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Production of extracellular halo-alkaline protease from a newly isolated Haloalkaliphilic *Bacillus* sp. isolated from seawater in Western India

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Summary

Haloalkaliphilic, gram positive, aerobic, coccoid *Bacillus* sp. Po2 was isolated from a seawater sample in Gujarat, India. On the basis of 16s rRNA gene homology, Po2 was 95% related to *Bacillus pseudofirmus*. A substantial level of extracellular alkaline protease was produced by Po2, which corresponded with the growth and reached a maximum level (264 U/ml) during the stationary phase at 24 h. The production thereafter remained nearly static at optimal level till 36 h. Po2 could grow in the range of 0–20% NaCl (w/v) and pH 7–9, optimally at 10% NaCl (w/v) and pH 8. The protease production was salt-dependent and optimum production required 15% NaCl (w/v) and pH 8. Among the organic nitrogen sources, optimum growth and protease production (260 U/ml) were supported by the combination of peptone and yeast extract. However, growth and protease production were highly suppressed by the inorganic nitrogen sources used; with the exception of potassium nitrate, which supported both growth and protease production to limited extent (24 U/ml). Strong inhibition of enzyme production was observed at above 1% glucose (w/v). Wheat flour served as both carbon and nitrogen source supporting growth and protease production.

Introduction

Microorganisms represent the most common candidates as sources of new enzymes because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. The exploration of the enzymatic potential of extremophiles has just begun and hitherto only a few enzymes have been explored and investigated (Herbert 1992; Eichler 2001; Rozzell 1999). Proteases are among the commercially most viable enzymes and exploration of further novel microbial sources of these enzymes for applications in many industries such as food and leather (Grebeshova *et al.* 1988) and as detergent additives (Denizci *et al.* 2004) has attracted considerable attention. Microbial alkaline proteases dominate the worldwide enzyme market, with a two-third share of the detergent industry (Niehaus *et al.* 1999; Gupta *et al.* 2002). Nowadays increasing emphasis is being laid on extremophiles for the presence of such enzymes, mainly due to the mechanisms and strategies that help them to function under stressful growth conditions (Madern *et al.* 2000; Margesin & Schinner 2001).

Alkaline proteases from extremophiles have been studied extensively in the recent years (Kim *et al.* 1991; Ferrero *et al.* 1996; Johnvesly & Naik 2001; Xiao 2001; Joseph *et al.* 2002; Kanekar *et al.* 2002; Saeki 2002; Bakhtiar *et al.* 2003; Beg & Gupta 2003; Ellaiah *et al.* 2003; Gessesse *et al.* 2003; Huang *et al.* 2003; Denizci 2004; Gupta *et al.* 2005). In comparison, haloalkaliphilic bacteria have been relatively less attended, as only few alkaline proteases are reported from these organisms (Studdert *et al.* 1997; Stan-Lotter *et al.* 1999; Gimenez *et al.* 2000; Studdert *et al.* 2001; Polosina *et al.* 2002). Besides the novel catalytic applications, the wide occurrence of many of such enzymes among the haloalkaliphilic bacteria holds ecological significance. The present work has focused on the production of extracellular alkaline protease from a new haloalkaliphilic bacterium Po2 that has 95% similarity with *Bacillus pseudofirmus*.

Materials and methods

Organism

Haloalkaliphilic bacterium, Po2, was isolated by enrichment culture technique from a seawater sample from Porbandar, in the coastal region of the Gujarat, Western India. The 1 ml of seawater was added to a complex medium (CMB) containing (g/l): glucose, 10;

peptone, 5; yeast extract, 5; KH_2PO_4, 5 and NaCl, 200. The pH of the medium was adjusted to 10 by adding separately autoclaved Na_2CO_3 (20%, w/v) and incubated at 37 °C for 48 h under shake flask conditions. After 48 h, a loopful of culture was streaked on above medium and based on colony characters, different organisms were selected. The potent strain, Po2 was further identified by 16S rRNA gene amplification and nucleotide sequencing of 1000 bp out of 1500 bp.

Inoculum preparation and protease production

The inoculum was prepared by adding a loopful of pure culture into 25 ml sterile CMB medium and incubated at 37 °C on a rotary shaker for 24 h. A 10% inoculum from the culture (at $A_{660;}$ 1.0) was added to gelatin broth (GB) containing (g/l); gelatin, 10; casein enzymatic hydrolysate, 10; NaCl (w/v), 100 and pH 9. After incubation for 48 h at 37 °C under shaking conditions (100 rev/min), the growth was measured at 660 nm and the cultures were harvested by centrifugation at $6000\times g$ for 10 min at 4 °C. The cell-free extract was used as crude preparation of enzyme to measure protease activity.

Enzyme assay

Alkaline protease activity was estimated by the Anson–Hagihara method (Hagihara 1958). The enzyme (0.5 ml) was added to 3.0 ml casein (0.6% w/v in 20 mM borax–NaOH buffer, pH 10) and the reaction mixture was incubated at 37 °C for 10 min before the addition of 3.2 ml of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid). The terminated reaction mixture was incubated for 30 min at room temperature. The precipitates were removed by filtration through Whatman no. 1 filter paper and the absorbance of the filtrate was measured at 280 nm. One unit of alkaline protease activity was defined as the amount of enzyme liberating 1 μg of tyrosine per minute under assay conditions. Enzyme units were measured using tyrosine (0–100 μg) as standard.

Growth kinetics and protease production

The kinetics of growth and enzyme production was followed at different time intervals. The Po2 culture was inoculated in GB medium (NaCl, 10% w/v; pH 9) and incubated at 37 °C under shaking conditions (100 rev/min). The culture samples were withdrawn aseptically every 3 h and cell density along with enzyme activity was monitored as described above.

Optimization of production medium for growth and protease production

Effect of NaCl

The effect of salt on growth and protease production was studied by varying the NaCl concentrations (0–20%, w/v) and at constant pH 9 in GB (describe above). The growth and enzyme activity were quantified after incubation for 66 h at 37 °C under shaking at 100 rev/min.

Effect of pH

In order to investigate the influence of pH on growth and protease production, the isolate, Po2 was grown in a GB medium at varying pH (7–10) and at NaCl concentration; 10% (w/v). After incubation for 66 h at 37 °C under shaking conditions at 100 rev/min, the growth and protease activity were quantified.

Effect of nitrogen sources

The organic nitrogen sources included soya peptone, tryptone, caseitone, gelatin, casamino acid, traders protein, peptone and yeast extract, while inorganic nitrogen sources included ammonium nitrate, ammonium chloride and potassium nitrate. The respective nitrogen sources were added as a sole source of nitrogen (0.5%, w/v). The growth and enzyme activity was monitored after 66 h growth at 37 °C.

Effect of glucose, peptone, yeast extract and inorganic phosphate on growth and protease production

To study the effect of glucose and inorganic phosphate (K_2HPO_4) on enzyme production, different concentrations (0–1.5%, w/v) were incorporated into the GB medium. The growth and enzyme activity were monitored after 66 h growth at 37 °C.

The effect of nitrogen source was examined in an experiment where various concentration of yeast extract (0–2.0% w/v) with 0.5% (w/v) peptone and different concentration of peptone (0–2.5% w/v) with 0.5% (w/v) yeast extract were formulated along with other component of CMB medium.

Enzyme production with molasses and wheat flour

Molasses (0.5–2% w/v) and wheat flour (0.25–1% w/v) with NaCl (10%, w/v) at pH 9 were used as sole source of carbon and nitrogen. After incubation for 66 h at 37 °C under shaking conditions (100 rev/min), the growth and protease activity were quantified.

Results and discussion

Literature suggested that the diversity of halophiles, alkaliphiles and haloalkaliphiles have been studied mostly from soda lakes. The present study exhibited the exploration of haloalkaliphilic bacteria from seawater. On the basis of the 16S rRNA gene sequencing, the isolate Po2 was found to be a *Bacillus* sp. For the identification of Po2, 1000 bp out of 1500 bp of 16s rRNA gene was sequenced. The homology-based results suggested 95% similarity of the Po2 strain to *Bacillus pseudofirmus*.

The growth kinetics of Po2 was studied along with alkaline protease production. It revealed that the growth

of the organism entered the exponential phase after 6 h and the stationary phase started after 18 h. Enzyme production was coherent with the growth pattern and it increased with the increasing growth, the optimum being at 24 h (264 U/ml). The production thereafter remained nearly static at optimal level along with the stationary phase up to 36 h (Figure 1). This trend was quite similar to the moderately halophilic *Pseudoalteromonas*, where optimum protease production was detected at the end of the exponential growth phase (Sanchez-Porro *et al.* 2003). In *B. sphaericus*, an obligate alkalophile, the major portion of the alkaline protease was secreted in post exponential phases (Singh *et al.* 2004). Similar studies on growth kinetics were also carried out with *Bacillus* sp. and other haloalkaliphilic organisms (Takii *et al.* 1990; Ferrero *et al.* 1996; Studdert *et al.* 1997). In case of *Aspergillus flavus*, maximum enzyme production was obtained during the stationary phase. (Malathi & Chakraborty 1991). These findings on the production of the enzyme during stationary phase clearly suggest the prominent role of extracellular proteases in ecological sustenance of the organisms.

Effect of salt and pH on growth and protease production

The isolate under study could grow in the range of 0–20 %(w/v) NaCl, the optimum being at 10% (w/v). These results indicated the halotolerant nature of the strain Po2. Similar trends were also evident in *Salinicoccus alkaliphilus* sp. nov., a moderately halophilic and alkaliphilic coccus isolated form Baer Soda Lake in Mongolia, which could grow over a wide range of NaCl, 0–25% (w/v) with optimum at 10% (w/v) (Zhang *et al.* 2002). Po2 produced protease in the range of 5–20% (w/v) NaCl, optimally (162–170 U/ml) at 10% (w/v) NaCl. The growth of the Po2 was reduced extensively in the absence of salt with no protease production, and reduction in protease production at 5 and 20 % (w/v) NaCl was also evident. The results clearly indicated the halophilic nature of the protease. Similar results have also been reflected by the haloalkalophilic archaeon, *Natronococcus occultus* in which protease secretion was optimum at 1–2 M NaCl (Studdert *et al.* 2001). However, in the case of the archaebacterium *Halobacterium mediterranei*, a much higher salt requirement (25%, w/v) for serine protease secretion was reported (Stepanov *et al.* 1992). Our isolate, Po2 grew and produced protease optimally (170 U/ml) at pH 8, with slightly suppressed growth at pH 9 and significantly reduced growth at pH 7 (Figure 2). A similar type of response has been observed on the growth of some haloalkaliphilic archaea such as a *Natronoincula* (Zhilina *et al.* 1998) and *Natronorubrum bangense* (Xu *et al.* 1999) where the optimum pH for growth was 9–9.5. Alkaline protease from *Bacillus subtilis* NCIM 2713 (Mane & Bapat 2001) was maximally active at pH 8 and from *Halomonas campisalis* at pH 8–11 and 0–15% (w/v) NaCl (Alva & Peyton 2003). The optimum pH range between 9 and 10 for growth and protease production is common among alkaliphilic and haloalkaliphilic organisms (Fujiwara & Yamamota 1987; Johnvesly & Naik 2001; Kaur *et al.* 2001; Studdert *et al.* 2001).

Effect of nitrogen sources

The alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole *et al.* 1988; Chu *et al.* 1992; Moon & Parulekar 1991). Thus, to optimize the conditions for production of protease, it was necessary to optimize the carbon and nitrogen sources. Among the organic nitrogen sources used in our study, all except casitone and gelatin were growth-promoting. The effect of various organic nitrogen sources was more pronounced on the enzyme production than on growth. Enzyme production was optimum with a combination of peptone and yeast extract (264 U/ml) followed by peptone (160 U/ml). Other organic nitrogen sources were not as effective towards enzyme production, though they supported growth comparable to peptone and yeast extract (Figure 3). Interestingly, according to one report, casaminoacids acted as an

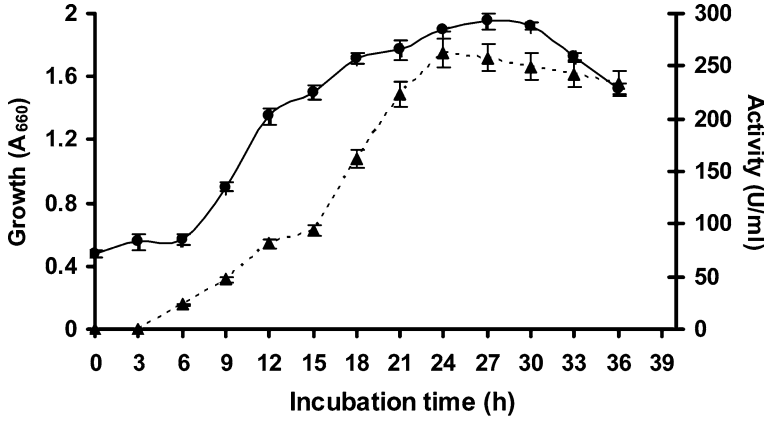

Figure 1. Growth kinetics along with alkaline protease production of Po2. Samples were withdrawn at 3 h intervals for the determination of cell growth (A_{660}) (●) and protease activity (▲).

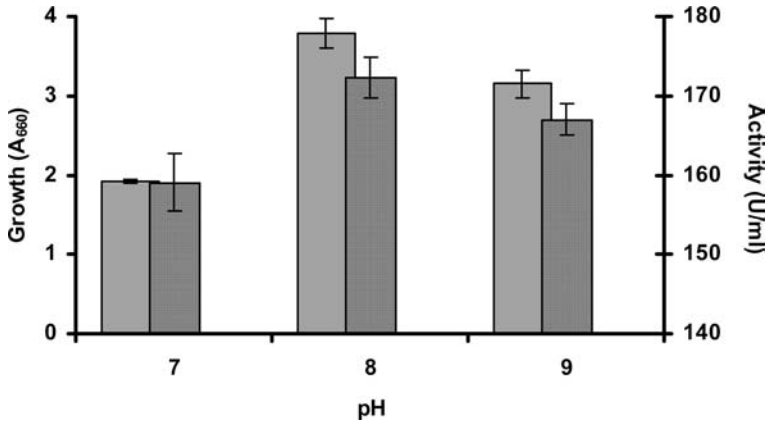

Figure 2. Effect of pH on growth and protease production of Po2. Samples were taken after incubation of 66 h at 37 °C under shaking conditions (100 rev/min), for the determination of cell growth (A_{660}) (□) and protease activity (■).

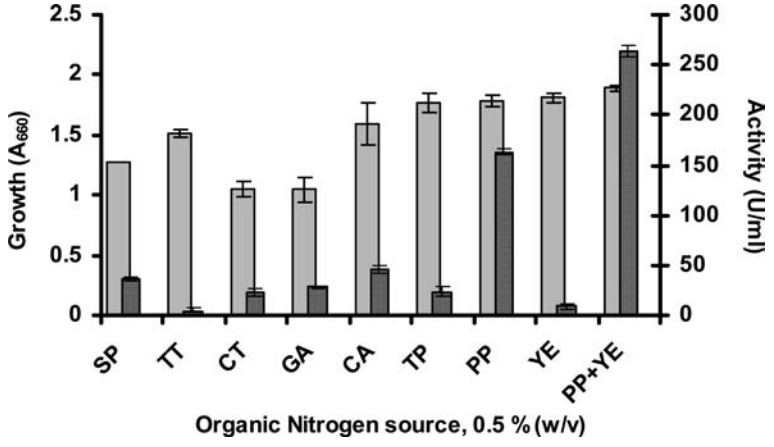

Figure 3. Effect of different organic nitrogen sources (0.5%, w/v). SP: Soya Peptone, TT: Tryptone, CT: Casitone, GA: Gelatin, CA: Casamino Acid, TP: Trader's Protein, PP: Peptone, YE: Yeast extract. After incubation of 66 h at 37 °C under shaking conditions (100 rev/min), the growth (□) and protease activity (■) was quantified.

inducer for protease production (Daatsellar & Harder 1974). However, in our case the protease production was highly repressed by casamino acids.

The inorganic nitrogen sources studied here were less favourable for both growth and enzyme production. While the enzyme production with inorganic nitrogen sources was highly reduced (0.4–24 U/ml), the growth was significantly supported (Figure 4). Among the inorganic nitrogen sources, however, there was significant variation in enzyme production with different sources. The growth, on the other hand, did not vary significantly. Among the sources, potassium nitrate was the best supporter for growth and enzyme production at 24 U/ml (Figure 4). These findings supported the phenomenon of repression of the growth and enzyme production by ammonium ions (Heineken & Connor 1972; Kole *et al*. 1988; Ferrero *et al*. 1996; Johnvesly & Naik 2001; Beg *et al*. 2002).

Effect of glucose concentrations

Positive correlation of glucose (0–2%, w/v) was observed on growth. The enzyme production increased from 120 to 250 U/ml with an increase in glucose concentration from 0 to 1% (w/v). However, beyond 1%, the enzyme production was highly suppressed. (Figure 5) suggesting a threshold level of glucose for optimum protease production. A similar relationship is reported in case of an alkaline protease from alkaliphilic *Bacillus* sp. where glucose repressed protease production (Kaur *et al*. 2001). Earlier, protease synthesis was demonstrated to be modulated by an inducer catabolite repression system, where glucose and ammonia repressed enzyme production (Reilly & Day 1983).

Effects of yeast extract and peptone concentrations

Yeast extract enhanced the growth as well as enzyme production when added in complex medium in the range of 0–2% (w/v). The steady increase in growth was observed up to 2% while the enzyme production was in the range of 120–190 U/ml, the maximum being at 0.5% yeast extract (w/v). While the growth was nearly unaffected by peptone concentrations in the range of 0–2.5% (w/v), the enzyme production increased from 30 to

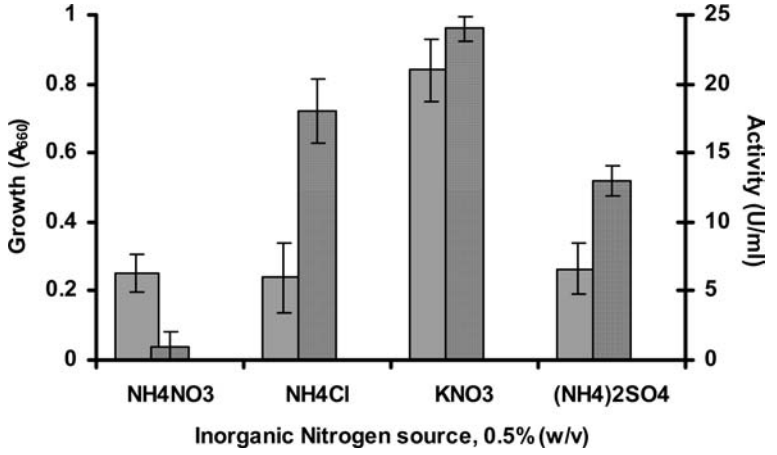

Figure 4. Effect of different inorganic nitrogen sources (0.5%, w/v). Ammonium nitrate (NH_4NO_3), Ammonium chloride (NH_4Cl), Ammonium sulphate ($(NH_4)_2SO_4$) and Potassium nitrate (KNO_3). After incubation of 66 h at 37 °C under shaking conditions (100 rev/min), the growth (■) and protease activity (■) was quantified.

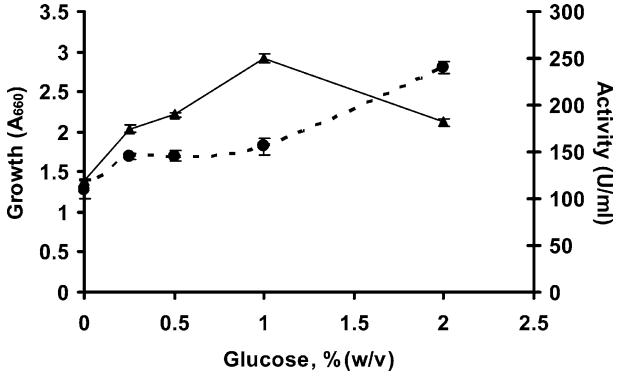

Figure 5. Effect of glucose concentration (0–2 %, w/v) on growth (●) and protease activity (▲). Samples were withdrawn after incubation of 66 h at 37 °C.

260 U/ml with increase in peptone concentration from 0 to 0.5% (w/v). However, drastic decrease in protease production was evident with further increase in the peptone concentration and at 2.5% (w/v), enzyme production fell to 105 U/ml (Figure 6).

Effect of K_2HPO_4

Inorganic phosphate slightly induced the growth with moderate suppression of enzyme production from 240 to 170 U (Figure 7). This effect was in contradiction to the alkaline protease production from *Bacillus firmus*, where an increased supply of nitrogen and phosphorus stimulated protease synthesis up to certain threshold levels (Moon & Parulekar 1991). This effect with our alkaline protease may be due to the difference in the production and secretion of the alkaline protease in haloalkaliphilic bacteria as compared to alkaliphilic organisms. So far, we have not come across with any particular reference relating to the protease production in haloalkaliphilic bacteria. The maximum enzyme production by our isolate, Po2 was achieved in the late exponential and early stationary phases that correspond to the secondary metabolism. Therefore, the enzyme production may not be solely dependent on the phosphate, usually known to assist primary metabolism.

Enzyme production from cheap sources

Cheaper sources for both carbon and nitrogen sources are the key attraction for commercialization of the production processes and thus, ability of the microorganisms to grow and produce enzymes using these sources has been arguably a point of interest (Kanekar 2002). Two cheap sources, wheat flour and molasses, were used for the production of alkaline protease from Po2. Among them, wheat flour (1%, w/v) proved a better source as it supported both growth and protease production (183 U/ml). The enzyme production paralleled growth with increasing concentrations of wheat flour up to 1% (w/v) (Figure 9). In contrast, molasses was relatively less effective towards growth and enzyme production. An inverse relationship between growth and protease production was evident with molasses (Figure 8).

In the literature, an extracellular alkaline protease from *Bacillus horikoshii*, was optimally produced when

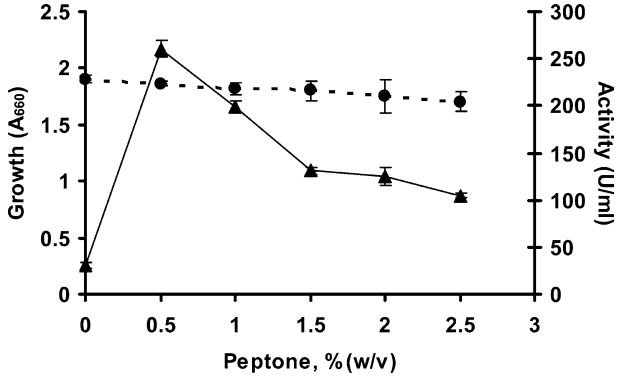

Figure 6. Effect of peptone (0–2.5%, w/v) on growth (●) and protease activity (▲). Samples were withdrawn after incubation of 66 h at 37 °C.

grown in soybean meal (1.5%, w/v) and casein (1%, w/v) (Joo *et al*. 2002). This feature was also observed in some earlier reports (Fujiwara & Yamamota 1987; Johnvesly & Naik 2001). More recently, alkaline protease production from *Bacillus* sp. was investigated in solid-state fermentation using wheat bran and lentil husk as carbon and nitrogen sources, where wheat bran was a better source (Uyar & Baysal 2004).

As our haloalkaliphilic *Bacillus* sp. Po2 described in this study, produced substantial amounts of alkaline protease, further investigations on the factors affecting its synthesis would be of great significance. Moreover, some of the novel features of the enzyme such as stability over the wide range of pH and salt make it an attractive candidate for future studies and process development.

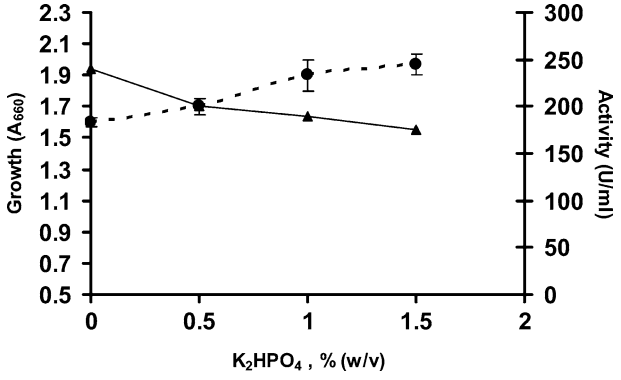

Figure 7. Effect of K_2HPO_4 (0–1.5%, w/v) on growth (●) and protease activity (▲). Samples were withdrawn after incubation of 66 h at 37 °C.

Acknowledgements

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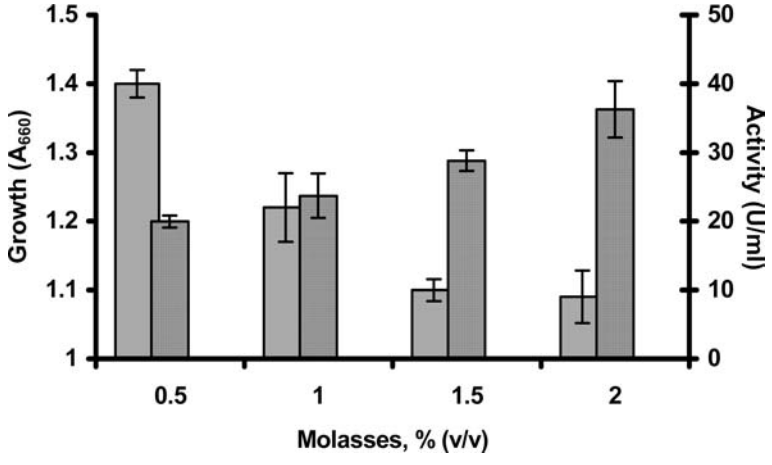

Figure 8. Effect of molasses (0.5–2%, w/v) on growth (□) and protease activity (■). Samples were withdrawn after incubation of 48 h at 37 °C.

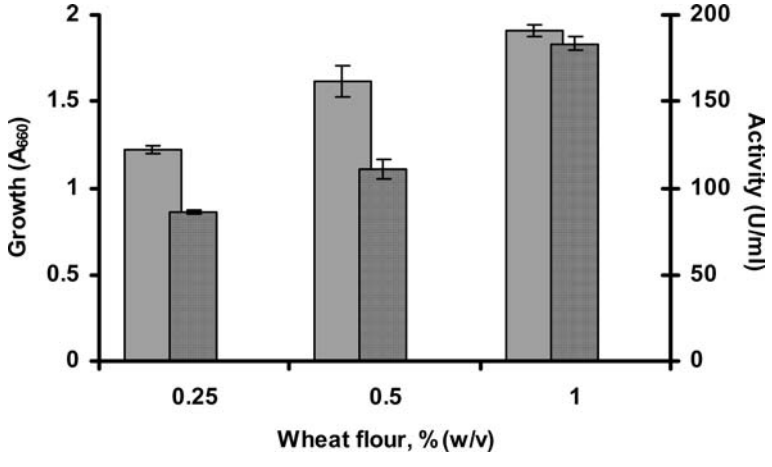

Figure 9. Effect of wheat flour (0.25–1%, w/v) on growth (□) and protease activity (■). Samples were withdrawn after incubation of 48 h at 37 °C.

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