

Full Length Research Paper

Production of pectinase by *Bacillus subtilis* EFRL 01 in a date syrup medium

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Accepted 28 June, 2012

A newly isolated *Bacillus subtilis* EFRL 01 was used to produce pectinases in batch fermentations on various inexpensive media. A mineral medium based on waste date syrup (15 g/L) as the carbon source and yeast extract (7.5 g/L) as the nitrogen source proved to be the most effective. A 48 h batch fermentation in this medium with a starting pH of 8.0 produced a pectinase titer of ~2,700 U/mL at the optimal fermentation temperature of 45°C. The optimal temperature, initial pH, the carbon source and its concentration, and the nitrogen source and its concentration, were identified after evaluation of multiple nutrient sources and fermentation conditions.

Key words: Polygalactouronase, *Bacillus subtilis*, fermentation, pectinases, date syrup.

INTRODUCTION

Pectinases hydrolyze pectins, the soluble complex polysaccharides that occur widely in plant cell walls. Pectinases are commercially used in many processes (Kashyap et al., 2001) and nearly 25% of the global enzyme sales are attributed to pectinases (Kaur et al., 2004). An emerging application of pectinases is in cotton scouring. Traditionally, cotton fibers are made absorbent and dyeable by treating with alkalis to remove the pectins and waxes that coat the fibers. This process is known as scouring. In view of its adverse environmental impact, alkali scouring is being replaced by enzymatic scouring using alkaline pectinases (Perkins, 1996; Wang et al., 2007; Dhiman et al., 2008). Alkaline pectinases that are active at alkaline pH values are also used in processing of pulp and paper (Sharma and Satyarnarayana, 2004; Favela-Torres et al., 2005).

As a bulk enzyme that is not recovered after use, pectinase must be produced cheaply. Several pectin rich substrates have been previously used to produce

microbial pectinases by fermentation (Blandino et al., 2002; Crotti et al., 1999; H et al., 1988; Patil and Dayanand, 2006; Freixo et al., 2008). Date syrup is a potential substrate that has been utilized for ethanol, citric acid and amylase production by some fungal strains (Acourene and Ammouche, 2012) but no report is available for pectinase production using date syrup. Here, we report on the use of date syrup for producing pectinases for possible use in cotton scouring.

Date (*Phoenix dactylifera* L.) is an important crop in desert regions of the Middle East and contributes significantly to human nutrition in some regions (Mohammed et al., 1983). Date fruit is highly nutritious and rich in calories (Al Hooti et al., 2002). Date fruit is boiled and then dried for storage. This process results in rich waste syrup that is potentially useful as a fermentation substrate. Large quantities of waste date syrup are produced, for example, in Sindh region of Pakistan. Pakistan also has a very substantial cotton textile industry. Use of the waste syrup to produce pectinases can potentially eliminate a pollution problem, improve revenues in date processing and reduce the cost of importing expensive pectinases for use in production of cotton textiles.

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Several fungal strains have shown great potential to produce different types of pectinolytic enzymes (Junwei et al., 1992; Junwei et al., 2000; Nitnikumar and Bhushan, 2010; Poonpairaj et al., 2001; Silva et al., 1993; Solis et al., 2009). There are a few reports of pectinase production by bacterial strains. Some of the bacterial species producing pectinases are *Agrobacterium tumefaciens*, *Bacteroides thetaiotamicron*, *Ralstonia solanacearum*, and *Bacillus* sp. (Jayani et al., 2005; Ranveer et al., 2010). However, selection of a commercial strain remains a laborious task and the choice gets tougher if commercially competent enzyme yields are to be achieved. Bacterial strain producing commercial enzymes are always preferred over fungal strains because of ease of fermentation process (for production) and implementation of strain improvement techniques or any modern technique to increase the yield of production (Prathyusha and Suneetha, 2011). In the present study, *Bacillus* sp was exploited for pectinase production from date syrup.

MATERIALS AND METHODS

Microorganism

Bacillus subtilis EFRL 01 was isolated from soil samples collected at Institute of Chemistry, University of Sindh, Jamshoro, Pakistan. The culture was identified using morphological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The bacterium was maintained on nutrient agar medium at 4°C and subcultured weekly. The agar medium contained (per liter of distilled water) the following: 3 g meat extract, 3 g peptone, 3 g sodium chloride, and 15 g agar.

Pectinase production

B. subtilis EFRL 01 was grown in a medium that contained the following (g per liter of distilled water): glucose, 10; peptone, 5; MgSO₄·7H₂O, 5; KH₂PO₄, 5 and Fe₂SO₄·7H₂O, 0.01. The initial pH was adjusted to 7.0. The culture medium (50 mL) was autoclaved (121°C, 20 min) in a 500 ml shake flask, cooled to the incubation temperature of 37 ± 2°C and inoculated with 0.5 ml of a culture that had grown for 90 h at 37°C and 120 rpm. After every 6-h of incubation, the pectinase containing supernatant was recovered by centrifugation (6000 g, 10 min, 4°C) and used for further analyses. The biomass was measured spectrophotometrically at 600 nm wavelength.

Effect of carbon source

The effect of carbon source (10 g/L) on enzyme production was assessed by replacing glucose in the above specified medium with an equal concentration of fructose, maltose, lactose, starch, molasses or date syrup. The inoculated flasks were incubated at the above specified conditions for 48 h. In one series of experiments, the concentration of the date syrup in the above specified medium was varied in the range of 5 to 30 g/L.

Effect of nitrogen source

Various nitrogen sources (corn steep liquor, yeast extract, sodium

nitrate, potassium nitrate and ammonium chloride) at a concentration of 5 g/L were used to replace peptone during enzyme production in the earlier specified basal medium. In one set of experiments, the yeast extract concentration was varied in the range of 2.5 to 15.0 g/L. Incubation conditions were 120 rpm, 37°C, for 48 h.

Influence of initial pH

Initial pH of the enzyme production medium was adjusted in the range of 4 to 10 with 0.1 M HCl or NaOH. The fermentations were carried out as specified above.

Effect of temperature

Fermentations with specified media were carried out at various temperatures ranging from 30°C to 60°C. The other conditions were as previously specified.

Pectinase assay

The pectinase activity was measured in the culture supernatant using a method adapted from Miller (1959). Thus, 1 ml of the cell-free supernatant was mixed with an equal volume of an aqueous solution of pectin (10 g/L) as the substrate. The mixture was incubated at 37°C for 15 min. Dinitrosalicylic acid reagent (2 mL) was then added and the reaction mixture was boiled for 5 min. The absorbance of the cooled reaction mixture was read at 540 nm against a blank. The latter was prepared exactly as the sample with the exception that pure water was added instead of the substrate. One unit of pectinase activity was defined as the amount of enzyme required for liberating 1 µg of galactouronic acid per minute under the assay conditions.

RESULTS AND DISCUSSION

Pectins occur widely in plants and therefore many microorganisms have developed the ability to produce pectinases. There are a few reports of pectinase production by bacterial strains (Jayani et al., 2005; Ranveer et al., 2010). This work focused on production of extracellular pectinases using a newly isolated soil bacterium *B. subtilis* EFRL 01 because bacteria grow rapidly. The *B. subtilis* pectinases are known to be useful in processing of cotton textiles (Ahlawat et al., 2009). The aim was to develop a really low cost batch production process for possible use in developing regions where much of the cotton textile industry is located. For operational simplicity and reduced expense, the culture pH and dissolved oxygen concentration were not controlled.

The fermentation profile of *B. subtilis* on a simple glucose based medium is shown in Figure 1. The maximum activity of pectinase was achieved after 48 h in a medium that contained 10 g/L glucose. The reduction in pectinase production after 48 h might be the result of change in pH during fermentation (Samriya et al., 2012), denaturation or decomposition of enzyme due to interaction with other components of medium (Soares et al., 1999), and depletion of nutrients in the medium

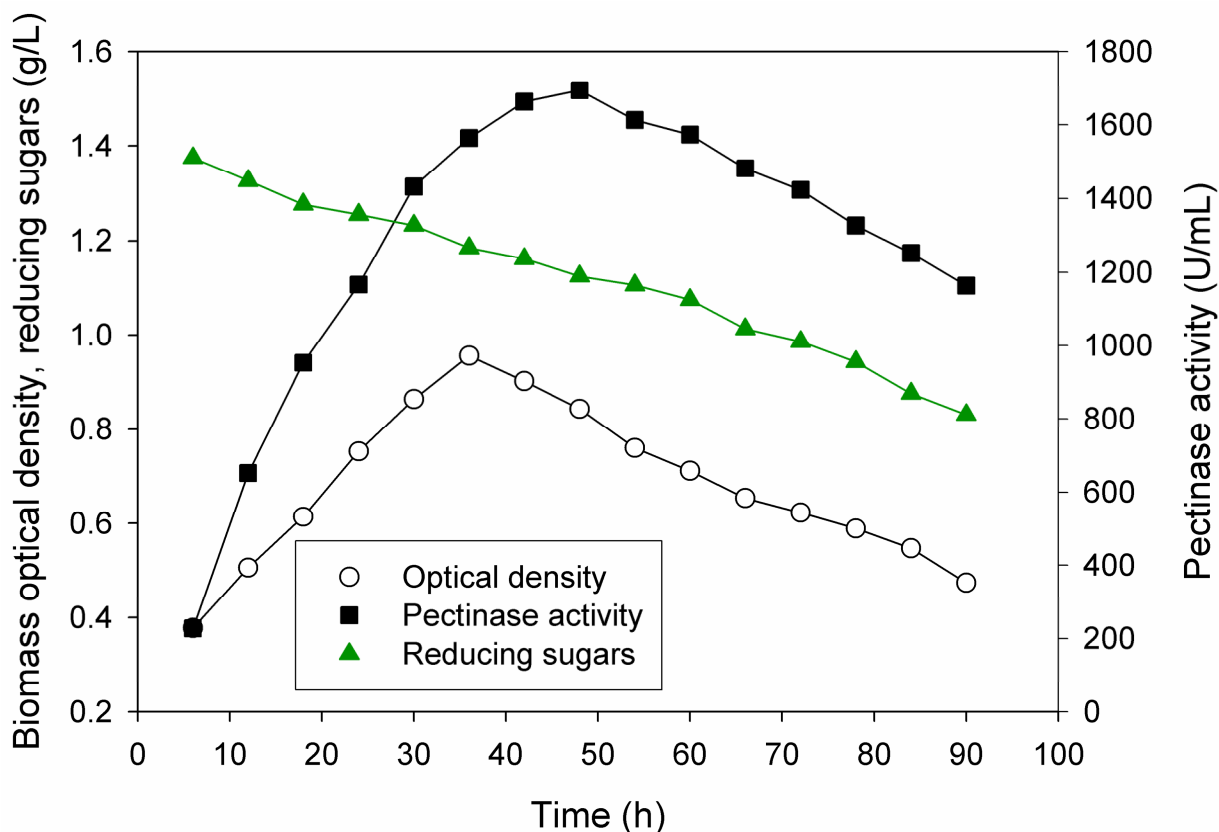


Figure 1. Batch profiles of biomass growth, pectinase activity and reducing sugar concentration during enzyme production (37°C, initial pH of 7.0). All experiments were in triplicate. The results are the mean.

(Palaniyappan et al., 2009). The time required to develop peak activity was significantly shorter than 72 h reported for peak pectinase activity in cultures of fungi such *Aspergillus niger* (Patil and Dayanand, 2006) and *Coriolus versicolor* (Freixo et al., 2008).

The effect of various carbon sources on pectinase production was tested by replacing glucose with the relevant carbon source. The other components of the medium were the same as in the glucose-based mineral medium. The results are shown in Figure 2. Compared to the other carbon sources, complex media based on molasses and date syrup gave the highest pectinase activity at 48 h of fermentation. The date syrup is liquid which is produced as a by-product of date industry contains (75% carbohydrates w/w) small amount of fats and proteins along with micro and macroelements (Al-Farsi et al., 2007; Al-Hooti et al., 2002) The date syrup based medium was the most effective probably because dates contain a significant quantity of pectin (22.4 mg/100g of fresh weight of dates) and this may have induced production of pectinases. In view of its effectiveness and low cost, date syrup was used as the carbon source in all subsequent work. Further tests were done to elucidate the effect of the initial concentration of date syrup in the medium on production of pectinase

within 48 h. The data are shown in Figure 3. Peak pectinase activity was produced at a date syrup concentration of 15 g/L. Higher concentrations severely inhibited the pectinase production. An elevated sugar concentration has been found to suppress pectinase production also in other microorganisms such as the fungus *Aspergillus japonicus* (Teixeira et al., 2000).

The effects of various inorganic and organic nitrogen sources (initial concentration of 5 g/L) on final pectinase activity and biomass concentration in a date syrup based mineral medium are shown in Figure 4. Yeast extract proved to be the best nitrogen source likely because it provided other stimulatory components such as vitamins. Yeast extract has previously proved superior to other nitrogen sources in the production of pectinases by the thermophilic fungus *Sporotrichum thermophile* (Kaur et al., 2004); *Bacillus shaericus* MTCC 7542 produced maximum polygalactouronase when grown on mineral medium containing yeast extract as sole nitrogen source (Ranveer et al., 2010). In view of its beneficial effect, the initial concentration of the yeast extract in the medium (15 g/L date syrup as carbon source) was varied to see how it might impact the production of the enzyme (Figure 5). Enzyme production at 48 h peaked when the initial concentration of the yeast extract was 7.5 g/L. Higher

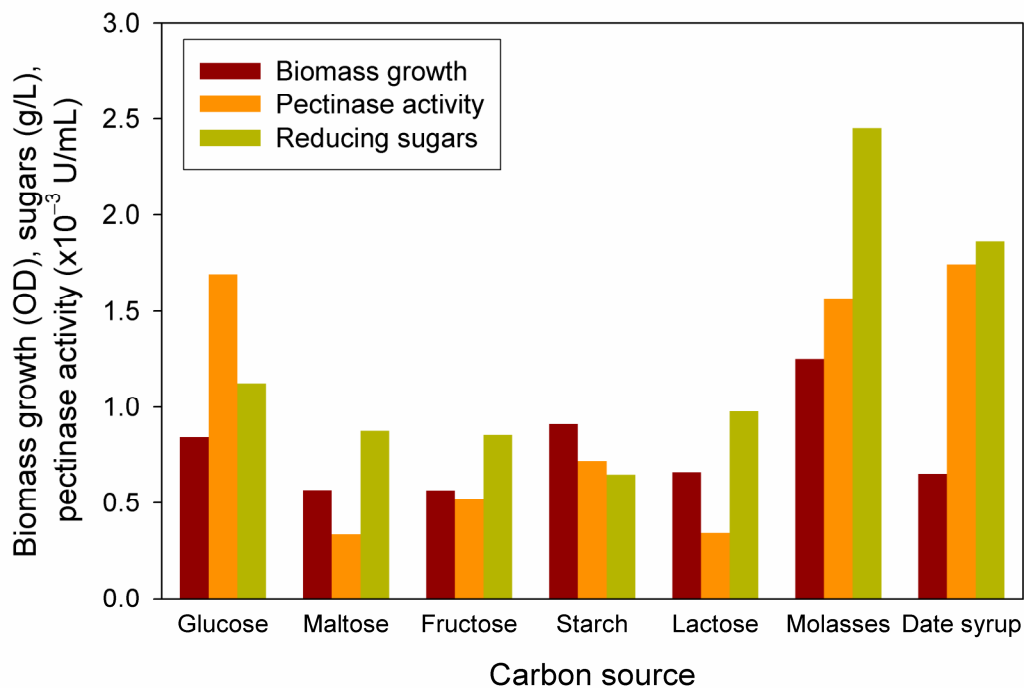


Figure 2. Effect of the carbon source (10 g/L initial concentration) on biomass concentration, pectinase activity and residual reducing sugar level at 48 h of fermentation (37°C, initial pH of 7.0). All experiments were in triplicate. The results are the mean.

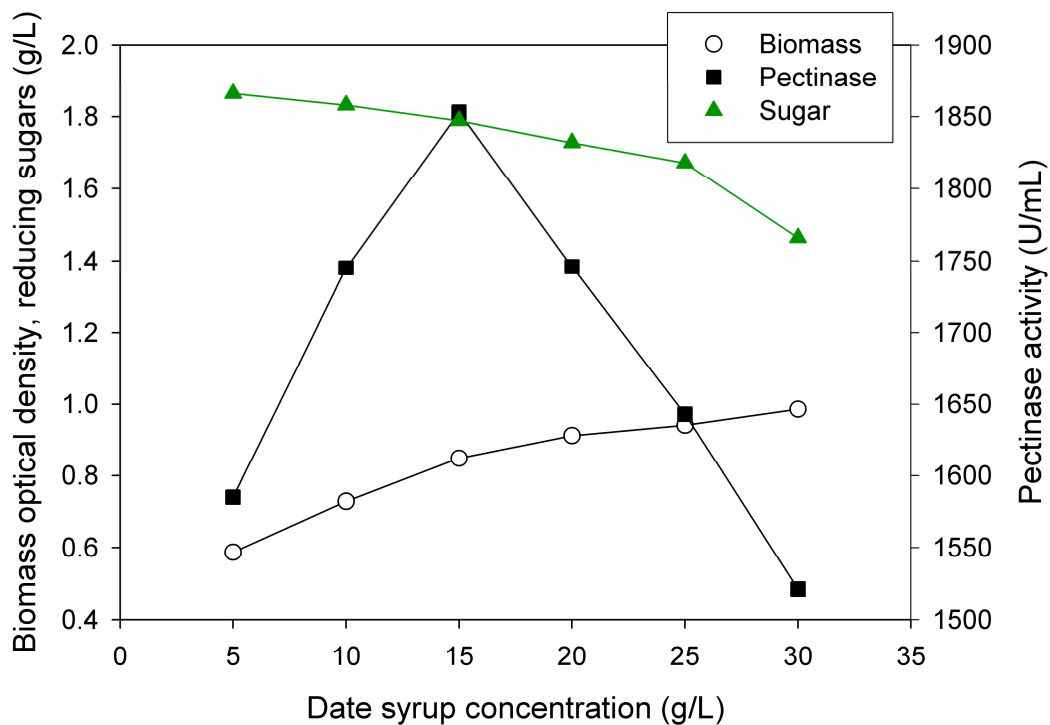


Figure 3. Effect of initial concentration of date syrup on final pectinase activity, biomass concentration and residual sugar concentration at 48 h of fermentation (37 °C, initial pH of 7.0). All experiments were in triplicate. The results are the mean.

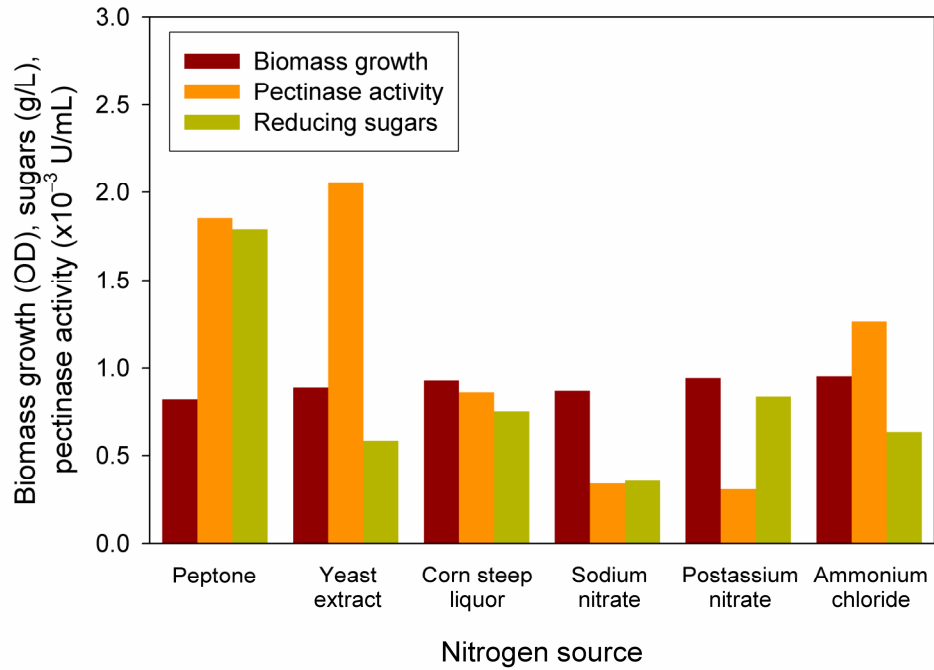


Figure 4. Effect of nitrogen source (5 g/L initial concentration) on pectinase production, biomass concentration and residual reducing sugar level at 48 h of fermentation (37 °C, initial pH of 7.0) in a date syrup (15 g/L initial concentration) mineral medium. All experiments were in triplicate. The results are the mean.

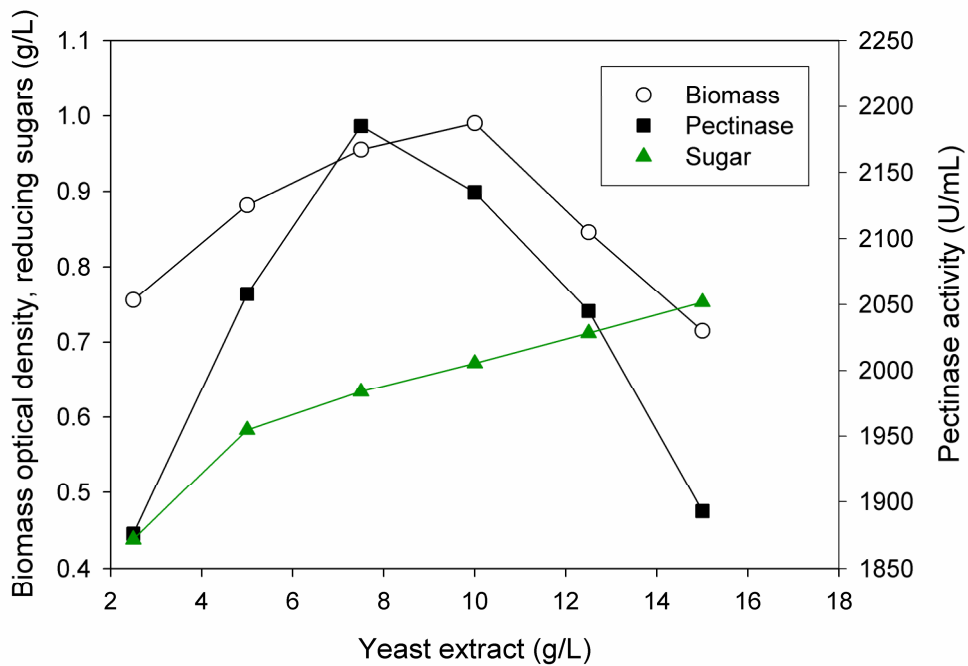


Figure 5. Effect of initial concentration of yeast extract on pectinase activity, biomass concentration and residual sugar concentration at 48 h of fermentation (37 °C, initial pH of 7.0). All experiments were in triplicate. The results are the mean.

concentrations of yeast extract inhibited both enzyme production and microbial growth (Figure 5).

The effects of initial pH of fermentation on enzyme production and microbial growth are shown in Figure 6.

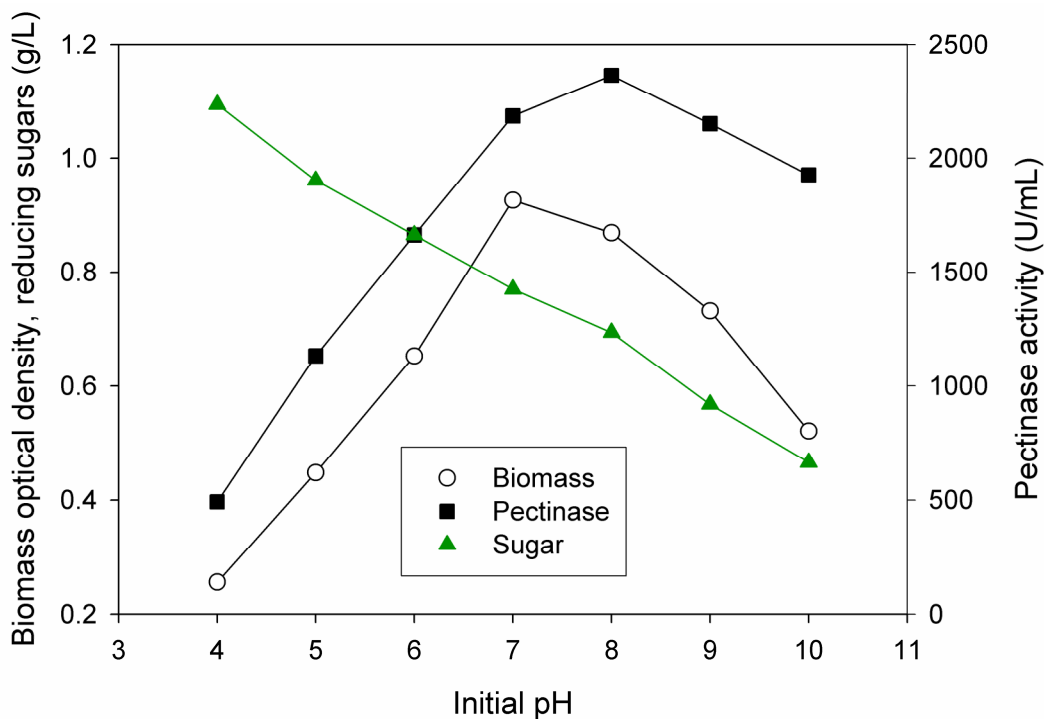


Figure 6. Effect of initial pH on pectinase production, biomass growth and residual sugar concentration at 48 h of fermentation (37 °C) in a mineral medium containing date syrup and yeast extract at initial concentrations of 15 g/L and 7.5 g/L, respectively. All experiments were in triplicate. The results are the mean.

Clearly, the optimal initial pH for pectinase production was 8 even though the optimal initial pH for biomass production was lower at 7.0 (Figure 6). Initial pH values of >8 adversely affected microbial growth and enzyme production. The reduction of pectinase production might be due to change in the pH of fermentation medium. Uenojo and Pastore (2007) and Cordeiro and Martins (2009) suggest that the reduction of pH is due to production of galactouronic acid by the action of pectinase on pectin which affect the pectinase production. The optimal initial pH value for producing pectinases of course depends on the microorganism nature. For example, a much higher initial optimal pH of 8.5 has been reported for pectinase production by *Bacillus pumilus* dcsr1 (Sharma and Satyanarayana, 2006); 8.0 pH has been noted for alkaline pectinase production by bacteria (Cocci Sps) (Ajit and Rita, 2012). In contrast, the optimal initial pH for pectinase synthesis by the thermophilic fungus *S. thermophile* has been found to be 7.0 (Kaur et al., 2004). The genes involved in the production of certain enzymes in at least some microorganisms are known to be pH regulated (Young et al., 1996).

The effect of fermentation temperature on production of biomass and pectinase activity is shown in Figure 7. Clearly, 45°C was the best fermentation temperature. It maximized both the enzyme titer and the final biomass

concentration. The optimal fermentation temperature of *B. subtilis* ERFL 01 is clearly much lower than the optimal temperature of 50°C reported for *B. pumilus* dcsr1 (Sharma and Satyanarayana, 2006). The observed optimum temperature in our work is comparable to a temperature of 45°C reported for the thermophilic mold *S. thermophile* (Kaur et al., 2004); 45 to 50°C has been reported for pectinase production by *Thermomucor indicae-seudaticae* N31 (Martin et al., 2010), and 50°C for thermostable exo-polygalactouronase from actinomycete (*Streptomyces erumpens* MTCC 7317 (Shaktimay and Ramesh, 2011).

Conclusion

Optimized batch fermentations conducted in a date syrup based medium using a newly isolated *Bacillus subtilis* EFRL 01 yielded a pectinase titer of ~2,700 U/mL. This peak titer was substantially greater than could be attained on media that used various other carbon sources. The optimal medium for producing the enzyme was a mineral medium formulated with 15 g/L of waste date syrup as the carbon source and 7.5 g/L yeast extract as the nitrogen source. At optimal fermentation temperature of 45°C, the peak enzyme titer was attained in 48 h in a medium with an optimal initial pH of 8.0. Waste date

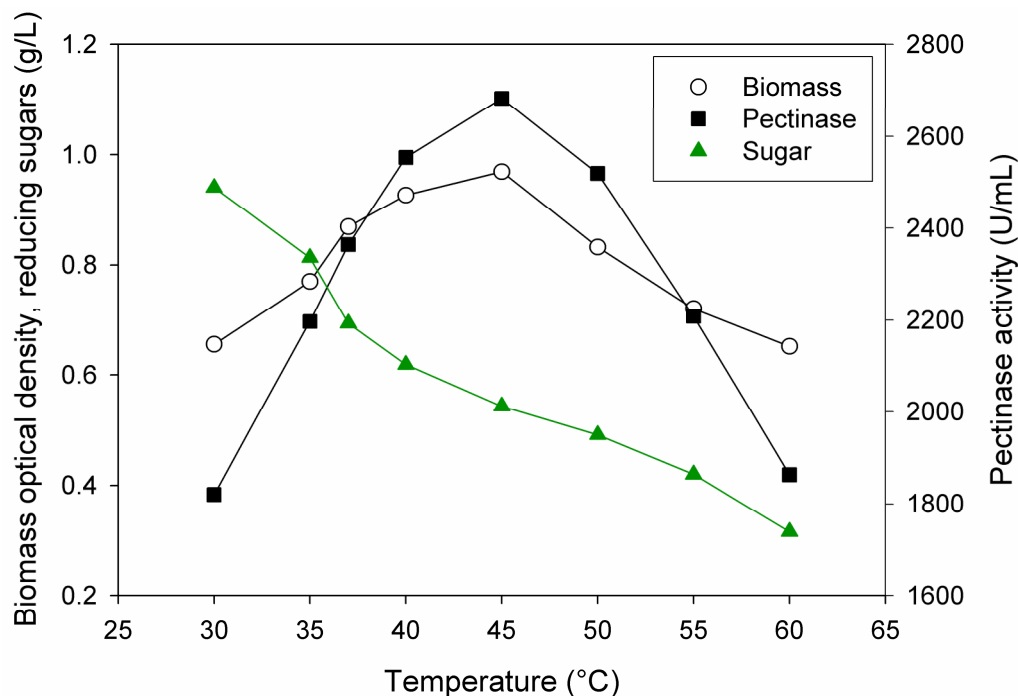


Figure 7. Effect of fermentation temperature on pectinase production, biomass concentration and residual sugar concentration at 48 h. The medium initially contained 15 g/L date syrup and 7.5 g/L yeast extract. The initial pH was 8.0. All experiments have been carried in triplicate. The results are the mean.

syrup can be used to inexpensively produce pectinases in batch fermentations using *B. subtilis* EFRL 01.

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