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ORIGINAL PAPER

Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces* sp. CN902

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Abstract The purpose of the present research is to study the production of thermophilic alkaline protease by a local isolate, *Streptomyces* sp. CN902, under solid state fermentation (SSF). Optimum SSF parameters for enzyme production have been determined. Various locally available agro-industrial residues have been screened individually or as mixtures for alkaline protease production in SSF. The combination of wheat bran (WB) with chopped date stones (CDS) (5:5) proved to be an efficient mixture for protease production as it gave the highest enzyme activity (90.50 U g^{-1}) when compared to individual WB (74.50 U g^{-1}) or CDS (69.50 U g^{-1}) substrates. This mixed solid substrate was used for the production of protease from *Streptomyces* sp. CN902 under SSF. Maximal protease production (220.50 U g^{-1}) was obtained with an initial moisture content of 60%, an inoculum level of 1×10^8 (spore g^{-1} substrate) when incubated at 45°C for 5 days. Supplementation of WB and CDS mixtures with yeast extract as a nitrogen source further increased protease production to 245.50 U g^{-1} under SSF. Our data demonstrated the usefulness of solid-state fermentation in the production of alkaline protease using WB and CDS mixtures as substrate. Moreover, this approach offered significant benefits due to abundant agro-industrial substrate availability and cheaper cost.

Keywords Solid-state fermentation · Protease *Streptomyces* sp. · Wheat bran · Date stone · Parameter optimization

Introduction

Proteases are among the most important industrial enzymes, accounting for nearly 60% of the industrial market in the world [1]. Several attempts have been made for the production of acid, neutral and alkaline proteases [2], which find potential applications in a number of biotechnological processes [3, 4]. Alkaline proteases are robust enzymes with considerable industrial potential [5]. These proteases are used in the detergent industry, leather processing, silver recovery, medical purposes, food processing, feeds and chemical industrial as well as waste treatment [6, 7]. Microorganisms are the most interesting source of proteases due to their broad biochemical diversity and bioengineering potentiality [8]. Microbial proteases account for approximately 40% of the total worldwide enzyme sales [9]. Currently, commercial proteases are mainly fungal [8] and eubacterial products. *Streptomyces* species producing protease include *S. griseus*, *S. rimosus* and *S. thermovulgaris* [10–13]. Protease production has been studied in submerged (SmF) and solid-state fermentation (SSF) [14, 15]. In search for cheaper fermentation processes with a high enzyme yield, SSF was found to be more attractive [16], with lower manufacturing costs and energy requirement. Moreover SSF has gained importance in the production of microbial antibiotics like cephamycin C [16], neomycin [17] and enzymes like proteases [18], xylanase [19], lipase [20] and amylase [21] due to several economic advantages over conventional SmF [17]. Metabolic processes of microorganisms are greatly influenced by

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temperature, pH, substrate, moisture content, air supply, inoculum concentration etc. Optimization of medium composition has been carried out to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. An important factor to be monitored while developing a production medium is its cost-effectiveness. This can be achieved by using cheaply available agro-industrial residues as unprocessed or moderately processed raw materials. Various substrates for optimal enzyme production have been tested including feather meal for thermophilic protease production by *Streptomyces* sp. 592 [22], wheat bran for α-amylase by *Penicillium chrysogenum* [21], red gram plant waste for α-galactosidase by *Aspergillus oryrae* [23]. In the present work, *Streptomyces* sp. CN902 strain, isolated from soil, was analysed for its ability to produce thermophilic alkaline protease. This strain was tested in SSF conditions by using abundant and low-cost substrates for optimal alkaline protease production.

Materials and methods

Microorganisms, maintenance and inoculum preparation

Streptomyces sp. CN902 strain was originally isolated from Tunisian soil and identified as a producer of thermophilic alkaline protease according to [24]. *Streptomyces* sp. CN902 was maintained on YEME medium [25]. For inoculum preparation, *Streptomyces* sp. CN902 was grown on nutrient agar slants at 28°C for 7 days until complete sporulation. Ten millilitres of sterile ringer solution was added to the slant, spores scraped and transferred into a sterile tube at a density of 1×10^{12} spores mL^{-1} [25].

Submerged culture

Production of protease from *Streptomyces* sp. CN902 was carried out in a minimal medium [25] containing (g L^{-1}): L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; glucose (added after autoclaving), 10. Medium pH was adjusted to 7.0. The broth was inoculated with 1×10^6 spores per ml and inoculated with shaking (Stuart, UK) at 160 rpm and 30°C for 5 days. After fermentation, supernatant was harvested by centrifugation (Sigma 2-16 K, Germany) at 10,000 rpm for 15 min at 4°C. The supernatant was filtered and used as crude enzyme extract to determine alkaline protease activity.

Solid-state fermentation

Different solid substrates such as wheat bran (WB), barley bran (BB), rice bran (RB), olive spinet (OS), oats bran (OB), chopped date stones (CDS) and chopped dried fish (CDF) obtained from local market and industrial by-products were tested for their effects on protease production. The OS, CDS and CDF were dried at 60°C prior to use and CDS and CDF were further chopped into smaller fragments. Ten grams of individual substrate was weighed into a 250-mL Erlenmeyer flask and moistened at 50% (w/v) with Tris–HCl buffer (20 mM, pH 7.0). The flasks were autoclaved for 20 min at 121°C. After cooling, 1×10^6 spores g^{-1} were added and the contents thoroughly mixed. Flasks were incubated at 30°C for 5 days. For each experiment, three flasks were used and withdrawn after the required time of incubation was attained.

Enzyme extraction

Protease extraction was conducted in Tris–HCl buffer (20 mM, pH 7.0) at a w/v ratio of 10 after vigorous shaking at room temperature for 5 min. The extract was centrifuged at 10,000 rpm at 4°C during 15 min, supernatant was filtered on 0.45 μm and used as enzyme source. All experiments were done in triplicate and data expressed as average values.

Protease activity assay

Protease activity was measured according to its action on casein [26] with modification on incubation temperature of 55°C instead of 42°C. Briefly, to 1 mL of 2% (w/v) casein solution, 1.9 mL of Tris–HCl buffer (100 mM, pH 8.0) and 0.1 mL of enzyme were added and mixture was incubated at 55°C for 30 min. After incubation, enzyme activity was stopped by addition of 2 mL of 2% (w/v) trichloroacetic acid. An enzyme blank was always included. The optical density of the trichloroacetic acid soluble materials was read at 280 nm and compared with a tyrosine standard. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 mg of tyrosine under assay conditions.

Optimization of solid-state fermentation conditions

Various process parameters influencing enzyme production during SSF were optimized. Different incubation periods (1–10 days) were studied for their effect on enzyme production. Initial moisture contents (40, 50, 60, 70, 80, 90 and 100%) of solid substrates (before autoclaving) were adjusted with Tris–HCl buffer (20 mM, pH 7.0). For initial pH optimization, substrate solutions were adjusted with 20 mM citrate-phosphate, Tris–HCl or glycine-NaOH buffers at pHs ranging from 3 to 10, respectively. Different incubation temperatures (20, 25, 30, 35, 40, 45 and 55°C) were tested for their effect on enzyme production. Different

inoculum levels (1×10^2, 1×10^4, 1×10^6, 1×10^8 and 1×10^{10} spores g^{-1}) were also tested on protease activity production. For each experimental variable all other parameters were kept at their optimal level. Data were expressed as the average of three replicates.

Effect of additional nutrients

The effect of additional nutrients on protease production was tested by adding carbon sources (1%, w/w) such as glucose, fructose, sucrose, maltose, raffinose or starch, and nitrogen sources (1% w/w) such as peptone, casein, sodium nitrate, ammonium nitrate or ammonium sulphate. Fermentation was carried out for 5 days and all other parameters were kept at their optimal level.

Results and discussion

Production of protease in submerged fermentation

Protease activity produced by *Streptomyces* sp. CN902 under SmF was detected in the culture supernatant from 16 h up to 5 days and the highest level (1.60 U mL^{-1}) was found on the second day (data not shown). Enzyme production gradually declined beyond the fourth day. Maximum proteolytic activity was fourfold higher than described for *S. rimosus* reported in [11] in which standard medium was used. It is noteworthy that optimized incubation time for maximal protease production (7.20 U mL^{-1}) by *Streptomyces* sp. 594 was obtained after 4 days [22]. In the case of *Bacillus subtilis* Y-108, maximum protease production (20.20 U mL^{-1}) was obtained at the third day [27] as it was the case for *Teredinobacter turnirae* [5]. The decline in enzyme production after its optimal level might reflect depletion of available nutrients.

Effect of agro-industrial wastes on enzyme production in solid-state fermentation

Solid-state fermentation processes are highly influenced by the nature of the solid substrate. Generally, substrates are water insoluble polymers of cellulosic or starch material [28]. Several solid substrates obtained from a local market and industrial by-products have been tested for protease production. As shown in Table 1, WB and CDS mixture (5:5) was the most effective substrate in SSF. Maximum protease activity production was 90.50 U g^{-1} under the following conditions: temperature 30°C, initial moisture content 50%, pH 7, for 5 days incubation using 1×10^6 spores g^{-1} inoculum level. Several reports described WB as a potent substrate for protease production by *Bacillus* sp. S4, *Pseudomonas* sp. S22 and *Apergillus oryzae* [8, 29]. WB also elicited protease production by *Aspergillus flavus* [26] as well as *Engyodontium album* [30]. WB is a rather complete nutrient for microorganisms [17, 31] and contains approximately 18% protein, 5% fat and 62% carbohydrate [32]. On the other hand CDS contains 5–7% protein, 4–10% fat and 12–27% carbohydrate and significant amount of minerals [33–35]. This substrate which is appropriate in supporting sustained microbial growth and protease production appeared rather adequate in SSF. To our knowledge CDS has never been used for protease production in SSF. Date palm stones of cultivars from Deglet Nour variety (*phoenix dactylifera*) constitute an abundant industrial waste by-product which can be used as an efficient nutrient for growth and protease production by *Streptomyces* sp. CN902. It is noteworthy that rice bran was previously found to be a suitable substrate for protease production by *Rhizopus oligosporus* [36].

Table 1 Effect of various agro-industrial substrates for thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a solid-state fermentation

Substrates	Weight (g)	Protease activity (U g^{-1})
WB	10	74.50
BB	10	60.20
RB	10	56.60
OB	10	20.50
CDS	10	69.50
OS	10	10.00
CDF	10	45.50
WB + BB	5 + 5	60.00
WB + RB	5 + 5	63.00
WB + CDS	5 + 5	90.50
WB + OB	5 + 5	62.50
WB + OS	5 + 5	50.50
WB + CDF	5 + 5	64.00

The culture was grown at 30°C; initial pH: 7.0; initial moisture: 50%; inoculum level: 1×10^6 spores g^{-1}; incubation period: 5 days

WB wheat bran, *BB* barley bran, *RB* rice bran, *OB* oats bran, *CDS* chopped date stones, *OS* olive spinet, *CDF* chopped dried fish

Effect of incubation period

Protease production in SSF was studied over a 10-day period. It is notable that in other studies, enzyme production was studied over an incubation time of 48 h for bacteria and 8–9 days for fungi [29, 37, 38]. Data of alkaline protease production versus incubation time using a WB and CDS mixture as substrate are shown in Fig. 1. Protease activity (6.50 U g^{-1}) was detected from the second day of

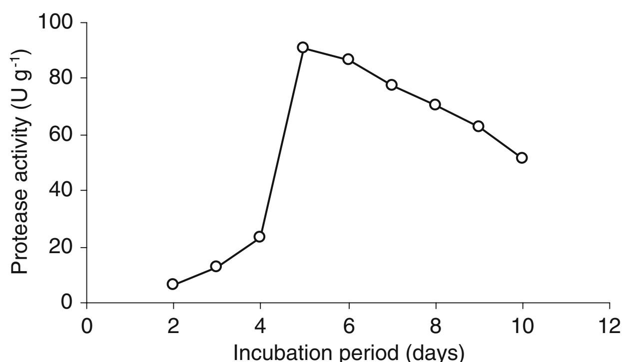

Fig. 1 The effect of incubation period on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a SSF system for mixture of WB + CDS (5:5). Initial pH: 7.0; initial moisture: 50%; inoculum level: 1×10^6 spores g^{-1}; incubation temperature: 30°C

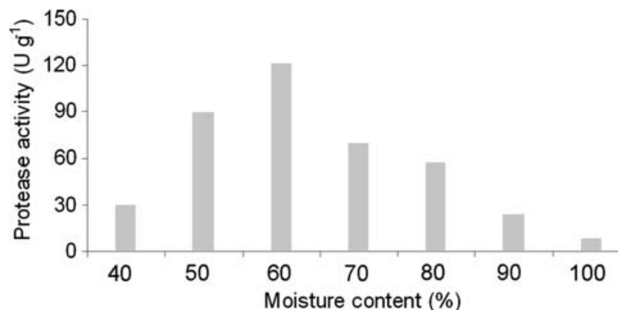

Fig. 2 The effect of Initial moisture content (%) on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a SSF system for mixture of WB + CDS (5:5). Initial pH: 7.0; inoculum level: 1×10^6 spores g^{-1}; incubation temperature: 30°C; incubation period: 5 days

incubation and culminated on day 5 reaching 90.5 U g^{-1} and then slightly declined till day 10 reaching 51.50 U g^{-1}. Moreover, it was previously reported that maximal protease activity by *Streptomyces* sp. 594 was attained after the fourth day, reaching a significantly lower level of 15.50 U g^{-1} [22] whereas in the case of *S. rimosus*, maximal protease activity (15.80 U g^{-1}) was attained on the ninth day [11]. In the latter case, maximum proteolytic activity was fourfold lower than found in our present case. It is remarkable that maximum protease activity production by *Aspergillus flavus* was obtained between the fifth and seventh day of incubation using SSF at 30°C [26]. Active mycelium growth which is closely linked to time incubation and culture conditions is crucial for high extracellular enzyme production [21, 39].

Effect of initial moisture content

Moisture content is an important factor in SSF process efficiency [40, 41]. The effect of initial moisture content on protease production is presented in Fig. 2. The highest enzyme production (121.50 U g^{-1}) was obtained at 60% initial moisture content which is twofold higher than described for *Bacillus* sp. [28]. A similar observation has been reported in the case of *Streptomyces* sp. 594 protease production [22]. Other reports indicated the requirement of 55 and 63% initial moisture content for maximum protease production by *Penicillium* LPB-9 [42] and *A. flavus* [26], respectively, in SSF. Generally, moisture level of the medium is considered as a fundamental parameter for microbial growth and metabolite production [43, 44]. Increase in SSF moisture content is believed to reduce the porosity of solid particles, thus limiting oxygen transfer. Conversely a decrease in SSF moisture content results in the reduction of substrate solubility and low degree of swelling [21, 45–47].

Effect of incubation temperature

The effect of varying temperatures on enzyme production is shown in Fig. 3. Maximum protease activity (156.50 U g^{-1}) was attained at 45°C. This optimal temperature is similar to those described for *Streptomyces* sp. 594 [22] or *S. rimosus* [11] which were also grown in SSF but quite different from *Teredinobacter turnirae* [5] or *Bacillus subtilis* PE-11 [28, 48]. In this latter case optimal temperature was around 30°C. Temperature is an essential factor affecting SSF performance because of its importance in microorganisms' growth and metabolite production [49, 50]. Overall, our present data seem to indicate that optimal temperature of 45°C is rather specific of Streptomyces whereas 30°C is rather optimal for Bacillus species.

Effect of initial pH

The effect of varying initial pH values on enzyme production is shown in Fig. 4. When initial pH was equal to 3.0, no enzyme production was detected. Protease activity gradually increased with pH reaching an optimum level at

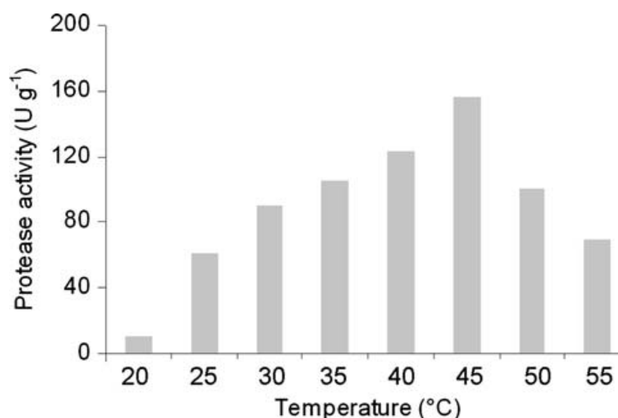

Fig. 3 The effect of temperature on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a SSF system for mixture of WB + CDS (5:5). Initial pH: 7.0; initial moisture: 60%; inoculum level: 1×10^6 spores g^{-1}; incubation period: 5 days

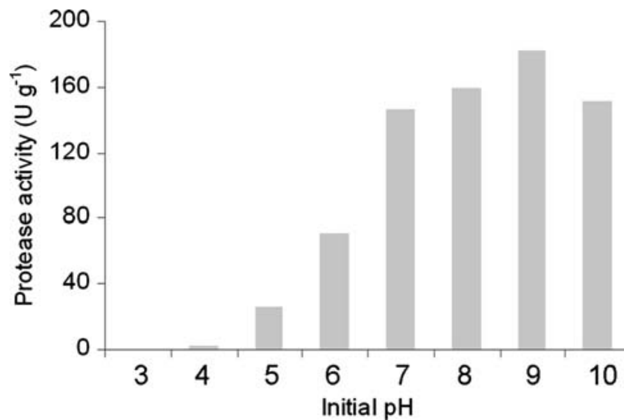

Fig. 4 The effect of initial pH on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a SSF system for mixture of WB + CDS (5:5). Initial moisture: 60%; inoculum level: 1×10^6 spores g^{-1}; incubation period: 5 days; incubation temperature: 45°C

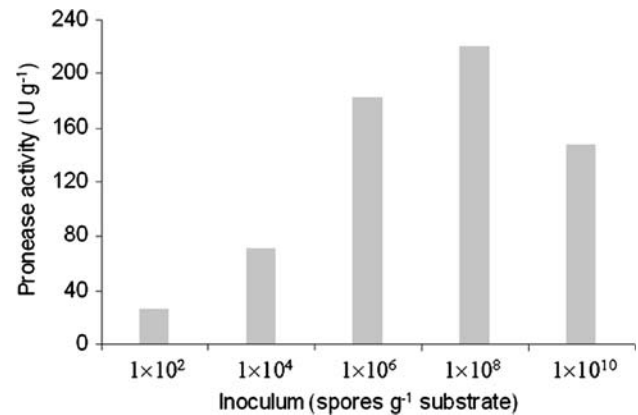

Fig. 5 The effect of inoculum level on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a SSF system for mixture of WB + CDS (5:5). Initial moisture: 60%; initial pH: 9.0; incubation period: 5 days; incubation temperature: 45°C

pH 9.0 (182.50 U g^{-1}). Thereafter, enzyme production decreased at all higher pH values reaching 151.30 U g^{-1} at pH 10.0. Data clearly indicated that protease production by the soil bacterium *Streptomyces* sp. CN902 under SSF is optimal at pH 9.0. Previous studies dealing with the effect of initial pH on protease production by other *Streptomyces* strains as *S. rimosus* and *Streptomyces* sp. 594 in SSF conditions indicated lower pH values, i.e., between 6.0 and 7.0 for optimal protease activity [11, 22]. Moreover, pH values between 7.0 and 9.0 were found to be suitable for optimal alkaline protease production by *T. turnirae*, and pH 10.0 in the case of *Bacillus* sp. [5, 28]. pH is important for any fermentation process as it may change with various metabolic activities through its effect on several components transferred across the cell membrane [51, 52].

Effect of inoculum level

The inoculum level is also an important factor for alkaline protease production. Using spores for inoculation offered several benefits when compared to vegetative cells. For instance they can serve as biocatalyst in bioconversion reactions because of their ability in carrying out the same reactions as the corresponding mycelium [51, 53]. As shown in Fig. 5, optimum enzyme activity (220.50 U g^{-1}) was obtained at an inoculum level of 1×10^8 spores g^{-1} substrate. Higher inoculum level did not increase protease production but rather decreased it reaching 147.30 U g^{-1} at 1×10^{10} spores g^{-1} substrate.

Effect of supplementation with nitrogen sources

The effect of various nitrogen sources (1% w/w) such as yeast extract, malt extract, peptone, ammonium nitrate, sodium nitrate, ammonium sulphate and casein on protease production is shown in Table 2. Each component was added to WB and CDS mixture in SSF. Ammonium sulphate and yeast extract increased protease activity to 233.50 and 245.50 U g^{-1}, respectively, versus 220.50 U g^{-1} for the control. Malt extract, peptone, ammonium nitrate, sodium nitrate and casein greatly reduced enzyme production. Yang et al. [27] also found that protease production by *B. subtilis* Y-108 was repressed by most of the nitrogen sources used with the exception of sodium nitrate which enhanced protease production. It has also been reported that casein increased protease production by *A. niger* var. *tieghem* when added to WB [54]. Conversely, both casein and gelatin decreased protease production by *A. flavus* [26].

Effect of carbohydrate addition

The effect of various sugars (1%) on protease activity is shown in Table 3. All sugars decreased protease production

Table 2 Effect of various nitrogen sources on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a solid-state fermentation

Organic nitrogen sources	%	Protease activity (U g^{-1})
Control	Nil	220.50
Yeast extract	1	245.50
Malt extract	1	193.20
Peptone	1	189.70
Ammonium nitrate	1	178.50
Sodium nitrate	1	172.60
Ammonium sulphate	1	233.50
Casein	1	170.60

The culture was grown at 45°C; initial pH: 9.0; initial moisture of mixture of WB + CDS: 60%; inoculum level: 1×10^8 spores g^{-1}; incubation period: 5 days

Table 3 Effect of various carbohydrates on thermophilic alkaline protease production by *Streptomyces* sp. CN902 in a solid-state fermentation

Carbohydrates	%	Protease activity (U g^{-1})
Control	Nil	220.50
Dextrin	1	175.40
Maltose	1	161.20
Starch	1	166.70
Glucose	1	182.50
Raffinose	1	198.30
Fructose	1	199.50
Sucrose	1	168.60

The culture was grown at 45°C; initial pH: 9.0; initial moisture of mixture of WB + CDS: 60%; inoculum level: 1×10^8 spores g^{-1}; incubation period: 5 days

and the lowest activity (161.20 U g^{-1}) was obtained with maltose and the highest activity (199.50 U g^{-1}) with fructose. This decrease may be attributed to the repression exerted by excessive amount of metabolizable sugars on protease production [5]. Another study reported that protease production by *B. subtilis* Y-108 was slightly enhanced by addition of 1% lactose or arabinose in SSF [24]. Moreover, sucrose and mannitol have a strong effect on protease production by *E. album* [27]. Overall, our data seem to indicate that the addition of any carbon source to WB and CDS mixture lead to the reduction in alkaline protease production by *Streptomyces* sp. CN902.

Conclusion

Production of a thermophilic alkaline protease by *Streptomyces* sp. CN902 strain under solid-state fermentation is influenced by the growth conditions of the bacteria in WB and CDS mixture. This mixture is an efficient substrate for optimal enzyme production in SSF. The use of waste raw materials is cheaper and more advantageous than conventional substrates for thermophilic alkaline protease production. Furthermore, a high enzyme level was reached by supplementation with yeast extract. These results open the way to the use of other substrate mixtures for optimal production of alkaline protease or other industrial enzymes by *Streptomyces* sp. CN902.

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