Fayoum University

Faculty Of Agriculture

STUDIES ON THE PRODUCTION OF PROTEASE BY MICROORGANISMS

By

ESRAA AHMED ISMAIL HASAN

B.Sc. Agric. Sci. (Food Science and Technology),

Fac. of Agric., Fayoum University, Egypt, ٢٠١١

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٥. SUMMARY

Sixteen proteolytic thermophilic bacteria were isolated from soil at Fayoum Governorate. The isolates were screened for their proteolytic activity on skim milk agar medium; the diameter of hydrolysis zone was the measurements from the level of proteolytic activity. The most active isolates were selected for the fermentation experiments and the determination of their productivity in the submerged culture. The seven selected isolates were used in fermentation experiments and the proteolytic activity was determined after ٤٨ hrs. The enzyme yields obtained in the fermentation medium were corresponding to the proteolytic level recorded by the diameter of hydrolysis zone on the skim milk agar medium. That indicates a positive relationship between the amount of the enzyme and its spreading in the skim milk agar medium. According to the results from the fermentation experiments, isolates S-٥, S-٨ and S-٩ which gave the highest enzymatic yields were chosen for studying the best environmental conditions for the enzyme production. The three isolates were identified based on morphological, biochemical and ١٦S rRNA gene sequencing analysis, isolates S-٥ was identified as *Brevibacillus panacihumi*; isolates S-٨ and S-٩ were identified as *Bacillus aerius.*

The environmental and nutritional conditions were tested for the highest production of enzymatic yield e.g. pH of medium, incubation temperature, fermentation period as well as carbon and nitrogen sources at different concentrations for *Bacillus stearothermophilus* ATCC٧٩٥٣, *Brevibacillus panacihumi* S-٥*, Bacillus aerius* S-٨ *and Bacillus aerius* S-٩.

The highest enzymatic yield was obtained in the fermentation medium at pH ٧,٠ for *Bacillus stearothermophilus* ATCC٧٩٥٣ and *Brevibacillus panacihumi* S-٥ while at ٨,٠ for both *Bacillus aerius* S-٨ and *Bacillus aerius* S-٩. Below or above this level showed a decrease in the enzyme production.

The fermentation at different temperatures i.e. ٤٠, ٥٠ and ٦٠ ºC showed that the optimum incubation temperature for the enzyme synthesis was ٥٠ ºC for all tested bacterial strains. The results, also, show that the enzyme production were decreased about ١٠,٢٤٪, ١١,٥٠٪, ٦,٢٢٪, ٨,٨٧٪, ٢٣.٠١٪, ٢٧,١٢٪, ٢٤,٢٥٪ and ٢٢,٥٤٪ for *Bacillus stearothermophilus* ATCC٧٩٥٣; *Brevibacillus panacihumi* S-٥; *Bacillus aerius* S-٨ and *Bacillus aerius* S-٩ at ٤٠ ºC and ٦٠ ºC, respectively.

Concerning the effect of the fermentation period on protease production, fermentation period were tried to detect the most favorable for the highest enzymatic yield. The maximum enzymatic activity was noticed, after ٧٢ hrs. fermentation period. Increasing the incubation period over ٧٢ hrs the enzyme activity decreased by ٣,٠٣٪, ٤٦,٧٠٪, ٣٤,٣٨٪, ٤٦,٨٣٪, ١٤,٤٣٪, ٢٣,٠٦٪, ١٠,٣٨٪ and ٢١,٤٠٪ for *Bacillus stearothermophilus* ATCC٧٩٥٣; *Brevibacillus panacihumi* S-٥; *Bacillus aerius* S-٨ and *Bacillus aerius* S-٩ at ٩٦ and ١٢٠ hrs. , respectively.

Dextrin, sucrose, maltose, glycerol and glucose were used as carbon source in the fermentation medium to evaluate the best one of carbon source which produces the highest enzymatic activity. These materials were tested at various concentrations to define the most suitable concentration of each source.

Glucose was the best carbon source followed by dextrin; sucrose; maltose and glycerol respectively for *Bacillus stearothermophilus* ATCC٧٩٥٣; *Bacillus aerius* S-٨ and *Bacillus aerius* S-٩. However dextrin as well as glucose gave the higher enzymatic activity than other carbon sources used for *Brevibacillus panacihumi* S-٥.

Accordingly, the concentration of these substrates also, affected the amount of protease production. The enzyme yield increased with the increase of substrate concentration to a certain level after which the activity was gradually decreased. This may be attributed to the substrate inhibition or repression.

Concerning the effect of nitrogen sources on enzyme production it may be concluded that, the highest activity from the media containing tryptone at the concentration of ١,٥٪ was ١٠٨,٣٥٪; ١١٨,١٥٪; ١٠٥,٧٩٪ and ١٠٥,٩٤٪ of that from the control medium by *Bacillus stearothermophilus* ATCC٧٩٥٣; *Brevibacillus panacihumi* S-٥; *Bacillus aerius* S-٨ *and Bacillus aerius* S-٩ respectively. However, it was found the concentration of ١,٠٪ yeast extract secured the highest enzymatic activity which presented ١٠١,٦٩٪,١٠٧,٥٥٪ for *Bacillus stearothermophilus*ATCC٧٩٥٣; *Brevibacillus panacihumi* S-٥ respectively. While control which contain ٠,٥ % yeast extract seemed to be the most suitable concentrations for *Bacilus aerius* S-٨ and *Bacillus aerius* S-٩.

Due to the fact that any waste product containing carbohydrates can serve as raw materials for enzyme synthesis f bacteria, cane sugar bagasse as well as rice straw was selected as a medium substrate for enzyme production by tested bacterial strains.

The maximum activity recorded in the cane sugar bagasse media at the concentration of ١,٣٤٪ (equivalent ٠. ٧٢% carbon) was only ٨١,٥٢٪ and ٨٣,٩٨٪ of the control medium for both strains of *Bacillus stearothermophilus* ATCC٧٩٥٣ and *Brevibacillus panacihumi* S-٥, respectively.

Regarding *Bacillus aerieus* S-٩, it was found that the best concentration of cane sugar bagasse was ١,٧٦٪ (equivalent ٠.٩٦ % carbon) which gave the enzymatic activity only ٦٥,١٠٪ from the control medium

Concerning the effect of different concentrations of rice straw on enzyme production, it may be concluded that, in general, all the investigated bacterial strains exhibited nearly the same trend. Increasing the rice straw concentration in the fermentation medium, positively affected most of the bacterial strains under examination. The highest enzymatic activity was produced in the medium contained ١,٩٨٪ (equivalent ٠,٩٦ % carbon) rice straw. At this concentration the enzymatic activity was about ١٠٠,٣٢ % of that produced in the control medium by *Bacillus stearothermophilus* ATCC٧٩٥٣.

Generally, we could be say that rice straw media resulted in somewhat higher enzymatic yields as those in media cane sugar bagasse.

Extraction of the enzyme by precipitation method was carried with the organic solvents, acetone, ethanol and isopropanol at different concentrations (fermentation liquor/solvent v/v). Also, enzyme was extracted by salting–out using different concentrations of ammonium sulfate.

Among the organic solvents, acetone was the best solvents followed by ethanol and isopropanol for extraction efficiency and purification for both *Bacillus stearothermophilus* ATCC٧٩٥٣ and *Brevibacillus panacihumi* S-٥. The highest recovery percentage (٣٠,٨٠٪) was ٥٠٪ saturation of acetone supernatant *Bacillus stearothermophilus* ATCC٧٩٥٣ culture while it was (٣٩,٥٧٪) at ٨٠٪ for *Brevibacillus panacihumi* S-٥. The highest recovery percentage was obtained at ٧٠٪ saturation of ethanol for *Bacillus aerieus* S-٨ while at ٨٠٪ saturation of isopropanol for *Bacillus aerieus* S-٩.

Extraction of the enzyme by salting –out with ammonium sulfate gave better extraction than that obtained by organic solvents with all tested bacterial strains. The best concentration for enzyme precipitation seems to be between ٧٠% and ٨٠٪ saturation of ammonium sulfate since the highest specific activity and recovery percentage were ٨٠٪ for all tested bacterial strain. Recovery percentage was ٩٩,١٪, ٧٩,١٧٪, ٧٧,٠١٪ and ٦٤,٨٥٪ for *Bacillus stearothermophilus* ATCC٧٩٥٣, *Brevibacillus panacihumi* S-٥, *Bacillus aerieus* S-٨ and *Bacillus aerieus* S-٩ respectively. Therefore, ammonium sulfate gave better results than that obtained by organic solvents for all tested bacterial strains. The best concentration for enzyme precipitation seemed to be between ٧٠٪ and ٨٠٪ saturation of ammonium sulfate science the highest specific activity and recovery ٥ were at ٨٠٪ for all tested bacterial strains. Analysis of molecular mass of the partially purified enzyme was carried out by SDS-PAGE which revealed four protein bands with different molecular weights ranged from ٧٥ to ٢٠ KDa. These bands were varied in molecular weight (٦٣, ٤٨, ٣٠ and ٢٥ KDa).

The influence of pH and temperature of the enzyme solution on the activity of partially purified protease was studied to detect the optimum conditions for the application of the enzyme.

A set of enzyme solution was adjusted to different pH values ranging from pH ٥,٠ to pH ١١,٠; another set was adjusted to the same pH values in a ٠,٠٠٥M $CaCl_2$ solution. It was found that the optimum pH which gave the highest activity was ٧,٠ for *Bacillus stearothermophilus* ATCC٧٩٥٣ while at pH ٨,٠ for *Brevibacillus panacihumi* S-٥, *Bacillus aerieus* S-٨ and *Bacillus aerieus* S-٩. However, below or above this levels, the activity of enzyme was gradually decreased.

With regard to the effect of Ca- ions on the enzyme activity, its presence increased the activity at various tested pH values from pH ٥,٠ to pH ١١,٠ by ٥,٧٣, ٤,٩٠ %, ٧,٢٢٪, and ٤,٥٦, ٥,١٥٪, ١,٨٤٪ and ١,٨٢٪ for *Bacillus stearothermophilus* ATTC٧٩٥٣; ٤,٣٠ %, ٥,٥٥٪, ٥,٦٨٪, ٦,٩٣ %, ٣,٤٢٪, ١,٥٥ % and ١,١٩ % for *Brevibacillus panacihumi* S-٥; ٥,٢٥٪, ٣,٧٠٪, ٥,٤١٪, ٨,٥٩٪, ٥,٩٠ %, ٣,٥١٪ and ١,٣٣٪ for *Bacillus aerius* S-٨ and ٣,٨٨٪, ٣,٣٣٪, ٦,٧٠ %, ٨,٢٠ %, ٧,٦٨ %, ٦,٦٣٪ and ٣,٣٠ % for *Bacillus aerius* S-٩.

The enzymatic activity was measured at different temperatures; the highest activity was recorded at ٧٠ ºC. In the presence of Ca-ions, the estimated activity was higher at different tested temperatures than that in enzyme solution without calcium for all tested bacterial strains.

The thermal stability of the enzyme was tested in the presence or absence Ca-ions at ٧٠ ºC for ١٢٠ minutes. The enzyme activity was estimated at ١٥ minutes intervals. In general, the retained activity percent was approximately the same of original activity after ١٥ and ٣٠ minutes (٩٩,٦٢٪ and ٩٩,٢٩٪) for *Bacillus stearothermophilus* ATCC ٧٩٥٣ , ١٥, ٣٠, ٤٥ and ٦٠ minutes (٩٨,٤٤٪ , ٩٧,٤٨٪ , ٩٤,٩١٪ and ٩١,٩٧٪) for *Brevibacillus panacihumi* S-٥ , ١٥ ,٣٠ ,and ٤٥minutes (٩٩,٩٦٪ , ٩٩,٧٧٪ and ٩٩,٦٨٪) for *Bacillus aeruis* S-٨ and ١٥ minutes (٩٩,٣٥٪) for *Bacillus aeruis* S-٩. However, in the presence of Ca-ions, the thermal stability of the enzyme was considerably enhanced with no apparent loss of enzyme activity after ٤٥ minutes for *Bacillus aeruis* S-٨ and ٣٠ minutes for *Bacillus stearothermophilus* ATCC ٧٩٥٣, *Brevibacillus panacihumi* S-٥ and *Bacillus aeruis* S-٩.