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Bioprocess Optimization of Halophilic Protease Production by a Halophilic Bacterial Strain JS4

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KEYWORDS

Halophilic protease, gelatin cup assay, Soya bean flour and Sucrose

A B S T R A C T

An extremely halophilic bacterial strain - *Methylophaga sp*. Strain JS4 producing halophilic extracellular protease was isolated from a hypersaline environment. The bacterial isolate JS4 hydrolyzed casein and gelatin, showing their proteolytic potential. The cell free extract of JS4 bacterial strain showed clear zones in gelatin cup assay. Both Bacterial biomass and enzyme production by *Methylophaga sp*. Strain JS4 was found to optimized at 40^{o}C, pH-7 (Neutral pH), after 72 hours of incubation at 150 rpm in the presence of 15% NaCl (W/V), Soya bean flour (Nitrogen source) and Sucrose (Carbon source).

Introduction

Extremophilic microorganisms dwelling in harsh environmental habitats have evolved unique properties; which can be of biotechnological and commercial significance. These non-conventional microorganisms living in such extremely hostile environmental habitats possess special adaptation strategies that make them interesting not and Schinner, 2001). Classical examples only for the fundamental research but also for biofermentation applications (Margesin for Extremophiles include halophiles, acidophiles, alkaliphiles, thermophiles, methanogens and haloalkaliphiles (Dodia *et al.*, 2006). Among the various extremophiles, halophilic organisms have evolved several adaptations to survive and function in extraordinary hypersaline ecosystems, such as solar salterns, the Dead Sea, solar lake and other hypersaline lakes (Oren, 2000). Modern interest in halobacteria is due to their unique characteristics at the genetic, biochemical, physiological and evolutionary levels (Oren, 2002).

Extreme halophiles act as an ideal source of extremozymes (halozymes) with extreme

stability, and the application of these enzymes as biocatalysts is attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials (Vijayanand *et al*., 2010). Most significantly halophiles secrete a wide range of extracellular hydrolytic enzymes such as nucleases, proteases, amylases, cellulases, lipases and xylanases of potential commercial values (Alqueres *et al.*, 2007).

Halozymes are distinguished from their homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and maintaining soluble and active conformations in high concentrations of salt upto 25% concentration (W/V) (Hough and Danson, 1999; Nascimento and Martins, 2006). This feature of halophilic microorganisms has showed several biotechnological applications (Ghosh *et al.*, 2010).

Halophilic proteases are active and highly stable in high salt concentrations which inhibit or even denature many conventional enzymes of non-halophilic organisms (Norberg and Hofsten, 1969). Probably the largest application of proteases is in laundry detergents, where they help in removing the protein based stains from clothing (Nijafi *et al.*, 2005).The present study is aimed to optimize the culture conditions for the production of haloalkaliphilic protease from an extreme halophile, *Methylophaga* sp. strain MJS_1 and to characterize the purified protease.

Materials and Methods

Halophilic Bacterial strain and culture media

An extremely halophilic bacterial strain JS4 was isolated from the salt samples of solar evaporated ponds and the bacterial strain was evaluated for their proteolytic potential on casein agar plates containing 20% NaCl (w/v). The isolate MJS_1 was aerobically cultured in a basal salt medium containing (g 1^{-1}) NaCl: 200; Casamino acids: 7.5; Yeast extract: 10; Trisodium citrate: 3.0; KCl: 2.0; $MgSO_4.7H_2O$: 20. and incubated at 100 rpm on shaker incubator at 37°C.

Halophilic Protease production

1 ml of a week old culture of *Methylophaga sp*. strain JS4 was inoculated into a sterilized 250 ml Erlenmeyer flask containing 50 ml of the above culture medium and incubated at 40^{o}C in an incubator shaker at 100 rpm for 4 days. The flasks were removed at regular intervals, the contents were centrifuged at 10,000 g for 10 min at 40˚C to remove cells and insoluble materials (sediment) and the cell free supernatant was used as the source of the crude enzyme (Vijayanand *et al*., 2010).

Qualitative and Quantitative protease assay

Protease production by the bacterial isolate JS4 was qualitatively investigated by Gelatin cup method (Vidyasagar *et al*., 2006). Protease activity was quantitatively assayed by the modified method of Anson (1938).The reaction mixture contained 1 ml of 1% casein and 0.5 ml of enzyme in the presence of 50 mM glycine. NaOH buffer at pH 10.0 containing 20% NaCl and incubated at 37^{o}C for 20 min and the reaction was stopped by adding 3 ml of 10% TCA. After standing for 1h the solution was filtered through Whatman filter paper. To 0.5 ml supernatant, 2.5 ml of Folin-ciocalteau reagent was added, the reaction mixture was incubated at room temperature for 30 min, and then the absorbance was measured at 660 nm.

Optimization of culture conditions for maximizing bacterial growth and protease production by JS4 isolate

Effect of temperature, salinity, pH and agitation rates

The influence of temperature, pH, NaCl concentrations and agitation rates on both bacterial biomass and halophilic protease production from *Methylophaga sp*. strain JS4 was studied. This was carried out by cultivating the halophilic bacteria at different temperatures (25°C-65°C), different initial pH values of the medium (pH 4.0-12.0), different NaCl concentrations 0-30%, (w/v) and different agitation speeds (0-250 rpm). Bacterial biomass and protease production was measured after 72 h of incubation.

Effect of various carbon and nitrogen sources

The bacterial strain was grown in the basal salt medium containing different soluble carbon sources (1% w/v) including glucose, glycerol, sucrose, starch, mannitol and maltose and different low cost-agro industrial residues including wheat bran, wheat flour and rice bran nitrogen sources (1% w/v) including organic nitrogen sources such as peptone, beef extract, casein, gelatin, yeast extract, corn steep liquor and skim milk powder, inorganic nitrogen sources such as NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl and different low cost agricultural by products such as soybean flour, cotton seed flour, corn seed flour and chick pea flour to investigate their effect on bacterial biomass and protease production (Vijayanand et al., 2010).

Result and Discussion

An extremely halophilic bacterial strain- JS4 isolate, producing halophilic extracellular protease was isolated from a hypersaline environment. The bacterial isolate JS4 hydrolyzed casein and gelatin, exhibiting their proteolytic potential. The cell free extract of JS4 bacterial strain showed clear zones in gelatin cup assay. The halophilic strain was identified as *Methylophaga sp*. Strain JS4.

Optimization of culture conditions for bacterial growth and protease production by JS4 isolate

Effect of incubation time

Considerable protease production by *Methylophaga sp*. Strain JS4 was noticed in the fermentation broth as soon as the bacteria entered the late exponential phase (~ 48 h), and the activity reached the maximum level in the stationary phase (~ 72 h) as shown in Fig.1. Hence, the optimum cultural conditions for growth and protease production in shake flasks were carried out after 72 h of incubation. Similar observations were reported in *Halogeometricum borinquense* TSS-101 (Vidyasagar *et al.*, 2006) and *Halobacterium* sp. JS_1 (Vijayanand *et al.*, 2010).

Effect of various incubation temperature

Both bacterial growth and enzyme production was maximized in between 30°C and 45°C, with optimum at 40°C after 72h of incubation (Fig.2). Bacterial growth and extracellular protease production by the isolate JS4 was found to be reduced at incubation temperatures below 35°C and above 45°C respectively. In contrast, the protease production by *Bacillus subtilis* strain 38 was found to be optimized at 47°C (Chantawannakula *et al.*, 2002). However, both bacterial biomass and protease production in many bacterial strains optimized at 37°C (Adhinarayana *et al.*, 2003; Ibrahim and Al-salamah, 2009).

Fig.1 Effect of Incubation Time on Protease Production by *Methylophaga sp*. JS4

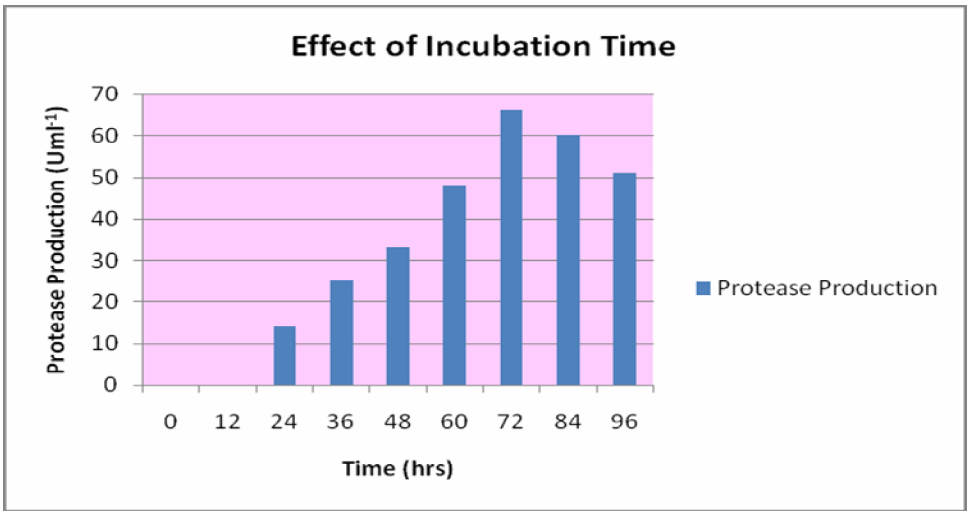


Fig.2 Effect of Incubation Temperature on Protease Production by *Methylophaga sp*. JS4

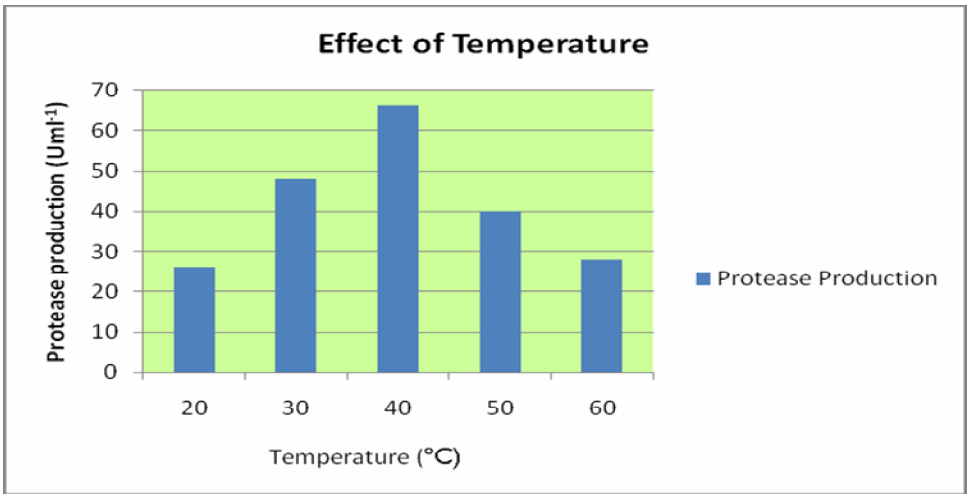


Fig.3 Effect of Incubation pH on Protease Production by *Methylophaga sp*. JS4

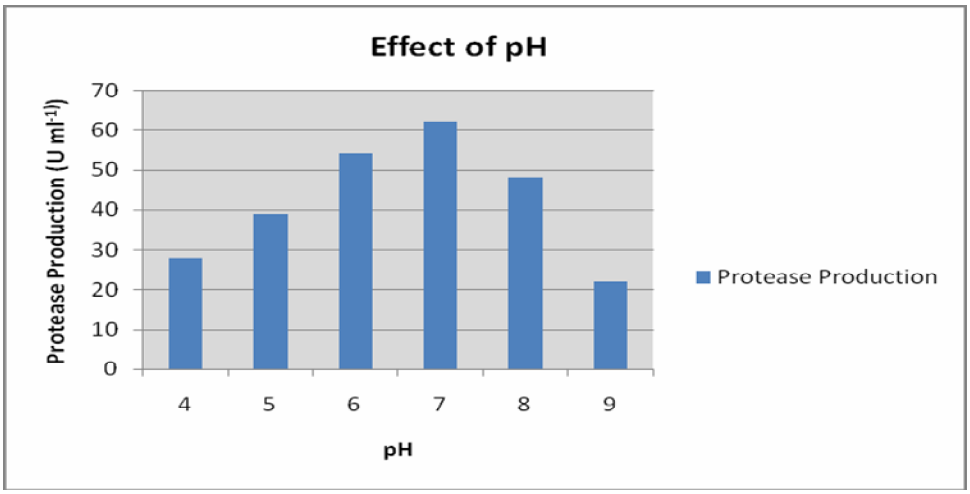


Fig.4 Effect of Salinity on Protease Production by *Methylophaga sp*. JS4

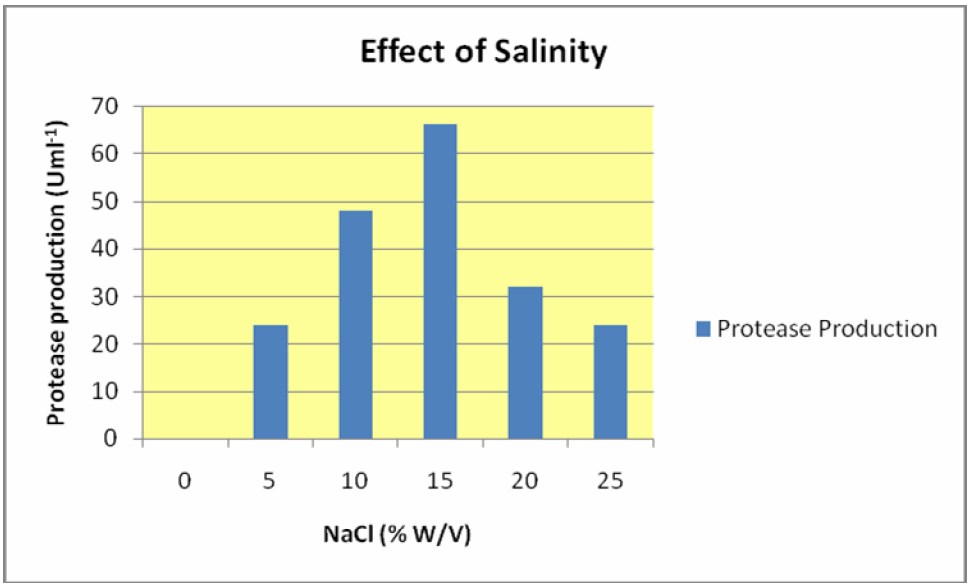
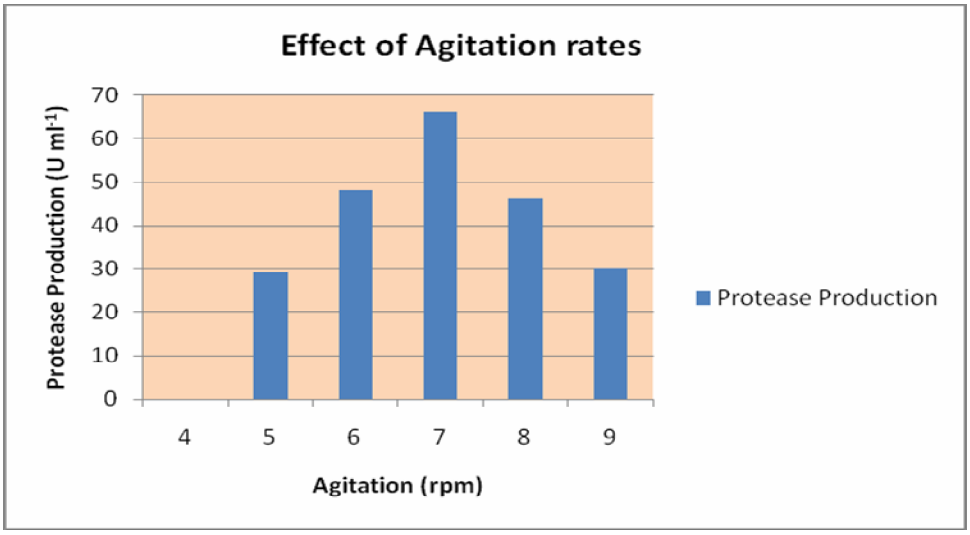


Fig.5 Effect of Agitation on Protease Production by *Methylophaga sp*. JS4



Effect of pH

Fluctuations in the hydrogen ion concentration of their environment greatly influence Halozyme production by bacterial strains. Bacterial biomass and protease production was found to be optimized at neutral pH (7.0), resembling *Halobacterium* sp. (Schmitt *et al.*, 1990), *Chromohalobacter* sp. TVSP-101 (Vidyasagar *et al.*, 2007) and *Halobacterium* sp. JS_1 (Vijayanand *et al.*, 2010). However, protease production by *Halobacterium salinarum* was found to be optimized at slightly alkaline pH (8.0) (Norberg and Hofsten, 1969). Bacterial biomass and protease production was found to be negatively regulated at extremes of pH (Fig 3).

Effect of salinity

The effect of various NaCl concentrations (0-30%, w/v) on bacterial biomass and protease production was investigated after 72 h of incubation. Both bacterial growth and extracellular protease production by the isolate JS4 was found to be maximized in the presence of 15% (w/v) NaCl concentration (Fig 4). In contrast, halophilic protease production by most of the halophilic bacterial strains including *Halobacterium salinarum* (Norberg and Hofsten, 1969), and *Halobacterium halobium* ATCC 43214 (Kim and Dordick, 1997) was found to be maximized at 4 M NaCl.

Effect of aeration/agitation

Bacterial biomass and protease production by *Methylophaga sp*. Strain MJS1 was investigated at different agitation rates, ranging in between 0 to 250 rpm. The variation in the agitation speed was found to influence the extent of mixing in the shake flasks and also affect the nutrient availability. The result represented in Fig.5 revealed that the bacterial growth and the enzyme production increased with increasing agitation rates and the maximum protease production were achieved at 150 rpm. In contrast, the protease production by *Bacillus licheniformis* strains Rand (Abusham *et al.*, 2009) and *Chromohalobacter* sp. TVSP-101 (Vidyasagar *et al.*, 2007) were found to be optimized at 200 rpm.

Effect of different carbon and nitrogen sources

The results obtained (Data not shown) showed that the supplementation of sucrose as carbon source instigated high biomass and proteolytic activity. This was in contrast to the previous reports which showed that the starch and glucose caused high level of enzyme expression in *Bacillus licheniformis* N-2 (Nadeem *et al.*, 2008) and *Bacillus* sp. PCSIR EA-3 (Qadar *et al.*, 2009). The most efficient natural nitrogen source for maximum enzyme production was found to be soybean flour. Similarly soybean flour was found to be a potent inducer for protease biosynthesis by various bacterial strains such as *Teredinobacter turniarae* (Elibola and Moreira, 2005) and *Halobacterium* sp. JS_1 (Vijayanand *et al.*, 2010).

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