were recorded in graphically illustrated in fig(1, 2 & 3). It indicated that the maximum enzyme activity (0.482u/ml with specific activity 0.48 U/mg protein) occurred when Bacillus subtiles was cultivated after 48hr at pH 8.0. The final pH

showed increase with pH from 5 to 8 and then decrease with alkaline pH from 9.0 to 11.0 except at 24 hr the final pH showed slightly decrease at initial pH 8.0.

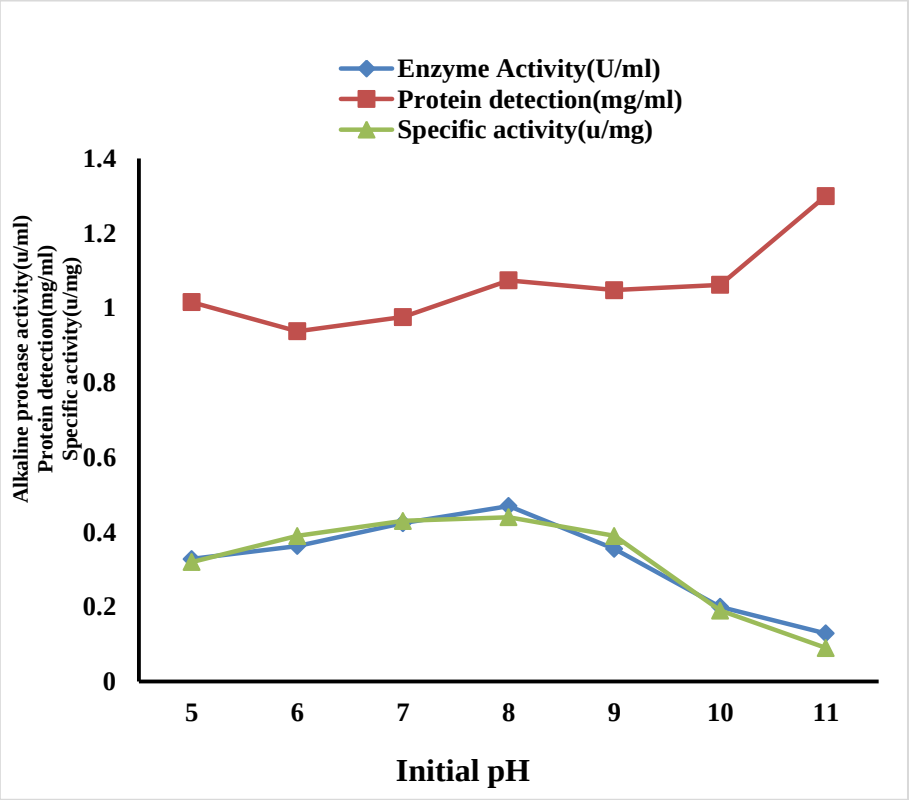

Fig .1. Effect of different initial pH values on alkaline protease production by Bacillus subtilis after 24 hr at 400c.

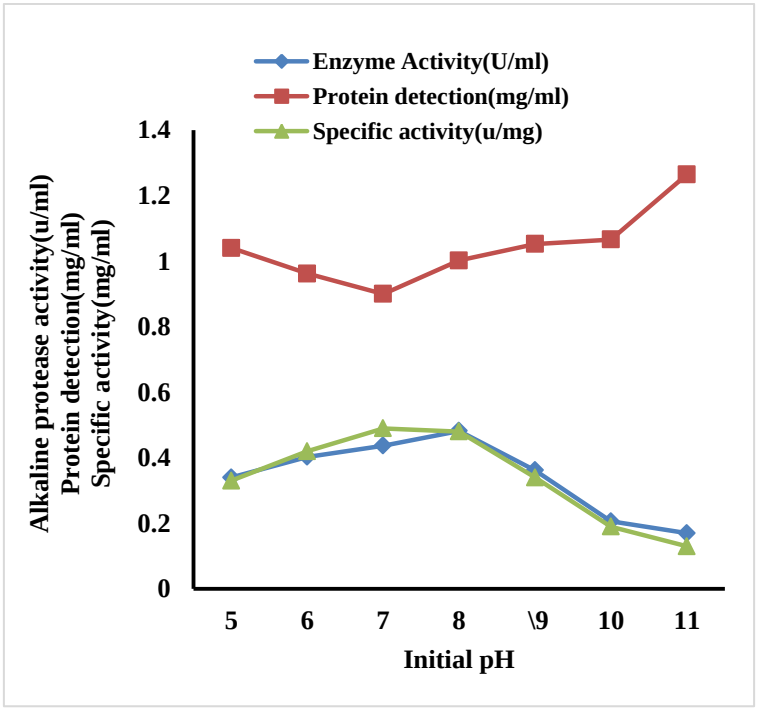

Fig. 2. Effect of different initial pH values on alkaline protease production by Bacillus subtiles after 48 hr

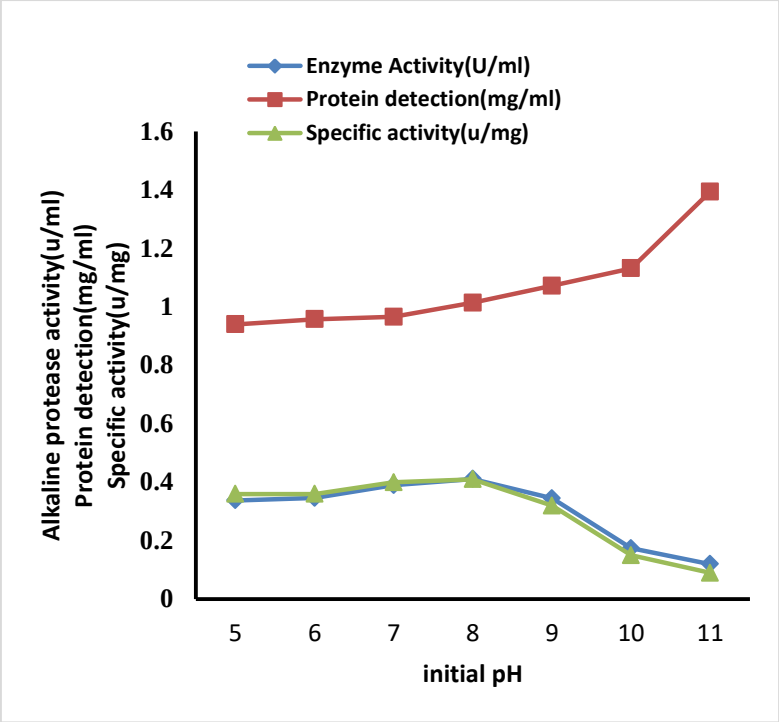

Fig.(3) . Effect of different initial pH values on alkaline protease production by Bacillus subtiles after 72 hr at 400c.

3.2 Effect of different incubation periods on alkaline protease production by Bacillus subtiles SFL on static conditions at free and skimmed nutrient broth media

This experiment had been done by two ways where alkaline nutrient broth media containing skimmed milk as substrate and broth media free from milk. In this experment, 100 ml of fermentation media were sterinlized and inoculated by2ml of spore suspension. The flasks were incubated at 400c in static incubator for 5 days. The sample were collected every day for determination of different parameters.

In tables (8 & 9), the data were recorded where the maximum enzyme activity at skim nutrient broth medium was 0.612U/ml with 0.59 U/mg protein in the second day of incubation and it was 0.256U/ml with specific activity 0.32U/mg protein at free skimmed milk nutrient broth. The results showed that the enzyme activity is two times at skimmed milk nutrient broth than at free skimmed mil; broth media.

Table (5) Effect of different incubation periods on alkaline protease produced by Bacillus subtiles on static condition at skim nutrient broth medium.

incubation periods(day)	Final pH	Enzyme activity(U/ml)	Protein determination(mg/ml)	Specific activity(U/mg protein)
1	7.61	0.400	1.094	0.36
2	8.19	0.612	1.096	0.59
3	7.97	0.435	1.080	0.40
4	8.23	0.402	1.068	0.37
5	8.47	0.382	0.954	0.40

For the selected organism No. 28 table showed the effect of different incubation periods in static conditions at 400c and at pH 8.02

Table (6) Effect of different incubation periods on alkaline protease produced by Bacillus subtiles SFL on static condition at free skim nutrient broth medium.

incubation periods (day)	Final pH	Enzyme activity (U/ml)	Protein determination (mg/ml)	Specific activity (U/mg protein)
1	7.87	0.245	0.936	0.26
2	9.21	0.256	0.798	0.32
3	8.44	0.230	0.880	0.0.26
4	9.07	0.248	0.856	0.0.29
5	9.31	0.184	0.916	0.0.20

For the selected organism No. 28 table showed the effect of different incubation periods in static conditions at 400c and at pH 8.01

3.3 Effect of different incubation temperature on alkaline protease production by Bacillus subtiles

In this experment, flasks of 100ml alkaline skimmed milk nutrient broth media were sterilized and inoculated with 2ml of spore suspension from 1slant of 24hr freshly well grown active culture , the flasks were incubated in static incubator at different temperature ranges(10-600c) for 48hrs.

The results presented in graphically illustrated in fig.(6). The results revealed that the highest alkaline protease activity(0.504 U/ml) and the highest specific activity(0.4 U/mg) were at 400c and the minium enzyme value was at 600c. The pH of the fermentation media media were initially adjusted to 9.0 and the final pH showed slightly decreased at temperatures(10,30), slightly increased at other temperatures and it was 8.74 at the best incubation temperature.

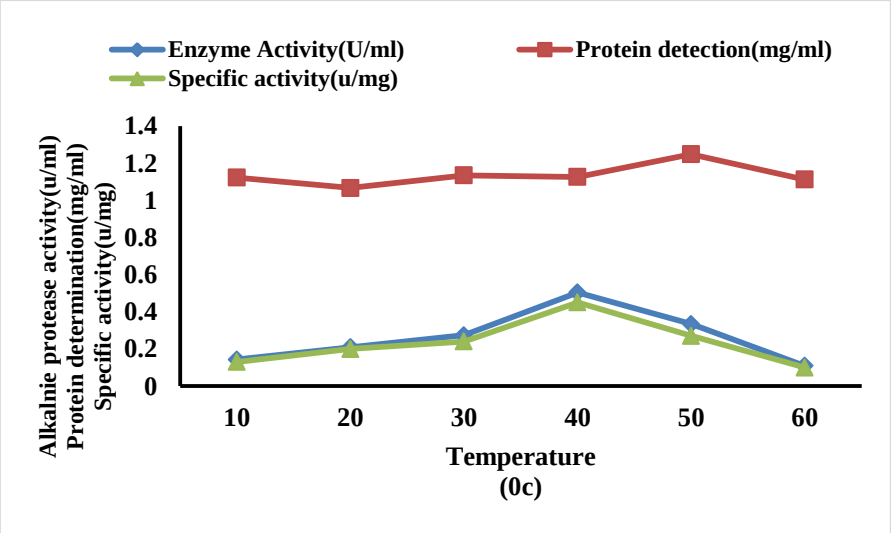

Fig. 4. effect of different incubation temperature on alkaline protease production by Bacillus subtiles.

3.4 Effect of different inoculum size on alkaline protease production by Bacillus subtiles

24 hr of freshly cultivated skimmed milk nutrient agar slanta was used to make spore suspension. Inocula of 1.0, 2.0, 4.0, 8.0 ml were used to inoculate 100ml of sterile alkaline skimmed milk nutrient broth flasks with pH 8.0. The flasks incubated for 48 hrs at 400c in static incubator. The results in graphically illustrated in fig.(7) showed that the maximum enzyme activity(0.351u/ml) was at 2.0 ml inoculum. The final pH decreased et all treatments.

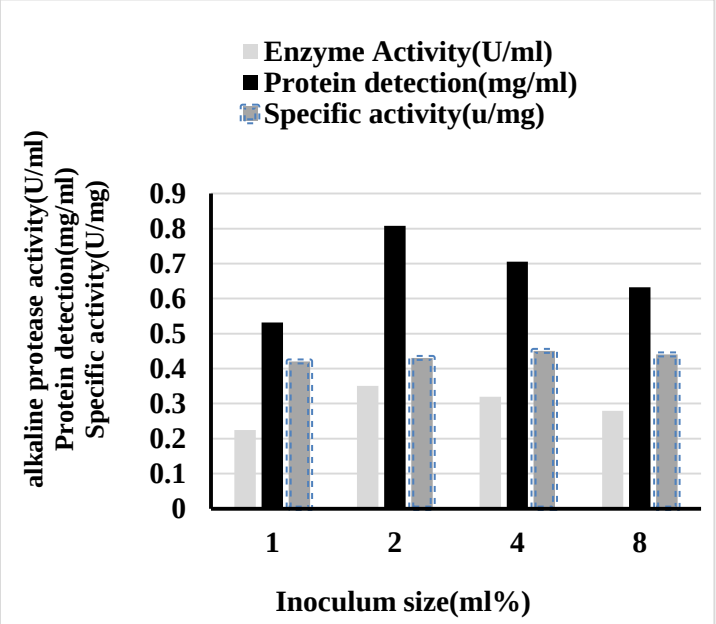

Fig. 5. Effect of different inoculm size on alkaline protease production by Bacillus subtiles

3.5. Effect of static and shaking conditions on alkaline protease production by Bacillus subtiles

The inoculated sterilized alkaline skimmed milk nutrient broth flasks were incubated in static incubator and in shaking incubator(150rpm) at pH 8.0 for 48hrs.

The data were recorded in illustrated in fig(6). It showed that the maximum enzyme activity(0.452u/ml) was at shaking conditions in compare to static incubator, it was 0.351u/ml. The final pH decreased at two conditions.

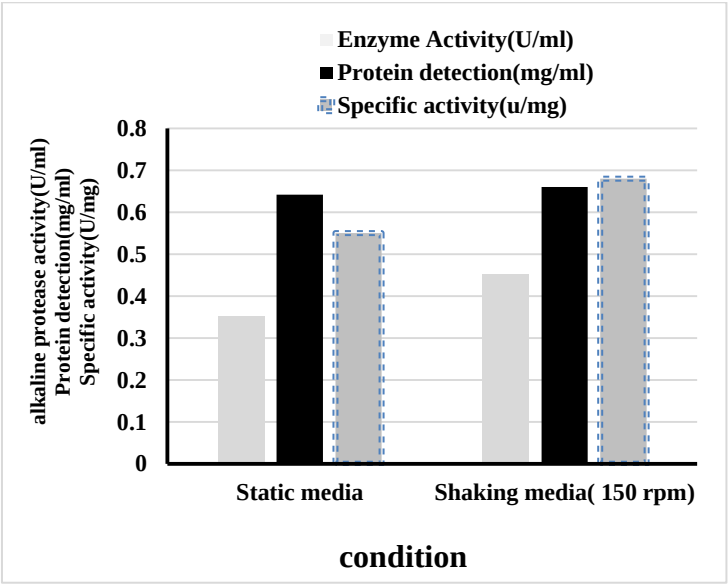

Fig. 6. Effect of static and shaking conditions on alkaline protease production by Bacillus subtiles.

Discussion

The current study was up on alkaline protease. Proteases are widely used in industrial production such as washing industry, leather industry, pharmaceutical industry and food industry Yang et al., (2016); Banerjee & Ray, (2017); Contesini et al.,(2018); Gurumallesh et al., (2019); Hammami et al., (2020).

In our search for alkaline protease, about 58 bacterial strains were isolated from different regions of Sharkia governorate, namely; Belbis, Zagzig, mithamel farms and Ismailia governorate. The collection take place from different sources including local soil, bean soil, bloody soil, vegetable water waste, poultry waste and animals waste. The isolation has been done on media described by Aftab et al., (2006). All bacterial isolates were screened qualitatively using Skim milk agar alkaline media containing NaOH(SMA) described by Ibrahim et al., (2007) as aspecific medium for alkaline protease production.

The bacterial isolates that showed the largest clear zone, which refer to higher bacterial ability for the production of alkaline protease than others, were selected for quantitative screening using skimmed milk broth media.

The selected bacterium, on the basis of the best production of alkaline protease, was subjected to morphological, biochemical and molecular identification and it was defined as Bacillus subtilis

In the current study, different experiments were done for optimization of the fermentation process for maximum production of alkaline protease where it was achieved by one factor optimization followed by applying statistical designs. the factors are pH, Incubation temperature, incubation periode and static or shaking condition.

The intial pH of the fermentation media is an important parameter where it affects all enzymatic process and tranportation of media components through the cell membrane. Singh et al.,(2010) reported that the relative metabolic effeciency is high under optimum pH range. By studying the effect of different initial pH value on alkaline protease production by Bacillus subtilis, it was founded that the optimum alkaline protease production was at pH 8.0. this result was in confirmation to Olajuyigbe et al., (2005) who reported that slightly alkaline medium (pH 8.0–8.5) is reported to be optimum for production of protease by B. subtilis IKBS 10. this result also confirmed with Yanga et al., (2000) & Karadag et al., (2009) who observed previously that use of casein as the substrate under the standard assay conditions gave the highest activity at pH 8.0.

Temperature is acritical parameter that vary from organism to another. The results in this study showing that the optimum incubation temperature for alkaline protease production by Bacillus subtilies was at 400c. Darani et al.,(2008) confirmed that temperature of 400C the best for production of protease by Bacillus sp. This result was in contrast to Shaheen et al .,(2008) who reported that the maximum protease production from Bacillus subtilies was at 500c

The production of enzyme is affected by incubation period and it vary from 24h to seven days depending on culture conditions and microorganism. The results in our search show that 48 h is the best incubation period for alkaline protease production. The result was confirmed by Adinarayana et al.,(2003) who observed that the maximum growth and enzyme production of Bacillus subtilis PE-11 was after 2 days and also confirmed by Younis et al., (2009) who reported that Bacillus subtilis KO strain showed its maximum production of protease within 48 h incubation period. In contrast to this Kumar et al., (2008) reported that the maximum protese production from Bacillus subtilies was after 72 h.

4. Conclusion

In the present study Among all isolates , An efficient alkaline protease producer was isolated from vegetable water waste and was identified as Bacillus subtilis SFL based on biochemical identification tests and the 16S rDNA sequence. Some environmental parameters were optimized for alkaline protease production by using alkaline skimmed milk nutrient broth media. We also determined the optimum growth factors for cultivating the organism: maximum production of alkaline protease was observed at pH8.0 and a temperature of 40 ◦C in shaking incubator for 48 h. the production of enzyme increased by using skimmed milk as substrate rather than free skimmed nmilk broth media.

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