Original Article

Optimization and characterization of bacterial proteinase enzyme using whey as a fermentation medium

Maged S. Ahmad[1], Baraka A. AbdEl-Salam[2], Manal M. Yaser[1], Safaa S. Taha[2*]

[1]Botany and Microbiology Department, Faculty of Science, Beni-Suef University, [2]Dairy Research Department, Food Technology Research Institute, Agricultural Research Centre, Giza, Egypt.

Correspondence: Safaa S. Taha, Dairy Research Department, Food Technology Research Institute, Agricultural Research Centre, Giza, Egypt. E_mail: safaasalah82@yahoo.com

ABSTRACT

Four lactic acid bacteria (LAB) isolated from sweet were screened for extracellular protease production in submerged fermentation medium using the dairy waste (whey) as a substrate. Different fermentation parameters such as media pH, temperature, carbon source and nitrogen source were studied for maximum production of protease enzyme. Two isolated lactic acid bacteria (LAB) showed maximum proteolytic activity and were identified as *Lactobacillus plantarum* and *Enterococcus faecium* using Biolog system. The highest protease activity of *L. plantarum* was (8.72Unit/ml), the protein content was (4.13mg/ml) and specific activity was (2.11Unit/mg) at optimum pH- 5 and 30°C, after 36 hrs of incubation period, fructose was used as carbon source, and yeast extract was used as nitrogen source, whereas in (10.2Unit/ml), the protein content was (4.8mg/ml) and the specific activity was (2.13Unit/mg) at optimum pH-5.5 and 45°C after 36hrs of incubation period, sucrose was used as a carbon source, and yeast extract was used as a nitrogen source for *Enterococcus faecium*.Two protease enzymes were partially purified using ammonium sulphate precipitation followed by sephadex G-100 chromatography, and then they were characterized.

Keywords: Protease, lactic acid bacteria, submerged fermentation, Whey.

Introduction

Proteases are a group of enzymes that hydrolyze or break down peptide bonds of proteins, and can break specific peptide bonds or link polypeptide chains to amino chain residues [1]. Proteases execute a large variety of functions and represent the most important industrial enzymes of interest accounting for about 60% of the total enzyme market in the world and for approximately 40% of the total worldwide enzyme sale [2, 3]. Proteases constitute a large and complex group of enzymes that play an important nutritional and regulatory role in nature. Proteases are found in a wide diversity of sources such as plants, animals and microorganisms, but they are mainly produced by microorganisms like bacteria and fungi [3].

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Proteases play important roles in physiological processes that is; they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogenicity and hydrolysis of large proteins to smaller molecules for transport and metabolism [4, 5].

Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular proteases have been exploited commercially. The current estimated value of the worldwide sales of industrial enzymes is expected to reach $8.5 billion in 2015 [6].

Biotechnology provided an unlimited and pure source of enzymes as an alternative to the plant and animal proteases to meet the current world demands. Microorganisms represent excellent sources of enzymes [7]. An attractive source of protease enzymes is offered by microorganisms, because by using authorized fermentation techniques, microorganisms can be produced in large amounts in a short period of time, they generate the desired product abundantly and regularly, and the genetic manipulation that is used to generate new enzymes with different characteristics which are demanded for miscellaneous applications, being easier in them than in plants and animals [8-10]. However, among all microbial sources, proteolytic enzymes which include almost all properties for biotechnological

applications, are preferred over the plant and animal- derived enzymes [11].

Microorganisms produce extracellular proteases naturally to break down large polypeptides in the medium into peptides and amino acids before cellular uptake. Such enzymes have been commercially used to degrade protein in miscellaneous industrial processes. Protease enzymes are one of the most important groups of industrial enzymes which are vastly used in the food, pharmaceutical, protein hydrolysis, detergent, cheese-making, brewing, photographic, baking, meat and leather industries and as digestive aids in animal and human food [12, 13].

There are differences in characteristics like substrate specificity, active site, catalytic mechanism, pH and temperature optima in proteases [14]. The nature of the amino acid and other functional groups (aromatic or aliphatic orsulphur-containing) which are close to the bond being hydrolyzed governs the specificity of proteolytic enzymes [15].

Since waste byproducts are generated in the industrial obtainment and manufacturing of food, high levels of pollution are produced. Significant liquid wastes are produced in dairy industries generate remarkable liquid waste, of which, cheese whey is the most abundant. Enormous pollution problems are caused if this industrial effluent is discarded in the water of rivers and lakes, because there are the large amounts of nitrogen, and phosphorus and high BOD (40 – 48,000 mg/L) and COD (89 -95,000 mg/L) contained in this by-product [16].

Thus, new biotechnological alternatives are sought by the scientists all around the world to utilize those by-products to get chemical compounds with potential applications in various industrial areas and decrease the environmental damage. Cheese-whey valorization has been examined in some research studies [17, 18].

Whey, which has been shown to be a potential substrate for protease production because of containing mainly lactose, protein and salts, is a waste byproduct of the dairy industry [19]. The whey consists of protein 0.36-0.46%, fat 0.26% and ash ranges from 0.84 to 0.95% [20]. These results demonstrated that the dairy waste (whey) was rich in nutrients and could be potentially used as a substrate for microbial fermentation.

Therefore, this study was designed to:

(1) Isolate bacterial strains from whey,

(2) Optimize the culture conditions for extracellular enzymes production using whey as a fermentation medium,

(3) Identify and characterize the isolates on the basis of their phenotypic characteristics, and

Purify and characterize the proteases produced from selected isolated lactic acid bacteria (LAB).

Materials and Methods

Microorganism

The bacterial strains were isolated from fresh sweet whey (Faculty of Agriculture, Cairo University, Giza, Egypt), using the pour plate Technique in MRS (De Man –Rogosa- Sharpe) and M17 agar media [21]. The plates were incubated at 37 °C for 48-72 hrs. The purified isolates were maintained in MRS andM17 broth and slants at 4°C.

Screening for protease production

The bacterial isolates were subjected to primary screening for extracellular protease production by plate assay using gelatin agar plate as described by [22].

Identification of proteolytic isolates

The bacterial isolates were examined microscopically and identified using new GEN III Micro Plate™ test panel of the identification in Biolog system [23]. Micro Plates were read in the Micro Station semi-automated reader after 20 hrs, and the results were interpreted by the identification systems software (GEN III database). The identification process was performed according to [24].

Lactic acid bacterial count

Lactic acid bacterial count was determined in MRS and M17 agar media, and the plates were incubated at 37°C for 48hr according to the method described by [25].

Fermentation medium

In the present study, the fresh sweet cheese whey (pH 6.3 and 4.3% lactose) was used as a fermentation medium for protease production. The whey proteins were precipitated by thermo coagulation under acidic conditions. Precipitated proteins were separated by filtration techniques (cheese cloth and Whatmann filter paper No.1). The supernatant was termed as pure whey and used as a fermentation medium after sterilization at 121°C for 20 min. The 250 ml Erlenmeyer flasks containing 100 ml of sterilized whey were inoculated separately with 5% of the bacterial isolates under aseptic conditions and incubated at different studied temperatures, pH conditions, and various incubation periods.

Optimization of culture conditions for protease production

Different process parameters such as pH, temperature, incubation period, and carbon and nitrogen sources were optimized by varying the respective parameters to enhance protease production using whey as a fermentation medium.

The effect of incubation Period

The effect of different incubation periods (12, 24, 36 and 48hrs) on protease production was studied.

The effect of pH on protease production

The effect of different pH values on the production of protease was investigated by adjusting the pH of the fermentation medium at different levels (5.0, 5.5, 6.0, and 6.5using a standard pH meter (Jenway, model 3510, UK).

The effect of temperature

The optimum temperature for protease production was determined by incubating the fermentation medium at different temperatures (i.e. 30, 37, 40, and 45°C) for each organism.
The pH, biomass (the bacterial cell concentration) and enzyme activity were determined after each interval of 12h of incubation at different studied pH and temperature values.

The effect of carbon source

For evaluation of the effect of different carbon sources on enzyme activity in fermentation media, three carbon sources viz. glucose, sucrose and fructose at different concentrations (1, 3 and 5% w/v) were added separately to fermentation medium as carbon sources. The fermentation medium flasks (sterilized whey) were inoculated with 5% (v/v), adjusted at the optimum pH and incubated separately at the optimum temperature and incubation period for each organism. At the end of the incubation periods, the fermentation media were centrifuged at 5.000 rpm for 20 min at 4°C. The supernatants were used for enzyme assay.

The effect of nitrogen source

For evaluation of the effect of different nitrogen sources on enzyme activity in fermentation media, three nitrogen sources viz. yeast extract, glutamic acid and potassium nitrate (0.1, 0.3 and 0.5% w/v) for each source were added to fermentation medium. The fermentation medium flasks (sterilized whey) were inoculated with 5% (v/v) and incubated separately at the best temperature, pH, and incubation period for each organism. At the end of incubation periods, the fermentation media were centrifuged at 5.000 rpm for20 min at 4°C. The supernatants were used for enzyme assay.

Analytical methods

Determination of pH

The pH value of fermentation media was measured using a standard pH meter (Jenway, model 3510, UK).

The determination of Bacterial cell concentration

The bacterial cell concentration (biomass) was determined by measuring the optical density at 600nm using a spectrophotometer (Shimadzu UV-vis spectrophotometer, Model UV-1201, Japan) according to [26].

The determination of protease activity

The protease activity was determined by method of [27] except for enzyme assay incubation time and temperature. One milliliter of culture filtrate was added with 1ml of 1% (w/v) casein solution in 0.05 M phosphate buffer, pH 7.0 and incubated at 37°C for 20min. The reaction was stopped by addition of 4 ml of 5% tri-chloroacetic acid. The reaction mixture was centrifuged at 4000 rpm for 10 min. Five ml of 0.4 M Na_2CO_3 was added to 1 ml of the supernatant followed by 0.5 ml FolinCiocalteu's phenol reagent (diluted with distilled H_2O ratio 1: 4). The reaction mixture was further incubated at 37°C for 20min, the amount of tyrosine released was determined using a spectrophotometer (ShimadzuUV-visspectrophotometer, ModelUV-1201, Japan) at 660 nm against the enzyme blank. One unit of protease activity was defined as the amount of enzyme required to release1µg of tyrosine/ml/min under standard assay conditions.

Protease enzyme production under the optimal conditions and extraction of crude enzyme

Protease production was carried out under submerged fermentation conditions using sweet whey as a fermentation medium. The selected bacterial strains were grown under the obtained optimal conditions. Bacterial cultures were centrifuged by centrifuging (Harrier18/180, Refrigerated, Model MSE, and UK) at 5000 rpm at 4 °C for 15 min to obtain culture supernatant. The culture supernatant is the crude extracellular enzyme extract. The pellet including cell debris was removed. The filtrate was then subjected for the purification.

Purification of protease enzyme

Purification of the proteases was done by standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, and Sephadex G-100 gel filtration chromatography. All purification steps were carried out at 4°C unless otherwise mentioned.

Ammonium sulphate precipitation

Protease precipitation was carried out by ammonium sulfate. The cell free extract (supernatant) was precipitated by adding solid ammonium sulphate at saturation levels (20-60% w/v). The precipitated protein was left overnight for 24hr at4°C, then separated by centrifugation at 5000 rpm for 30 min at 4°c, and dissolved in 40 ml of 0.05M phosphate buffer, pH 7, to get the concentrated enzyme solution [28]. Both enzyme activity and protein content were determined.

Dialysis

The obtained ammonium sulfate precipitate (enzyme suspension) was dialyzed against distilled water using acetylated cellophane tubing prepared from Viking dialysis tube for 24 hr. at 4°Cas described by [29]. Both enzyme activity and protein content were determined.

Gel Filtration Chromatography Technique

The dialyzed concentrated enzyme samples of selected strains were further purified on Sephadex G-100 column (2.5×37 cm) Sigma Aldrich, the USA. The sephadex G-100 column was equilibrated with 0.05 M phosphate buffer of pH 7.0. The dialyzed enzyme samples of selected bacterial strains were loaded onto Sephadex G-100 (Sigma Aldrich, USA) column separately and then eluted with the same buffer. The eluted fractions of 5 ml volume were collected at a flow rate of 1.0 ml/minimum in calibrated 5ml tubes. The absorbance of the

fractions (50fractions) for each isolated strain was measured at 280 nm for protease activities using spectrophotometer (Shimadzu UV-vis spectrophotometer, Model UV-1201, Japan). The purified enzymes were stored at -20°C for further studies. Both enzyme activity and protein content were determined. Preparation of the gel column and the fractionation procedure was performed according to the method described by [30].

Determination of Protein Content

The total protein content of the samples was determined according to the method described by [31], using Bovine Serum Albumin (BSA) as a standard protein. The absorbance was measured with spectrophotometer (Shimadzu UV-vis spectrophotometer, Model UV-1201, Japan) at 660 nm against a blank control.

Characterization of Protease enzyme:

The effect of pH on protease activity

The effect of pH on activities of resulted purified proteases were measured at different pH values (4, 5, 6, 7, 8 and 9) using 1 % (w/v) solution of casein as a substrate dissolved in different buffers (0.05 M). Reaction mixtures were incubated separately at 37°C for 30 min, and then the protease activities and protein contents were measured.

The effect of temperature on Protease activity

The influence of temperature on activities of purified proteases of selected isolates were studied by incubating reaction mixtures separately at different temperatures (25, 30, 35, 37, 40, 45 and 50°C using 1 % casein solution as a substrate dissolved in pH 0.7. The relative activities of the obtained proteases were measured.

The effect of substrate concentration on protease activity

This experiment was carried out to study the effect of different concentrations of casein substrate (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 %) on purified protease activity. The protease activity and protein content were determined.

Statistical analysis

Statistical analysis was performed using the GLM procedure with SAS software [32], analysis was carried out by Duncan multiple ranges test to determine the differences between means of the treatments. A probability to $P < 0.05$ was used to establish the statistical significance.

Results and Discussion

Isolation of Bacterial strains

Four bacterial strains were isolated from the fresh sweet whey. The best growth was shown on the MRS and M17agar media for two strains and other strains, respectively. Four strains of varying morphology were selected and tested for qualitative analysis (zone of hydrolysis) for protease production.

Screening for protease production

The proteolytic activities of bacterial isolates were evaluated using gelatin agar medium. It was expressed as appearance of clear zone (zone of hydrolysis) around bacterial colonies. All bacterial isolates showed clear zones of hydrolysis around disc blank on the nutrient gelatin agar medium after incubation at 37 °C for 2 days, and the diameters of the zones were measured. The screening of 6 bacterial isolates was performed on skim milk agar plate and gelatin agar plate [33].

The identification of proteolytic isolates

The four bacterial isolates were identified by Gram staining and optical microscopy. Gram stain indicated that all bacterial isolates were Gram-positive, but two isolates (strains No.1 &2) were of cocoidal form, while other strains (strains No.3 & 4) were rod shaped, occurring singly or in chains.

Optimization of culture conditions for protease production

Optimization of culture conditions is very important for maximum microbial growth and enzyme production by microorganisms [34]. Culture conditions such as pH, incubation period, temperature and sources of carbon and nitrogen are known to influence the synthesis and secretion of extracellular enzymes by microorganisms [35]. The pH, biomass and protease activity changes were used as indicators of aforementioned parameters effects.

The effect of pH, temperature and Incubation period on pH changes

The influence of pH (5.0, 5.5, 6.0, and 6.5), incubation period (zero, 12, 24, 36 and 48hrs) and temperatures (30, 37, 40 and 45°C) for bacterial isolates on fermentation media and pH changes are shown in (Tables 1:4). Microbial strains depend on the extracellular pH because culture pH strongly influences many enzymatic processes and transports various components across the cell membranes, which in turn support the cell growth and product formation [36]. It could be seen from the results that the pH values of four bacterial isolates gradually decreased at different studied temperatures with increasing incubation periods. The lowest pH values were recorded for strain 4 at all studied temperatures except for 45°C at the end of the incubation periods. The strain 2 showed lower pH changes compared to the other strains at 45°C during the incubation period.

Table 1: The effect of pH and incubation periods on fermentation medium pH changes at 30°C

Strain	pH	Incubation periods (hr.)

		Zero time	12	24	36	48
St.1	5	4.95^{A}	4.88^{B}	4.76^{C}	4.67^{C}	4.65^{C}
	5.5	5.40^{A}	5.16^{B}	4.72^{C}	4.72^{C}	4.63^{D}
	6	5.65^{A}	5.26^{B}	4.84^{C}	4.71^{D}	4.64^{D}
	6.5	6.21^{A}	5.72^{B}	4.95^{C}	4.73^{D}	4.64^{E}
St.2	5	4.95^{A}	4.85^{B}	4.72^{C}	4.60^{D}	4.53^{E}
	5.5	5.37^{A}	5.11^{B}	4.86^{C}	4.74^{D}	4.69^{E}
	6	5.72^{A}	5.33^{B}	5.03^{C}	4.88^{D}	4.75^{E}
	6.5	6.27^{A}	5.76^{B}	5.53^{C}	5.21^{D}	4.80^{E}
St.3	5	4.92^{A}	4.78^{B}	4.64^{C}	4.53^{D}	4.48^{E}
	5.5	5.24^{A}	4.91^{B}	4.77^{C}	4.64^{D}	4.58^{E}
	6	5.65^{A}	5.12^{B}	4.82^{C}	4.69^{D}	4.62^{E}
	6.5	5.97^{A}	5.23^{B}	4.84^{C}	4.72^{D}	4.68^{E}
St.4	5	4.93^{A}	4.72^{B}	4.45^{C}	4.10^{D}	4.00^{E}
	5.5	5.40^{A}	4.70^{B}	4.43^{C}	4.12^{D}	4.00^{E}
	6	5.72^{A}	4.71^{B}	4.40^{C}	4.13^{D}	4.02^{E}
	6.5	5.96^{A}	4.73^{B}	4.44^{C}	4.16^{D}	4.06^{E}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

Table 2: The effect of pH and incubation periods on fermentation medium, pH changes at 37°C

Strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	4.95^{A}	4.87^{B}	4.76^{C}	4.63^{D}	4.62^{D}
	5.5	5.40^{A}	5.00^{B}	4.79^{C}	4.67^{D}	4.62^{D}
	6	5.65^{A}	5.23^{B}	4.81^{C}	4.66^{D}	4.60^{E}
	6.5	6.21^{A}	5.28^{B}	4.92^{C}	4.66^{D}	4.60^{E}
Str.2	5	4.95^{A}	4.86^{B}	4.74^{C}	4.63^{D}	4.61^{D}
	5.5	5.37^{A}	4.92^{B}	4.81^{C}	4.66^{D}	4.61^{E}
	6	5.72^{A}	5.22^{B}	4.87^{C}	4.70^{D}	4.66^{E}
	6.5	6.27^{A}	5.63^{B}	5.23^{C}	4.84^{D}	4.72^{E}
Str.3	5	4.92^{A}	4.76^{B}	4.63^{C}	4.51^{D}	4.50^{D}
	5.5	5.24^{A}	4.88^{B}	4.74^{C}	4.60^{D}	4.57^{D}
	6	5.65^{A}	5.18^{B}	4.85^{C}	4.74^{D}	4.71^{D}
	6.5	5.97^{A}	5.26^{B}	4.92^{C}	4.69^{D}	4.66^{D}
Str.4	5	4.93^{A}	4.55^{B}	4.39^{C}	4.08^{D}	4.05^{E}
	5.5	5.40^{A}	4.58^{B}	4.34^{C}	4.07^{D}	4.02^{E}
	6	5.72^{A}	4.54^{B}	4.34^{C}	4.06^{D}	4.01^{E}
	6.5	5.96^{A}	4.58^{B}	4.31^{C}	4.06^{D}	4.00^{E}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

Table 3: The effect of pH and incubation periods on fermentation medium pH changes at 40°C

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	4.95^{A}	4.82^{B}	4.74^{BC}	4.70^{CD}	4.61^{E}
	5.5	5.40^{A}	4.83^{B}	4.73^{C}	4.61^{D}	4.60^{D}
	6	5.65^{A}	5.14^{B}	4.86^{C}	4.73^{D}	4.70^{D}
	6.5	6.21^{A}	5.04^{B}	4.82^{C}	4.68^{D}	4.66^{D}
Str.2	5	4.95^{A}	4.68^{B}	4.46^{C}	4.40^{C}	4.34^{CD}
	5.5	5.37^{A}	4.75^{B}	4.55^{C}	4.39^{D}	4.32^{D}
	6	5.72^{A}	4.68^{B}	4.54^{BC}	4.40^{CD}	4.36^{D}
	6.5	6.27^{A}	5.72^{B}	5.38^{C}	4.82^{D}	4.48^{E}
Str.3	5	4.92^{A}	4.80^{B}	4.72^{C}	4.65^{D}	4.64^{D}
	5.5	5.24^{A}	4.89^{B}	4.78^{C}	4.67^{D}	4.65^{D}
	6	5.65^{A}	5.21^{B}	5.09^{C}	4.82^{D}	4.80^{D}
	6.5	5.97^{A}	5.26^{B}	5.03^{C}	4.75^{D}	4.72^{D}
Str.4	5	4.93^{A}	4.81^{B}	4.71^{C}	4.30^{D}	4.21^{E}
	5.5	5.40^{A}	5.15^{B}	4.63^{C}	4.26^{D}	4.25^{D}
	6	5.72^{A}	5.29^{B}	4.64^{C}	4.34^{D}	4.33^{D}
	6.5	5.96^{A}	5.26^{B}	4.43^{C}	4.03^{D}	4.02^{D}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

Table 4: The effect of pH and incubation periods on fermentation medium, pH changes at 45°C

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	4.95^{A}	4.82^{B}	4.71^{C}	4.67^{D}	4.67^{D}
	5.5	5.40^{A}	4.93^{B}	4.84^{C}	4.75^{D}	4.74^{D}
	6	5.65^{A}	4.94^{B}	4.86^{C}	4.75^{D}	4.73^{D}
	6.5	6.21^{A}	5.14^{B}	4.92^{C}	4.82^{D}	4.77^{E}
Str.2	5	4.95^{A}	4.64^{B}	4.49^{C}	4.43^{D}	4.38^{E}
	5.5	5.37^{A}	4.65^{B}	4.48^{C}	4.40^{D}	4.35^{E}
	6	5.72^{A}	4.63^{B}	4.47^{C}	4.40^{D}	4.33^{E}
	6.5	6.27^{A}	4.68^{B}	4.49^{C}	4.43^{D}	4.39^{E}
Str.3	5	4.92^{A}	4.79^{B}	4.74^{C}	4.70^{D}	4.68^{D}
	5.5	5.24^{A}	4.92^{B}	4.80^{C}	4.71^{D}	4.70^{D}
	6	5.65^{A}	5.22^{B}	5.15^{C}	4.85^{D}	4.84^{D}
	6.5	5.97^{A}	5.39^{B}	5.27^{C}	5.17^{D}	5.17^{D}
Str.4	5	4.93^{A}	4.82^{B}	4.78^{BC}	4.73^{D}	4.71^{D}
	5.5	5.40^{A}	5.28^{B}	5.14^{C}	5.02^{D}	5.01^{D}
	6	5.72^{A}	5.35^{B}	5.14^{C}	5.03^{D}	5.02^{D}
	6.5	5.96^{A}	5.32^{B}	5.12^{C}	5.00^{D}	4.88^{E}

Different letters on the same row differ significantly at $p<0.05$, during incubation period

The effect of pH, temperature and Incubation Period on bacterial biomass changes:

The bacterial biomass changes after incubation of bacterial isolates at different temperatures, incubation periods, pH conditions and inoculum volume (5%) which are presented in Tables (5:8). It could be observed that the bacterial biomass of all the bacterial isolates increased at all studied temperatures with progressing the incubation periods until 36hr.The bacterial biomass of strains1, 2 & 4, 1& 3 and finally 1& 2 decreased at 30, 37, 40 and 45°C, respectively, at the end of the incubation period (48hr.). Also, it could be noticed that the highest value of bacterial biomass (2.358) was recorded for Strain 4 at 37°C and pH 6.5 after 36hr. of incubation periods.

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Table 5: The effect of pH and incubation periods on the bacterial biomass at 30°C

strain	pH	Incubation periods (hr.)				
		zero time	12	24	36	48
Str.1	5	0.425^{E}	0.812^{D}	0.913^{B}	1.023^{A}	0.816^{C}
	5.5	0.728^{E}	0.868^{D}	1.005^{B}	1.247^{A}	0.914^{C}
	6	0.713^{E}	0.953^{C}	1.083^{B}	1.214^{A}	0.895^{D}
	6.5	0.803^{E}	1.090^{C}	1.151^{B}	1.454^{A}	1.016^{D}
Str.2	5	0.211^{D}	0.337^{C}	0.458^{B}	0.539^{A}	0.549^{A}
	5.5	0.217^{E}	0.538^{D}	0.600^{C}	0.777^{B}	0.797^{A}
	6	0.225^{E}	0.624^{D}	0.772^{C}	0.851^{B}	0.864^{A}
	6.5	0.215^{E}	0.655^{D}	0.833^{C}	0.934^{B}	0.964^{A}
Str.3	5	0.279^{E}	0.566^{D}	0.716^{C}	0.816^{B}	0.881^{A}
	5.5	0.285^{E}	0.661^{D}	0.808^{C}	0.902^{B}	0.986^{A}
	6	0.292^{E}	0.709^{D}	0.996^{C}	1.092^{B}	1.196^{A}
	6.5	0.309^{E}	1.281^{D}	1.341^{C}	1.434^{B}	1.707^{A}
Str.4	5	0.605^{E}	1.328^{D}	1.747^{C}	2.069^{B}	2.121^{A}
	5.5	0.612^{E}	1.587^{D}	1.896^{C}	2.142^{B}	2.189^{A}
	6	0.615^{E}	1.813^{D}	2.016^{C}	2.189^{B}	2.241^{A}
	6.5	0.607^{E}	1.845^{D}	2.045^{C}	2.197^{B}	2.251^{A}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

Table 6:The effect of pH and incubation periods on the bacterial biomass at 37°C

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	0.425^{E}	0.705^{D}	0.821^{C}	0.926^{B}	0.998^{A}
	5.5	0.728^{E}	0.853^{D}	0.916^{C}	0.965^{B}	1.083^{A}
	6	0.713^{E}	1.025^{D}	1.217^{C}	1.280^{B}	1.287^{A}
	6.5	0.803^{E}	0.954^{D}	1.251^{C}	1.377^{B}	1.386^{A}
Str.2	5	0.211^{E}	0.641^{D}	0.792^{C}	0.867^{A}	0.845^{B}
	5.5	0.217^{E}	0.854^{D}	0.930^{C}	0.988^{A}	0.977^{B}
	6	0.225^{D}	1.031^{C}	1.103^{B}	1.251^{A}	1.237^{A}
	6.5	0.215^{E}	1.012^{D}	1.147^{C}	1.204^{A}	1.177^{B}
Str.3	5	0.279^{E}	0.542^{D}	0.568^{C}	0.602^{B}	0.661^{A}
	5.5	0.285^{E}	0.592^{D}	0.630^{C}	0.712^{B}	0.772^{A}
	6	0.292^{E}	0.766^{D}	0.840^{C}	0.913^{B}	1.026^{A}
	6.5	0.308^{E}	0.823^{D}	0.909^{C}	0.935^{B}	1.139^{A}
Str.4	5	0.605^{E}	1.221^{D}	1.503^{B}	1.724^{A}	1.345^{C}
	5.5	0.612^{E}	1.603^{D}	2.016^{B}	2.181^{A}	1.984^{C}
	6	0.615^{E}	1.945^{D}	2.135^{C}	2.312^{A}	2.189^{B}
	6.5	0.608^{E}	2.045^{D}	2.214^{C}	2.358^{A}	2.260^{B}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

Table 7. The effect of pH and incubation periods on the bacterial biomass at 40°C

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	0.425^{E}	0.645^{D}	0.872^{B}	0.983^{A}	0.963^{C}
	5.5	0.728^{E}	0.853^{D}	0.962^{B}	0.994^{A}	0.944^{C}
	6	0.713^{D}	1.310^{C}	1.437^{B}	1.490^{A}	1.485^{A}
	6.5	0.803^{D}	0.852^{C}	0.952^{B}	0.986^{A}	0.964^{C}
Str.2	5	0.211^{D}	0.690^{C}	0.714^{B}	0.835^{A}	0.842^{A}
	5.5	0.217^{D}	0.719^{C}	0.853^{B}	0.992^{A}	0.993^{A}
	6	0.225^{D}	0.803^{C}	0.879^{B}	0.975^{A}	0.983^{A}
	6.5	0.215^{E}	0.897^{D}	1.003^{C}	1.021^{B}	1.043^{A}
Str.3	5	0.279^{E}	0.321^{D}	0.465^{B}	0.545^{A}	0.433^{C}
	5.5	0.285^{E}	0.343^{D}	0.465^{C}	0.638^{A}	0.536^{B}
	6	0.292^{E}	0.475^{D}	0.672^{B}	0.766^{A}	0.611^{C}
	6.5	0.308^{E}	0.552^{D}	0.615^{C}	0.896^{A}	0.802^{B}
Str.4	5	0.605^{E}	0.646^{D}	0.750^{C}	0.875^{B}	0.948^{A}
	5.5	0.612^{E}	0.725^{D}	1.163^{C}	1.258^{B}	1.332^{A}
	6	0.615^{E}	1.022^{D}	1.514^{C}	1.628^{B}	1.670^{A}
	6.5	0.608^{E}	1.431^{D}	2.027^{C}	2.181^{B}	2.251^{A}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

Table 8:The effect of pH and incubation periods on the bacterial biomass at 45°C

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	0.425^{D}	0.467^{C}	0.547^{B}	0.593^{A}	0.463^{E}
	5.5	0.728^{E}	0.854^{D}	1.080^{B}	1.109^{A}	1.061^{C}
	6	0.713^{E}	0.999^{C}	1.131^{B}	1.181^{A}	1.144^{B}
	6.5	0.803^{E}	0.945^{C}	0.964^{B}	0.994^{A}	0.866^{D}
Str.2	5	0.211^{E}	0.531^{D}	0.632^{C}	0.735^{A}	0.687^{B}
	5.5	0.218^{E}	0.658^{D}	0.771^{C}	0.881^{A}	0.861^{B}
	6	0.225^{D}	0.752^{C}	0.870^{B}	0.948^{A}	0.883^{B}
	6.5	0.215^{D}	0.831^{C}	0.958^{B}	1.027^{A}	0.981^{B}
Str.3	5	0.279^{E}	0.1000^{D}	1.058^{C}	1.133^{B}	1.155^{A}
	5.5	0.285^{E}	1.072^{D}	1.167^{C}	1.192^{B}	1.297^{A}
	6	0.292^{E}	1.170^{D}	1.260^{C}	1.263^{B}	1.337^{A}
	6.5	0.308^{E}	1.227^{D}	1.294^{C}	1.497^{B}	1.653^{A}
Str.4	5	0.605^{E}	0.614^{D}	0.627^{C}	0.647^{B}	0.677^{A}
	5.5	0.612^{E}	0.623^{D}	0.639^{C}	0.670^{B}	0.698^{A}
	6	0.615^{D}	0.625^{C}	0.640^{B}	0.654^{A}	0.655^{A}
	6.5	0.608^{E}	0.636^{D}	0.707^{C}	1.207^{B}	1.332^{A}

Different letters on the same row differ significantly at $p<0.05$, during incubation period

The effect of pH, temperature and incubation Period on enzyme activity

The protease activity of bacterial isolates at different temperatures, incubation periods and various pH conditions with inoculum volume (5%) is indicated in Tables (9:12). It could be seen that the enzyme activity increased with increasing of the incubation periods until 36 hr. for all the bacterial isolates at different studied pHs and temperatures,

clearly suggesting that the enzyme production is growth associated in nature [37, 38]. Where maximum enzyme production was observed at the late exponential phase and early stationary phase of the growth. These results are in accordance with observations made by [39]. The strain No.1 showed high enzyme activity at 40°C, pH 5 and after 36hr.of incubation period (Table 11). Similar observation was reported by [40] who reported that the 40°C supported maximum protease production from B. subtilis isolated from sea water. While, both strains (No.2 and 3) at pH5 and 6, respectively, recorded higher enzyme activity at 45°C after 36hr.of incubation period, as shown in (Table 12). On his study, [41] found 45°C was optimum for a Gram+ve strain producing protease. On the other hand, [42] reported that the optimum pH and temperature for L. delbrueckii was 6.5 at 45°C. The maximum protease enzyme was achieved at 30°C and pH 5 for strain No.4 after 36hr.of incubation period (Table 9). The optimum temperature for action of protease isolated from a LAB, Enterococcus faecalisTN-9 was 30 °C [43]. Based on the previous results, the statistical analysis showed that, the protease production was highly and significantly affected ($p<0.05$) by the medium temperature, pH and incubation period.

Table 9: The effect of incubation of isolated strains at 30°C on the enzyme activityμ/ml at different pH conditions

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	0.734^{D}	0.852^{C}	0.906^{BC}	0.978^{B}	0.960^{A}
	5.5	0.734^{E}	0.833^{D}	0.915^{C}	0.978^{B}	0.960^{A}
	6	0.725^{E}	0.843^{D}	0.897^{C}	0.960^{A}	0.942^{B}
	6.5	0.752^{D}	0.843^{C}	0.924^{AB}	0.969^{A}	0.969^{A}
Str.2	5	0.761^{E}	0.843^{D}	0.888^{C}	0.933^{A}	0.897^{B}
	5.5	0.770^{E}	0.852^{D}	0.888^{C}	0.924^{A}	0.906^{B}
	6	0.761^{D}	0.833^{C}	0.870^{B}	0.906^{A}	0.906^{B}
	6.5	0.752^{D}	0.861^{C}	0.924^{A}	0.924^{A}	0.897^{B}
Str.3	5	0.707^{D}	0.797^{C}	0.879^{B}	0.978^{A}	0.978^{A}
	5.5	0.725^{D}	0.806^{C}	0.870^{B}	0.969^{A}	0.969^{A}
	6	0.734^{E}	0.815^{D}	0.879^{C}	0.951^{B}	0.960^{A}
	6.5	0.743^{E}	0.824^{D}	0.897^{C}	0.933^{B}	0.942^{A}
Str.4	5	0.806^{D}	1.04^{C}	1.12^{B}	1.21^{A}	1.22^{A}
	5.5	0.815^{D}	0.978^{C}	1.02^{B}	1.05^{A}	1.07^{A}
	6	0.824^{D}	1.01^{C}	1.04^{B}	1.08^{A}	1.1^{A}
	6.5	0.797^{D}	0.960^{C}	1.01^{B}	1.06^{A}	1.08^{A}

Different letters on the same row differ significantly at $p<0.05$, during incubation period

Table 10: The effect of incubation of isolated strains at 37°C on enzyme activityμ/ml at different pH conditions

strain	pH	Incubation periods (hr.)				
		Zero time	12	24	36	48
Str.1	5	0.734^{E}	0.870^{D}	0.924^{C}	0.978^{A}	0.960^{B}
	5.5	0.734^{E}	0.879^{D}	0.915^{C}	0.969^{A}	0.933^{B}
	6	0.725^{E}	0.861^{D}	0.915^{C}	0.978^{A}	0.960^{B}
	6.5	0.752^{E}	0.879^{D}	0.924^{C}	0.987^{A}	0.960^{B}
Str.2	5	0.761^{E}	0.861^{D}	0.897^{C}	0.942^{A}	0.933^{B}
	5.5	0.770^{E}	0.870^{D}	0.906^{C}	0.960^{A}	0.942^{B}
	6	0.761^{E}	0.852^{D}	0.888^{C}	0.933^{A}	0.897^{B}
	6.5	0.752^{E}	0.861^{D}	0.933^{C}	0.960^{A}	0.942^{B}
Str.3	5	0.707^{E}	0.824^{D}	0.879^{C}	0.924^{B}	0.933^{A}
	5.5	0.725^{D}	0.815^{C}	0.861^{B}	0.906^{B}	0.924^{A}
	6	0.734^{E}	0.824^{D}	0.870^{C}	0.942^{B}	0.951^{A}
	6.5	0.743^{E}	0.833^{D}	0.879^{C}	0.933^{B}	0.951^{A}
Str.4	5	0.806^{D}	0.978^{C}	1.02^{B}	1.06^{A}	1.07^{A}
	5.5	0.815^{D}	0.987^{C}	1.07^{B}	1.08^{A}	1.09^{A}
	6	0.824^{C}	0.978^{B}	1.03^{A}	1.03^{A}	1.04^{A}
	6.5	0.797^{D}	0.987^{C}	1.01^{B}	1.08^{A}	1.08^{A}

Different letters on the same row differ significantly at $p<0.05$, during incubation period

Table11: The effect of incubation of isolated strains at 40°C on enzyme activityμ/ml at different pH conditions

strain	pH	Incubation periods(hr.)				
		zero time	12	24	36	48
Str.1	5	0.734^{D}	0.861^{C}	0.942^{B}	1.02^{A}	1.01^{A}
	5.5	0.734^{E}	0.870^{D}	0.924^{C}	1.01^{A}	0.987^{B}
	6	0.725^{D}	0.870^{C}	0.933^{B}	1.01^{A}	1.01^{A}
	6.5	0.752^{E}	0.879^{D}	0.942^{C}	1.01^{A}	0.997^{B}
Str.2	5	0.761^{E}	0.888^{D}	0.933^{C}	0.987^{A}	0.978^{B}
	5.5	0.770^{E}	0.906^{D}	0.951^{C}	0.987^{A}	0.978^{B}
	6	0.761^{D}	0.888^{C}	0.933^{B}	1.01^{A}	1.01^{A}
	6.5	0.752^{D}	0.897^{C}	0.942^{B}	0.987^{A}	0.987^{A}
Str.3	5	0.707^{D}	0.888^{C}	0.933^{B}	0.978^{A}	0.987^{A}
	5.5	0.725^{D}	0.879^{C}	0.924^{B}	0.969^{A}	0.969^{A}
	6	0.734^{D}	0.897^{C}	0.942^{B}	0.978^{A}	0.987^{A}
	6.5	0.743^{E}	0.870^{D}	0.915^{C}	0.951^{B}	0.960^{A}
Str.4	5	0.806^{D}	0.924^{C}	0.960^{B}	0.987^{A}	0.997^{A}
	5.5	0.815^{D}	0.933^{C}	0.978^{B}	1.02^{A}	1.03^{A}
	6	0.824^{D}	0.942^{C}	0.987^{B}	1.03^{A}	1.04^{A}
	6.5	0.797^{D}	0.924^{C}	0.960^{B}	1.01^{A}	1.02^{A}

Different letters on the same row differ significantly at $p<0.05$, during incubation period

Table 12:The effect of incubation of isolated strains at 45°C on the enzyme activityμ/ml at different pH conditions

Strain	pH	Incubation periods(hr.)				
		zero time	12	24	36	48
Str.1	5	0.734^{E}	0.861^{D}	0.906^{C}	0.960^{A}	0.924^{B}
	5.5	0.734^{E}	0.852^{D}	0.897^{C}	0.942^{A}	0.933^{B}
	6	0.725^{E}	0.870^{D}	0.906^{C}	0.951^{A}	0.942^{B}
	6.5	0.752^{E}	0.870^{D}	0.915^{B}	0.960^{A}	0.960^{C}
Str.2	5	0.761^{D}	0.906^{C}	0.978^{B}	1.06^{A}	1.04^{A}
	5.5	0.770^{D}	0.906^{C}	0.960^{B}	1.05^{A}	1.05^{A}
	6	0.761^{D}	0.897^{C}	0.969^{B}	1.05^{A}	1.04^{A}
	6.5	0.752^{D}	0.906^{C}	0.951^{B}	1.04^{A}	1.04^{A}
Str.3	5	0.707^{D}	0.897^{C}	0.951^{B}	0.987^{A}	0.997^{A}
	5.5	0.725^{D}	0.897^{C}	0.942^{B}	0.987^{A}	0.987^{A}

	6	0.734^{D}	0.906^{C}	0.960^{B}	1.01^{A}	1.01^{A}
	6.5	0.743^{D}	0.915^{C}	0.951^{B}	0.987^{A}	0.997^{A}
Str.4	5	0.806^{D}	0.879^{C}	0.924^{B}	0.960^{B}	0.969^{A}
	5.5	0.815^{D}	0.888^{C}	0.933^{B}	0.987^{A}	0.997^{A}
	6	0.824^{D}	0.897^{C}	0.942^{B}	0.978^{A}	0.978^{A}
	6.5	0.797^{D}	0.870^{C}	0.906^{B}	0.933^{A}	0.942^{A}

Different letters on the same row differ significantly at $p<0.05$, during the incubation period

The effect of carbon sources on protease production

Carbon sources greatly influence the enzyme production, and a wide range of them serve as energy sources. The effect of various carbon sources on enzyme production was measured using the optimum temp, pH and incubation period for each isolate from aforementioned results, and presented in Figs. (1:4). From the obtained results, sucrose showed higher enzyme activity than glucose and fructose for three isolates (No.1, 2 and3), whereas fructose showed maximum enzyme activity for strain No.4 (Fig.4) at level of 5%. These findings indicated that sucrose is the best carbon source for protease production for three isolates, and the fructose is the best carbon source for the fourth isolate. This observation was in disagreement with previous studies which suggested that higher amount of protease was synthesized when carbon sources were poorly utilized for growth purposes [44]. However, [45] recoded that the sucrose is a good substrate for production of extracellular proteases. Increased yields of alkaline proteases were also reported by several workers in the presence of different sugars such as lactose [46], sucrose [47] and fructose [48].

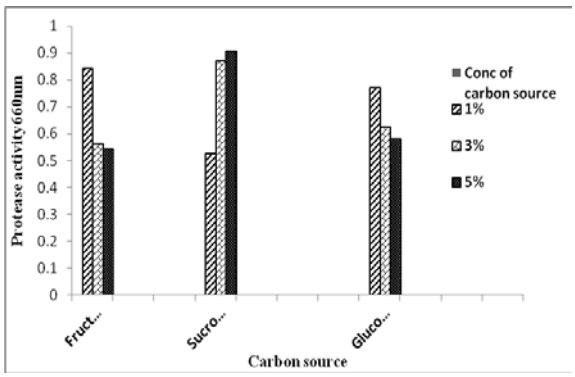

Figure 1. The effect of carbon source on Protease activity by bacterial isolate.1

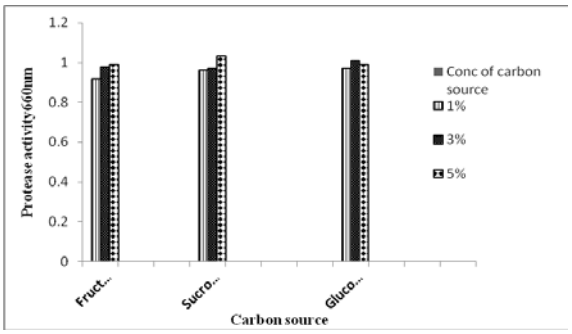

Figure 2. The effect of carbon source on Protease activity by bacterial isolate.2

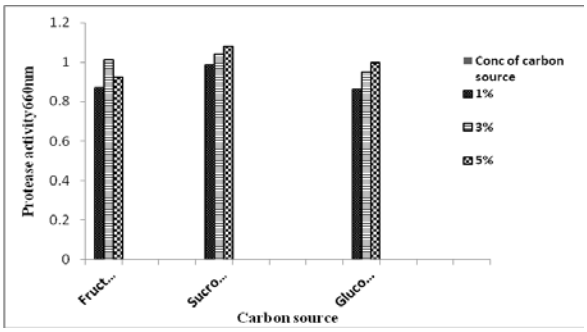

Figure 3. The effect of carbon source on Protease activity by the bacterial isolate.3

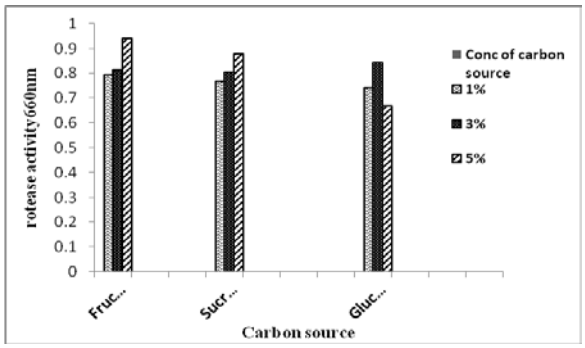

Figure 4. The effect of carbon source on Protease activity by the bacterial isolate.4

The effect of Nitrogen Sources on Protease production

Most of the microorganism can utilize nitrogen sources either inorganic or organic forms or sometimes both, which are required to produce amino acids, nucleic acids, proteins and other cell wall components. The medium supplemented with organic nitrogen sources supported higher protease production when compared to inorganic nitrogen sources [49]. The alkaline protease production was better with organic nitrogen sources than with the inorganic nitrogen sources [1]. The effect of various nitrogen sources (yeast extract, potassium nitrate and glutamic acid) at different concentrations (0.1,0.3 and 0.5%) on enzyme production was studied. From the obtained results, it was observed that the growth medium containing yeast extract at 0.5%yielded higher protease activity in all selected isolates. This was followed by potassium nitrate and glutamic acid. These results indicated that yeast extract is the best

organic nitrogen source for protease production by four selected isolates. This trend is in a good agreement with the previous reports [50, 51].

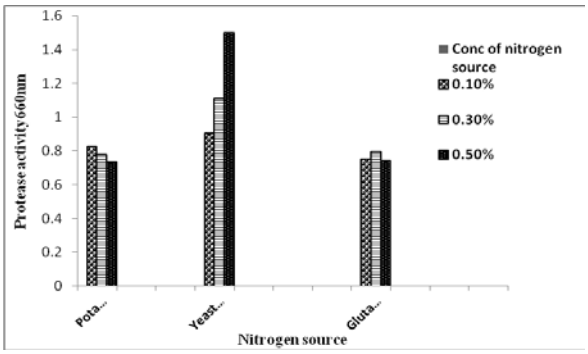

Figure 5. The effect of nitrogen source on Protease activity in bacterial isolate.1

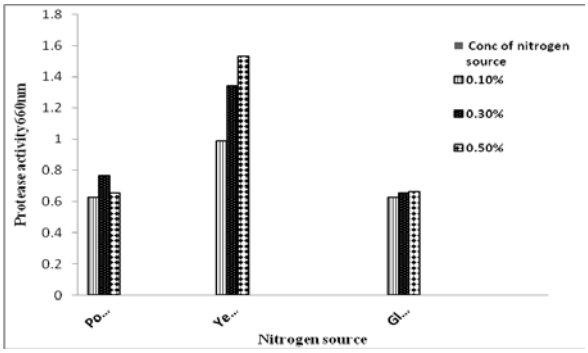

Figure 6. The effect of nitrogen source on Protease activity in bacterial isolate.2

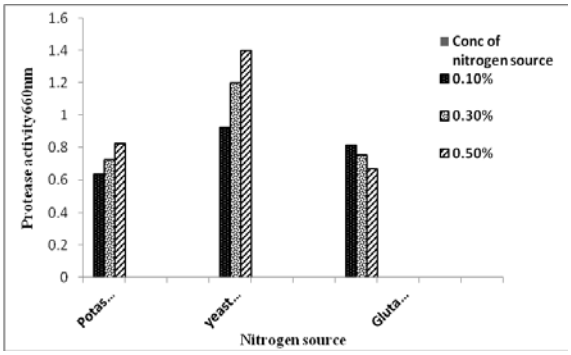

Figure 7. The effect of nitrogen source on Protease activity in bacterial isolate.3

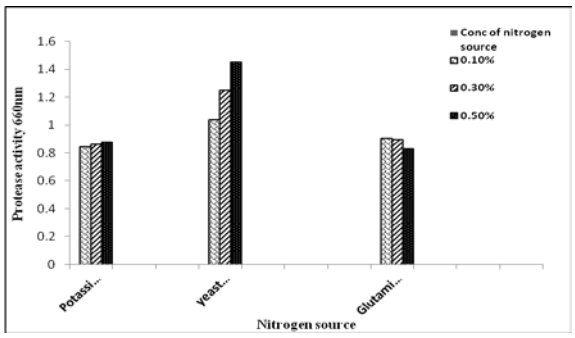

Figure 8. The effect of nitrogen source on Protease activity in bacterial isolate.4

Protease enzyme production under the optimal conditions and extraction of crude enzyme

The previous results showed that isolated strains No.2 and No.4 had higher protease activity, so that they were chosen for protease production under the obtained optimal conditions. The two selected isolates were cultured separately in submerged fermentation medium containing the sweet whey under the optimum conditions. After complete fermentation, the fermented broth for each isolate was centrifuged at 5000 rpm for 15minutes at 4°C to obtain supernatant extract containing crude enzyme. The total protease activity and protein content were measured in the culture filtrate (crude enzyme). The maximum protease activity (8.72U/mL) and protein content (4.13 mg/ml) with specific activity(2.11U/mg) were obtained for strain No.4 at 30 °C after 48 hr. of incubation with inoculum volume 5%,fructose (5%) and yeast extract (0.5%) at pH (5.0) and the maximum protease activity (10.2U/mL) and protein content (4.8mg/ml) with specific activity(2.13U/mg) were obtained for strain No.2 at 45 °C after 36 hr. of incubation with inoculum volume 5% ,sucrose (5%) and yeast extract (0.5%) at pH(5.5). According to [52], the highest protease activity (124.2 U/mL) was observed in *Bacillus* sp. SP-5 after 24 h of incubation at 25 °C using yeast extract as a nitrogen source and sucrose as a carbon source. Fructose was found to be the best carbon source [53].

3.8. The identification by Biolog system

The two bacterial isolates (strains No.2 and No.4) with the highest enzyme activity were further identified by Biolog system (Table 13) The results showed that the two bacterial isolates (2&4) were *Enterococcus faecium*and *Lactobacillus plantarum*, respectively using Biolog system. The new GEN III micro plates were applicable to an unprecedented range of both Gram-negative and Gram-positive bacteria. The GEN III micro plates can be used to evaluate the ability of the cell to metabolize all major classes of biochemical. In addition to determining other important physiological properties such as pH, salt, and lactic acid tolerance; reducing power and chemical sensitivity were examined. Biolog's technology gives a possibility to identify the environmental and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" from discrete test reactions, performed within a 96 wells microplate. More than 2650 identification profiles of the environmental and fastidious organisms of interest in diverse fields of microbiology can be determined by the system. The end result of Biology GEN III is a pattern of colored wells on the micro plate that is a characteristic of that bacterial species.

Table 13: Layout of assays in the Micro Plate

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 Sodium Bromate	A12 pH 5
B1 D-Raffinose	B2 D-Lactose	B3 D-Melibiose	B4 Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-D-Mannosamine	B8 N-Acetyl-DGalactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galacto	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocyclin e
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid F10	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 Amino-Butryric Acid	H3 Hydroxy-Butyric Acid	H4 β-Hydroxy-D, LButyric Acid	H5 Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

The purification of protease enzyme

The purification of the proteases was done by standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, and Sephadex G-100 gel filtration chromatography.

The results of partial purification of both proteases are summarized in Table (14&15). Approximately 1.4-fold purification was achieved during ammonium sulphate precipitation (20%-60%) with a yield of14.46% enzyme and specific activity (2.91 U/mg) of the final partially purified enzyme from L. plantarum strain and fold purification (1.06) with yield of 11.96% enzyme, and specific activity of (2.26) U/mg for Enterococcus faecium. According to [54], partially purified was thermos table alkaline protease from Bacillus pumilus D-6 by ammonium sulphate precipitation at 40-60% level of saturation.

The obtained ammonium sulfate precipitate (enzyme suspension) was dialyzed against distilled water with three to four changes of water using acetylated cellophane tubing to remove ammonium sulfate ions from protein molecules at 4°C.The dialyzed enzyme solutions of both L. plantrum and Enterococcus faecium strains were loaded separately on sephadex G –100 gel filtration column. By gel filtration, the

protease enzyme was purified to (1.98) fold with a yield of (2.11%) and the specific activity of (4.18) U/mg for strain L. plantarum (Table 14). Similarly, the purification fold (2.84) with a yield of (1.74%) and specific activity of (6.05) U/mg were measured for strain Enterococcus faecium (Table 14). On their studies [55, 56], have reported the purification of protease enzymes by gel filtration chromatography with Sephadex G-200 and Sephadex G-100 columns, respectively. Purified fractions achieved having specific activity of 905.7 using sephadex G-100 column chromatography [57]. These variations in results might be due to the nature of protease, column used and microbial sources. The gel filtration fractions with protease activities were pooled and collected separately, and stored at -20°C separately for further characterization of the proteases.

Table 14: The purification results of extracellular protease enzyme from L. plantarum

Fraction	Volume (ml)	activity (unit/ml)	Total activity (unit/m)	protein (mg /ml)	Total protein (mg/ml)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	200	8.72	1748	4.13	826	2.11	100	1
Ammonium sulphate precipitation (20-60%)	40	6.32	252.8	2.17	86.8	2.91	14.46	1.4
Sephadex G-100	100	0.368	36.8	0.088	8.8	4.18	2.11	1.98

Table 15: Purification results of extracellular protease enzyme from Enterococcus faecium

Fraction	Volume (ml)	activity (unit/ml)	Total activity (unit/ml)	protein (mg/ml)	Total protein (mg/ml)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	200	10.2	2040	4.80	960	2.13	100	1

Ammonium sulphate precipitation (20-60%)	40	6.10	244	2.70	108	2.26	11.96	1.06
Sephadex G-100	75	0.472	35.4	0.078	5.85	6.05	1.74	2.84

The characterization of protease enzyme

The effect of pH on protease activity

The effect of pH on activities of resulted purified proteases were studied by incubating the purified protease extracts separately at different pH values ranging from (4-9), and a temperature, 37°C for 30 min using1 % casein as a substrate to find the most suitable pH. The results in (Fig.9) showed that the enzyme activity for both strains decreased with increasing of pH. The highest protease activity for both enzymes was at pH 4.0. These results were in accordance with [58] who reported that protease from Pedicoccusacidilactici had the maximum activity at pH4.0.

These results suggest that the protease enzymes of this study belong to the acidic protease group. Acidic proteases have extensive applications in the dairy industry for the coagulation of milk protein in cheese production [15] and generally in the improvement of food flavors [59].

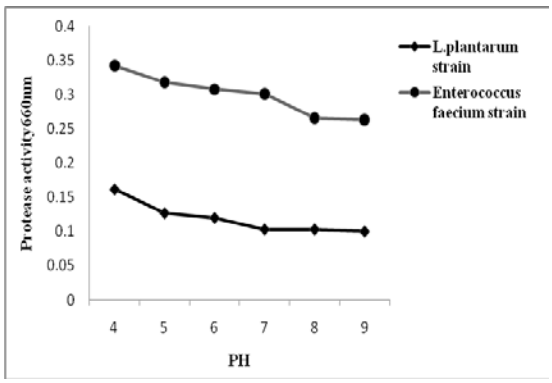

Figure 9: The effect of pH on protease activity of L. plantarum and E. faecium

The effect of temperature on protease activity

The influence of temperature (25: 50°C) on activities of purified proteases of selected isolates using 1% casein as a substrate and at pH 4 for 30 min. are presented in Fig. (10). The highest protease activity was recorded at 35°C for L. plantarum strain, similarly. Protease enzyme produced by B. anthracis, S-44 exhibited an optimum incubation temperature for purified enzyme activity, 35 °C [60]. Whereas the highest protease activity for Enterococcus faeciums train was at 37°C. According to [61], the optimum temperature for Streptococcus thermophiles CNRZ 385 was 37°C. The results showed that protease activity increased with increasing the temperature until reached to optimum, then, decreased for both isolated strains.

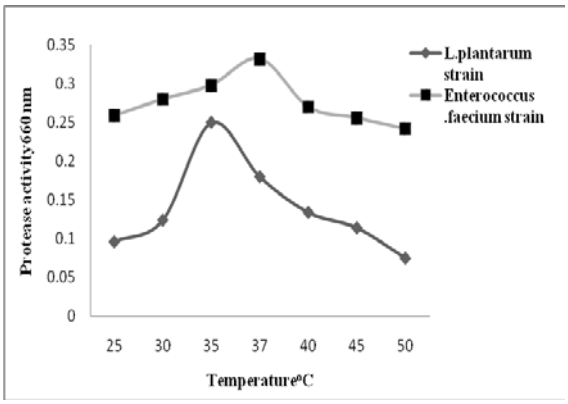

Figure 10: Theeffect of temperature on protease activity inboth of L. plantarumand E. faecium

The effect of substrate concentration on protease activity

The effect of substrate (casein) concentration (w/v), viz. (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0%) on the activities of purified proteases of selected isolates wereindicated in Fig. (11). The results showed that the protease activity gradually increased with increasing the substrate concentration in L. plantarum strain until reached to 2.5% and, then decreased and gradually decreased with increasing substrate concentration for Enterococcus. faecium strain. The results indicated that the optimum substrate concentration for purified protease enzyme was 2.5% for L. plantarum strain. Whereas for Enterococcus faecium strain, it was 0.5%. Considering other investigations, a study carried out by [62] indicated that protease from Lactobacillus acidophilus had maximum activity at 2% casein concentration. The protease has a high level of hydrolytic activity against casein as substrate and poor to moderate hydrolysis of BSA and egg albumin, respectively [55].

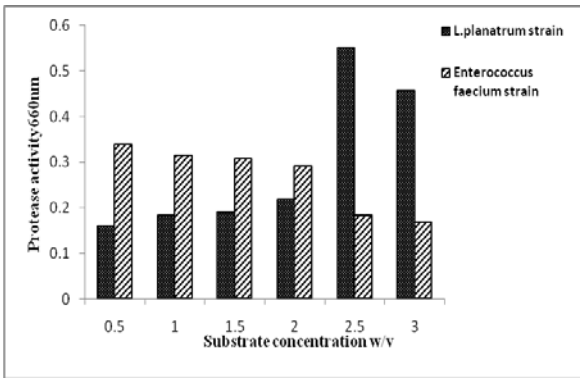

Figure 11: The effect of substrate concentration on protease activity of L. plantarum and E. faecium

Conclusion

Proteases are the leading enzymes with immense commercial potentials which are widely used in industrial and therapeutic applications. The dairy industry disposes sewage water causing inherent dangers of water pollution. This problem can be efficiently reduced by converting this hazardous by-product into an industrially important metabolite by microbial cell factories. In the present study, whey as a dairy industry waste was used as a substrate for extracellular protease production by isolated lactic acid bacteria. The optimization of growth conditions for maximum protease production by isolates was carried out. Under the optimum conditions, the enzyme yields were higher. Using Biolog system, two bacterial isolates with the highest enzyme activity were selected to identify and study characteristics of their proteases after purification. The results indicated that proteases were acidic and mesophilic. It has promising potentials for application in processes such as cheese making and improvement of food flavors.

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