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PRODUCTION OF PROTEASE ON WHEAT BRAN BY A NEWLY ISOLATED *STREPTOMYCES* SP. UNDER SOLID STATE FERMENTATION

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Abstract

Proteases are a group of enzymes that catalyse the degradation of proteins resulting in the production of their amino acid constituents. In the present study newly isolated *Streptomyces* sp. was subjected to produce proteases through solid state fermentation while wheat bran was used as substrate. To produce proteases, a local strain *Streptomyces* sp. was isolated from a soil sample of Ezzemouls saltpan, located in Ain M'lila (East of Algeria). The phenotypic and phylogenetic studies of this strain showed that it represents probably a new species. The SSF production medium for *Streptomyces* sp. was optimized using Plackett and Burman statistical methods. The results showed a maximum activity on basal wheat bran medium supplemented with 1% fructose. The best SSF humidifying solution was pH 9.0 phosphate buffer at 50% moisture. Protease has an optimum at pH 7.0, which is a typical characteristic of neutral proteases. The optimum temperature was 60°C and proved stable up to 80°C. The results showed that the novel *Streptomyces* sp. isolate is a good producer of extracellular neutral protease on wheat bran, which can be beneficial for industries.

Key words: Optimization, Protease, Solid state fermentation, *Streptomyces*, Wheat bran

Introduction

Proteases (EC) are the most important industrial enzymes and widespread in nature. Microbial proteases belong to acid, neutral or alkaline based on their pH optimum for activity, active sites and may depend on the composition of the medium. Culture conditions play significant role on growth and production of protease by bacteria (Padmapriya and Christudhas 2012). Although protease production is an inherent capability of all microorganisms, the microbes that produce a substantial amount of extracellular proteases are of industrial importance (Bhunia et al. 2012) that can have application in various fields such as leather processing, food processing, pharmaceutical and bioremediation process (Sankaralingam et al. 2012). Bacterial proteases for industrial uses are produced and studied mainly from *Bacillus* and *Streptomyces* species. The possibility of using *Streptomyces* for protease production has been investigated because of their capacity to secrete the proteases into extra cellular media, which can generally be regarded as safe (Jayasree et al. 2009). Further the use of solid state fermentation (SSF) for production of enzymes and other products has many advantages over submerged fermentation (Lonsane and Ramesh 1990). These advantages include: easier recovery of products, the absence of foam formation and contamination risks can significantly reduce due to the low water contents. Moreover, it permits the use of agricultural and agro-industrial residues, as

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substrates which are converted into products with high commercial value (Lonsane and Ramesh 1990, Pandey 1992). Therefore, the present work aimed to characterise *Streptomyces* sp. and then focus on optimizing the production of extracellular protease in solid state fermentation on wheat bran.

Materials and Methods

Isolation and screening of proteolytic bacterial strain

Soil samples were taken from Ezzemoul saltpan (located in East of Algeria 006º 34'E, 036º 02'N) and used for the isolation of bacteria on M4 medium (Agar (18 g/l, DIFCO, USA), yeast extract (3 g/l, DIFCO, USA), malt extract (3 g/l, DIFCO, USA), peptone (5 g/l, DIFCO, USA) and glucose (10 g/l, DIFCO, USA). A total of 5 isolates of actinobacteria were isolated and purified by streaking on agar plates. The isolates were examined for various morphological and biochemical characteristics as per Bergey's Manual of determinative Bacteriology (1994). Isolated strains were tested for protease production on skim milk agar plates. After 7days of incubation at 30°C, the proteolytic activity was confirmed by clear zone formation around the bacterial growth. The most potential strain was chosen for the rest of the study.

Strain identification

Morphological study

Streptomyces sp. strain colonies were characterized morphologically on different media (ISP2, ISP3, ISP4, ISP5; ISP: International *Streptomyces* Project), Starch casein agar, Glucose asparagine agar, Hickey and Tresner agar) following the directions given by the International *Streptomyces* Project (Shirling and Gottlieb 1966). Cultural characteristics such as growth importance, aerial and substrate mycelium colour and diffusible pigment production, were recorded after incubation for 7, 14 and 21 days at 28°C. Micro-morphological observations were carried out with a light microscope using two different methods: slide culture technique (Zaitlin et al. 2003) and inclined coverslips technique (Williams and Cross 1971, Holt et al. 1994).

Phylogenetic study

Studied strain DNA was extracted using DNA extraction kit (Ultraclean Microbial DNA Isolation (Mol. Biol). 16S rRNA genomic regions were then analyzed using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') as forward and reverse primers, respectively. Amplification was carried out by AB Applied Biosystems Veriti 96 well thermal cycler, using *Taq* DNA polymerase. Polymerase chain reaction (PCR) program was 95°C/10 min for initial denaturing, 95°C/45 s, 56°C/45 s, 72°C/1 min for 36 cycles and 72°C/10 min for final extension. PCR products were electrophorized on agarose gel with Tris Borate EDTA in Embi Tec Runone TM electrophoresis cell, under 100 volts. The gel was finally photographed on a UV Imager Master® VDS (Pharmacia Biotech). Purified products were subjected to 16S rRNA sequencing by automated Sanger method (Sanger et al. 1977) using 1387R primer, at (GATC Biotech AG, Germany) laboratory. The sequences were finally corrected using the software (Sequencer v. 4.1.4, Gene Codes). Sequences were analysed by MEGA 5 software (Tamura et al. 2011) and nBLAST tool at NCBI. The 16S rRNA nucleotide sequence of the isolate was aligned with homologous regions from various actinobacteria, and the phylogenetic tree was constructed by neighbour-joining method (Saitou and Nei 1987). A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained.

Physiological and biochemical studies

The physiological and biochemical characteristics were determined according to the methods of Shirling and Gottlieb (1966), Crawford et al. (1993), Chaphalkar and Dey (1996) and Singleton (1999).

Solid state fermentation (SSF) and enzyme assay

5 g of wheat bran from the mill (Esalaam–JIJEL) were introduced into 250 ml Erlenmeyer flasks then moistened with 10 ml of humidifying solution. The medium was inoculated with 10^6 spores/ ml. Incubation is carried out in a shaking water bath at two temperatures (30°C and 37°C) for two time intervals (2 days and 5 days). After fermentation, the fermented substrate was ground manually and then mixed with 40 ml of buffer solutions. The mixture was homogenised in an agitator for 30 min and then centrifuged at 11000 g for 20 min at 4°C. The clear supernatant was used as crude enzyme. Protease activity was measured following by the methods of Tsuchida et al. (1986).

Optimization of wheat bran based medium for protease production

Optimization of humidifying solution

The influence of the humidifying agent in SSF on protease production was evaluated by the use of three different solutions at neutral pH (distilled water, phosphate buffer, salt solution) for moistening wheat bran. The fermentation was carried out under the same conditions mentioned above. In the second stage, the pH of the humidifying solutions was varied from 7 to 10. Finally, the moisture content of solid fermentation was optimized by incubation of the studied strain at different volumes of the humidifying solution, which correspond to the different moisture levels: 40, 50, 60, 70 and 80%.

Effect of supplementation carbon and nitrogen sources and salts on protease production

The influence of supplementary carbon and nitrogen sources and mineral salts to wheat bran, on production of protease by selected *Streptomyces* sp. strain was investigated following Plackett and Burman design (Plackett and Burman 1946). The 7 independent variables (including 2 dummy variables) were organized in 8 combinations. Each variable was examined at a high level (coded as +1) and a low level (coded as -1). The experimental values of the coded levels are shown in table1. Plackett and Burman (1946) design is based on the first order polynomial model: $Y = \beta 0 + \Sigma \beta i Xi$ where Y is the response (protease enzyme production), βo is the models intercept, βi is the linear coefficient and Xi is the level of the independent variable. All the experiments were carried out in triplicates.

Table 1. Experimental description for the Plackett and Burman design

Factors	Low level (-1)*	High level (+1)*
X1 : glucose	0	1%
X2 : fructose	0	1%
X3 : error	-	-
X4 : casein	0	1%
X5 : gelatin	0	1%
X6 : error	-	-
X7 : NaCl	0	0.1%

* Concentrations in (g/l).

pH and temperature effects on protease activity

For determination of optimum pH of the enzyme, the reaction mixture buffer was varied over the pH range 3.0 to 12.0. Similarly, enzyme production was also monitored at temperature in the range of 20 to 90°C (in increment of 10°C).

Protease thermal stability

The enzyme was incubated at different temperature values (60, 70, 80 and 90°C) for 120 min. The samples were submitted to determination of protease activity every 30 min.

Results and Discussion

Isolation and screening of protease producing strain

The actinobacterial strain (coded SS) was isolated from the soil of Ezzemoul Saltpan under sterile conditions. After purification, the proteolytic activity was confirmed by clear zone formation around the bacterial growth due to the casein hydrolysis.

Identification of protease producing strain

Morphology and cultural characteristics

The studied *Streptomyces* strain has a very good growth on all media tested except for ISP5 medium (Table 2). This microorganism will be classified in the grey series because of the predominance of this colour in the aerial mycelium, it is only the ISP5 medium that gives a greyish white colour of this mycelium. The back of the colony (vegetative mycelium) varied from white, greyish pink to grey. This pigment was intracellular but has diffused on ISP2, ISP7, casein starch and Hickey and Tresner media and was insensitive to pH changes. By studying the morphological properties, it was found that SS strain aerial mycelia formed unfragmented, branched, straight hyphae bearing non motile cylindrical spores. Spores chains were related to Rectiflexible (RF) category and contained up to 40 spores per chain.

Table 2. Cultural characteristics of SS strain

Time (day)	Culture media	Growth characteristics	Pigmentation	
			Substrate mycelium	Aerial mycelium
7th	ISP2	Good	Greyish pink	Grey (dark medium)
	ISP3	Good	Dark grey	Grey
	ISP4	Good	Grey	Grey
	ISP5	Moderate	Light pink	White
	ISP6	Good	White	Light grey
	ISP7	Good	Brown beige	Grey (Grey pink)
	Glucose asparagine	Moderate	Red	Light grey
	Starch casein	Good	White	Grey (dark medium)
	Hickey and Tresner	Good	Dark grey pink	Grey (+pigment)

Contd. Table 2

14th	ISP2	Good	Greyish pink	Grey (dark medium)
	ISP3	Good	Dark grey	Grey
	ISP4	Good	Grey	Grey
	ISP5	Moderate	Light pink	Greyish white
	ISP6	Good	White	Light grey
	ISP7	Good	Beige	Grey with white center
	Glucose asparagine	Good	Grey white	Light grey
	Starch casein	Good	White	Grey (dark medium)
	Hickey and Tresner	Good	Dark grey pink	Dark grey (+pigment)
21th	ISP2	Good	Greyish pink	Grey (dark medium)
	ISP3	Good	Dark grey	Grey
	ISP4	Good	Grey	Grey
	ISP5	Moderate	Light pink	Greyish white
	ISP6	Good	White	Light grey
	ISP7	Good	Beige	Grey with white center
	Glucose asparagine	Good	Grey white	Light grey
	Starch casein	Good	White	Grey (dark medium)
	Hickey and Tresner	Good	Dark grey pink	Dark grey (+pigment)

Molecular identification

16S rRNA gene sequence of SS strain was submitted in the GenBank database with accession number SUB6916285. Comparative analysis of this sequence with the corresponding sequences of other microorganisms obtained from the same database, found 24 most closely related species with a high degree of relatedness. The phylogenetic tree constructed by the Neighbor-Joining distance method is shown in Fig.1, *Streptomyces roseolus* NBRC 12816^T is the closest species occupying the same phylogenetic line with high similitude degree (98.97), 30% bootstrap support and a genetic distance of 0.74. These two last observations assume that they are probably two different species despite the high degree of similarity. It is therefore necessary to use phenotypic characters and DNA-DNA hybridization to confirm.

Streptomyces roseolus NBRC 12816^T = ATCC 23210 was first described by Preobrazhenskaya and Sveshnikova (1957) whose various phenotypic characters were detailed by Shirling and Gottlieb (1968a). According to the phylogenetic classification of Labeda et al. (2012), it is classified in group 43 with 100% bootstrap percentage with *Streptomyces roseosporus* CGMCC 4.1871^T, both belonging to class 5 of the digital taxonomy phenotype of Williams et al. (1983). They have been considered synonymous in the Bergey's Manual of Systematic Bacteriology (Williams et al. 1989).

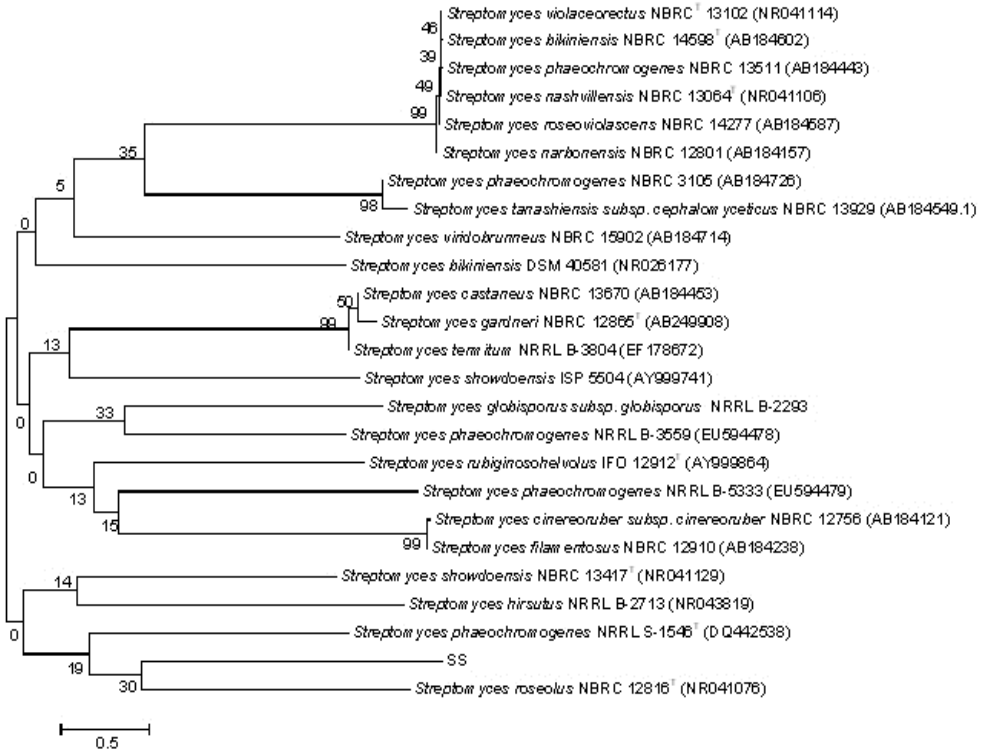

Fig. 1. Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequences showing SS strain and the nearest related taxa. (Numbers at nodes indicate percentages of 1000 bootstrap resampling. Bar equals 0.5 nucleotide substitutions per site).

Physiological and biochemical characteristics

SS strain had assimilated glucose, mannose, xylose, dextrins, galactose, arabinose, maltose and lactose (Table 3). It produced a melanoid pigment on ISP7, degraded starch, gelatin, casein, peptonised milk, reduced nitrates to nitrites and tolerated up to 3% NaCl. For the other characters, our strain did not use citrate, produced neither H_2S nor indole nor tryptophan deaminase but had urease and arginine decarboxylase. It did not develop at extreme temperatures (4°C and 45°C) and showed optimal growth at 37°C and pH 6.0 to 6.5 (Table 3).

Table 3. Physiological and biochemical characteristics of SS strain

Tests	Results
Production of melanoid pigments	
ISP 6	-(1)
ISP 7	+
Hydrolysis	
Starch	+
Gelatin	+
Casein	+
Skimmed milk hydrolysis	
Peptonisation	+
Coagulation	-
Nitrate reduction	+
NaCl tolerance	3 %
Growth at different temperature values	(Dry weight/40 ml)
4°C	-
22°C	+ (27.2 mg)
28°C	+ (42.7 mg)
37°C	+ (155 mg)
45°C	-
Growth at different pH values	
5.5	+ (2)
6.0	+++
6.5	+++
7.0	++
7.5	++
8.0	+
Citrate utilization	-
H_2S production	-
Indole production	-
Tryptophane desaminase test	-
Decarboxylase test	
Arginine	+
Lysine	-
Ornithine	-
Urease test	+

Contd. Table 3

Tests	Results
Degradation	
D - glucose	+
L - rhamnose	-
D - mannitol	-
Mannose	+
D - xylose	+
Dextrine	+
D - galactose	+
Sucrose	-
L - arabinose	+
D - fructose	-
Maltose	+
Lactose	+
Raffinose	-
I - inositol	-
Cellulose	-

(1) For tests: (+): Strain growth or positive test reaction; (-), No growth or negative test reaction, (2) Growth at different pH values: (+++): Good, (++): moderate, (+): poor.

Table 4 reports a comparison of SS strain phenotypic characteristics with the two most closely related species. Our strain had the same type of spore chain (rectiflexible) as the two species (*Streptomyces roseolus, Streptomyces roseosporus*), but aerial and substrate mycelia colours and pigments production were completely different. Also, strain SS differed from the two listed species in the assimilation of rhamnose. These ascertainments were corroborated by the low bootstrap value and the significant genetic distance between SS strain and *Streptomyces roseolus.* Finally, it can be concluded that this strain, isolated from a poorly exploited environment, probably corresponds to a new species. Nevertheless, the DNA-DNA hybridization method must be used with the closely related species to confirm our assumption.

Table 4. Comparison of SS strain phenotypic characteristics with related species of the group 43

Phenotypic characteristics	**SS strain**	***Streptomyces roseolus*** (Shirling & Gottlieb 1968a)	***Streptomyces roseosporus*** (Shirling & Gottlieb 1968b)
Morphology			
Aerial mycelium colour	Light grey to dark grey	Red to yellow	Red, yellow or white
Substrate mycelium colour	Beige to dark grey pink	No pigment	Greyish yellow to yellowish brown
Diffusible pigment	Brown	Absence	Absence
Melanoide pigment	Presence	Absence	Absence
Spore chaine type	RF	RF	RF
Spore number per chain	40	10 à 50	More than 50
Spore form	Cylindrical	Cylindrical	Cylindrical
Hydrolysis			
D-glucose	+	+	+
L-rhamnose	-	+	+
D-mannitol	-	-	-
Mannose	+	nd	nd
D-xylose	+	+	+
Dextrine	+	nd	nd
D-galactose	+	nd	nd
Sucrose	-	-	nd
L-arabinose	+	+	+
D-fructose	-	+/-	+/-
Maltose	+	nd	nd
Lactose	+	nd	nd
Raffinose	-	-	-
I-inositol	-	-	-
Cellulose	-	nd	nd

nd: not determined.

Optimization of wheat bran based medium for protease production

The effect of humidifying solution nature

The addition of buffer solution to wheat bran, gave better protease production, followed by distilled water then salt solution (Fig. 2). This is probably due to the buffering capacity of the solution that was used as a partial pH control system. Indeed, during the culture, bacterial metabolic activity modifies the pH of the medium either by acidifying it (by the production of acids or by the absorption of ammonium ions), or by alkalinizing it (by the release of ammonia from the degradation of proteins, urea or other amines) (Manpreet et al. 2005).

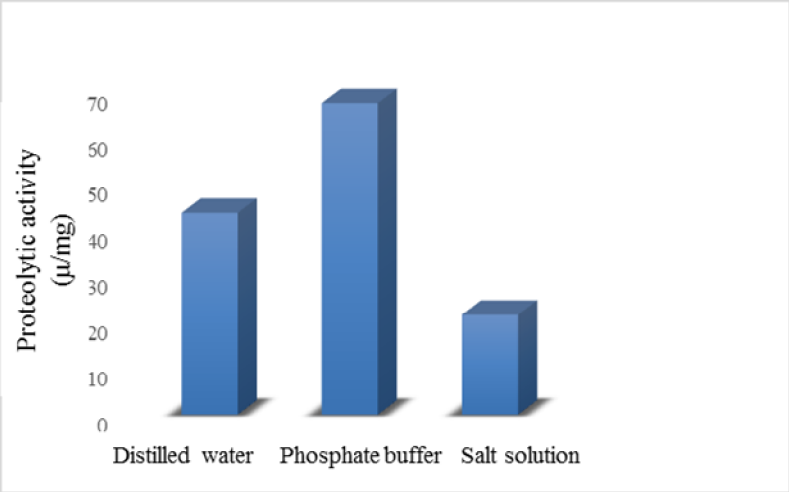

Fig. 2: Effect of humidifying solution nature on protease production in SSF using wheat bran as substrate.

The effect of initial pH buffer solutions

The results of this study revealed that buffer solution at pH 9 added to the wheat bran, has ensured a better protease production (Fig. 3) followed by buffer solution at pH 10. However, a number of reports suggest pH 7.0 - 7.5 to be optimal for protease production by bacteria and fungi. It has been reported that *E. album* BTMFS10 prefers both acidic (pH 4.0 and 5.0) and alkaline (pH 10.0) for protease production. A little acidic pH of 6.0 was found to be optimum for protease production by *P. aeruginosa* PseA. For *B. circulans*, pH 10 has been reported to be suitable for protease production. Protease production by *B. subtilis* remained approximately the same irrespective of the initial pH in the whole range tested (5.0 - 10.0) (Sharma et al. 2017).

In fact, microorganism's metabolic activities are very sensitive to pH variations that affect strongly the different enzymatic processes as well as nutrients transport through the cell membrane (Sandhya et al. 2005, Lazim et al. 2009, Paranthaman et al. 2009). These metabolic activities lead in turn, to a change in the hydrogen ions balance and therefore the pH of the culture medium (Elibol and Moreira 2005).

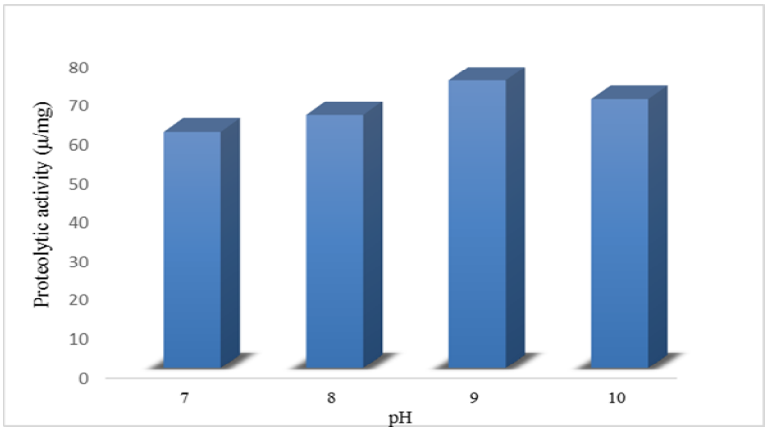

Fig. 3: Effect of initial pH buffer solutions on protease production in SSF using wheat bran as substrate.

The effect of initial moisture content

In SSF, the moisture content is an important factor that influences the growth and product yield of microbes (Ramesh and Lonsane 1990). Moisture was reported to cause swelling of the substrates, thereby facilitating better utilization of the substrate by microorganisms (Kim et al. 1985, Nagendra and Chandrasekharan 1996). The data presented in Fig. 4, indicated clearly a maximum production of the enzymes at 50% moisture. Agrawal et al. (2005) reported 50% of initial moisture content to be best for protease production by *Penicillium* sp. Similar observation has been reported by other workers for *A. oryzae* NRRL 2217, *A. oryzae* CFR305 and *P. aeruginosa* PseA (Sharma et al. 2017). The water content, or rather the amount of water available, is really important since low humidity would limit substrate hydrolysis, solubilisation and nutrient diffusion, while high humidity would reduce porosity (interparticular space), gas volume and gas exchange and also promote bacterial contamination.

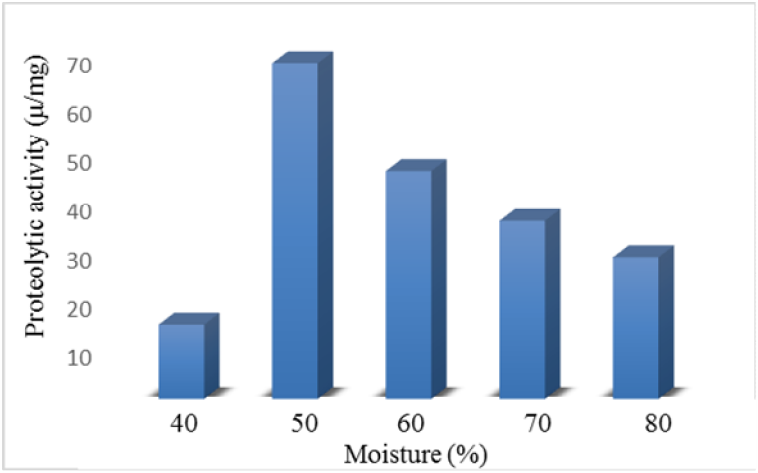

Fig. 4: Effect of moisture % on the production of protease in SSF using wheat bran as substrate.

The effect of supplementation carbon and nitrogen sources and salts on protease production

The statistical analysis of the results, carried out by the Minitab 17 software and presented in Table 5, showed a significant effect of the factor X2 (Fructose) with a Student value of 2.99. It is therefore the only factor having a positive effect on the production of the protease by the strain SS, while the other source of carbon (glucose) and nitrogen sources (casein and gelatin) and NaCl showed no significant effects. Much research has shown that the addition of fructose to the culture medium increases greatly the protease production by certain molds such as *Alternaria* (Vandakini and Shastri 1983) and *Aspergillus* sp. (Radha et al. 2012). Our results also corroborate those of Eman et al. (2012), who demonstrated that carbon sources have a significant effect on protease production by *Streptomyces griseus*. On the other hand, casein which can serve as a substrate for proteolytic enzymes did not show a significant effect on protease production by the strain. This protein is a source of nitrogen which has a low solubility, which gives a low yield of proteolytic activity in solid medium. Several studies of microbial protease production in solid medium have confirmed our results (Jignasha and Satya 2007, Eman et al. 2012, Abdelwahed et al. 2014). In addition, Lazim et al. (2009) showed that the addition of casein in wheat bran solid culture medium did not increase protease production in *Streptomyces* sp.

Table 5. Statistical parameters for Plackett and Burman design (Minitab17 software)

Term	Effect	Coefficient	T value	P value
X_1	0.73	0.36	0.06	0.956
X_2*	35.18	17.59	2.99	0.096
X_4	0.15	0.08	0.01	0.991
X_5	6.19	3.10	0.53	0.651
X_7	2.16	1.08	0.18	0.871

T: Student test, p: probability, *: Statistically significant, R^2 = 65 %. Regression equation: Activity = 57.29 + 0.36 X_1 + 17.59 X_2 + 0.08 X_4 + 3.10 X_5 + 1.08 X_7

The effect of pH on protease activity

According to Fig. 5, an increase in the proteolytic activity from pH 3.0 to pH 7.0 was observed, beyond this value, the activity decreases considerably, which supposes the presence of a neutral protease with an optimum pH of 7.0, these results corroborate with those of Jignasha and Satya (2007) who demonstrated that actinobacteria and mainly those belonging to the genus *Streptomyces* are large producers of neutral proteases.

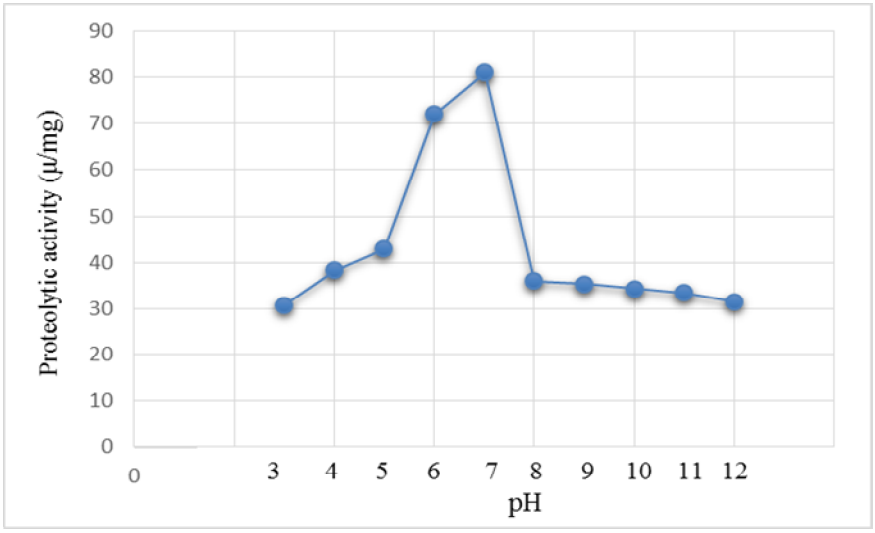

Fig. 5: Effect of pH on protease activity.

The effect of temperature and thermal stability of the enzyme

The enzyme activity increases in the temperature range between 20 to 60°C to reach the maximum activity at 60°C (Fig. 6). Beyond this temperature, the proteolytic activity decreases considerably. At 90°C, the enzyme loses most of its activity. It is important to note that the enzyme has activity over a wide temperature range (significant activity between 40 and 80°C), which is similar to the work reported by Sanchez-Porro et al. (2003), who showed that the *Pseudoaltermonas* sp. protease is active in a temperature range of 25 to 75°C.

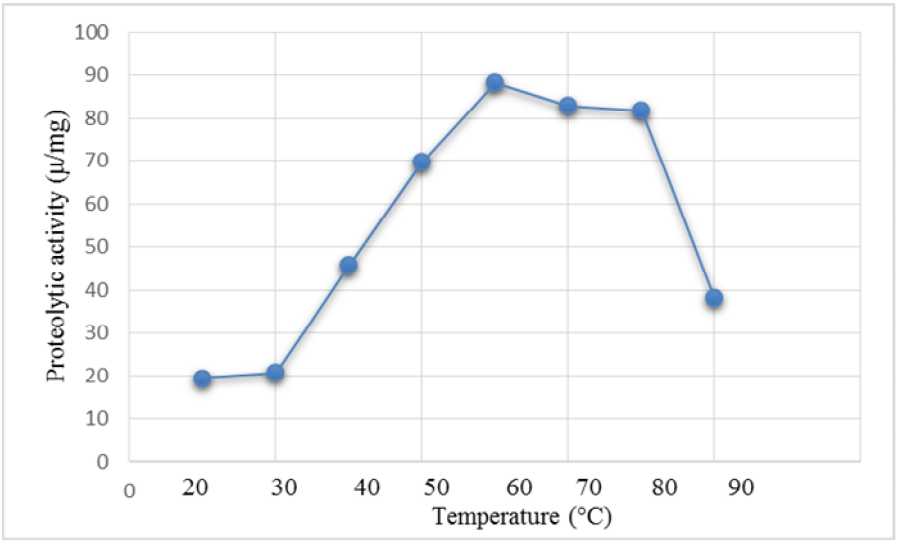

Fig. 6: Effect of temperature on protease activity.

On the other hand, our results showed a significant thermal stability (Fig. 7). Indeed, at 60, 70 and 80°C this enzyme has kept more than 90% of its activity after 120 min. At 90°C the proteolytic activity has decreased considerably but it was persisting even after 90 min of heating.

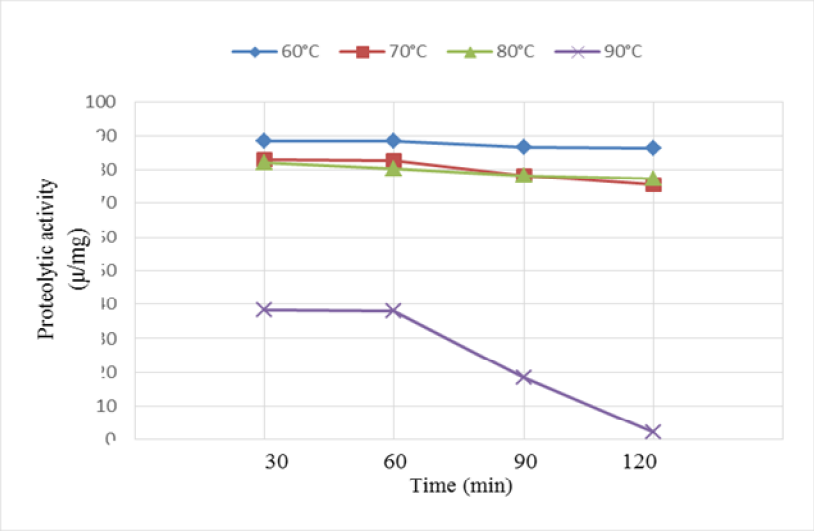

Fig. 7: Protease thermal stability study.

Conclusion

In conclusion, the newly *Streptomyces* sp. SS strain isolated from Saltpans which are rare regions in the world, was able to produce protease enzyme by SSF on wheat bran. It belongs probably to a new species within the genus *Streptomyces* according to genotypic and phenotypic data. The best protease producing medium based on wheat bran was moistened with 50% of pH 9.0 buffer solution and supplemented with 1% fructose. Protease had an optimum at pH 7.0, which is a typical characteristic of neutral proteases. The optimum temperature was 60°C and proved stable up to 80°C. All these results show the high biotechnological potential of this *Streptomyces* strain for the production of the thermostable neutral protease since they could greatly reduce the overall cost of enzyme production.

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Conflict of interests: The authors declare that there is no conflict of interests.

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