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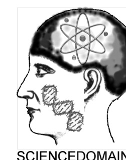

Proteases Production by a Bacterial Isolate *Bacillus amyloliquefaciens* 35s Obtained from Soil of the Nile Delta of Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present work was designed to investigate the production of proteases from an Egyptian bacterial isolate.
Study Design: Samples were collected from soil, water and food, and screened for the isolation of proteases producing bacteria. The highest proteases producing isolate was selected for proteases production.
Place and Duration of Study: Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, between March 2014 and September 2014.
Methodology: Twenty samples were obtained. Primary screening of proteolytic bacteria was done by inoculating plates of skim milk agar. Secondary screening of proteolytic bacteria was done by quantitative determination of proteases concentration. One isolate from soil was chosen due its high proteolytic activity. Identification of the selected isolated was done 16S rRNA gene sequencing. Optimization of proteases production was conducted using a two-step approach. First, a quick identification of the important factors by simple screening experiments (this manuscript)

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including inoculum size, incubation period, carbon and nitrogen sources, temperature and pH. In a subsequent manuscript, application of complex response surface methodology for further optimization will be studied. Data generated from the above experiments were analyzed using one-way ANOVA with post hoc multiple comparison analysis performed using Tukey's HSD.
Results: In the first optimization step, the production of extracellular proteases was favored in the presence of starch, and peptone. A 2.1 fold increase in proteases production was obtained using the design space in the optimized medium as compared with the un-optimized basal medium. Enzyme production increased significantly with optimized medium (845 U/ml) compared to un-optimized medium (405 U/ml). Optimum production conditions were composed of incubation period of 24 h at 37ºC, pH 8 and agitation speeds of 120 rpm. Optimum medium for proteases production was composed of (g/l): peptone (10), starch (10), KH_2PO_4, $MgSO_4.7H_2O$, and $CaCl_2$. Interaction between the variables and optimizing these variables will be studied in a later manuscript using a Plackett-Burman design and further with the Face Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM).
Conclusion: This work succeeded to obtain a novel bacterial isolate capable of producing proteases and the production process was optimized by screening the physical and nutritional parameters influencing proteases production by employing one-variable-at-time approach.

Keywords: Proteases production; optimization; identification; Bacillus amyloliquefaciens.

1. INTRODUCTION

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Proteases dominate the worldwide enzyme market, accounting for a two-third share of the detergent industry [1]. They can be produced in large quantities in relatively short time by established fermentation methods and produce an abundant, regular supply of the desired product [2]. Members of the genus *Bacillus*, from many different environments, have been explored and exploited for proteases production. Some of the most potent proteases producing bacilli strains are *Bacillus licheniformis*, *B. subtilis*, *B.amyloliquefaciens* and *B. mojavensis*. Proteases are largely produced during stationary phase and thus they are generally regulated by carbon and nitrogen stress, also strongly influenced by media components, such as variation in C/N ratio, presence of some easily metabolized sugars, such as glucose [3]. Several other physical factors, such as temperature, pH, and agitation speed inoculum size and incubation period have been also observed to influence proteases production.

The optimization of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation [4]. Generally, no defined medium has been established for the best production of proteases from different microbial sources [5]. Glucose and lactose were reported as effective carbon sources for proteases production in some strains of *Bacillus* [6]. There are few reports where glucose was found to enhance proteases production [7]. Though sugars like maltose, sucrose and lactose have been found to increase proteases production, repression in enzyme synthesis was observed with these ingredients at high concentrations [4].

Alkaline proteases comprise 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen sources in the medium [8]. At the same time there are reports that the use of sodium nitrate [9] had resulted in an increase or stimulation in proteases production. Contradictory to this are the observations of Rao et al. [10], who found organic nitrogen sources better than inorganic ones for proteases production.

A study by Frankena et al. [11] showed that a link existed between enzyme synthesis and energy metabolism in bacteria, which was controlled by temperature and oxygen uptake. Temperature as high as 55ºC was found optimum for production of thermostable proteases from thermophilic and alkaliphilic *Bacillus* sp. [12], whereas maximum production of proteases by *B. licheniformis* MZK 03 was achieved at 37°C [13]. Several studies reported maximum proteases production is often in the mesophilic range [14].

Media pH strongly affects many enzymatic processes and transportation of various components across the cell membrane. pH as

high as 10.5 has been found ideal for proteases production by *Vibrio metschnikovii* DL 33-51 [15] while pH 7 was found optimal for *V. fluvialis* VM 10 [16]. Abu-Sayem et al. [13] reported that pH of 8.5 was the most efficient pH for proteases production by *B. licheniformis* MZK 03. Neutral and alkaline proteases production is often reported in the neutral to alkaline range which ranges from 7 to 11, e.g. serine proteases [14].

The growth of microorganisms and thus enzyme production are significantly affected by agitation and aeration [17]. For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation. Therefore, it is essential to establish optimum combination of air flow and agitation for maximum yield [18]. Agitation rate required for maximum proteases production varies with the organism used. Agitation rates that have been found optimal for proteases production from bacteria such as *V. fluvialis* VM 10, *Pseudomonas aeruginosa*, *B. cereus* and *Bacillus* sp.103 were 100, 140, 200 and 250 rpm respectively [16]. Abu-Sayem et al. [13] found that agitation rate of 120 rpm has been found to be optimal for production of proteases from *Bacillus* sp. RKY3.

The classical method of experimental optimization involves changing one variable at a time keeping the others constant, helps to firstly predict and optimize the major factors to influence a fermentation process prior to the application of a statistical design of optimization [19].

The scope of this work was to optimize the environmental and nutritional requirements influencing microbial production of proteases using one variable at time approach produced by bacteria isolated from natural habitats of delta Egypt. The produced proteases will be later (in a subsequent manuscript) purified and used in various applications.

2. MATERIALS AND METHODS

2.1 Microorganisms

Bacillus subtilis NRRL14, used as a reference strain, was obtained from Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. All of the other proteases producing isolates mentioned in this study were isolated from different sources at Faculty of Agriculture, Ain Shams University, Cairo, Egypt. All cultures were maintained on nutrient agar slants (med.1) at 4ºC and sub-cultured at monthly intervals.

2.2 Media Used

2.2.1 Maintenance medium

Med. 1 (Nutrient agar medium, [20]) was used for stock cultures maintenance of proteolytic bacterial isolates and inoculums preparation. It has the following composition (g /l of distilled water): meat extract 3, peptone 5, pH 7.

2.2.2 Screening medium

Med. 2 (Skim milk agar [21]) was used for screening of proteolytic bacterial isolates based on the coagulation and proteolysis of casein. It has the following composition (g/l of distilled water): meat extract 3, peptone 5, skim milk 100, agar 20, and pH 7.0.

2.2.3 Proteases production media

All the following media were adjusted at pH 7:

Medium 3. [22] (g/l): Gelatin 15, glycerol 3 ml/l, $MgSO_4$ 1.12. Medium 4. L.B. medium [23] (g/l): Peptone 10, yeast extract 5, NaCl 5.Medium 5. [24] (g/l): Glucose 10, casein 5, yeast extract 5. Medium 6. Tryptone yeast extarct gluscose, (TGY) [25] (g/l): Yeast extract 5, tryptone 10, glucose 10. Medium 7. [22] (g/l): Gelatin 30, casein 5, glycerol 200 ml/l. Medium 8. [26] (g/l): Yeast extract 5, casein 1, K_2HPO_4 0.2, Na_2CO_3 0.12. Medium 9. [20] (g/l): Tryptone 5, yeast extract 15, NaCl 5.Medium 10. [26] (g/l): Glucose 10, peptone 5.0, yeast extract 5.0, $MgSO_4$ 0.1, K_2HPO_4 0.1.

2.3 Sampling and Isolation of Proteolytic Bacteria

For isolation of proteolytic bacteria, twenty samples were collected from soil, water and food products in Qalyobia Governorate (located in the Delta of the Nile north of Cairo). Soil samples (grass lawn) were collected at 20 cm depth using pre-sterilized plastic scoops, placed into sterile plastic bags and stored in ice boxes during transport to the laboratory. Water samples were collected in pre-sterilized bottles from tap water after cleaning and disinfecting the

tap outlets using 1% hypochlorite solution. Tap was turned on to a steady stream and let run for five minutes before collecting samples. Food samples (cheese and minced meat) were collected in pre-sterilized plastic bags and stored in ice boxes during transport to the laboratory. At the laboratory, all samples were kept refrigerated until further study. Collected soil or food samples were thoroughly mixed to ensure consistency.

2.4 Screening of Proteases Producing Isolates

Soil and food samples were suspended in sterilized water at a constant ratio of (1 part sample: 9 part sterilized waster) and used for the screening process. Primary screening of proteolytic bacteria was done by inoculating plates of skim milk agar (med. 2) with 1 ml from the suspended sample and plates were incubated at 37ºC for 24h. Formation of clear zones around the colony indicated hydrolysis of skim milk and thus production of proteases enzymes. Depending on the zone of clearness; isolates were selected for secondary screening.

Secondary screening of proteolytic bacteria was done by quantitative determination of proteases concentration (U/ml) (as described later) by inoculating 50 ml of TGY in 250 ml Erlenmeyer flasks with 5 ml of 24h culture of the selected isolates from the primary screening. Inocula of the selected isolates were prepared by inoculating TGY broth for 24h at 37ºC and agitation speed of 120 rpm. The best isolate which produced the highest proteases concentration was selected to run all the following experiments. The purified bacterial isolates were transferred on nutrient agar (med. 1) slants and then preserved at 4ºC and sub-cultured at monthly intervals as stock cultures.

2.5 Standard Inoculum

Standard inoculum was prepared by inoculating 50 ml of nutrient broth medium in 250 ml Erlenmeyer flasks with a loop of tested culture. The inoculated flask was incubated on a rotary shaking incubator (Lab-line Ltd.) at the rate of 120 rpm for 24h at 30ºC and considered as the standard inoculum (7.0 x 10^5/ml viable cells) for shake flasks and bioreactor experiments.

2.6 16S rRNA Gene Sequencing and Analysis

Pure culture of the selected isolate from secondary screening was grown overnight on nutrient broth. The DNA was isolated using Cell Lysis method. 16S rDNA was amplified by Thermocycler (PTC-100 TM Programmable Thermal Controller) using the following primers, Forward: 5’-AGAGTTTGATCCTGGCTCAG-3’ and Reverse: 5’-TACCTTGTTACGACTT-3’. The amplified 16S rDNA PCR product was sequenced using automated sequencer (Synergy scientific, Chennai). Sequence similarity for the 16S rDNA sequence was done using BLAST online service (http://www.ncbi.nlm. nih.gov/ blast/). The unknown organism was identified using the maximum aligned sequence through BLAST search [27]. The acquired sequences were used for a gene homology search, with the 16S rRNA sequences available in the public databases from BLAST http://www.ncbi.nlm.nih.gov/ BLAST/, NCBI, Bethesda, MD, USA), and were identified to the generic level. Using the CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France), the 16S rRNA sequences of the isolated strains were aligned with sequences of related organisms obtained from Gen Bank. Phylogenetic analysis was performed with PHYLIP, and a phylogenetic tree was constructed via the neighbor-joining method using the “TreeView” program. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

2.7 Optimization of Proteases Production

The most efficient proteases production isolate obtained from the secondary screening, was used in optimization experiments and thereafter, which were all run in triplicates and average response was taken. TGY medium (med. 6) was used as the basal medium for all experiments. Unless otherwise mentioned, for all the following experiments, 50 ml of tested medium were placed in 250 ml Erlenmeyer flasks, and after sterilization, flasks were inoculated with 5 ml of 24h culture of standard inoculum. All media were adjusted to pH 7.0 and flasks were incubated at 30°C in a rotary shaking incubator at 120 rpm. Samples of 10 ml were taken after 24h of incubation and used for determination of biomass formation and proteases concentration as described later. Optimized factors were adjusted continuously for ongoing experiments.

2.8 Screening of Factors Affecting the Production of Proteases by One-variable at-a-Time Approach

2.8.1 Effect of different proteases production media

Eight broth media (med. 2 to med. 9) were tested to select the most suitable media for proteases production.

2.8.2 Effect of inoculum size

To determine the optimum inoculum size that gives the highest proteases production, 250 ml Erlenmeyer flasks containing 50 ml of TGY broth were inoculated with different inoculum sizes (1, 3, 5 control, and 7 ml). Depending on the results obtained from this test, the inoculum size was regulated in the following experiments.

2.8.3 Effect of incubation period

To determine the optimum incubation period for proteases production, proteases were produced under the conditions mentioned previously. Ten ml sample was taken at 2h intervals for the determination of cells mass and proteases production.

2.8.4 Nutritional factors

2.8.4.1 Carbon sources

This experiment was conducted to study the effect of different carbon sources on proteases production. Glucose, as the main carbon source, was replaced by one of nine different sources of carbon, i.e. starch, mannitol, glycerol, sorbitol, maltose, lactose, sucrose, mannose or fructose and glucose was used as the control treatment. The amount of tested carbon source added to TGY medium was equivalent to its respective carbon content in glucose as the control. The pH of the medium was adjusted to 7.0 using 1 N sodium hydroxide solution or 1 N hydrochloric acid before autoclaving. Carbon sources, except for monosaccharides, were separately sterilized by filtration and added aseptically to the respective sterilized medium prior to inoculation. Flasks were inoculated and incubated as described before. After determining the most efficient carbon source, concentration levels (0, 8, 10 control and 15 g/l) were tested under the same previously mentioned conditions.

2.8.4.2 Nitrogen sources

To test the effect of nitrogen source on proteases production, 13 sources of nitrogen were tested using TGY basal medium (med. 6) by the selected isolate. Tested nitrogen sources were casamino acids, soybean meal, peptone, gelatin, tryptone, urea, yeast extract, casein, sodium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate and potassium nitrate. These sources were added as concentration so that they all have the same equivalent amount of total nitrogen concentration, which was 14% w/w nitrogen as in peptone. The range of each medium component was decided based on the experimental results of the first step optimization done using the traditional “one variable at a time” approach. To determine the most efficient concentration of nitrogen source, concentration levels (0, 5, 10 control and 15 g/l) were conducted.

2.8.5 Physical factors

To detect the optimum temperature for microbial growth and proteases production, TGY (med. 6) basal medium was inoculated and incubated at 25, 30, 37 and 40ºC in a rotary shaking incubator (Lab-line Ltd.) at 120 rpm. To study the effect of medium pH on proteases production, five levels of initial pH values of TGY (med. 6) basal medium, ranging from 5 to 9 adjusted by a pH meter (HANNA), were chosen to study their effects on proteases production by selected bacterial isolate. To study the effect of shaking level on proteases production, five agitation speeds ranged from 0 to 200 rpm were applied.

2.9 Extraction and Determination of Biomass and Proteases Concentration

For the extraction of crude proteases, the fermented medium was centrifuged at 10000 rpm for 10 min at 4ºC to obtain proteases rich broth. Cell pellet was collected for cells dry weight determination. The supernatant was preserved at 4ºC for analysis and the assay was done within 24h [13]. The total protein content of different enzyme preparations was determined by the method of [28]. Proteases production was determined according to the modified method of Anson [29].

2.10 Proteases Production Parameters

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$.

Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1}) = Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1}) [30].

2.11 Statistical Analysis

Data generated from the above experiments were analyzed using one-way ANOVA with post hoc multiple comparison analysis performed using Tukey's HSD. In shake flask experiments, mean of three replicates were compared using SPSS 16.0 for windows at a significance level of $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Proteases Producing isolates

From samples of soil, water and food (dairy and meat products), 40 proteases producing isolates were obtained on skim milk agar medium (med. 2). Out of these isolates, 11 were selected based on the diameter of their clear zone. The selected isolates were 4s, 5s, 10s, 33s, 34s, 35s, 39s, 41s, 15w, 16w and 19w with diameter of hydrolysis zone ranged from 3.5 to 4.5 cm diameter as illustrated in (Table 1) and (Fig. 1). Based on quantitative determination of proteases production by the selected 11 isolates, one isolate (35s) was selected regarding its highest proteases production (454 U/ml) as shown in Fig. 2.

3.2 Identification of the Selected Isolate

Based on morphological characteristics, Gram staining, endospore formation and further confirmation by sequencing the 16S rRNA gene, the isolate 35s was identified as *Bacillus amyloliquefaciens* 35s [27]. Upon the amplification of 16S rRNA sequence, using specific primer, an amplified product of 1500bp was obtained, sequenced and compared with the Gen Bank data bases using BLASTN software by the Finch TV program (http://www.geospiza.com/products/finchtv.shtml) as shown in (Fig. 3). The 16S rRNA sequence of the isolate revealed a close relatedness to *B. amyloliquefaciens* with 95% similarity. The phylogenetic analysis of nucleotide sequences on the basis of 16s rRNA by revealed most closely to *B. amyloliquefaciens* 35s (Fig. 4). Hence the strain was confirmed as *B. amyloliquefaciens* 35s, and the sequence was submitted to GenBank. Amplified product was subjected to DNA sequencing with automatic sequencer.

Table 1. Screening of proteolytic gram positive bacteria on skim milk agar (med. 2) depending on diameter of hydrolysis zone

Shape morphology and endospore formation	Isolate number	Diameter zone (cm)	Isolate number	Diameter zone (cm)
Long rods endospore formers	1s	3.1	40s	2.0
	2s	3.3	41s*	3.8
	3s	3.1	42s	2.6
	4s*	3.5	42s	2.9
	5s*	4.0	43s	3.0
	6s	1.9	14w	3.2
	10s*	3.9	15w*	4.0
	13s	3.0	16w*	4.0
	14s	2.1	17w	3.1
	30s	4.0	18w	2.9
	31s	3.1	19w*	4.0
	32s	1.7	21w	3.7
	33s*	4.0	22w	2.0
	34s*	4.2	23w	3.0
	35s**	4.5	1f	3.1
	36s	1.8	2f	3.6
	36s	3.0	3f	3.1
Non sporulatedcocci	37s	3.0	4f	2.7
	38s	3.5	5f	1.6
	39s*	4.2	6f	3.2

S: Refers to isolates obtained from soil samples,W: Refers to isolates obtained from water samples.
*F: Refers to isolates obtained from food samples, *: The selected 11 isolates according to their highest zone of casein hydrolysis, **: The most efficient proteases producing isolate*

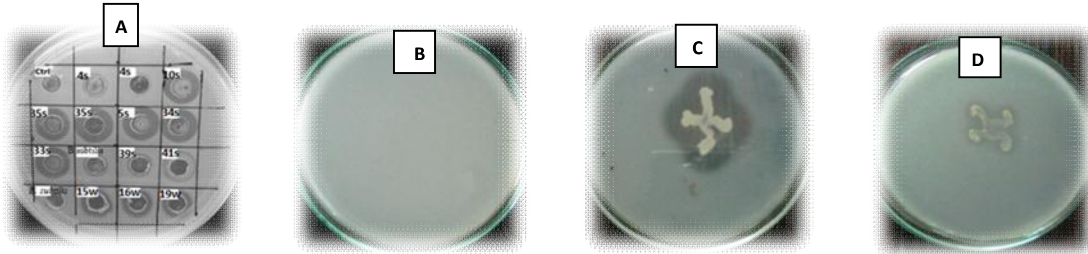

Fig. 1. Screening for proteases production from 11 selected proteolytic bacterial isolates on 10% (w/v) skimmed-milk agar plates; A) Extracts from 11 selected bacterial proteolytic isolates; B) Control plate showing negative hydrolysis; C) Hydrolysis of skim milk by strain 35s; D) hydrolysis of skim milk by *B. subtilis*NRRL14

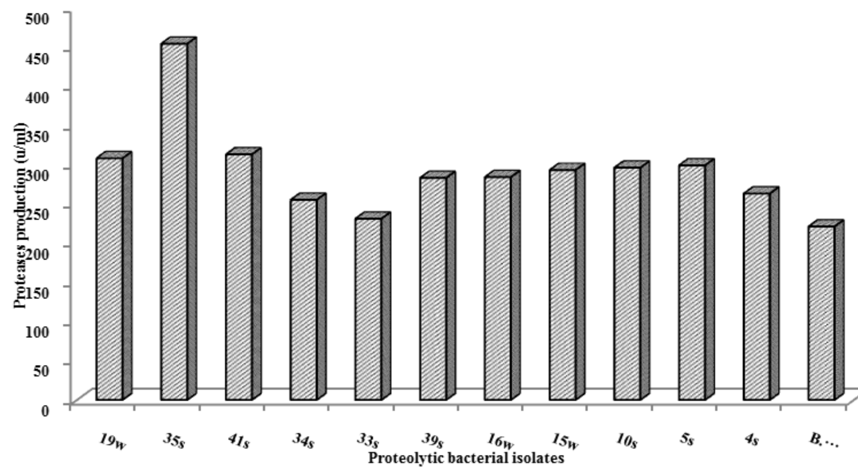

Fig. 2. Quantitative determination of proteases production by selected eleven proteolytic bacterial isolates in TGY (med. 6) liquid medium

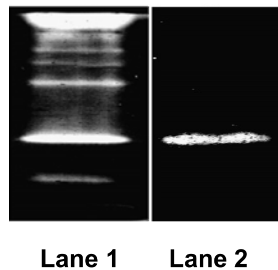

Fig. 3. Photographic representation of PCR amplified product in agarose gel (Lane 1, 1500 bp ladder; Lane 2, PCR amplified sample)

3.3 Production of Proteases

3.3.1 Effect of different media

TGY (med. 6) medium was used as the basal medium to study the effect of nutritional and physical factors on proteases production by *B. amyloliquefaciens* 35s in screening experiments. This experiment was carried out to study the effect of different media on proteases production. In view of available literature, eight different media were selected to carry out this study (Table 2). TGY (med. 6) medium was the most suitable medium for proteases production reached 432 U/ml, productivity of 18 $uml^{-1}h^{-1}$, biomass of 0.81 g/l and proteases yield coefficient relative to biomass of 533 (u/g), while medium (5), containing glucose, casein and yeast extract, showed the lowest production and productivity of proteases, reaching 191 u/g and 4.79 $uml^{-1}h^{-1}$, respectively. In a similar study, Wretlind and Wadstrom [25] found that *Pseudomonas aeruginosa* strain PAKS-I produced proteases highest activity (20 to 30 uml^{-1}) on TYG medium which was chosen as a production medium since all three types of proteases including neutral, alkaline and acidic proteases were produced in this medium. Thus, TGY (med. 6) medium was used as the basal medium for further studies.

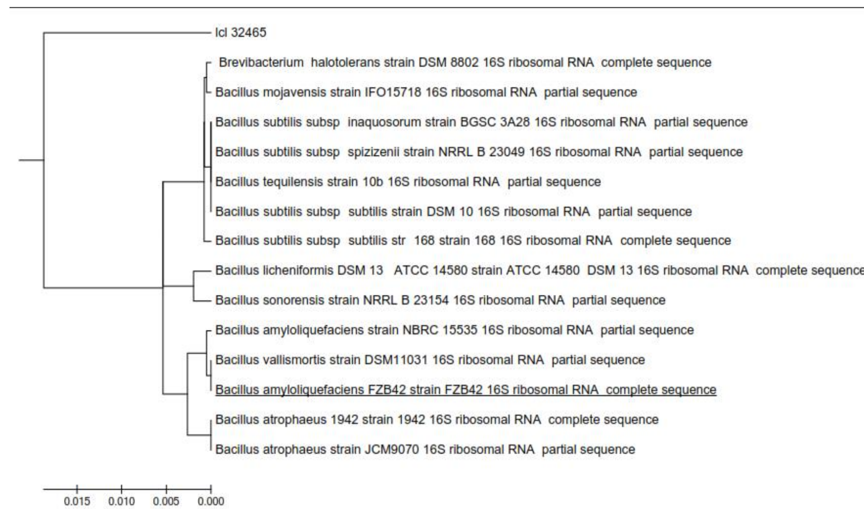

Fig. 4.Phylogenetic analysis of 16S rRNA genes of the strain *B. amyloliquefaciens FZB42* strain; It was shown as underlined. This tree was constructed by crustal method using BLAST software. The origins and Gen Bank accession numbers are as follows: *Brevibacterium halotolerans* strain DSM 8802, *B. subtilis* subsp. *subtilis* str.168, *B. amyloliquefaciens* FZB42 strain, *B. subtilis* subsp. *inaquosorum* strain BGSC 3A28, *B. tequilensis* strain 10b, *B. subtilis* subsp. *subtilis* strain DSM 10, *B. vallismortis* strain DSM11031, *B. mojavensis* strain IFO15718, *B. subtilis* subsp. s*pizizenii*strain NRRL B-23049, *B. amyloliquefaciens* strain NBRC 15535, *B. atrophaeus* 1942 strain 1942, *B. atrophaeus* strain JCM9070, *B. licheniformis* DSM 13 = ATCC 1458: DSM 13, *B. sonorensis* NRRL B-23154

Table 2. Effect of different media on proteases and biomass production by *B. amyloliquefaciens* 35s after 24 h of incubation at 30ºC using shake flasks as a batch culture

Medium number	Biomass (g/l)	Proteases production (U/ml)	Productivity ($uml^{-1}h^{-1}$)	Proteases yield coefficient relative to biomass (u/g)	Medium Ref.
3	0.75	210	8.75	280	[22]
4	0.713	350	14.5	490	[23]
5	0.6	115	4.79	191	[24]
6	0.81	432	18	533	[25]
7	0.78	215	8.95	275	[22]
8	0.75	219	9.125	292	[26]
9	0.916	290	12.08	316	[20]
10	0.935	385	16.04	411	[26]

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$.
Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1})= Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1})

3.3.2 Effect of inoculum size

The inoculum size (ranged from 1 to 7% v/v) was found to affect proteases production by *B. amyloliquefaciens* 35s on TGY medium as the productive medium (Table 3). High inoculum size 7% v/v decreased proteases production by 8% comparing with 5% v/v as the control in which, 5% v/v was the optimal inoculum size for proteases production, achieving the highest proteases concentration, productivity, biomass formation (578.7 U/ml, 24 $uml^{-1}h^{-1}$,1.95 g/l respectively) and proteases yield coefficient relative to biomass of 296.8 (u/g). Thus, 5% v/v inoculum size was applied for the rest of experiments. In similar study, Borah et al. [31] found that 5% v/v inoculum size gave higher proteases production (6500 U/ml) than that given by 1% v/v inoculum size (3800 U/ml). Mabrouk et al. [6] found that the maximum production of proteases by *B. licheniformis* ATCC 21415 was achieved with an inoculum size of 5% v/v, and a

higher inoculum of 11% v/v gave less proteases than a lower inoculum size of 1% v/v did. Therefore, high inoculum sizes do not necessarily give higher proteases yield. An explanation could be due to the higher surface area to volume ratio, which resulted in the increased production of proteases [32]. In addition, an improved distribution of dissolve oxygen and more effective uptake of nutrient also contributed to a higher proteases production. If the inoculum sizes are too small, insufficient number of bacteria would then lead to reduce the amount of secreted proteases [33].

3.3.3 Effect of incubation period

This experiment was carried out to investigate the optimum incubation period for proteases production by *B. amyloliquefaciens* 35s. TGY (med. 6) basal medium was inoculated and incubated at 30ºC for 48 h. *B. amyloliquefaciens* 35s exhibited the maximum proteases production (460 U/ml), productivity of 19.16 ($uml^{-1}h^{-1}$) and biomass of 2.5 (g/l) after 24h of incubation. The production of the enzyme decreased after 30h of incubation as illustrated in Fig. 5. Similar studies done by Soundra et al. [34] reported that optimum incubation period for maximum proteases production by *Bacillus sp.* isolated from soil sample was 24h, while, Borah et al. [31] found that 48h was the optimum incubation period for proteases production by *B. altitudinis.*

3.4 Optimization of Proteases Production

Proteases production optimization was carried out using a two-stage strategy. In the first stage, screening of carbon and nitrogen sources, physical factors and statistical screening of physical factors of proteases production by *B. amyloliquefaciens* 35s was done. In the second stage, interaction among factors was studied subsequently in Plackett-Burman and multi-factorial response surface approach of Face Centered Central Composite Design (FCCCD) (data of the second stage optimization will be published latterly). Multi-factorial response surface approach is a quick and effective tool to study the effect of both the primary factors and their mutual interactions on extracellular proteases production for optimization process [35].

Table 3. Effect of inoculum size on proteases and biomass production by *B. amyloliquefaciens* 35s on TGY medium after 24h of incubation at 30ºC using shake flasks as a batch culture

Inoculum size (% v/v)	Biomass (g/l)	Proteases production (U/ml)	Productivity ($uml^{-1}h^{-1}$)	Proteases yield coefficient relative to biomass (u/g)
1	1.5	$415.\pm1.15^{b}$	17.3	277
3	1.75	450.8 ± 0.60^{c}	18.75	257.6
5 (control)	1.95	578.7 ± 0.50^{d}	24	296.8
7	2.3	530.7 ± 0.37^{a}	22	230.8

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$.
Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1})= Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1}), Values with same superscripts did not vary significantly according to Tukey test at 5% level.
The values are mean of three replicates±standard deviation

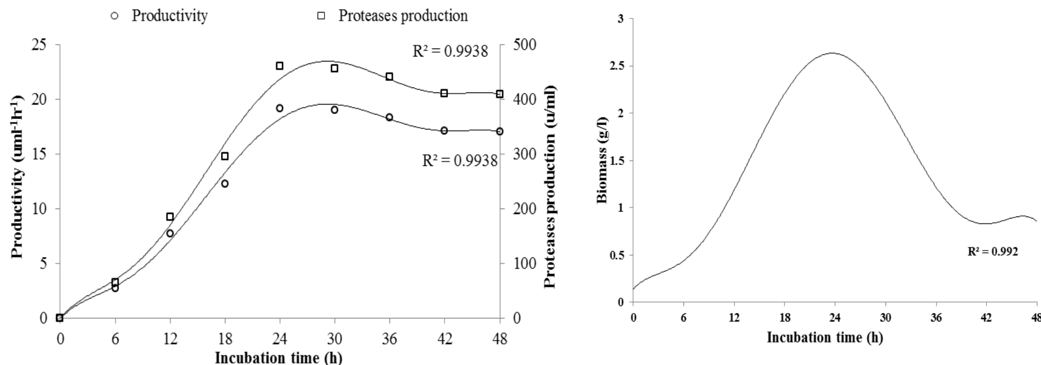

Fig. 5. Effect of incubation period on proteases and biomass production by *B. amyloliquefaciens* 35s on TGY medium during 48 h of incubation period at 30°C

3.5 Screening of Factors Affecting the Production of Proteases by One-variable-at-a-time Approach

3.5.1 Nutritional factors

3.5.1.1 Carbon and nitrogen sources

Ten carbon sources and thirteen nitrogen sources were tested for proteases production by *B. amyloliquefaciens* 35s on TGY medium to select the most efficient carbon and nitrogen source for proteases production. Glucose and tryptone in the previously reported medium were replaced one-by-one with different carbon or nitrogen sources at their respective equivalent concentrations, considering 40% carbon contenting lucose and 14% nitrogen content in tryptone.

B. amyloliquefaciens 35s produced maximum proteases in the presence of starch (454 U/ml) with productivity of 18.9 ($uml^{-1}h^{-1}$) and proteases yield coefficient relative to biomass of 181.6 (U/g), followed by sorbitol to record proteases concentration of (420 U/ml), mannitol (403.5 U/ml) and glycerol (377 U/ml), as shown in Table 4. The readily assailable simple carbon sources, e.g., lactose, sucrose, glucose, mannose and fructose lead to less proteases production as, 223, 236, 247, 218, 203 U/ml, respectively. Starch increased the proteases production by 1.83 fold over that obtained by glucose as control. Similar findings were reported by Chauhan and Gupta [36], where *Bacillus* sp. produced maximum proteases in the presence of starch (1265 U/ml) followed by mannitol (1113 U/ml), maltose (1086 U/ml) and glycerol (970 U/ml). Also, Akhavan and Jabalameli [37] found that starch and maltose were the best substrates for proteases production while other sugars such as fructose, glucose, and sucrose could not influence production of proteases.

With regards to nitrogen sources, complex organic nitrogen sources induced high proteases production, where peptone gave 474 U/ml, soy bean meal gave 230 U/ml then casamino acid gave 223 U/ml, while simple inorganic sources gave poor proteases production (Table 5). Moreover, peptone induced proteases production 1.88 fold over that obtained by tryptone (control).Soybean meal, as a complex natural nitrogen source, may give non-homogeneous media formulations leading to variability and reduced reproducibility in production experiments. Using of complex organic nitrogen sources, as yeast extract (734 U/ml) and tryptone (484 U/ml) were observed to induce high proteases production while simple inorganic sources decreased the proteases production [38]. Findings of Shikha and Darwal [39] were in agreement with these observations, in which medium containing organic sources in the form of molasses, as carbon source, plus wheat bran as nitrogen sources induced the production of 285 U/ml proteases, which was more than that obtained by molasses only (242 U/ml). However, Mehrotra et al. [40] achieved maximum enzyme production from alkaliphilic *Bacillus* sp. isolate in the presence of glucose (1% w/v) and ammonium chloride (1% w/v) at pH 10.5. Thus, starch and peptone were used as carbon and nitrogen sources in basal medium TGY and were chosen for further optimization.

A closely similar study [41] tested the effect of carbon and nitrogen sources on protease production by *Bacillus amyloliquefaciens* B7 using one-factor-at-a-time experiment, and found that higher level of protease was achieved in the presence of D-fructose (10 g L–1), giving 198.5 U mL–1 of protease. As a nitrogen source, yeast extract at 5 g L–1, induced higher protease production (222.45 U mL–1) compared to peptone, beef extract, ammonium nitrate, diammonium phosphate, ammonium chloride and urea (188.58,106.59, 44.79, 45.33, 52.54, 63.53 U mL–1 respectively).

To optimize the levels of starch and peptone, the first step of optimization (the traditional one-variable-at-a-time approach) was applied to select the most efficient starch and peptone concentrations for proteases production by *B. amyloliquefaciens* 35s in modified TGY medium. Results shown in Table 6illustrate the levels of starch and peptone. Starch, ranged from 8–10 g/l, was selected due to the highest proteases production and productivity which reached 454 U/ml, 18.9 $uml^{-1}h^{-1}$, respectively. When adding 10 g of starch. Peptone, ranged from 5–15 g/l, induced the highest proteases production when added at 10 g/l, giving 474 U/ml and 19.75 $uml^{-1}h^{-1}$ of productivity. These results were in agreement with Shah et al. [42] in which they observed that peptone was found to be the most favorable, followed by meat and yeast extract, for proteases production. They reported that 1% peptone concentration was the optimum concentration for maximum proteases production and that addition of peptone to the fermentation medium shortened the lag period and increased

the exponential period that resulted in enhanced enzyme production.

3.5.2 Physical factors

Different environmental conditions were screened to find the most efficient levels for maximum proteases production by *B. amyloliquefaciens* 35s on modified TGY medium as a basal medium.

3.5.2.1 Effect of incubation temperature and initial pH

Four different temperatures, 25, 30 (control), 37 and 40ºC and five pH values of 5, 6, 7 (control), 8 and 9 were investigated for their influence on proteases production by *B. amyloliquefaciens* 35s. Results (Table 6) indicated that maximum production of proteases were obtained at 37ºC and pH 8 that increased the proteases production to 384.22 U/ml, which is 1.6 fold over that obtained at 30ºC and pH 7 as controls, productivity of 16 $uml^{-1}h^{-1}$, biomass of 2.5 g/l and yield productivity coefficient relative to biomass of 153.6 (u/g).The lowest production was obtained at 25ºC (233 U/ml) and pH 5 (289 U/ml). Similarly, Padmapryia and Williams [43] found that maximum proteases production was obtained by *B. subtilis*at 37°C and pH 7. Borah et al. [31] found that the production of proteases by *B. altitudinis* was not affected by temperature in the range studied (7-45ºC) but proteases production was affected by pH to reach maximum production at pH 9 and minimum production at pH 5. The incubation at 30, 40ºC, was found to decrease the production of proteases, and the optimum temperature for proteases, produced from *B. subtilis*strain 38, was 47ºC [44]. Another study showed that optimum temperature for proteases production by *Bacillus* sp. MIG was 30ºC [45]. Studies by Frankena et al. [11] showed that temperature and oxygen content of the medium can control enzyme synthesis and energy metabolism in bacteria. As for the extra-cellular enzymes, temperature was found to influence their secretion, possibly by changing the physical properties of the cell membrane [32]. On the other hand, a lower growth of *Bacillus* sp. strain at high temperatures could be due to the fact that protein conformation changes or degraded at higher temperatures, and hence, causes a decrease in proteases production [12]. Horikoshi [46] reported that highest proteases production by *Bacillus* sp. strain CR-179 were detected at pH 8, suggesting that *this strain* can be classified as alkaliphilic *Bacilli,* since alkaliphiles are defined as organisms that grow optimally at alkaline pH, i.e. at pH above 8 and some being even capable of growing at pH>11. Also, Das and Prasad [47] reported that pH of 8.0 was the best for proteases production by *B. subtilis*.

3.5.2.2 Effect of agitation speed

The effect of agitation speed (0, 100, 120, 140 and 200) rpm on proteases production was investigated by *B. amyloliquefaciens* 35s. The highest proteases production (384.23 U/ml) was observed by agitation speed of 120 rpm (control), productivity of 16 $uml^{-1}h^{-1}$, biomass of 2.5 g/l and yield coefficient relative to biomass of 153.6 (u/g) as shown in Table 6. Extreme levels of agitation, i.e. zero rpm and 200 rpm, were found to decrease proteases production due to decreasing or increasing aeration very much. Microorganisms vary in their oxygen requirement. In particular, O_2 acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in agitation speed has been found to influence the extent of mixing in shake flasks and also affects the nutrient availability [48]. In an investigation by Akhavan and Jabalameli [37], *Bacillus* sp. strain CR-179 grown in culture media containing starch and corn steep liquor showed maximum proteases production at 150 rpm agitation speed after 24h incubation. At this speed, aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media [4]. Nutrient uptake by bacteria also was increased resulting in increased proteases production [49]. At 180 rpm proteases production was found to be reduced. This was perhaps due to denaturation of enzymes caused by high agitation speed. Also, it could damage bacterial cells, so that reduction of proteases producers resulted in decreased proteases production [50].

The scope of this study was to obtain a bacterial isolate capable of the production of proteases and optimize the physical and nutritional parameters influencing proteases production by employing a two stage optimization process. The first stage includes the optimization by one variable at time approach, while the second includes the statistical approach of Response Surface Methodology (RSM) of Face Centered Central Composite Design (FCCCD) (data of the second stage optimization will be published latterly in a separate manuscript).

Table 4. Screening of different carbon and nitrogen sources on proteases production by *B. amyloliquefaciens* 35s on TGY medium by one variable at a time approach

	Carbon or nitrogen sources	Biomass (g/l)	Proteases production (U/ml)	Productivity ($uml^{-1}h^{-1}$)	Proteases yield coefficient relative to biomass (u/g)
Carbon sources	Starch*	2.5	454 ± 1.1^{i}	18.9	181.6
	Mannitol	2.3	403.5 ± 1.32^{g}	16.8	175.4
	Glycerol	1.45	377 ± 0.72^{f}	15.7	260
	Sorbitol	2.1	420 ± 1.90^{h}	17.5	200
	Maltose	1.35	280 ± 2.62^{e}	11.7	207.4
	Lactose	1.16	223 ± 0.53^{b}	9.25	192.2
	Sucrose	1.19	236 ± 0.85^{c}	9.8	198.3
	Glucose (control)	1.95	247 ± 2.25^{d}	10.3	200.8
	Mannose	1.14	218 ± 2.83^{b}	9.08	191.2
	Fructose	1.1	203 ± 1.12^{a}	8.45	184.5
Nitrogen sources	Casamino acids	1.19	223 ± 0.96^{h}	9.29	187.4
	Soybean meal	1.15	230 ± 0.92^{i}	9.6	200
	Peptone*	2.4	474 ± 0.58^{l}	19.75	197.5
	Gelatine	2.2	128 ± 1.17^{d}	5.33	58.1
	Tryptone (control)	2.3	252 ± 1.06^{j}	10.5	109.5
	Urea	0.95	165 ± 0.75^{f}	6.875	173.7
	Y.E	1.20	271 ± 1.17^{k}	11.29	225.8
	Casein	1.21	153 ± 0.21^{e}	6.375	126.4
	$NaNO_3$	1.17	227 ± 1.11^{i}	9.45	194
	NH_4NO_3	1.15	139 ± 1.90^{g}	5.79	120.8
	NH_4Cl	1.1	107 ± 0.98^{c}	4.45	97.2
	$(NH_4)_2SO_4$	0.96	73 ± 1.60^{b}	3.04	76.04
	KNO_3	0.95	62 ± 1.25^{a}	2.58	65.2

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$, Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1}) = Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1}), Values with same superscripts did not vary significantly according to Tukey test at 5% level, Carbon or nitrogen sources were added to med. TGY as basal medium.

**:The most suitable carbon and nitrogen source for proteases production, The values are mean of three replicates±standard deviation*

Table 5. First step optimization of starch and peptone concentrations and their effect on proteases production by *B. amyloliquefaciens* 35s in one variable at a time approach

Ingredient	Concentration (g/l)	Biomass (g/l)	Proteases production (U/ml)	Productivity ($uml^{-1}h^{-1}$)	Proteases yield coefficient relative to biomass (u/g)
Starch	0	0.65	60 ± 0.26^{a}	2.5	92.30
	8	2.45	420 ± 0.77^{b}	17.5	171.42
	10 (control)	2.5	454 ± 0.45^{c}	18.9	181.6
	15	0.83	63 ± 0.25^{a}	2.65	75.90
Peptone	0	0.96	65 ± 0.26^{a}	2.8	67.70
	5	1.9	220 ± 0.26^{b}	9.16	115.78
	10 (control)	2.4	474 ± 0.45^{c}	19.75	197.5
	15	2.1	465 ± 0.45^{c}	19.35	221.42

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$.
Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1}) = Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1}),Values with same superscripts did not vary significantly according to Tukey test at 5% level.
Carbon or nitrogen sources were added to medium TGY as basal medium.
The values are mean of three replicates±standard deviation

Table 6. Screening of different physical factors on proteases production by *B. amyloliquefaciens* 35s using one variable at a time approach

Physical factors	levels	Biomass (g/l)	Proteases production (U/ml)	Productivity ($uml^{-1}h^{-1}$)	Proteases yield coefficient relative to biomass (u/g)
Incubation temperature (°C)	25	0.78	$233.02±0.36^d$	9.70	298.7
	30(control)	1.15	$253.49±0.43^c$	10.56	220.4
	37	2.5	$384.22±0.23^a$	16.00	153.6
	40	0.85	$258.5±0.70^b$	10.77	304.16
Initial pH	5	0.55	$298.10±0.65^d$	12.42	542
	6	0.63	$324.37±0.40^c$	13.51	514.8
	7(control)	2.4	$253.49±0.40^b$	14.33	143.4
	8	2.5	$384.23±0.29^a$	16.00	153.6
	9	1.88	$210.30±0.26^e$	8.76	111.8
Agitation speed (rpm)	0*	0.56	$66±1.44^a$	2.75	117.8
	100	1.13	$201±1.5^c$	8.35	177.8
	120(control)	2.5	$384.23±0.62^e$	16.00	153.6
	140	1.47	$255±0.96^d$	10.625	173.4
	200*	0.97	$167±0.42^b$	6.96;l	172.16

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$.
Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1}) = Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1}), Values with same superscripts did not vary significantly according to Tukey test at 5% level.
**Proteases production under extreme agitation conditions.*
The values are mean of three replicates±standard deviation

4. CONCLUSION

Results of this study succeeded to obtain a bacterial isolate capable of producing proteases and production process was optimized by screening the physical and nutritional parameters influencing proteases production by employing one-variable-at-time approach. The optimum conditions for protease production by *Bacillus amyloliquefaciens* isolate 35s are medium; TGY, Inoculum size: 5% v/v, incubation period: 24h, incubation temperature and pH: 30ºC and pH 7, respectively, carbon source: starch (10g/L), nitrogen source: peptone (10g/L) and agitation speed: 120 rpm.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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