Advanced Journal of Microbiology Research Vol. 2013
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Production dynamics of *Bacillus subtilis* strain AG-1 and EAG-2, producing moderately alkaline proteases

Afia Ghafoor* and Shahida Hasnain

Department of Microbiology and Molecular Genetics, Quaid-e-Azam Campus University of the Punjab. 54590. Lahore, Pakistan.

Accepted 9 April, 2013

Bacillus **strains isolated from different habitats were screened and identified for high protease activity. Two of the *bacillus* strains genetically identified as *Bacillus subtilis* AG-1 and *Bacillus subtilis* EAG-2 exhibited tremendous potential for protease production. The strains were optimized with respect to production media and its components. Culture conditions were critically investigated with reference to other key determinants, involving inoculum concentration, incubation time, initial pH of the medium and temperature. The optimum production pH and temperature was found to be 7.0 and 35 ^{o}C for *B. subtilis* AG-1, while 7.2 and 37 ^{o}C were the optimum conditions for *B. subtilis* EAG-2. The inoculation ratio and incubation time were determined for both of the strains as 1% (v/v) and 24-32 h respectively. The study reveals that, high protease yield can be achieved over a moderate fermentation period with relatively economical production costs. Hence these strains might be useful for commercial exploitation.**

Key words: *Bacillus subtilis*, protease, production medium, pharmamedia, optimum growth.

INTRODUCTION

Nowadays proteases have achieved a central position in the enzyme industry by crossing the figure of 60% sale share of enzyme market. Microbial proteases find numerous applications in industrial sectors such as detergents, leather, animal feed, food processing, silk degumming and waste water treatment (Beg and Gupta, 2003; Dayanandan et al., 2003; Gessesse and Gashe 1997, Gupta et al., 2002). *Bacillus* strains (Gupta et al., 2002; Joo et al., 2002; Kirk et al., 2002; Manachini and Fortina, 1998; Masui et al., 2004) are the main source of industrial proteases among micro-organisms, although fungi have also been utilized in some with greater emphasis in food industry. However a marked increase in the production of *Bacillus* derived proteases is due to their production capacities and high catalytic activity (Freddi et al., 2003; Kirk et al., 2002). Thus based upon the geographic diversity of our local environment, chances exists that, there is a significant potential for *Bacillus* species producing proteases with interesting properties. So with these aims and objectives a study design was prepared to search out for protease producers. Total of 65 isolates were screened for proteolytic activity. Out of 25 positive protease producers, two of the bacillus strains encoded initially as AG-1 and EAG-2 were found to be highly active. The goal was to identify these strains and to further optimize the fermentation conditions so as to determine their commercial significance.

*Corresponding author. E-mail: afia_ghafoor@yahoo.com. Tel: +92-42-9231238. Fax: +92-42-9230481.

MATERIALS AND METHODS

Screening for protease producing bacterial strains

For isolation of protease producing microorganism, soil samples were collected from different vicinities including protein rich crop fields, ornamental plant nurseries, garbage dump and slaughter-houses in Islamabad, Nowshera and Lahore (Pakistan). The soil samples were dissolved in sterile water and heated to 80 ^{o}C for 10 min so as to avoid the possible contaminating organisms other than *Bacillus* sp. The samples were later serially diluted in sterile distilled water and plated onto skim milk agar plates containing: skim milk, 100 ml/l; yeast extract, 1.5 g/l; NaH_2PO_4, 1 g/l; bacteriological agar, 12 g/l; NaCl, 1 g/l; pH 7.2. Plates were incubated at 37 ^{o}C for 24 h. A zone of skim milk hydrolysis around the colonies gave a clear indication of protease producing organisms. Clones exhibiting the largest cleared zones around their colonies were purified and maintained on nutrient agar slants at 4 ^{o}C.

Genotypic study

Among the highly active protease producers, two *bacillus* strains encoded as AG- 1 and EAG- 2 were selected for further study. The two strains were subjected to 16S rDNA analysis. Amplification was performed according to method previously described by Hasnain and Thomas (1996). The rRNA gene was amplified by means of forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1522r (5′-AAGGAGGTGATCCA (AG) CCGCA-3′). Reaction was set up with 0.5 µg of chromosomal DNA template, 0.5 µM of each primer, 200 µM deoxynucleoside triphosphate and 1 unit of *Taq* polymerase. After an initial denaturation at 94°C for 5 min, samples were subjected to 30 cycles of 20 s at 94°C, 20 s at 50°C and 2 min at 72°C. Final extension step was performed at 72°C for 5 min. Products were eluted in 1.0% (w/v) agarose gels by means of Aqua pure DNA extraction kit (*BIORAD*) and subjected to automatic sequencer (ABI 3100) for sequencing.

Nucleotide accession number

The nucleotide sequences obtained here has been submitted to Gene bank database and assigned accession numbers, EU158264 and EU338361. The strains were identified as *Bacillus subtilis* AG-1 and *B. subtilis* EAG-2.

Protease production

Media used and culture conditions

In order to check the maximum enzyme production, organisms were cultivated in four different types of media, Luria- Bertani broth (LB), Tryptic soy broth (TSB), Pharmamedia (Traders Protein USA) and protease production medium comprising of glucose (separately sterilized), 1.0% (w/v); yeast extract, 0.3% (w/v); casein (separately dissolved), 0.60% (w/v); Na_2HPO_4, 0.20% (w/v); KH_2PO_4, 0.10% (w/v); $MgSO_4.7H_2O$, 0.05% (w/v). Organisms were grown at pH 7.0, 7.2, 8.0 and 8.5 in the respective media. Cultivations were conducted in 50 ml of medium in 250 ml Erlenmeyer flasks, while incubations were carried out at 30, 35, 37 and 40°C at each pH. The agitation speed was kept constant at 150 rpm for 24-32 h in all sets of experiment.

Optimization of the selective medium

To screen out the most favorable carbon source, different sources were studied at variable concentrations ranging from 0.5- 4%. It includes glucose, fructose, sucrose, maltose and lactose, while different nitrogen sources were also employed involving casein, peptone, tryptone, beef extract and yeast extract to achieve the maximum enzyme production.

Culture harvesting

Sample aliquots were withdrawn from all sets of experiments to determine the biomass at regular intervals. However for protease activity measurement, cultures were centrifuged at 13,000 rpm for 20 min at 4°C. The cell pellet was discarded and the supernatant was preserved at 4°C for further analysis.

Enzyme assay

Protease activity was monitored by modification of casein digestion method of Kunitz (1947). A 100 l aliquot of suitably diluted enzyme solution was added to 1 ml of casein (1%) prepared in Tris buffer (100 mM, pH 8.0) and incubated for 20 min at 37°C.The reaction was truncated after the addition of 3 ml of 0.3 M trichloroacetic acid (TCA) and undigested protein was precipitated. The precipitates were removed after centrifugation at 7,000 rpm for 20 min and filtrate was collected. The acid soluble material was estimated spectophotometrically at 280 nm. A suitable control (prepared by adding TCA to the substrate prior to the addition of the enzyme) was run simultaneously. Enzyme activity units were calculated by using the equation A_{280} of 0.001 = 1 Kunitz - Units.

RESULTS

Growth characteristics of protease producing strains

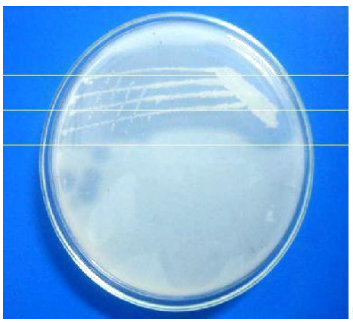
Both of the *bacillus* strains exhibited high protease activity on the selective screening medium however a clear variation is observable in their growth pattern on nutrient agar. The AG- 1 strain shows a spreading mucoidal growth whereas rather restricted and smaller dry colonies can be seen in case of EAG-2 (Figure 1).

Selection of most favorable media

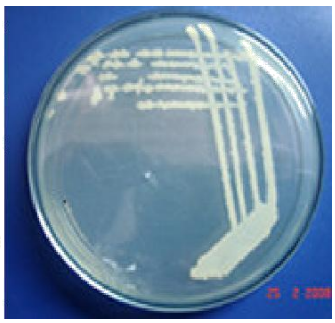
The effect of different media on enzyme production was analyzed. The pre-cultures were prepared by transferring a loop full of culture of each strain into 25 ml of the nutrient broth incubated at 37°C and 150 rpm for 24 h. Main cultures of each medium were inoculated at 1% ratio (v/v) to that of total volume. The results shown in the Figure 2 gave an idea of biomass/enzyme production capacity of media used in the study. A sharp increase in cell density was observed, when tryptic soy broth (TSB) was used for cultivation of strain EAG-2, followed by protease production medium and Luria-Bertani broth (LB). While Pharmamedia proved to be the least active. However when these results interpreted in terms of enzyme production capability again pharmamedia was the least active medium followed by TSB and LB; hence protease production medium exhibited the highest enzyme productivity. In case of *B. subtilis* AG-1, protease specific medium showed better enzyme production than growth, when compared with others. TSB proved to be the second favorite medium in terms of growth /enzyme rate followed by LB and Pharmamedia.

Optimization of key physico-chemical factors

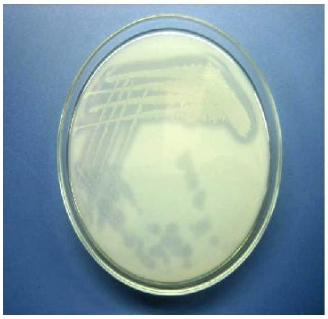
Production experiments were performed with inoculation ratios from 0.5-1.5% under different sets of pH and temperature. Incubations were conducted over a period of 24-32 h, while optical density was monitored at 600 nm at regular interval. An increase in the optical density was observed from 0.5-1.0% followed by a decline in cell growth. An explanation for this would be that, from 0.5-1.0% inoculation ratio cells did not face any nutrient or oxygen limitation, but beyond this, there was an overload



*Bacillus subtilis*EAG-2 (*a*)



*Bacillus subtilis*EAG-2 (*c*)



*Bacillus subtilis*AG-1 (*b*)

*Bacillus subtilis*AG-1 (*d*)

Figure 1. Morphological growth characteristics of *Bacillus subtilis* EAG-2 and AG-1(*a*) and (*b*) represent selective medium whereas (*c*) and (*d*) refer towards nutrient agar.

leading to faster consumption of nutrients and ultimately resulted in lessen growth (Table 1). According to the results monitored at different time intervals; the optimum growth period for both strains was around 24-32 h. The two strains lie in close relation with respect to growth related enzyme production. However Table 1 shows that pH 7.0 and 7.2 was the optimum production pH for both strains, while the optimum fermentation temperature for *B. subtilis* EAG-2 was 37^{o}C. Instead of this *B. subtilis* AG-1 showed promising results at 35^{o}C.

Selection and optimization of carbon and nitrogen sources

Protease specific medium was further optimized for efficient reproducibility by supplementing with different carbon and nitrogen sources. These experiments were performed in 250 ml, 500 ml, 1 and 2 L Erlenmeyer flasks with a culture volume from 50-450 ml. However highest production rate was observed in 1 L flasks. Among the tested carbon reservoirs, using 1% glucose (v/v) was most efficient monosaccharide for enzyme production. Both the strains were triggered efficiently by sugars indicating their stimulating effect on enzyme production. In case of strain AG-1 peak enzyme yield was observed when supplemented with 1% glucose followed by maltose, fructose and sucrose with narrow margins in between. However maltose 1% appeared as the best carbon source when utilized for EAG-2. Data shown in Figure 3 highlights the common utility of glucose as monomer and dimmer, while lactose was the least active among tested disaccharides, indicating the suppressing role of galactose subunit on enzyme production. The strains were simultaneously checked against different nitrogenous substances for enzyme production either alone or in combination with one another (Table 2) . None of the single source tried, proved much efficient. The combination of casein with yeast extract exhibited better enzyme production followed by casein and peptone. However a combination of casein with peptone and yeast extract in a ratio of 1:1:3 further favored the enzyme production.

DISCUSSION

Current study highlights the role of different media and culture conditions on biomass/enzyme yield of the *Bacillus* strains. It is quite evident that protease specific

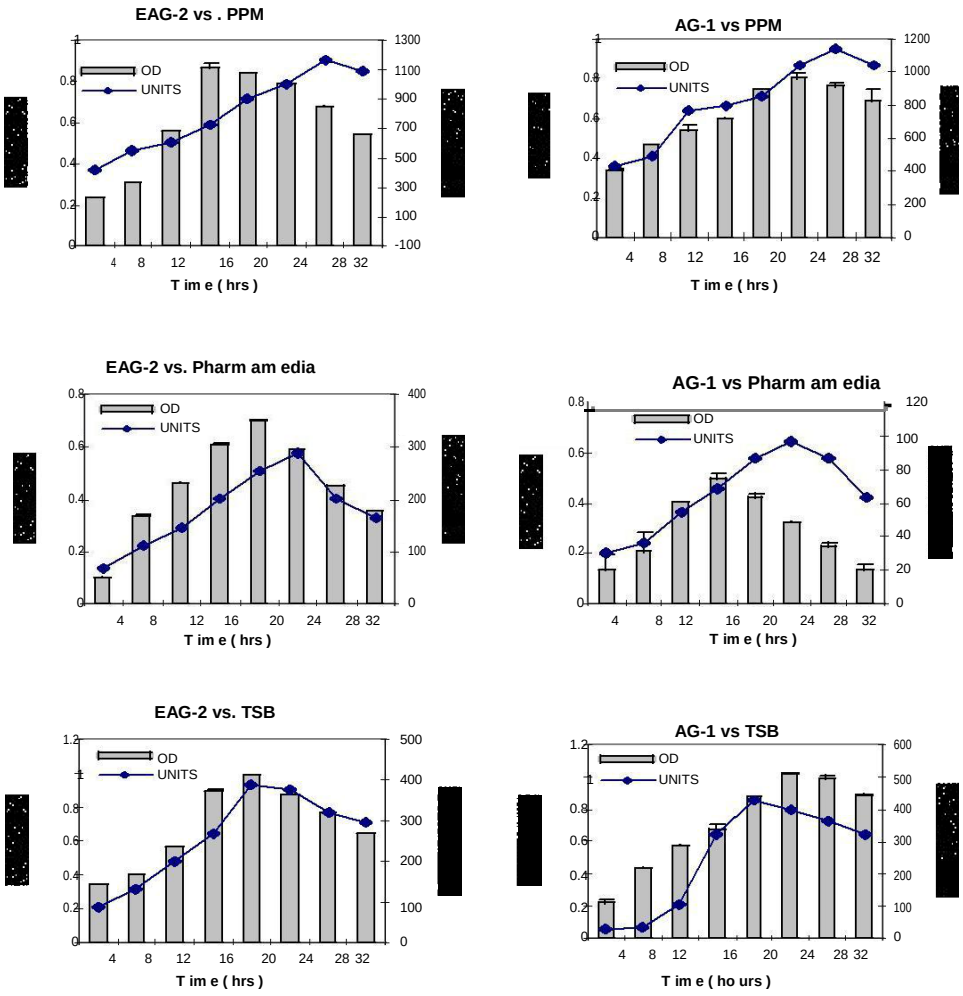


Figure 2. Growth kinetics of *Bacillus subtilis* EAG- 2 and AG-1 in different media with respect to their optimum conditions. All the experiments were performed in triplicates.
Legends: PSM (protease specific medium), TSB (tryptic soy broth) and LB (Luria-Bertani broth)

Table 1. Describes the Effect of various physico-chemical parameters on biomass production of *Bacillus subtilis* AG-1 and EAG-2 strains.

Parameters studied	AG-1 (OD 600 nm)	EAG-2 (OD 600 nm)
Inoculum		
0.5	5.34	6.02
1	8.0	8.45
1.5	6.4	7.12
Temperature		
30	5.5	4.1
35	8.0	7.29
37	6.94	8.45
40	3.22	8.0
pH		
7	8.0	7.32
7.2	7.11	8.45
8	5.61	7.97
8.5	4.1	6.0

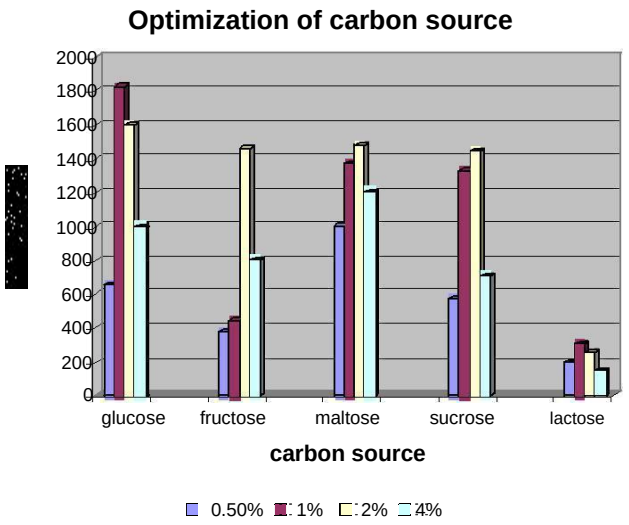


Figure 3a. The data shows the effect of sugars on enzyme activity for *Bacillus subtilis* AG-1.

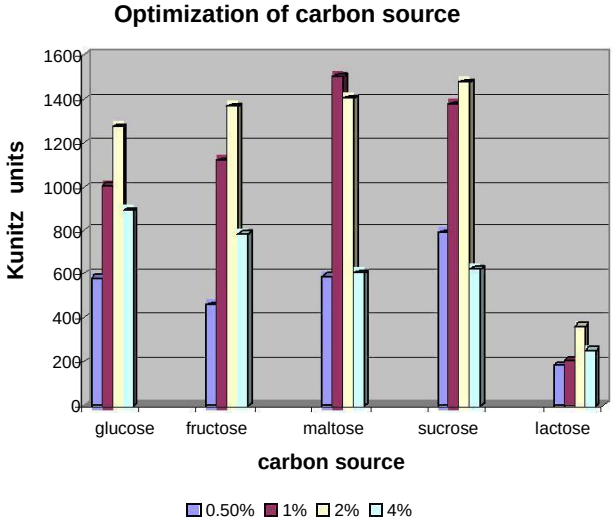


Figure 3b. The data shows the effect of sugars on enzyme activity for *Bacillus subtilis* EAG-2.

medium appeared as the most suitable for both having intact casein as the major nitrogenous source that played a role in active induction for protease production which was absent in rest of the media.. Thus it can be predicted that protease production medium might be the medium of choice for cultivating the protease producers reported here, although TSB showed a pronounced increase in cell density in case of EAG -2 but it failed to maintain the enzyme production at the same level. This could be explained as; tryptic soy broth is highly rich in free amino acids, which may hinder the enzyme production. Pharmamedia had not shown any remarkable effect on growth as well as on enzyme, perhaps it is a complex medium rich in nitrogenous components and may give a

Table 2. Define the effect and relationship of different nitrogen sources to choose out the best combination for enzyme/ biomass production.

Nitrogen source	AG-1 U/ml	EAG-2 U/ml
Peptone	401	322
Casein	863	792
Yeast extract	670	546
Beef extract	578	368
Tryptone	330	234
Peptone, Casein	900	956
Beef extract, Casein	586	495
Yeast extract, Casein	912	1003
Casein, peptone and Yeast extract	1102	1389
Casein, peptone and Beef extract	954	984

non-homogenous formulation leading to variability and reduced reproducibility in production experiments. This can be further supported by the repressive role of organic nitrogen sources and excessive amino acids in alkaline protease production described previously (Gibbs et al., 2004; Joo and Chang, 2005). However it may be useful for other metabolites (Al-Ajlani et al., 2007). Both strains exhibited variable growth charac-teristics with reference to physico-chemical parameters; hence they lie in the mesophilic range. But their enzyme performance is interestingly comparable with reference to pH and temperature. The optimum activity temperature for strain AG-1 was found to be 60 ^{o}C at pH 8.0, whereas EAG-2 showed peak response at pH 8.5 and 65 ^{o}C (Data not shown). From nutritional aspects glucose played an essential role being a potent enzyme inducer for *B. subtilis*EAG-2, as well as *B. subtilis*AG-1.

In a nutshell it can be assumed that, both of the *B. subtilis* strains are active protease producers with high biomass/enzyme productivity ratio. Efficient enzyme per-formance at thermo-alkaline range is another indis-pensable property. A unique feature of these strains was their high enzyme yield over a relatively moderate fermentation period sharply comparable to earlier reports (Joo et al., 2002; Joo and Chang, 2005), and hence might be a good addition to enzyme industry with less processing costs.

ACKNOWLEDGEMENT

We are highly grateful to Dr Joachim Schindler (Germany) for technical support and guidance for this work.

REFERENCES

Al-Ajlani MM, Sheikh AM, Ahmad Z, Hasnain S (2007). Production of

surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. Microb. Cell. Fact. 6: 17.
Beg QK, Gupta R (2003). Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis.* Enzyme. Microb. Technol. 32: 294-304.
Dayanandan A, Kanagaraj J, Sounderraj L, Rajkumar GS (2003). Application of an alkaline protease in leather processing: an eco-friendly approach. J. Cleaner. Prod. 11: 533–536.
Freddi G, Mossotti R, Innocenti R (2003). Degumming of silk fabric with several proteases. J. Biotechnol. 106:101–112.
Gessesse A, Gashe BA (1997). Production of alkaline protease by alkaliphilic bacteria isolated from an alkaline soda lake. Biotechnol, Lett. 19: 479-481.
Gibbs BF, Zougman A, Masse R, Mulligan C (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy fermented food. Food. Res. Int. 37:123–131.
Gupta R, Beg QK, Lorenz P (2002). Bacterial alkaline proteases molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59:15–32.
Hasnain S, Thomas CM (1996). Two related rolling circle replication plasmids from salt tolerant bacteria. Plasmid. 36:191-199.
Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2002). Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process. Biochem. 38:155–159.
Joo HS, Chang CS (2005). Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: Optimization and some properties. Process. Biochem. 40: 1263–1270.
Kirk O, Borchert TV, Fuglsang CC (2002). Industrial enzyme applications. Curr. Opinions. Biotechnol. 13: 345–351.
Kunitz M (1947). Crystalline soybean trypsin inhibitor. Part II. General properties. J. Gen. Physiol. 30: 291–310.
Manachini PL, Fortina MG (1998). Production in sea-water of thermostable alkaline proteases by a halo tolerant strain of *Bacillus licheniformis*. Biotechnol. Lett. 20: 565-568.
Masui A, Yasuda M, Fujiwara N, Takagi M, Ishikawa H (2004). Enzymatic hydrolysis of gelatin layers on used lith film using thermostable alkaline protease for recovery of silver and PET film. Biotechnol. Prog. 20: 1267–1269.