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Full Length Research Paper

Isolation, optimization and characterization of protease producing bacteria from soil and water in Gondar town, North West Ethiopia

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Microorganisms can produce protease that shares nearly 40% of the total worldwide enzyme market. Proteases have many applications in different industries and environmental bioremediations. However, there is no scientific report on protease producing microorganisms in Gondar. The objective the study was to isolate, optimize and characterize protease producing bacteria. One gram soil or 1ml water from each collected sample was added in test tube containing 9 ml saline water and mixed well using vortex mixer. Each test tube containing the suspensions was used as stock culture. Serial dilution was made up to 10^{-6} for each sample. Hundred micro-liter suspensions were taken from each test tube containing 10^{-6}, spread over milk agar plates and incubated at 37^0C for 24 hrs. Morphologically distinct colonies were subcultured in to nutrient agar slant for incubation at 37^0C for 24 hrs and maintained at 4^0C for further analysis. Isolated colonies were screened on tryptone yeast extract dexterous agar plate for proteolytic activity. The promising isolates were selected for optimization and characterization. From a total of 32 isolates, 9.5 % (ATCSs, DSs and DWs) isolates were shown proteolytic activity. The optimum pH and temperature for growth and protease productions by the isolates were found to be 8-10 and 37^0C-45^0C, respectively. Based on morphological and biochemical characterization isolate ATCSs was identified as genus *Staphylococcus*. Proteolytic activity is influenced by the type of microorganism used chemical and physical parameters. Therefore, a significant task should be done to get good proteolytic activity on identification and selection of potential microorganisms and optimization of physical and chemical conditions for protease producing isolates.

Keywords: Proteolytic activity, protease producing microorganisms, optimization, characterization

INTRODUCTION

To produce environmental eco-friendly products and product out puts chemical process are being replaced by enzymes like proteases (Nigam, et al., 2012). Proteases from microbial sources are preferred over the enzymes from plant or animal sources (Mukesh Kuma et al., 2012). Because microbial proteases have a good source of enzymes due to their broad biochemical diversity, their rapid growth, the limited space required for cell cultivation and the ease with which the enzymes can be genetically manipulated to generate new enzymes for various application (Nigam, et al., 2012). Microbial proteases are the largest groups that take a share of nearly 60 % of the total worldwide enzyme market (Rayda et al., 2012). Thus an extensive search of proteases from varies microbial sources has been an angling process for many years.

Proteases useful for industrial application are influenced by different temperature, incubation period, and quantity of inoculums, medium pH, NaCl concentration and the composition and type of media. The majority of industrial processes are capable under harsh conditions; it would be of great importance to have microbial enzymes that demonstrate optimal activities at wide ranges of pH, temperature and salt concentration (Nigam et al., 2012). Protease can be acidic, neutral or alkaline depending on their activities at different

temperature (Narasimha et al., 2011). Alkalophilic proteases play an important role in the detergent industry. On the other hand acidophilic proteases have significant role in leather tanning process, food industry and x-ray films (for removal of sliver) (Habib et al., 2012; Rayda et al., 2012; Badgujar and Mahajan, 2013).

Although a wide range of fungi (such as genus *Aspergillus*, *Mucor* and *Rhizopus*) and bacteria (such as genus *Clostridium*, *Bacillus* and *Pseudomonas*) are known to date to produce proteases, a large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains. *Bacillus* strains have the ability to secret large amounts of alkaline proteases having significant proteolysis activity and stability at considerably high pH and temperatures (Kuberan et al., 2010; Ghasemi Asl et al., 2011). The genus *Bacillus* has been widely recognized as sources of the most commercial protease mainly nutrient alkaline extracellular proteases for an enzyme to be used as a detergent additive. It should be stable and active in the presence of typical detergent ingredient, such as surfactants, builders, bleaching agent, bleach activators, fillers, fabric softeners and various other formulation aids (Abou-Elela et al., 2011; Nigam, et al., 2012; Maal et al., 2009).

The optimum conditions for *Bacillus cereus* to produce protease that shows enzymatic maximum activity are at 50^0C and 10 pH. The alkaline proteases hydrolyze peptide bonds and have many applications of food complementary of beasts and poultries, bakery, leathering, oil manufacturing industries, alcohol production industries, beer production industries (Abou-Elela et al., 2011; Nigam, et al., 2012;). Proteases produced from *Bacillus subtitles* group are used in the treatment of bums and wounds. Oral administration of proteases produces an anti inflammatory response in burns patient and speeds up the process of healing. One of the major drawbacks affecting the stability at alkaline pH of enzymes form alkaliphile confers stability in a wide pH range but is usually thermo-labile. Thus, it is desirable to search for new protease with novel properties form as many different sources as possible (Abdelnasser et al., 2007).

Proteases produced from microorganisms have many roles in different industries (such as detergent, leather, food and textile industries) and environmental bioremediation. Proteases have the ability to degrade or remove complex proteins from polluted environment (Das and Prasad, 2010; Vishwanatha.T et. Al., 2010). Despite the above importance of proteases, there is no a scientific search on protease producing microorganisms in Gondar. To accomplish this task, there should be an activity of searching protease producing microorganisms in this particular area. Therefore, the present study can be important in giving direction for researchers this unexplored research area for finding protein degrading agents from microorganisms.

MATERIALS AND METHODS

Study area and design

Gondar town, particularly Kebele 18 (Alfora of Gondar, Dashin Beer factory and Atse Tewodros cafae waste disposal areas) was our study area. Gondar town is found in North West Ethiopia, 730 km away from North West direction Addis Ababa. The town has $12^0$36' North latitude and $37^0$28' East longitude with an elevation of 2133 Meter above sea level. Average maximum and minimum temperature is 29 ^{0}C (in March and May) and 10 ^{0}C (in January and December), respectively. The mean relative humidity for an average year is recorded as 55.7 % and on monthly basis it ranges from 40 % in January to 79 % in July.

The study was performed by experimental analysis using different chemicals and materials following sequential procedures to isolate and characterize protease producing bacteria from water and soil near Dashin beer waste disposal, slaughter house and Atse Tewodros cafae. During the study, enzyme producing bacteria as potential tools for the detection and identification of protein hydrolysis was developed and tested. The experiment was conducted at university of Gondar, Faculty of Natural and computational Sciences, Department of Biology in microbiology laboratory from January to June, 2013.

Sample collection

Soil (5cm depth) and water samples were collected aseptically from different areas of Gondar town using sterilized flasks. Soil samples were collected from Ats Tewodros, Maraki and Bridge cafe where the waste materials of food is disposed, Alfora of Gondar and Dashen beer industry waste disposal area, respectively. The water samples were collected from the Dashen beer industry where the waste product of liquid is released. The samples were taken in to microbiology libratory where the entire activities were performed.

Isolation and screening of protease producing bacteria

All of the samples were serially diluted in distill water which contains 0.85% of NaCl salt. Serial dilution was done up to 10^{-6} in ordered to get pure colonies. Then milk agar plate media was prepared and serially diluted samples were spread on to milk agar plat media from test tube containing 10^{-6} serially diluted samples. Then the spread plates were incubated at 37^0C for 48hrs. Then, the selected colonies were sub cultured in to test tube which contains nutrient agar and preserved under 4^0C for further analysis (Ghasemi Asl et al., 2011).

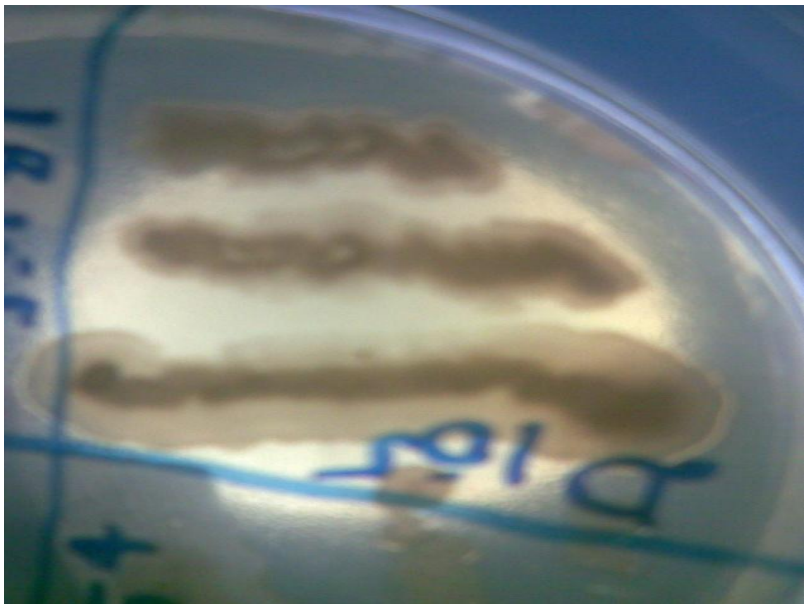

Figure 1. Screening and protecolytic activity of Dashin soil sample isolate

Each isolated single colonies were streaked on to tryptone yeast extract dexterous agar plate (tryptone 1%, dextrose 0.1%, yeast extract 0.5%, agar 2%) and incubated at 37 ^{0}c for 48 hrs for determination of proteolytic activity. After 48 hrs, the formation of clear zone around the colonies resulting from casein hydrolysis was taken as evidence of proteolytic activity (Vidya Pallavi et al., 2012).

Optimization and characterization of selected isolates

To optimize the activity of protease producing bacteria, selected isolates were streaked in to tryptone yeast extract dextrose plate and incubated at different pH and temperature. To determine the effect of temperature and alkalinity on proteolysis activity, isolates were subcultured on tryptone yeast extract dextrose plate adjusted at pH values 8, 9, 10, and 11 and incubated at 37^{0}C, 50^{0}C, and 60^{0}C for 48 hrs (Ghasemi Asl et al., 2011; Narasimha et al., 2011; Vidya Pallavi et al.,2012). After incubation the result was recorded.

Selected isolates were gram stained and characterized using different biochemical tests (catalase, coagulase, TSI test, indole, and urease test). The catalase test was performed on the Gram positive cocci. This was done by mixing a dense culture with two drops of H_2O_2 and looking for bubbles. Organisms positive (produced bubbles) in the test were considered to be *Staphylococci*. The *Staphylococci* were further tested with the coagulase test. The coagulase test was performed by mixing a dense suspension of the culture with plasma contained in a small test tube. The set up was incubated overnight, and then observed for fibrin clot. Those positive in the coagulase test were identified to be *Staphylococcus aureus*, and those negative were coagulase negative staphylococci (Srinivas Naik etal., 2013).

Triple sugar iron (TSI) agar was used for the differentiation of the *Enterobacteriaceae.* Using a sterile straight wire the TSI was stabbed deep to the bottom and the surface of the agar slant was streaked with the test organism. By the different three sugar fermentation, gas accumulation and hydrogen sulfide production abilities the *Enterobacteriaceae* were identified. The indole test was performed by inoculating peptone water, and incubating it overnight. The detection of indole was by the addition of Kovac‘s reagent Srinivas Naik etal., 2013).

RESULTS

Isolation and screening of protease producing bacteria

From a total of 15 collected samples, 32 colonies were isolated. All of isolate colonies were streaked on tryptone yeast extract dextrose plate media. Among 32 isolates, 3 (9.5%) were shown clear zone around streaked isolates (Figure 1). Those of the 3 isolates were obtained from Atse Tewodros Café soil sample, Dashin soil Sample and Dashin water samples. Then, these 3 isolates were selected for further analysis and characterization. Prote-

Table 1. Proteolytic activity and growth rate of isolates at different pH and temperature

Parameters		Proteolytic/ Growth activities of isolates		
`pH	Temperature	ATCSsi	DSsi	DWsi
8	37^0c	**+++/+++**	**+++/+++**	**+++/+++**
	45^0c	**++/++**	**++/+++**	**++/++**
	60^0c	**-/-**	**-/-**	**-/-**
9	37^0c	**++/+++**	**++/+++**	**+++/+++**
	45^0c	**+++/++**	**+/++**	**++/+++**
	60^0c	**-/-**	**-/-**	**-/-**
10	37^0c	**++/+++**	**+/++**	**+/++**
	45^0c	**+/+**	**++/+**	**++/+**
	60^0c	**-/-**	**-/-**	**-/-**
11	37^0c	**+/++**	**-/+**	**+/++**
	45^0c	**-/++**	**-/+**	**-/+**
	60^0c	**-/-**	**-/-**	**-/-**

Key: +++/+++ (More protolytic activity/ growth rate), **++/++** (Moderate proteolytic activity/ growth rate), **+/+** (good proteolytic activity/ growth rate), **(**No proteolytic activity/ growth rate)

Table 2. Morphological and biochemical characterization of selected isolates

Characteristics	**Isolates**		
	ATCSsi	DSsi	DWsi
Colony shape/ color	Circular/whitish	Irregular/yellow	Irregular/whitish
Gram reaction	+	+	-
Cell shape	Cocci	Rod	Rod
Catalase	+	-	
Coagulase	+	+	
TSI			K/AH_2S^+
MR/VP			-/+
Indole			-

Key: + (positive), - (negative), ATCSsi (Atse Tewodrose café soil sample isolate), DSsi (Dashen soil sample isolate), DWsi (Dashen water sample isolate), K/A (red slant/yellow but), H_2S^+ (Hydrogen peroxide production)

olytic activity of DSSi

Optimization and characterization of selected isolates

As the result indicate that the three isolates were shown good proteolytic activities and grown well at 37^0C and 45^0C both in 8 and 10 pH. There was no growth and proteolytic activity of all the three isolates at 60^0C in all adjusted different pH (Table1).

According to the gram staining result two (ATCSs and DSs) isolates were gram positive. ATCSs isolate was shown cocci cell morphology, catalase and coagulase positive result. The two (DSs and DWs) isolates were shown rod cell morphology. But DSs isolate was shown a negative result for catalase test and positive for coagulase test. One (DWs) isolate was gram negative, produce H_2S and ferment glucose (Table 2).

Based on the above morphological and biochemical characterization, ATCSs isolate was grouped under genus *Staphylococcus*. Other two (DSs and DWs) isolates need more characterization for identification at genus level.

DISCUSSION

Proteolytic bacteria are wide spread in nature and are able to grow under various growth conditions such as in different pH and temperature. The protolytic activity is also varying in different media type, pH and temperature. In the present study the optimum growth and proteolytic activity of the three isolates were shown between 37^0C and 45^0C. No proteolytic activity was seen at 60^0C. In the similar study Abdel Nasser et al (2007 reported that

the optimum temperature for proteolytic activity of protease producing bacteria was 37^0C-50^0C. Thus, the result was also related with Abdel Nasser et al (2007) result. Ghasemi Asl et al. (2011) reported that optimum pH for proteolytic activity of protease producing bacteria was observed between pH 8 and 10. Similar result was also observed in the present study. These indicate that for the growth of protease producing bacteria, alkaline environment is more suitable. This idea is also supported by Kuberan et al (2010).

In the present study, 3 bacterial isolates were able to produce protolytic enzyme. Of this 66.7% (2) strain were gram positive rod shape and staphylococcus species and 33.3% (1) was rod shape gram negative bacteria.

CONCLUSION

Protease producing microorganisms have many roles in different industries and environmental bioremediation by degrading protein containing compounds. The proteolytic activity and growth condition of these microorganisms are affected by different chemical (the type of media used) and physical (pH and temperature) parameters. Not only the physical and chemical conditions but also the type of microorganisms used can affect the proteolytic activity. To get a good proteolytic activity, identification and selection of potential isolates; and optimization of physical and chemical conditions (temperature, pH and the type of media used) for potential protease producing microorganisms should be a significant task. Therefore, the three isolates have good proteolytic activity in alkaline (ranged from 8- 10 pH) and ambient thermophilic conditions (ranged from 37^0C- 45^0C).

Conflict of interest statement

We declare that we have no conflict of interest.

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