Utilization of Some Agro Wests for the Production of Acid Protease by Aspergillus niger

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Abstract

Acid protease production under solid-state fermentation was investigated using isolated Aspergillus niger, which produced a higher amount of extracellular acid protease (130 U/ml) than other locally isolated fungal specie under submerged liquid fermentation. Among all agro-industrial waste materials evaluated, dried melon peel supported maximum protease production. The addition of carbon and nitrogen sources to the solid medium increased the yield and synthesis of enzymes. Using casein as a nitrogen source and fructose as a carbon source, maximum enzyme synthesis was accomplished, increasing enzyme activity to (750U/gm). The synthesis of acid protease was greatly influenced by physiological fermentation variables, with 105 (spore/ml) being the ideal inoculum size. These factors included the pH of the medium (pH 6.0), moisture ratio (1:0.75), incubation duration (120 h), temperature (35 °C), and inoculum level.

Keywords: *Milk-clotting enzymes, Acid Proteases, Solid State Fermentation, Optimization, Aspergillus spp.*

INTRODUCTION

Rennet, a common milk-clotting protease that has been used extensively in cheese production since antiquity, is often taken from the stomach of newborn calves.The main milk-clotting protease in rennet, chymosin (EC: 3.4.23.4), is a pepsin-like aspartic protease that has a strong specificity for cleaving kappa casein at Phe105-Met106 to produce para-kappa casein and glycomacropeptide. As a result, rising cheese-making and a shortage of calf rennet are driving up the demand for alternative milk-coagulating sources [1].

Rennet has milk clotting activity in addition to being essential for the balanced development of flavor and cheese texture due to its high proteolytic activity to clotting activity ratio. However, calf rennet only supplies 20–30% of the world's need for milk-clotting preparation today [2], due to this and a variety of factors (vegetarianism, religious beliefs, the high price of rennet, etc.), the attention is being turned to the use of coagulants extracted from plants, Bacteria, animal, genetically engineered chymosin, and microbial Proteases [3], however [4] reported that 90% of commercially available proteases are obtained from microbial sources, because of their biochemical diversity, quick growth, thermal stability, high catalytic activity, and fast and safe production, giving consequent, higher economically feasible, which are mostly produced by fungi they are the main producers of acid proteases, with the members of the genera Aspergillus, Penicillium, Endothia, Mucor, etc. and microbial Proteases produced

by different bacteria such as Bacillus spp [5, 6].

Solid-state fermentation, which is more helpful to the environment and has a number of financial advantages over traditional submerged fermentation, has recently gained prominence in the synthesis of microbial enzymes (SmF). Since they can be employed as the cheapest raw materials while boosting product recovery and more, SSF technologies are consequently of particular economic relevance to nations with an abundance of biomass and agro-industrial leftovers [7, 8].

One of the most significant microbes employed in biotechnology is Aspergillus niger. Citric acid and extracellular enzymes are already produced using it, and have been for many years. In fact, the US Food and Drug Administration (USFDA) considers citric acid and numerous A. niger enzymes to be generally recognized as safe (GRAS) [9]. One of the most significant microbes employed in biotechnology is Aspergillus niger. Citric acid and extracellular enzymes are already produced using it, and have been for many years. In fact, the US Food and Drug Administration (USFDA) considers citric acid and numerous A. niger enzymes to be generally recognized as safe (GRAS)[10].

Changes in pH, temperature, substrate, water content, inoculum concentration, etc. have a significant impact on the metabolic activities of microorganisms. For every species, these parameters vary greatly from species to species. Knowing the optimal conditions for microorganisms to produce the most enzymes is therefore essential [11].

MATERIALS AND METHODS

The Fungi Used in the Experiments

The Cultures of Fungi which includes Aspergillus niger, Aspergillus oryza, Aspergillus flavus and Aspergillus fumigatus were obtained from mycology bank\ Biology Department, Collage of Science, Salahaddin University.

Agro-industrial Wastes Used in the Experiments

Wheat bran, orange peel, potato peel, banana peel, expired skim milk powder, carrot husks, raisin husks, green bean, green bean peel and melon peel were obtained from Erbil local markets.

Pre-treatment of Wastes

Preparation of wastes was achieved which included, dehydration by using oven at 70 ºC for 24 hrs., size reduction by grinding, sieving by 1mm pore size, moisturization by addition minerals solution and sterilization.

Milk Clotting Enzyme Production Using Solid State Fermentation

In 250 ml conical flasks, 10 ml of mineral salt solution (g/l00g: 2.0, MgSO47H2O and 2.0; FeSO4) were added to ten grams of solid substrate and pH adjusted to (6.0). After cooling, the medium was inoculated with 1 ml of spore suspension (106 spores/ml) and autoclaved at 121.5 Co for 20 minutes. The medium was then incubated at 35 Co in a shaking bath for 120 hours. Same procedure was followed for all other substrates [12].

Enzyme extraction

Following 120 hours of incubation, the solid medium was extracted with 30 ml of distilled water using 150 rpm for 0.5 hours at 35 Co while shaking in a shaker bath. At 4 Co for 15 minutes, the filtrate was centrifuged at 8,000 rpm. The milk clotting protease activity and the proteolytic activity were tested using the supernatant as a crude enzyme source.

Enzyme activity

1-Clotting activity

Milk-clotting enzyme activity (MCA) was determined according to [13], with some modifications using 12% (w/v) dried skim milk in 0.01 M calcium chloride solution as substrate. The reaction mixture contained 0.5 ml of crud enzyme extract and 1ml of substrate and incubated at 35 °C. The enzyme activity was calculated according to [13]

as follow:

{Milk- clotting activity units = 2400/T*S/E}

Where:

- T = time necessary for crude fragment formation,
- S = the volume of milk
- E = the volume of enzyme.

2- Determination of Proteolytic activity

The method of [13]was used to measure the proteolytic activity. 2.5 ml of 1% (w/v) soluble casein in distilled water was mixed with one milliliter of enzyme extract. For 10 minutes, the reaction mixture was incubated at 38°C. By adding 5 ml of 12% (w/v) trichloroacetic acid, the reaction was stopped. Filtration was used to get rid of the generated precipitate. The supernatant's absorbance at 280 nm was measured. The amount of enzyme that, under these circumstances, causes a rise in absorbance of 0.001 per minute is referred to as one unit.

3-Protein Determination

Proteins concentration was estimated according to the procedure of [14], bovine serum albumin (BSA) was used as standard.

4-Determination of Enzyme specific activity

The specific activity measured according to the following question:

Specific activity (enzyme unit\ mg protein. By calculating specific activities of four spices of Aspergillus, The results observed that A. niger has highest specific activity and A. oryza has lowest specific activity, then the A.niger used for MCE production and optimization under solid state fermentation (SSF) system.

Optimization of various parameters influencing maximum enzyme production by Aspergillus niger using Solid State Fermentation

The impact of substrates

Different substrates (wheat bran, orange peel, potato peel, banana peel, expired skim milk powder, carrot husks, raisin husks, green bean, green bean peel and melon peel) were tested for enzyme production. Then highest enzyme-producing substrates combination was selected from above-mentioned substrates and mixed such as 4 gm Wheat bran and 5 gm dried melon and 1gm expired skim milk powder. Enzyme activity was monitored at 120 hrs. The highest enzyme-producing combinations of substrates were used in SSF for further optimization [15]. studied the MCE production from B. subtilis MK775302 under SSF utilizing some agro-industrial wastes. Plackett-Burman (PB) and central composite designs were used to optimize the production medium including agricultural and industrial wastes.[16] Results revealed that the combination of wheat bran, casein (1% w/w), and glucose (0.5% w/w) made for the best fermentation medium.

The impact of pH:

SSF was carried out using a moisturizing agent with different pH ranges of 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, adjusted by using (0.1N) NaOH and (0.1N) HCl. The flasks were incubated at 35 ºC for 120 hrs. and the enzyme activity and protein concentration were measured as described earlier.

The impact of incubation temperature:

The SSF was carried out at different temperatures (30 °C , 35 °C , 40 °C , and 45

°C) for 120 hrs. and the enzyme activity and protein concentration were monitored.

The impact of the initial moisture content

To determine the best ratio for enzyme production under SSF, the substrate was moistened using mineral salt solutions (g/l: 2.0, MgSO47H2O; 2.0; FeSO4) in a range of ratios (w/v), starting from 1:0.5, 1:7.5, 1:1, 1:1.5, 1:1.75, and 1:2. Then, the enzyme activity and protein concentration were monitored.

The impact of size of inoculum:

The size of inoculum was used were 103,104,105,106,107and 108, and the counting made by hemocytometer under the microscope.

The impact of incubation time:

Following inoculation, the flasks were incubated at 30 °C for varying lengths of time (from 72 to 192 hours), during which enzyme activity and protein content were analyzed.

The impact of nitrogen source supplementation

Several N sources, such as Skim milk powder, peptone, yeast extract, casein, and ammonium sulfate, were added to the SSF production medium in amounts ranging from 1% to 2%. The flasks were then incubated at 35 °C for 120 hours while the enzyme activity and protein content were monitored.

The impact of supplementation with a carbon source

Different carbon sources, including glucose, fructose, soluble starch, lactose, maltose, and sucrose, were added to the SSF production medium. The flasks were incubated at 35 °C for 120 hours with an addition of 1% of the carbon sources, after which the enzyme activity and protein content were monitored.

RESULTS AND DISCUSSION

Milk Clotting Enzyme Production by Aspergillus spp

Pure cultures of four various species (A. oryza, A. flavus, A. niger, and A. fumigatus) were evaluated for milk-clotting enzyme production by submerged fermentation conditions and the A.niger showed the highest Milk-clotting activity 100 (U/ml) and specific activity 71.42 (U/mg) while A. oryza had the lowest Milk-clotting activity 1.53 (U/ml) and specific activity 1.09 (U/mg) as shown in Figure (1).

A. niger was chosen for additional research on solid-state fermentation's ability to produce MCE. All other isolates demonstrated some MCE production, but not as much as A. niger. Similar results were obtained by [17] when the MCE produced from A. niger solid state fermentation, also when [18] Compared the screening rate of acid protease between thirteen different strains of A. niger, of all the isolates, A. niger IHG9 was found to be the best producer of protease with enzyme activity 4.7±0.32 (U/g), and [19] [20] reported that A. niger I1 is a producer of acid protease. While [21] and [6] reported the Milk-clotting and aspartic protease synthesis from Aspergillus oryzae in solid-state fermentation (SSF).

Effect of culture conditions on Milk-clotting Enzyme production by A. niger under SSF

A solid-state fermentation experiment to determine the optimal substrate for milk clotting enzyme

Various substrates have been evaluated for MCE production. It is evident from Figure. (2) That the maximum MCA and SPA 300 (U/gmM) and SPA 136.3636 (U/mg) were obtained when a mixture of {(4gm) wheat bran, (1gm) expired skim milk powder and (5gm) dried melon peel}, was used as a substrate, other substrates combinations showing less MCA and SPA. On the other

hand, these (expired skim milk powder, orange peel, banana peel, carrot pulp, melon peel, raisin pulp, potato peel, green bean, green bean peel, and wheat bran) substrates showed no clotting activity alone in 30 minutes when were used alone as SSF substrates.

It is necessary to investigate a number of industrial residues in order to choose the best one for enzyme production in an SSF process because it depends on a number of factors, primarily those related to cost, handling simplicity, dependability, and substrate material availability [9, 22] The reports described wheat bran as a potent substrate for production of MCE by A.niger FFB1, A.niger ATCC 16404, A.flavus and Rhizomucor naninitalensis [23] Also, wheat bran combination with other agricultural residues was used by [24-26] and had combined wheat bran with soybean (8:2), rice bran 75% and rice bran (7:3) respectively which showed best productivity of enzyme. But no articles were found that used expired skim milk powder or dried melon (cantaloupe) peel as substrate for enzyme production under SSF. Dried cantaloupe peel is a great source of reducing sugars, vitamin C, calcium, and phenolic compound [27] with rich protein and minerals content in wheat bran and carbohydrate of expired skim milk. Which together enhance the growth and MCE production of A. niger [15].

Effect of pH

Figure (3) shows that the highest level of enzyme production was observed at pH 6 (MCA: 324U/gm) and (MCA: 294.6U/gm) at pH 5. Same result obtained by [21] when he produced MCE from Aspergillus oryzae DRDFS13 under solid-state fermentation (SSF)

[28] indicated that, with the exception of the initial pH of the substrate, which is adjusted before inoculation, changes in pH during the SSF technique are not easily controlled. Because pH variations have a significant impact on numerous enzymatic processes and the movement of different nutrients through the cell membrane, which promotes growth and the generation of metabolites, microorganisms are extremely sensitive to pH changes.

Figure (1): MCE production by different Aspergillus spp under submerged fermentation

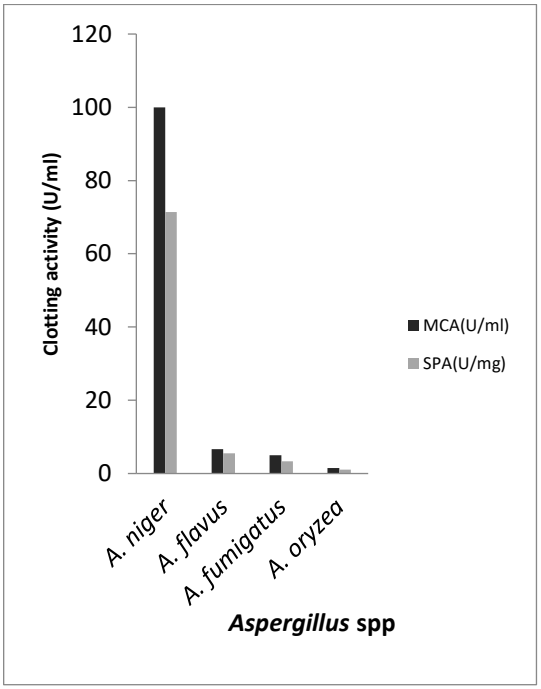


Figure (2): Milk Clotting Enzyme production on different substrate

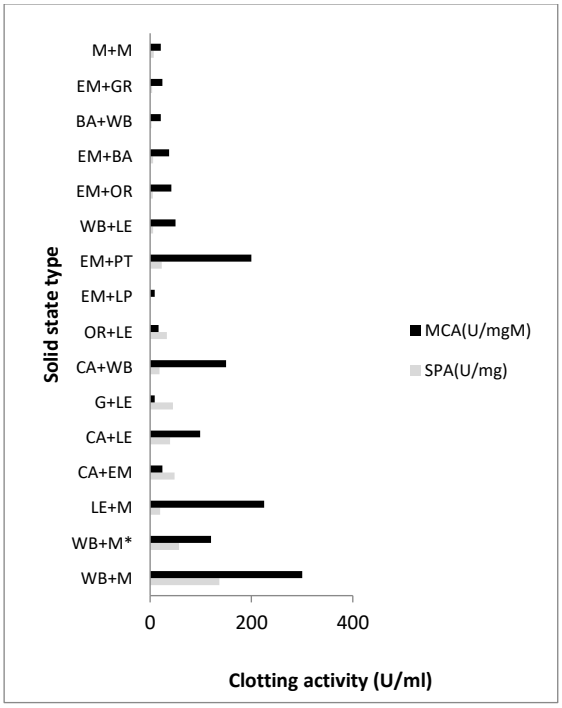


Figure (3): Effect of different pH on Milk Clotting Enzyme production

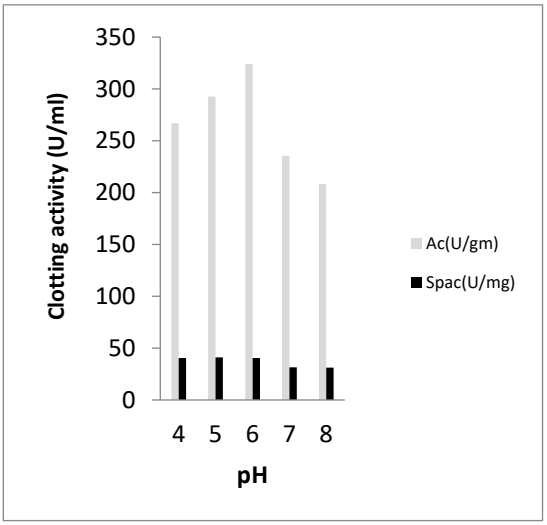
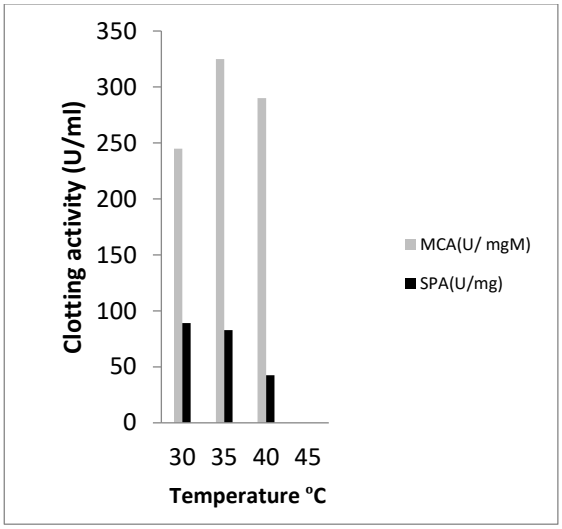


Figure (4): Effect of different incubation temperatures on MCE production



These processes inexorably result in a metabolic change in the hydrogen ion balance, which affects the pH of the culture medium [29]. The enzyme may become denaturated, losing its catalytic activity, as a result of pH changes. The ionic state of the substrate may also change, forming charged particles that may not match the ionic active sites of the enzyme. As a result, no enzyme-substrate combination will develop, and the microbe will no longer have access to the substrate. So, each enzyme has a certain pH range where it functions at its best, and any significant fall or increase in that range causes the enzyme's growth and activity to slow down [30].

Insignificant results were reported by [31, 32] they have studied acid protease production from different A. spp with an optimum pH range of 7.5-4.5 and 7.5 respectively. While the significant result was obtained by [33] who mentioned that the maximum MCE production by Rhizomucor miehei occurs in solid state medium at pH 6.0. However,[34] found that the optimum pH for the production the acidic protease from A. niger was pH 5.0 using SmF.

Incubation Temperature:

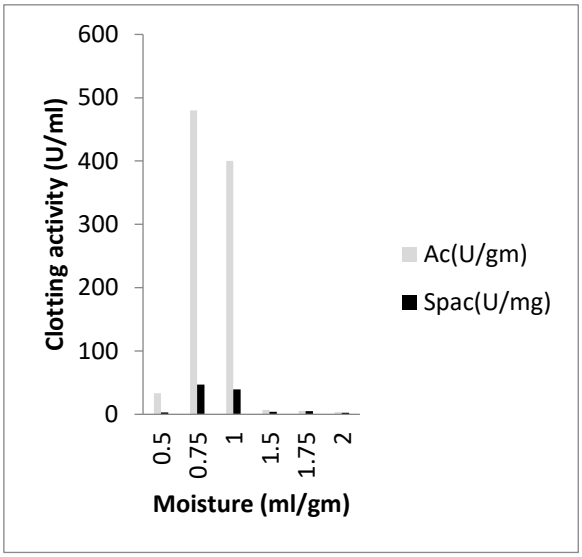
Figure (4) shows maximum enzyme production at 35 °C (325 U/gm) MCA with (82.91 U/mg) SPA. Above 35ºC there was a decrease in the enzyme production in at 40ºC the SPA was (42.95 U/mg).

The advantage of A. niger producing protease at a lower temperature is that low rates of evaporation prevent the fermentation temperature from rising during the incubation period. However, the opposite phenomenon occurs at high temperatures, where the fermentable mass's temperature rises due to a release of metabolic heat causes drying of the culture, a decrease in water activity (aw), and a reduction in the availability of nutrients, which restricts aeration and induces limited growth. It is well recognized that one of the most important factors that must be regulated in a bioprocess is temperature. This finding demonstrates that the strain of A. niger is mesophilic. As the incubation temperature was raised or lowered, the activity dropped until it totally vanished at 45°C. This may be because higher temperatures cause denaturation of proteins, enzyme inhibition, and reduced fungal growth.[35, 36] have noted that the generation of milk-clotting proteases by A. niger during solid-state fermentation occurs best at this temperature (30°C). The appropriate incubation temperature for the protease enzyme from A. spp., according to numerous authors, is between 31 and 35 degrees Celsius [37, 38].

Initial Moisture Content

Figure (5) shows a ratio of 1:0.75 was found to be the best ratio for MCE production from A.niger with MCA (480 U/gm.) and SPA (47.05 U/mg) and good productivity of MCE at 1:1 with MCA (400 U/gm.) and SPA (43.05 U/mg) but lowest MCA (3.5U/gm) at the ratio of 1:2 [39]. Observed that the mold development and the synthesis of enzymes in solid state fermentations are known to be significantly influenced by the substrate's initial moisture level [40] stated that when there is water present in the substrate, mold may more quickly reach the nutrients. Water also changes the physico-chemical characteristics of the substrate, which has an impact on the synthesis of enzymes.

Figure (5): Effect of different moisture on Milk Clotting Enzyme production



[41] reported that the substrate's ability to diffuse oxygen is negatively impacted by too much water [42] claimed that low water content significantly minimizes the substrate's solubility and degree of swelling, which limits the accessibility of the material to fungi while also preventing bacterial contamination, which demands higher moisture yields. A decrease in the substrate's porosity, the loss of particle structure, the development of stiffness, which reduces gas exchange (the transfer of O2 and CO2), and an increase in the formation of aerial mycelium, on the other hand, have all been observed at higher levels. The optimum moisture content for fungal growth and substrate utilization was largely dependent on the organism and substrate used for cultivation. Similar results have been published by other workers [43] and [21] reported that 1:1.6, 1:1 (milliliter distilled water for gram media) and moisture content of

55.0%for maximum MCE production by Rhizomucor miehei and A. oryzae respectively. While [44, 45]

used same genus A.spp for acid protease production but they obtained different optimum moisture content of 60% and 20%.

Effect of Size of Inoculums

The 105(spore/ml) size of inoculum showed highest enzyme production MCA (520 U/gm.) and SPA (53.45 U/mg) and decreased with decrease and increase of inoculum size 103,104,106,107and 108 (spore/ml).

The findings indicated that as inoculum size was increased, milk clotting enzyme production gradually decreased. A highly concentrated inoculum may produce an excessive amount of biomass, which will reduce the amount of nutrients available for the culture's faster and greater biomass growth. While a reduced inoculum density could result in insufficient biomass and low product. A similar observation has been reported in the case of [34] in which 106 (spores/ml) is the optimum inoculum size with using Rhizopus stolonifer for MCE production under SSF.

[23] and [46] reported that an essential biological component that affects fermentation's ability to produce biomass is the size of the inoculum. Most frequently, spore suspensions are used for inoculating solid cultures because they have a number of benefits over vegetative cells, including a longer lifespan than mycelium, reduced sensitivity to environmental factors, and easier storage.

According to the results of [21, 33, 47] an inoculum concentration ranging from 108 CFU/g - 106 (spore/g) is optimal for producing the aspartic proteases of, Rhizomucor miehei NRRL 2034 and Rhizomucor miehei and inoculum of 0.5*106 spores/ml by A. oryzae DRDFS 13 under SSF system respectively. Moreover,[28] and [20] cite data showing that acid protease synthesis by Rhizomucor nainitalensis and Penicillium sp. LCJ228 is maximum when inoculum concentrations of 103 (spore/g)) and 2 g/kg solid substrate were applied respectively.

Incubation Time:

After 72 hours of incubation, enzyme synthesis began and grew throughout time, reaching a peak at 5 days (120 hours) (533.3 U/gm). Following that, the production of the enzyme began to decline, and at 8 days of incubation, the clotting activity had fallen to (333.3 U/gm). obtained the same result with A. oryzae DRDFS 13 under SSF.

After 120 hours of incubation, production may decline as a result of the accumulation of waste products that hinder the development of milk clotting enzymes, the buildup of toxic metabolites produced during fermentation, or the depletion of nutrients accessible to bacteria [6, 15] Similar results were observed by [28, 32, 45] when they produced a high amount of acid protease under solid-state fermentation after 7,5 and 6 day incubation using different fungi (A. niger MTCC 281, A.terreus and Penicillium sp. LCJ228) respectively, but under submerged fermentation, it needs less time as founded by [34] that the maximum milk-clotting enzyme activity was at 3 days of incubation.

Impact of Different Nitrogen Sources

Results in Figure (6) indicated that optimum MCA of 720 (U/g) was achieved by casein also increase in product yield was obtained with the skim milk, peptone but less MCE production was observed when the inorganic source of nitrogen was ammonium sulfate with MCA of 197 (U/g). The yield of the enzyme was higher when organic sources of nitrogen were employed than when an inorganic source of nitrogen was introduced to the system, but it was lower when compared to the control. Because nitrogen is one of the

essential components for all fermentation processes and is necessary for all organisms' genetic makeup, its availability is a vital factor in the growth of microorganisms and the creation of enzymes. Extracellular protease's function is to convert complex proteins found in nutritional media into forms that may be transported and attacked in order to promote growth and vital processes [35].

Figure (6): Impact of different nitrogen sources on Milk Clotting Enzyme production (AS: Ammonium sulfate)

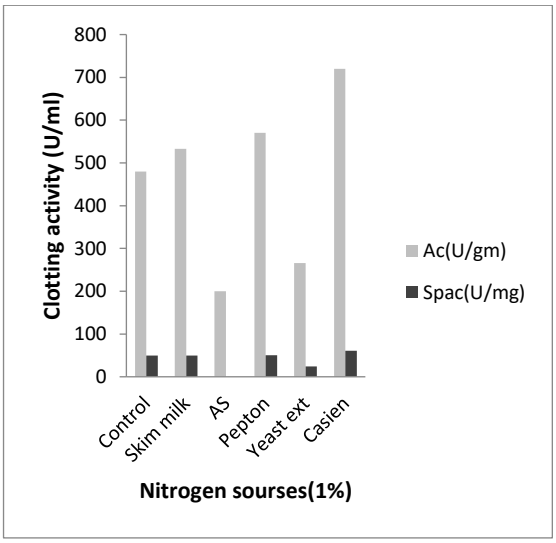
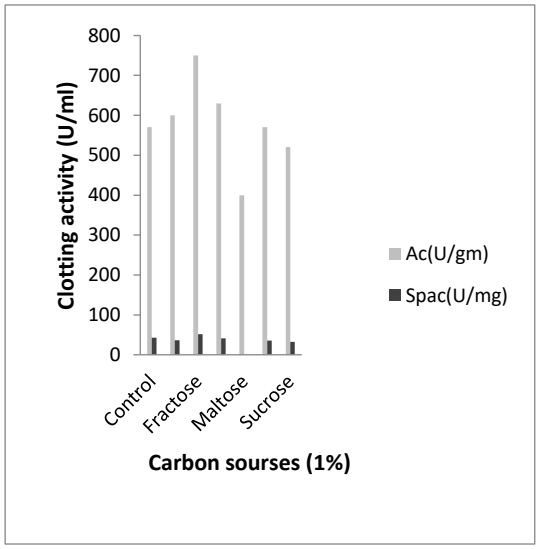


Figure (7): Effect of different carbon sources on Milk Clotting Enzyme production



These results are similar to those reported by [35, 48] they showed that casein is inducer of the protease production of A.spp, Rhizomucor nainitalensis, and A. fumigatus respectively.

Impact of Different Carbon Sources

Results in Figure (7) indicated that maximum MC activity (750U/g) was obtained for monosaccharide, and fructose as shown in Figure (7). Except for maltose, MCE's activity with disaccharides (sucrose and lactose) and polysaccharides (starch) was comparable to that of the control. By adding 1% of certain carbs to the medium, the MC activity can be improved. Compared to fructose, glucose had a less significant effect on MCE. Among monosaccharides, disaccharides and [21] polysaccharides used in the study, the monosaccharide improved the clotting activity. However, it was observed that the medium supplemented with 1% Maltose favored biomass growth rather than acid protease production by the fungal culture.

Many earlier researchers have also discovered that the generation of enzymes is influenced by carbon sources [11] and [21] reported that the optimal carbon source for producing acid protease by A. niger and A. spp. was fructose. Whereas cellulose and starch significantly reduced enzyme activity.

It has frequently been stated that an enzyme cannot be generated in a specified media without a protein supply. But still, it has also been observed that the production of enzymes is dramatically reduced when the right kind of carbohydrate (C-source) is not present in the medium [37], hence, a C-source is always necessary for a fermentation medium. While [15, 38]. In submerged and solid-state cultures of Bacillus subtilis (natto) and Rhizomucor nainitalens, it was found that using glucose as a carbon source resulted in higher MCA than utilizing other sources of carbon.

CONCLUSIONS

The milk clotting activity of Aspergillus niger was higher than that of the other three Aspergillus species. The optimum substrate for Aspergillus niger's ability to produce MCE was a mixture of 50% melon peel, 40% wheat bran, and 10% after it had expired. skim milk powder. Fructose and casein were the ideal sources of carbon and nitrogen for the synthesis of MCE.

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