academicJournals

Vol. 15(47), pp. 2698-2702, 23 November, 2016
DOI: 10.5897/AJB2016.15430
Article Number: ED35D9561763
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Production of alkaline proteases by alkalophilic *Bacillus subtilis* during recycling animal and plant wastes

Amira Hassan Al-Abdalall* and Eida Marshid Al-Khaldi

Department of Biology, Faculty of Science, University of Dammam, El-Dammam, Kingdom of Saudi Arabia.

Received 24 April, 2016; Accepted 24 October, 2016

The production of extracellular alkaline protease by *Bacillus subtilis* was studied with submerged fermentation. A new strain of *Bacillus* sp. was isolated from alkaline soil, which was able to produce extracellular alkaline protease. The production of alkaline protease involved the use of agricultural or animal wastes at pH 8 and temperatures at 37°C. Results showed that growing *B. subtilis* sub sp. *subtilis* under optimized growth resulted in production of alkaline protease with enzyme activity of 1412.5 U/ml, while with pomegranate peel at a concentration of 3%, the enzyme activity reached 3600 U/ml; further increase in pomegranate concentration did not however, lead to additional enzyme activity. Among various nitrogen sources, yeast extract was found to be the best inducer of alkaline protease. Among metal salts, KNO_3 and NH_4Cl were found to increase protease production. The maximum enzyme production (3600 U/ml) was observed with pomegranate peels of fermentation medium in the presence of yeast extract, potassium nitrate and ammonium chloride.

Key words: Production, alkaline protease, *Bacillus subtilis*, animal wastes, enzyme activity.

INTRODUCTION

Marketable proteases have reached up to 60% of the total industrial enzymes' market, and they represent one of the three largest groups of industrial enzymes known. The proteolytic enzyme has been severally applied in industries including the pharmaceutical, food and detergent industries (Moon and Parulekar, 1991). Proteases of commercial importance are produced from microbial, animal and plant sources (Patel, 1985). Almost all living organisms can produce alkaline protease at 32 to 45°C and pH 8 to 9 (Akcan and Uyar, 2011). Microbial proteases are produced from high yielding strains, including species of *Bacillus* sp., which is the most important group of bacteria that are involved in the enzyme industry. This bacterium is known to produce proteolytic enzymes quite effectively (Kaur et al., 1998). Generally, alkaline proteases are produced using submerged fermentation due to apparent advantages in enzyme production characterized by defined medium, and process conditions, and advantageous in downstream despite the cost-intensiveness for medium components

*Corresponding author. E-mail: aalabdalall@uod.edu.sa.

(Prakasham et al., 2006). The researchers of microbial proteases production are always in search of new and cheaper methods to improve the protease production as well as to reduce the market cost of this enzyme (Mukherjee et al., 2008). Recently, efforts have been made to explore new resources to reduce the protease production cost by improving the yield, and using cost-free or low-cost feedstocks or agricultural byproducts as substrate(s) for protease production (Sandhya et al., 2005). Currently, production of the enzyme by using cheap materials has attracted attention of many researchers such as Shaheen et al. (2008) and Boominadhan et al. (2009) who used crushed crab shells, acid wastes (Johnvesly et al., 2002), fish wastes (Ellouz et al., 2001), chicken-feather (Fakhfakh et al., 2009), maize extract with molase (Maal et al., 2009), wastes of rice and dates (Khosravi-Darani et al., 2008), protein by-products from lather industry (Kumar et al., 2008), wheat wastes (Gouda, 2006), that prepared peptone from sardine wastes with wastes of flour industry (Haddar et al., 2010). Researchers are always trying to find the most appropriate alternatives that are also cheaper. In view of this, the aim of the present study was to estimate the feasibility of utilizing easily available substrates in submerged fermentation for the production of alkaline protease by *B. subtilis* by using abundant natural resources and cheaper wastes of growth conditions of bacterium to enhance protease enzyme production and the effect of addition of nitrogen and metal salts sources on enzyme activity.

MATERIALS AND METHODS

Bacterial isolate

Bacillus subtilis isolated from soil during November 2009 at the Eastern Province of Saudi Arabia, were used in this research. The isolate was identified in the Plant Protection Department, Faculty of Science and Agriculture in King Sauod University by Biolog Systems by Al-Yahya et al. (2007). Identified isolates were evaluated for their ability to produce alkaline protease by Al-Khaldi (2014). *B. subtilis* sub sp. *subtilis* was the highest active isolate in alkaline protease activity and thus used for the study.

Source of natural wastes

Kitchen wastes as well as agricultural and animal wastes were collected from farms in sterile plastic bags (Sonia Sethi et al., 2012). The following wastes were used, bran, seeds of linen, date, olive, peels of mango, carrot, orange, banana, pomegranate and potatoes were also used. Corn husks, onion peel, egg shells, sheep bones and manure, camel manure and pigeon feathers were taken by replacing casein in the growth media.

Media preparation

After washing wastes, it was left to dry air in an oven at 70°C for 5 days followed by grinding. 0.75 g of each type was kept in a flask with 25.0 ml H_2O and inoculated with 500 µl of the isolate, then incubated with shaking water bath (90 rpm) at 37°C for 5 days (Al-Khaldi, 2014). The enzyme was extracted and examined for protease production.

Protease assay

Proteolytic activity in the bacterial cultural media was determined by using the spectrophotometric method (Rao and Narasu, 2007). 500 µl of filtrate, representing the enzyme, was added to 100 µl of 1% w/v casein (substrate) soluble in phosphate buffer at pH 8. 50°C for 30 min and the reaction was stopped by the addition of 2.0 ml trichloroacetic acid (10%) and the reaction was left for 30 min at room temperature in the dark. The precipitate was separated by centrifugation at 10000 rpm. 1000 µl from the filtrate was added to 5.0 ml NaOH (0.5 mol/l) and 500 ml folin Ciocalteau Phenol reagent. The resulted color was read using UV-1800 spectrophotometer (model no a1145806148cd). The absorbance was read at 660 nm. One unit of proteolytic enzyme activity was defined as the amount of casein that hydrolyzed during 30 min incubation at 37°C for milliliter of solution of extract. All experiments were conducted in triplicate and the mean at three with standard deviation (SD) was represented.

Effect of different waste concentrations on enzyme production

To test the best kind of waste as substrate of enzyme production, 0.1, 0.5, 1.0, 3.0, 6.0 and 12 g/100 ml H_2O were distributed in flasks contain 250 distilled water and cultured with the bacteria; the flasks were shaken at 90 rpm in a water bath at 37°C for 5 days, the enzyme activity was measured for enzyme production (Al-Khaldi, 2014).

Effect of some salts and nitrogenous sources on the enzyme activity

0.5 g/100 ml H_2O of the following salts (NaCl, $ZnCl_2$, cobalt chloride, yeast extract, peptone, potassium nitrate and ammonium chloride) were dissolved and poured into flasks containing the waste and inoculated with 500 µl of the bacterium. Flasks were incubated at 37°C for 5 days and then examined for alkaline protease production (Al-Khaldi, 2014).

Produced enzyme in rotein media or in natural wastes

The optimized media of enzyme production (10 g fructose, 5 g KNO_3, 150 g NaCl, 5 g K_2HPO_4, 0.4 g $MgSO_4$, 0.2 g $CaCl_2$ and 10 g Tween-80) dissolved in one liter of distilled water, was prepared (Al-Khaldi, 2014). The optimized media and natural waste media and mixing media of both were inoculated with 25.0 µl of bacteria with incubation at 37°C for 5 days. The produced enzyme was separated by centrifugation at 10000 rpm (Al-Khaldi, 2014).

Statistical analysis

The results obtained were analyzed statistically using version of SPSS16 program where transaction averages were compared at the abstract level (0.05) using the least significant difference test (LSD) (Norusis, 1999).

RESULTS

Pomegranate peel proved to be the best media used for enzyme production; as shown in Table 1, the activity

Table 1. Effect of natural wastes on alkaline protease activity.

Plant and animal wastes (3 g/100 ml)	Optical density (OD)	Protease activity (U/ml)
Bran	0.3316	157±5.57
Linen seeds	0.4108	208.5±16.73
Date seeds	0.3380	159±16.04
Olive seeds	0.2172	102±4.58
Mango peel	1.2512	1007.5±35.00
Carrots Peel	0.8630	640±90.04
Orange peel	1.0723	840±48.80
Banana peels	0.4322	227.5±15.21
Pomegranate peel	3.9524	3075±525.00
Potatoes peel	0.6120	400±30.41
Corn husks	0.6745	455±44.23
Onions peel	0.9147	687.5±41.61
Eggshells	0.0832	39±1.50
Pulp peel	0.1666	80±5.00
Shrimp peel	0.4976	287.5±19.53
Fish guts	0.1865	86±5.90
Sheep bones	0.2965	139±4.00
Sheep manure	0.2064	97.5±7.70
Camel manure	0.1680	80±5.29
Pigeon feathers	0.3111	143.5±10.48

Table 2. Effect of pomegranate peel on alkaline protease activity.

Pomegranate peel concentration (g/100 ml)	Optical density (OD)	Protease activity (U/ml)
0.1	0.2948	137.5±2.50
0.5	0.9988	767.5±50.19
1	2.1409	1837.5±121.24
3	4.0000	3600±0.00
6	4.0000	3600±0.00
12	4.0000	3600±0.00

Table 3. Effect of some salts and nitrogen sources to the best of pomegranate peel concentration on alkaline protease activity.

Some salt and nitrogen sources (0.5 g/100 ml)	Optical density (OD)	Protease activity (U/ml)
NaCl	3.9422	3540±60.00
$CoCl_2$	1.8934	1600±5.00
$ZnCl_2$	3.7352	3352.5±52.50
Yeast extract	4.0000	3600±0.00
Peptone	3.6877	3312.5±130.00
KNO_3	4.0000	3600±0.00
NH_4Cl	4.0000	3600±0.00

measured was 3075 U/ml, followed by 1007.5 U/ml for mango peel. Orange peel yielded protease enzyme with an activity of 840 U/ml followed by onion peel, carrot peel, corn husks and potato peels with 687.5, 640, 455 and 400 U/ml as enzyme activities, respectively. Shrimp peel yielded enzyme with activity of 287.5 U/ml, followed by banana peel recording 227.5 U/ml. Among all the plant and animal wastes investigated in this study, egg shells produced alkaline protease enzyme with the lowest activity of 39 U/ml (Table 1).

The effect of various concentrations of pomegranate peel on alkaline protease activity is shown in Table 2. The results revealed that the alkaline protease activity progressively increased from 137.5 U/ml at concentration 0.1 g to 3600 U/ml at concentration 3 g of pomegranate peel; further increase in pomegranate peel did not however, alter the activity of the enzyme (Table 2).

Table 3 revealed the effect of some salts and nitrogen sources of pomegranate peel concentration on alkaline protease activity. The result indicated that adding yeast extract with potassium nitrate and ammonium chloride increased enzyme activity to 3600 U/ml. Among all salts and nitrogen sources investigated, the alkaline protease enzyme obtained in the presence of cobalt chloride had the lowest activity (1600 U/ml) (Table 3).

More so, the alkaline protease enzyme activity of 3600 U/ml obtained after growing *B. subtilis* subsp. *subtilis* in media containing pomegranate peels (3 g/100 ml) (Table 4) was significantly higher than that recorded after growing in optimized medium (1412.5 U/ml). The enzyme

Table 4. Comparing of activity alkaline protease in the optimal medium with/without pomegranate peel, and in medium with pomegranate peel only.

Type of medium	Optical density (OD)	Protease activity (U/ml)
Optimal medium	1.6863	1412.5±59.11
Optimal medium with Pomegranate peel	4.0000	3600±0.00
Pomegranate peel medium	2.3164	2010±76.61

activity after mixing pomegranate with the optimized media was, however, 2010 U/ml.

DISCUSSION

Bacillus species are considered to be the most important sources of protease and have been used for enzyme production using submerged fermentation (Gaurav et al., 2015). Several substrates obtained from a kitchen wastes as well as agricultural and animal wastes were collected from farms and tested for protease production (Table 1). Adding natural and cheap nutrients, considered as wastes, to bacterial growing media led to high production of alkaline protease enzymes with increasing activity. Alkaline protease production by *B. subtilis* using submerged fermentation was influenced by physiological and chemical nature of substrates and associated with growth of the microbial strain (Abid et al., 2017). In this study, maximum enzyme production (3075 U/ml) was observed with pomegranate peels; further studies were carried out using it as a substrate. Results proved that pomegranate peels were the best additive.

The principal components of primary cell wall are composed of the cellulose microfibrils and pectins, and they play a significant role in the structuration (Agoda-Tandjawa et al., 2012). It is understood that the mechanical properties of pomegranate peel gels are administered mainly by pectins and/or fibrous material. Also, the presence of calcium and/or sodium ions and the biopolymer concentrations were reported to have an influence on the properties of the mixed cellulose and pectins.

Pomegranate peel at concentration of 3% recorded high enzyme activity reaching 3600 U/ml (Table 2). With increase in concentration, there is no difference in the rate of the enzyme activity production. This steadiness in activity may be due to change of enzyme. Units ranging from free to conjugated, which contains the active sites on the enzyme are totally occupied by the substrate. Results proved that adding yeast extract and potassium nitrate increased enzyme activity (Table 3). Generally, adding nitrogenous compounds stimulate microorganism growth and enzyme production. Some microorganisms use these compounds in enzyme production and not in growth. Growing *B. subtilis* subsp. *subtilis* under optimized medium (10 g fructose, 5 g KNO_3, 150 g NaCl, 5 g K_2HPO_4, 0.4 g $MgSO_4$, 0.2 g $CaCl_2$ and 10 g Tween-80) dissolved in one liter of distilled water, enzyme activity reached 1412.5 U/ml (Table 4), while with pomegranate peel, high enzyme activity reaching 3600 U/ml was recorded. The pomegranate peel contains many ions of metals necessary for the growth of bacteria, and contributes to the stability of the enzyme protease. Kushwaha et al. (2013) pointed out that pomegranate peel powder was evaluated for its minerals value: Sodium (mg/kg) 362.74, potassium (mg/kg) 6679.50, calcium (mg/kg) 728.23, magnesium (mg/kg) 524.80, phosphorus (mg/kg) 57.01, iron (µg/g) 18.33, copper (µg/gm) 4.67, zinc (µg/g) 9.63. The effect of nitrogen sources on protease production is shown in Table 3. Yeast extract increased protease production by *B. subtilis*, while Yang et al. (2000) found that protease production by *B. subtilis* Y-108 was repressed by most of the nitrogen sources. Addition of metal salts sources was investigated. Among these metal salts, KNO_3 and NH_4Cl were found to increase protease production (Table 3). For maximum alkaline protease activity, there is a need for a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations. These cations were also found to enhance the thermal stability of *Bacillus* alkaline protease (Paliwal et al., 1994). These cations are believed to protect the enzyme against thermal denaturation, and also play a vital role in maintaining the active conformation of the enzyme at high temperatures. In addition, specific Ca^{2+} binding sites influence the protein activity and stability apart from the catalytic site described for proteinase K (Maheshwari and Saraf, 2015).

Conflict of Interests

The authors did not declare any conflict of interests.

REFERENCES

Abid M, Cheikhrouhou S, Cuvelier G, Leverrier C, Renard CM, Attia H, Ayadi MA (2017). Rheological properties of pomegranate peel suspensions: The effect of fibrous material and low-methoxyl pectin at acidic pH. Food Hydrocoll. 62:174-181.

Agoda-Tandjawa G, Durand S, Gaillard C, Garnier C, Doublier JL (2012). Properties of cellulose/pectins composites: Implication for structural and mechanical properties of cell wall. Carbohydr. Polym. 90:1081-1091.

Akcan N, Uyar F (2011). Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation. Eurasia J. Biosci. 5(8):64-72.

Al-Khaldi E (2014). Studies on Alkaline Protease Enzyme Produced by

a Halophilic Bacillus isolate from Kingdom of Saudi Arabia. PhD. Thesis in Microbiology. University of Dammam, KSA.
Al-Yahya A, El-Hussieni M, Ibrahim A, Ibrhaim EA (2007). New biocontrol fungus *Trichoderma kongii* in the kingdom of Saudi Arabia isolation and identification. Saudi J. Biol. Sci. 14(2):137-144.
Boominadhan U, Rajakumar R, Sivakumaar V, Joe M (2009). Optimization of protease enzyme production using *Bacillus* sp. isolated from different wastes. Bot. Res. Int. 2(2):83-87.
Ellouz Y, Bayoudh A, Kammoun S, Gharsallah N, Nasri M (2001). Production of protease by *Bacillus subtilis* grown on sardinella heads and viscera flour. Bioresour. Technol. 80:49-51.
Fakhfakh N, Kanoun S, Manni L, Nasri M (2009). Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather-degrading *Bacillus licheniformis* RPk. Can. J. Microbiol. 55:427-436.
Gaurav P, Anil P, Pavani JVP, Sayantan B, Deviram GVNS, Ajay K, Mitali P, Ravi Gyana P (2015). Production, optimization and partial purification of protease from *Bacillus subtilis*. J. Taibah Univ. Sci. 9(1):50-55.
Gouda M (2006). Optimization and purification of alkaline Proteases produced by marine *Bacillus* sp. MIG newly isolated from eastern harbour of Alexandria. Pol. J. Microbiol. 55(2):119-126.
Haddar O, Zaghloul I, Saeed M (2010). Expression of alkaline proteinase gene in two recombinant *Bacillus cereus* feather-degrading strains. Folia Microbiol. 55:23-27.
Johnvesly B, Manjunath R, Naik R (2002). Pigeon pea waste as a novel, inexpensive, substrate for production of a thermostable alkaline protease from thermoalkalophilic *Bacillus sp*. JB-99. Bioresour. Technol. 82:61-64.
Kaur M, Dhillon S, Chaudhary K, Singh R (1998). Production, purification and characterization of a thermostable alkaline protease from Bacillus polymyxa. Indian J. Microbiol. 38:63-67.
Khosravi-Darani K, Falahatpishe R, Jalali M (2008). Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil. Afr. J. Biotechnol. 7:1536-1542.
Kumar A, Swarnalatha S, Sairam B, Sekaran G (2008). production of alkaline protease by *Pseudomonas aeruginosa* using proteinaceous solid waste generated from leather manufacturing industries. Bioresour. Technol. 99:1939-1944.
Kushwaha SC, Bera MB, Kumar P (2013). Nutritional Composition of Detanninated and Fresh Pomegranate Peel Powder. IOSR J. Environ. Sci. Toxicol. Food Technol. 7:38-42.
Maal B, Emtiazi G, Nahvi I (2009). Production of alkaline protease by *Bacillus cereus* and *Bacillus polymixa* in new industrial culture mediums and its immobilization. Afr. J. Microbiol. Res. 3(9):491-497.
Maheshwari DK, Saraf M (2015). Halophiles: Biodiversity and Sustainable Exploitation. Springer.
Mukherjee PK, Das P, Rao PSS (2008). Time trends in MB-PB ratio among untreated leprosy patients attending a referral hospital in UP, India during 2001 to 2010. Indian J Lepr. 85:59-64.
Moon SH, Parulekar SJ (1991). A parametric study of protease production in batch and fed-batch cultures of Bacillus firmus. Biotechnol. Bioeng. 37:467-483.
Norusis MJ (1999). SPSS/PC + Statistics 6.0 for the IBM PC/XT/AT and PS/2. Library of Congress, USA.
Paliwal N, Singh SP, Garg SK (1994). Cation-induced thermal stability of an alkaline protease from a *Bacillus* sp. Bioresour. Technol. 50:209-211.
Patel PR (1985). Enzyme isolation and purification. In: Biotechnology: Applications and Research. Technomic Publishing Co. Inc., USA. pp. 534-564.
Prakasham RS, Rao CS, Sarma PN (2006). Green gram husk - An inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation, Biosensors. Technol. 97: 1449-1454.
Rao K, Narasu M (2007). Alkaline Protease from *Bacillus firmus* 7728. Afr. J. Biotechnol. 6(21):2493-2496.
Shaheen M, Shah A, Hameed A, Hasan F (2008). Influence of culture conditions on production and activity of protease from *Bacillus subtilis* BS1. Pak. J. Bot. 40(5):2161-2169.
Sandhya C, Madhavan NK, Pandey A (2005). Microbial proteases. In: Barredo JL, editor. Microbial enzymes and biotransformations. New Jersey, USA. The Human Press Inc. 17:165-180.
Sonia Sethi B, Palsaniya P, Mishra R, Beejawat N (2012). Optimization of alkaline protease production from bacteria isolated from soil. J. Micro. Biotechnol. Res. 2(6):858-865.
Yang JK, Shih IL, Tzeng YM, Wang SL (2000). Production and purification of protease from a Bacillus subtilis that can deproteinise crustacean wastes. Enzyme Microb. Technol. 26:406-413.