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ORIGINAL ARTICLE

Production of pectinolytic enzymes pectinase and pectin lyase by *Bacillus subtilis* SAV-21 in solid state fermentation

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Abstract Pectin-degrading enzymes (pectinase and pectin lyase) were produced in solid state fermentation by *Bacillus subtilis* SAV-21 isolated from fruit and vegetable market waste soil of Yamuna Nagar, Haryana, India, and identified by 16S rDNA sequencing. Under optimized conditions, maximum production of pectinase (3315 U/gds) and pectin lyase (10.5 U/gds) was recorded in the presence of a combination of orange peel and coconut fiber (4:1), with a moisture content of 60% at 35 °C and pH 4.0 after 4 days and 8 days of incubation, respectively. Pectinase yield was enhanced upon supplementation with galactose and yeast extract, whereas pectin lyase production was unaffected by adding carbon and nitrogen source to the basal medium. Thus, *B. subtilis* SAV-21 can be exploited for cost-effective production of pectinase and pectin lyase using agro-residues.

Keywords Coconut fiber · Pectinase · Pectin lyase · Solid state fermentation · Optimization

Introduction

Polysaccharide-degrading enzymes have wide applications in industry. Among them, pectinases contribute to more than 25% of global enzyme sales (Jayani et al. 2005). Pectinases are a group of enzymes involved in the depolymerization of pectic polymers. Based on their mode of action, pectinases include polygalacturonase (PG), pectin esterase (PE), pectin lyase (PL), and pectate lyase (Ahlawat et al. 2009). Pectinase is involved in the hydrolysis of pectin containing a certain degree of esterified groups, while PG acts on unesterified polygalacturonic acid. Among all pectinases, pectin lyases are the only enzymes capable of depolymerizing highly esterified pectin into small molecules without prior action of other enzymes. PL cleaves pectin by a β-elimination mechanism to form 4,5-unsaturated oligogalacturonates without affecting polymer chain ester content, which is responsible for the specific aroma of fruits (Yadav et al. 2009).

Pectinases are used in fruit juice extraction and clarification, scouring of cotton, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, alcoholic beverages, and in food industries (Jayani et al. 2005). In order to meet this high demand, it is important to produce pectin-depolymerizing enzymes at a large scale in a cost effective manner. Solid state fermentation (SSF) has gained more popularity in recent years in the production of many enzymes due to its lower operation costs and energy requirements, simpler plant and equipment projects, higher enzyme production and lower effluent generation as compared to submerged fermentation (SmF) (Pandey et al. 1999; Couto and Sanroman 2006). The selection of substrate is important in SSF and depends on several factors, mainly the cost of availability, and this may involve screening of numerous agro-industrial residues.

Being both economical and ecofriendly, agro-industrial residues are the prime choice of substrate for enzyme production. Several agro-residues including, wheat bran, sugar beet, sugar cane bagasse, corn cob, wheat straw and citrus wastes have been utilized for pectinolytic enzymes production (Bai et al. 2004; Maller et al. 2011; Khan et al. 2012). Orange peel, a rich source of pectin containing about 20–30% pectic substances (May 1990), is a good inducer of pectinase. Coconut fiber, which contains 3–4% pectic content (Franck 2000), has

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not been exploited for pectinase and PL production in SSF. Currently, the global annual production of coconut fiber is about 350,000 metric tons, of which 90% is accounted for by India along with Sri Lanka. But still, this resource is underutilized and it accumulates as waste during coconut processing, which can cause a threat to the environment upon disposal (Coir Board, India 2016). Coconut fiber could thus be utilized in production of enzymes such as pectinase and pectin lyase. A survey of the literature revealed that PLs have been produced mainly from fungal genera such as *Aspergillus*, *Penicillium* and *Fusarium*. Since only a few reports are available on bacterial PLs (Nadaroglu et al. 2010; Demir et al. 2011, 2014; Gopinath and Suneetha 2012; Li et al. 2012), it is pertinent to isolate, produce and characterize these industrially important enzymes from bacterial sources using pectin containing agro-residual wastes in SSF. This will not only help utilize these agro-residues but also lead to cost-effective production of enzymes.

Materials and methods

Microorganism and culture conditions

The bacterial strain *Bacillus subtilis* SAV-21 was isolated from fruit and vegetable market waste soil of Yamunanagar, Haryana, India, in our laboratory for pectinase and PL production (Rehman et al. 2012). The strain was identified on the basis of 16S rRNA gene sequence (1375 bp) and submitted to NCBI with accession number KU145146. It was maintained on nutrient agar slants in a refrigerator and subcultured every week.

Inoculum preparation

The inoculum was prepared by transferring a loop full of *B. subtilis* SAV-21 culture (grown on a nutrient agar plate) into 50 mL autoclaved nutrient broth taken in a 250 mL flask, which was then incubated at 37 °C under agitation at 200 rpm on a rotary shaker for 18 h (Sharma and Satyanarayana 2012).

Processing of agro-industrial residues for use as substrates

Various agro-residues (wheat bran, rice bran, paddy straw, corn cob, sugarcane bagasse, mustard oil cake, saw dust, mustard straw, cotton straw, groundnut peel, wheat straw and cottonseed oil cake) and fruit peel wastes (lemon peel, mosambi peel, pineapple peel, papaya peel, banana peel, mango peel, coconut fiber, pomengranate peel, orange peel, cheeku peel and kinnow peel) were procured from local market of Yamuna Nagar, Haryana, India. These agro-residues were washed several times with tap water to remove all water-soluble compounds, and sun dried until they were completely dehydrated. Dried agro-residues were grounded to about 2–3 mm particle size in a grinder and used for the production of pectinase and pectin lyase (Rao et al. 2014).

SSF for the production of pectinase and PL

SSF was performed in Erlenmeyer flasks (250 mL) each carrying 5.0 g of one of the above mentioned substrates (alone or in combination) moistened with 70% distilled water before sterilization. The basal production medium in flasks was autoclaved, cooled and inoculated aseptically with 1.0 mL (20% v/w) of 18-h-old inoculum of *B. subtilis* SAV-21 (CFU = 1×10^9 mL^{-1}). Flasks were then incubated at 37 °C for 48 h in a bacteriological incubator (Kashyap et al. 2003).

Enzyme extraction

Pectinase and PL were extracted from the fermented substrate twice with 50 mL distilled water (pH 7.0) by constant shaking in an orbital shaker at 200 rpm for 10 min (Sharma and Satyanarayana 2012). The extract was squeezed through muslin cloth and centrifuged at 10,000 *g* for 15 min at 4 °C. The clear supernatant was used as a source of crude pectinase and PL.

Assay of pectinase activity

Pectinase activity was assayed by measuring the amount of D-galacturonic acid liberated from pectin. The reaction mixture containing 50 μL appropriately diluted enzyme and 450 μL 0.5% pectin (having 65–70% degree of esterification; dissolved in 50 mM glycine-NaOH buffer, pH 11.0) was incubated for 10 min at 60 °C, and the end products were quantified by using DNSA reagent (Miller 1959). A standard curve of D-galacturonic acid was prepared to convert absorbance values to μmol. One unit of pectinase was defined as the amount of enzyme required to liberate 1 μmol D-galacturonic acid per minute under the assay conditions.

Assay of PL activity

The assay of PL activity was based on measuring the absorption of colored derivatives obtained by the reaction of unsaturated uronic acid ester and thiobarbituric acid at 550 nm (Nedjma et al. 2001). A 250 μL aliquot of suitably diluted enzyme solution was incubated with 250 μL 0.5% (w/v) pectin (dissolved in 50 mM Tris–HCl buffer, pH 9.0) at 60 °C for 10 min and then 50 μL 1.0 *N* NaOH was added. The mixture was shaken and heated at 80 °C in a water bath for 5 min. After cooling, 600 μL 1.0 *N* HCl was added to the mixture and shaken. This resulted in the disappearance of the yellow color due to acidification of the medium. Then 500 μL 0.04 M thiobarbituric acid solution was added and incubated at

80 °C for 5 min in a water bath. The solution was cooled and its absorbance was measured at 550 nm. A control was prepared by adding 1% NaCl solution instead of the enzyme in the reaction mixture. The enzyme activity was expressed in terms of μmoles of unsaturated galacturonide released per minute, based on the molar extinction coefficient value of 5500 M^{-1} cm^{-1} of the unsaturated product.

Optimization of parameters affecting the enzyme production

Pectinase and PL were produced in SSF using basal medium containing a combination of orange peel and coconut fiber in 4:1 ratio (the best substrate selected after screening) as described above, and various parameters affecting the enzyme production were optimized by one varying one factor at a time keeping all other factors constant. All the experiments were run in triplicate.

Effect of temperature and pH

The effects of cultivation temperature and pH on enzyme production were studied by incubating the flasks containing 5.0 g orange peel and coconut fiber (4:1) at different temperatures (30–50 °C) and over a pH range of 3.0 to 10.0 for 48 h (Phutela et al. 2005).

Effect of nitrogen source

The best nitrogen source for the production of pectinase and PL by *B. subtilis* SAV-21 was determined by supplementing different organic (yeast extract, beef extract, peptone, tryptone, glycine, urea, lysine, ornithine and arginine) and inorganic nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate) separately in solid media to a final concentration of 1% (w/v) after dissolving them in distilled water used for adjusting the moisture content (Kashyap et al. 2003). In the control, no exogenous nitrogen source was added.

Effect of carbon source

The effect of different carbon sources (glucose, mannitol, pectin, galactose, sucrose, lactose, maltose, sodium acetate and xylose) was evaluated by supplementing these in solid media separately to a final concentration of 1% (w/v) after dissolving them in distilled water used for adjusting the moisture content (Kashyap et al. 2003). In the control, no exogenous carbon source was added.

Incubation period

To determine the optimum fermentation time, enzyme production was monitored over a time period of 2–8 days for pectinase and 2–10 days for PL (Bayoumi et al. 2008).

Effect of different metal salts and moisture content

The effect of different metal salts ($CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 2H_2O$, H_3BO_3, $ZnCl_2$, KCl and NaCl) at a final concentration of 1 mM was studied by adding these to solid medium after dissolving in the distilled water used to adjust the moisture level of the substrate (Kashyap et al. 2003) followed by monitoring the production of enzymes.

The effect of moisture level on pectinase and PL production was investigated by adding different volumes of distilled water (moistening agent) to the solid substrate to give a final moisture content of 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% according to El-Shishtawy et al. (2014).

Results and discussion

Screening of different agro-residues for use as substrates in SSF

In the present study, the pectin-degrading bacterial strain was isolated from fruit and vegetable waste soil after screening many bacterial isolates. It was identified as *Bacillus subtilis* SAV-21, and assigned the accession number KUK145146 by NCBI. For screening of the best substrate, 5.0 g each of 24 different agro-residues was used as substrates individually as well as in combinations for the production of pectinase and PL by *B. subtilis* SAV-21 at pH 7.0 and 35 °C. The results shown in Fig. 1a revealed the highest production of pectinase with orange peel (OP) (450.50 ± 12.8 U/gds) followed by cheeku peel (325.19 ± 10.33 U/gds) and coconut fiber (CF) (324.25 ± 9.35 U/gds), whereas the production of PL was maximum in OP (2.77 ± 0.15 U/gds) followed by CF (2.39 ± 0.32 U/gds) and mosambi peel (2.21 ± 0.33 U/gds). Agro-residues such as paddy straw, mustard straw and mustard oil cake did not induce pectinase production but showed PL production. With most of the substrates, the titer of PL was much lower as compared to pectinase.

Combinations of OP and rice bran each with other substrates in equal proportions were also evaluated as substrates for production of both the enzymes to examine whether the combination of substrates could increase enzyme production. The results showed the highest production of pectinase with a mixture of OP and lemon peel (LP) (524.91 ± 22.4 U/gds) ,closely followed by a mixture of OP and CF (503.95 ± 20.58 U/gds). Further, the enzyme production obtained with the combination of substrates was higher than that in the

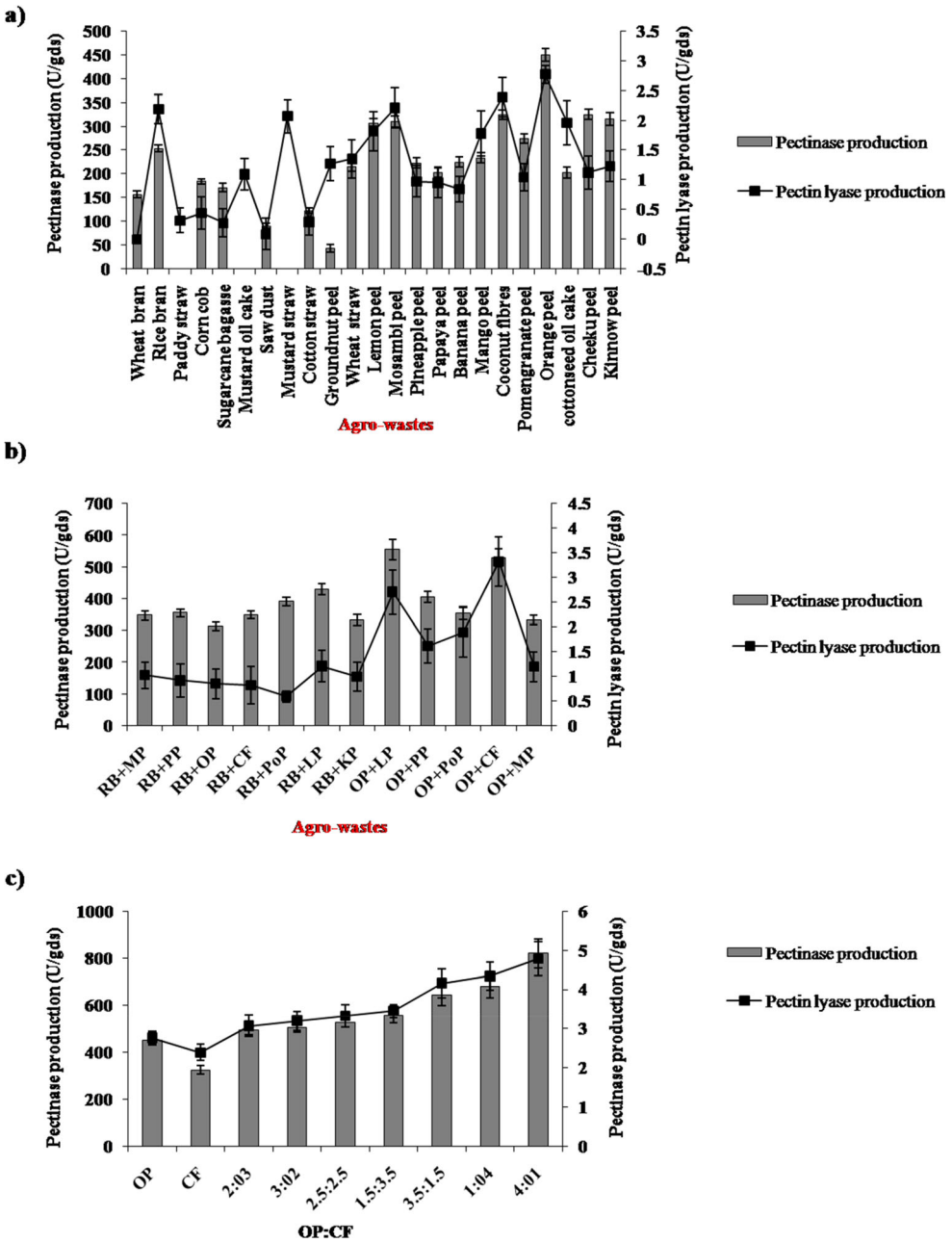

Fig. 1a–c Screening of different agro-wastes for use as substrates in solid state fermentation (SSF). **a** Pectinase and pectin lyase production by *Bacillus subtilis* SAV-21 using different agro-industrial residues as substrates. **b** Effect of combinations of rice bran and orange peel with other fruit peel wastes on pectinase and pectin lyase production by *Bacillus subtilis* SAV-21. *RB* Rice bran, *MB* mosambi peel, *OP* orange peel, *PP* pineapple peel, *PoP* pomengranate peel, *KP* kinnow peel, *CF* coconut fiber, *LP* lemon peel. **c** Combined effect of OP and CF substrate usage in different proportions on pectinase and pectin lyase production by *B. subtilis* SAV-21

presence of OP (450.09 ± 12.8 U/gds), LP (306.95 ± 9.24 U/gds) or CF (324.25 ± 9.35 U/gds) alone. On the other hand, the production of PL was maximum (3.32 ± 0.49 U/gds) with a combination of OP and CF (Fig. 1b). In a further step of screening the most appropriate substrate combination, enzyme production was carried out using different ratios of OP and LP as well as OP and CF. The effect of varying the ratio of OP and LP revealed the highest production of pectinase (582.27 ± 40.75 U/gds) and PL (3.49 ± 0.20 U/gds) at a ratio of 3.5:1.5, while OP and CF combination showed maximum production of pectinase (820.11 ± 73.38 U/gds) and PL (4.78 ± 0.44 U/gds) at a ratio of 4:1 (Fig. 1c). A comparison of the highest enzyme titers showed that the production of pectinase and PL was 71.0% and 73.2% higher with the combination of OP and CF than with the OP and LP mixture, respectively. Hence, the 4:1 mixture of OP and CF was selected as the best substrate for optimizing production of both the enzymes in SSF.

The nature of the solid substrate is the most crucial factor in SSF, as it not only provides nutrients to the microbial culture growing on it, but also serves as an anchorage for growth of microbial cells. Therefore, it becomes necessary to select a solid substrate or a mixture of substrates for optimal enzyme production. In this study, the mixture of OP and CF (4:1) supported maximum production of both pectinase and PL, which might be attributed to its higher pectin content.

Though, pectinolytic enzymes have been produced in SSF by many researchers using different agro-residues, to date there is no report on the use of CF as the substrate. Pectinase production has been reported using wheat bran by *Bacillus* sp. MG-cp-2 (Kapoor et al. 2000), *Bacillus* sp. DT7 (Kashyap et al. 2003) and *B. megatherium* (El-Shishtawy et al. 2014); decorticated ramie fiber by *Bacillus* sp. MG-cp-2 (Kapoor et al. 2000); a mixture of sesame oilseed cake, wheat bran and citrus pectin in the ratio of 1:1:0.01 by *B. pumilus* dcsr1 (Sharma and Satyanarayana 2012) and sugar beet pulp by *Bacillus gibsonii* S-2 (Li et al. 2005). PL has been produced using sugar beet pulp by *Bacillus clausii* (Li et al. 2012) and wheat bran by *B. pumilus* (Nadaroglu et al. 2010), *Geobacillus stearothermophilus* Ah22 (Demir et al. 2011), *Bacillus* sp. VITSG-1 (Gopinath and Suneetha 2012) and *Brevibacillus borstelensis* (Demir et al. 2014). PG production has been reported using *Solanum tuberosum* peel by *Bacillus firmus*-I-4071 (Bayoumi et al. 2008) and *B. licheniformis* (Dharmik and Gomashe 2013) and wheat bran by *Bacillus* sp. (Soares et al. 1999), *B. licheniformis* (Rehman et al. 2012) and *Bacillus tequilensis* SV-11 (Chiliveri et al. 2016).

Effect of temperature

Temperature is one of the important parameters that determines the success of the fermentation process because it directly influences the microbial growth and metabolic activity of enzymes. The effect of temperature on production of both the enzymes was tested in the range of 30–50 °C. The highest production of both pectinase (820.11 ± 61.38 U/gds) and PL (4.78 ± 0.48 U/gds) was observed at 35 °C but declined at higher temperatures (Fig. 2). Hence, the optimum temperature for production of both the enzymes was taken as 35 °C. At 50 °C, pectinase and PL production by *B. subtilis* SAV-21 was 63.54 and 67.57% of the maximum, respectively. The lower enzyme production at below and above the optimal temperatures could be due partly to reduced growth of the bacterial strain and a decrease in the moisture content of the substrate, respectively.

In consonance with the present observation, the optimal temperature for pectinolytic enzyme production by *Bacillus* sp. in SSF has been reported in the range of 30–50 °C in most cases. For pectinase production, the optimum temperature was reported as 30 °C for *Bacillus sp*. MG-cp-2 (Kapoor et al. 2000); 35 °C for *Bacillus* sp. DT7 (Kashyap et al. 2003); 37 °C for *B. firmus*-I-10104 (Bayoumi et al. 2008) and *B. megatherium* (El-Shishtawy et al. 2014), and 40 °C for *B. pumilus* dcsr1 (Sharma and Satyanarayana 2012). PL production in SSF was found to be optimum at 27 °C for *B. pumilus* and *G. stearothermophilus* Ah 22 (Nadaroglu et al. 2010; Demir et al. 2011); 30 °C for *Bacillus* VITSG-1 (Gopinath and Suneetha 2012) and 40 °C for *B. clausii* (Li et al. 2012). In contrast, the optimal temperature for PL production by *B. borstelensis* (P35) was recorded at 50 °C (Demir et al. 2014).

Effect of pH of the moistening agent

The effect of pH of the moistening agent (distilled water) was evaluated on the production of pectinase and PL in the range of 3.0–10.0. The enzyme titer was significantly affected by the pH of the moistening agent during fermentation. It was noted that pectinase and PL production by *B. subtilis* SAV- 21 increased with increase in pH up to 4.0, followed by a continuous decline with further increase in pH up to 10.0, indicating that the optimum pH for enzyme production was 4.0. The production of pectinase and PL at the optimum pH was 976.15 ± 48.15 U/gds and 6.29 ± 0.48 U/gds, whereas at pH 10.0, it was 452.22 ± 27.86 U/gds and 2.88 ± 0.16 U/gds, respectively (Fig. 3). The pH regulates the growth and synthesis of extracellular enzymes by several microorganisms. It may also affect the membrane permeability of microbes and the stability of enzymes. Since the *B. subtilis* SAV- 21 is acidophilic, the decrease in enzyme titer at pH values above the

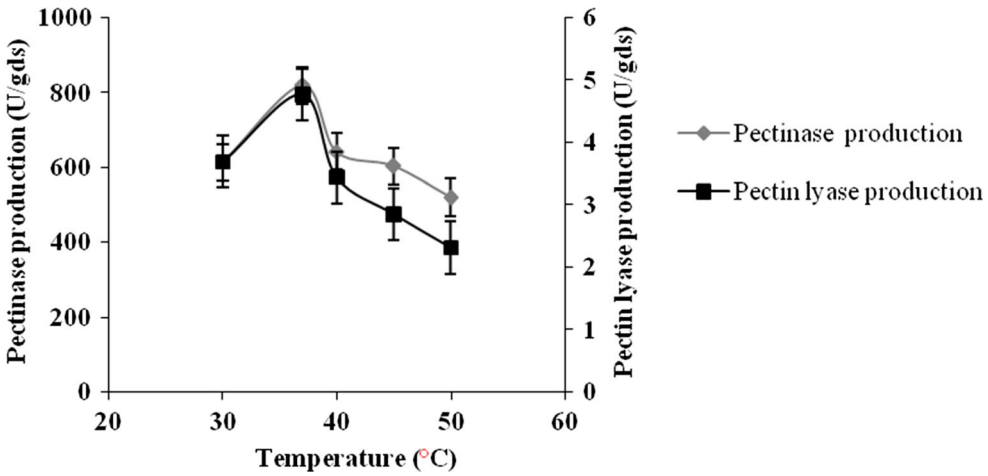

Fig. 2 Effect of fermentation temperature on pectinase and pectin lyase production by *B. subtilis* SAV-21

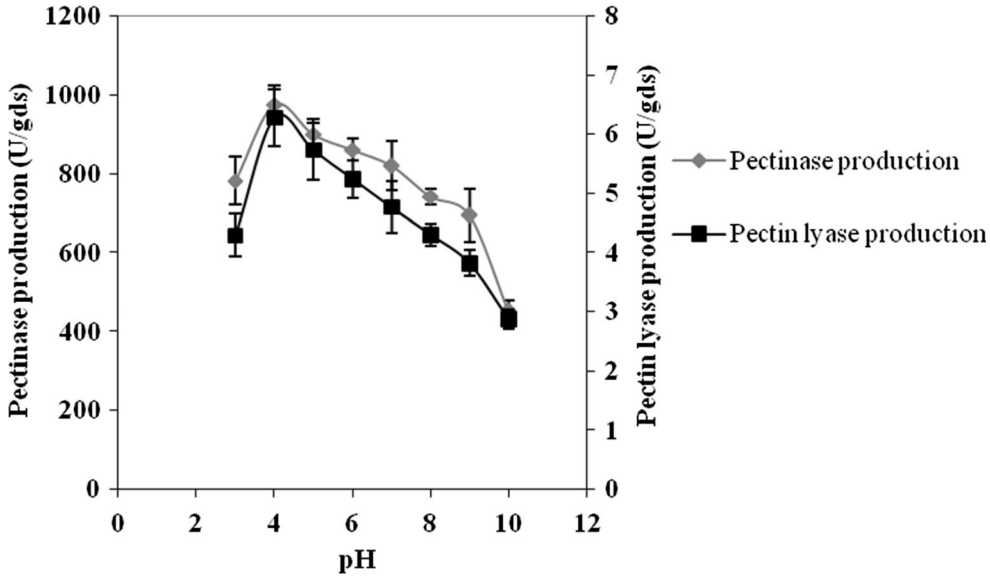

Fig. 3 Effect of pH of the moistening agent on pectinase and pectin lyase production by *B. subtilis* SAV-21

optimum value might be due to lesser growth of the bacterial strain. Although the maximum enzyme production occurred at pH 4.0, the isolated enzyme was active over a wide pH range with optimum pH at 11.0 (data not shown) indicating that the change in medium pH might have influenced the growth or permeability of the bacterial strain rather than enzyme stability. The optimal pH observed in this study was in line with that reported for pectinase production from *Aspergillus* sp. in the range of 4.0 to 5.25 (Phutela et al. 2005; Rao et al. 2014). However, the optimum pH for pectinase production by other bacterial strains has been reported to be higher than 4.0; for instance, it was 9.0 for *B. pumilus* dcsr1 (Sharma and Satyanarayana 2012). Similarly, the highest PG production from *Bacillus* sp. has been reported over a pH range of 6.0–9.0 (Kobayashi et al. 2001; Dharmik and Gomashe 2013; Chiliveri et al. 2016).

Effect of nitrogen source

Nitrogen is an essential constituent of proteins and hence is required for microbial growth and enzyme production. Supplementation of a nitrogen source in the basal SSF medium (containing a mixture of OP and CF in 4:1 ratio) raised the pectinase production in comparison to a control containing only the solid substrate (Fig. 4), thereby implying that nitrogen is an important requisite for increasing pectinase production in SSF. Among the various nitrogen sources used in this study, yeast extract supplementation enhanced the pectinase production maximally (1019.43 ± 78.21 U/gds) followed by $(NH_4)_2SO_4$ and NH_4Cl (Fig. 4). In contrast, PL production did not exhibit any increase; rather, it declined with nitrogen supplementation (Fig. 4), indicating that the solid substrate provided sufficient nitrogen for the production of this enzyme. In agreement with the present findings, maximum pectinase production in SSF by *Bacillus* sp. has been reported on supplementation with yeast extract (Kashyap et al. 2003; Ahlawat et al. 2008) and NH_4Cl (El-Shishtawy et al. 2014). PL production by *Bacillus* sp. was found to be enhanced in the presence of $(NH_4)_2SO_4$ (Nadaroglu et al. 2010; Demir et al. 2014). Peptone, gelatin and ammonium chloride were recorded as the best PG inducers for *B. firmus*-I-10104 (Bayoumi et al. 2008). Supplementation of the basal medium with yeast extract and KNO_3 has also been reported to enhance PG production (Rehman et al. 2012; Dharmik and Gomashe 2013).

Effect of carbon source

Microbial production of primary metabolites is highly influenced by their growth, which in turn is determined by the availability of nutrients in the substrate. It may, therefore, be inferred that the improvement of nutritional value of solid medium by its supplementation with a carbon source will increase the growth of the bacterial strain, resulting in higher enzyme production (Khan et al. 2012). On adding various carbon sources to the basal medium, pectinase production by *B. subtilis* SAV-21 was observed to be slightly lower in the presence of lactose, xylose and glucose as compared to the control, while the level of enzyme production was unaffected by other carbon sources (Fig. 5). The observed decrease in production of both the pectinolytic enzymes on adding carbon sources to the basal medium indicated that the solid substrate was sufficient to support the carbon requirement of the bacterial strain. A decline in bacterial pectinase production has also been recorded earlier on supplementation of solid substrate with sugars such as lactose and glucose (Ahlawat et al. 2008; Rehman et al. 2012; El-Shishtawy et al. 2014), and starch, sucrose and maltose (El-Shishtawy et al. 2014). In contrast,

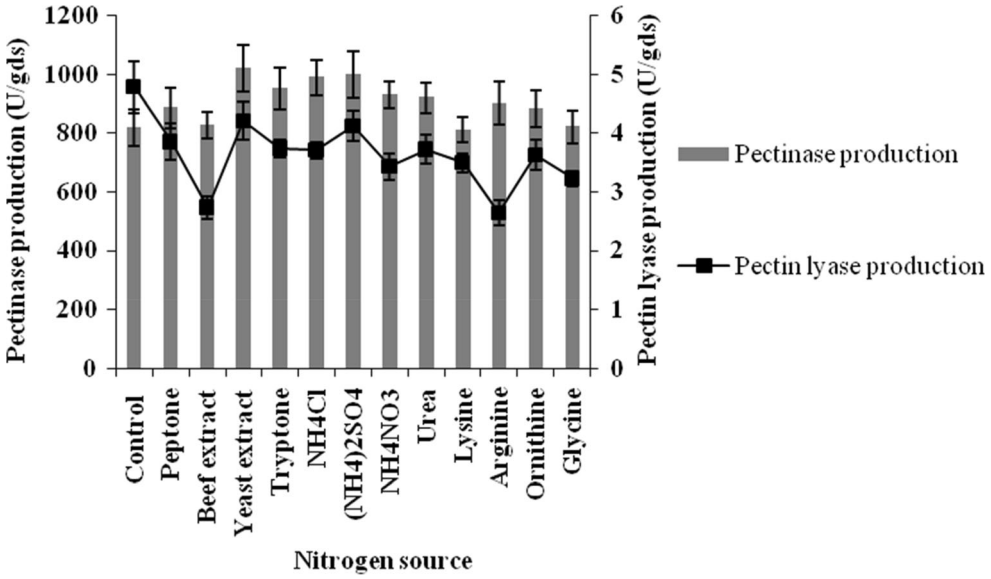

Fig. 4 Effect of nitrogen sources on pectinase and pectin lyase production by *B. subtilis* SAV-21

fungal pectinase showed enhanced pectinase production on supplementation of the basal SSF medium with sucrose (Phutela et al. 2005). In this study, the production of PL by *B. subtilis* SAV-21 was higher in the control than in the presence of different carbon sources during an incubation period of 48 h (Fig. 5). In contrast, citrus pectin was found to enhance the PL production in *B. pumilus* P9 (Nadaroglu et al. 2010) and *G. stearothermophilus* Ah22 (Demir et al. 2011). The differential effect of carbon sources on various bacterial strains might be related to their genetic differences.

Effect of fermentation time

The effect of varying fermentation time on the production of pectinase and PL was evaluated for 2–8 days and 2–10 days, respectively. The data showed that production of PL by *B. subtilis* SAV-21 took a much longer time and was lower as compared to pectinase. The maximum production of pectinase (1002.89 ± 95.12 U/gds) and PL (8.49 ± 0.73 U/gds) occurred after a fermentation period of 4 and 8 days of incubation, respectively (Table 1). Any further increase in the fermentation period decreased enzyme production, which

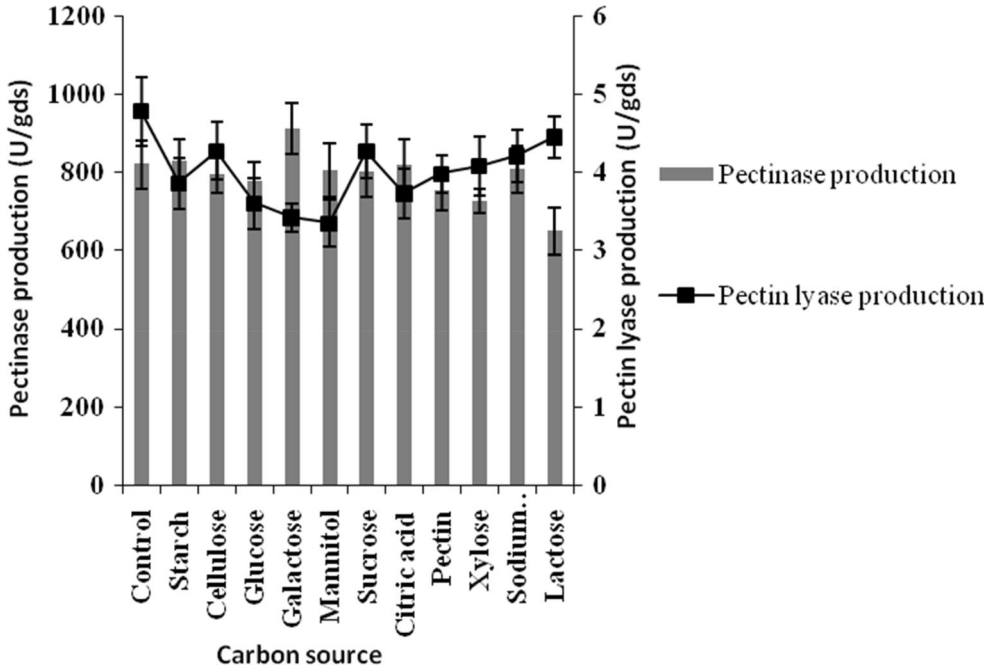

Fig. 5 Effect of carbon sources on pectinase and pectin lyase production by *B. subtilis* SAV-21

Table 1 Effect of incubation period on pectinase and pectin lyase production by *Bacillus subtilis* SAV-21

Incubation period (days)	Pectinase activity (U/gds)	Pectin lyase activity (U/gds)
2	820.11 ± 73.38	4.78 ± 0.44
3	895.22 ± 82.15	5.31 ± 0.46
4	1002.89 ± 95.12	5.89 ± 0.42
5	818.39 ± 74.83	6.48 ± 0.57
6	764.72 ± 68.11	6.93 ± 0.63
7	741.54 ± 62.11	7.52 ± 0.61
8	618.34 ± 59.06	8.49 ± 0.73
9	–	5.23 ± 0.42
10	–	3.6 ± 0.27

could be due partly to depletion of the nutrients required for bacterial growth and reproduction.

Similar results of maximum pectinase production were obtained after an incubation period of 4 days in the case of *B. firmus*-I-10104 (Bayoumi et al. 2008) and *Sporotrichum thermophile* (Kaur et al. 2003). However, *B. pumilus* dcsr1 needed a longer duration of 144 h for pectinase production in SSF (Sharma and Satyanarayana 2012). On the other hand, pectinase production occurred maximally after a fermentation time of 48 h from *B. gibsonii* S-2 (Li et al. 2005) and 72 h from *B. tequilensis* SV11 (Chiliveri et al. 2016). An optimal incubation period of 96 h for maximum PG production by *B. licheniformis* in SSF (Dharmik and Gomashe 2013) was also in agreement with the present findings.

Contrary to the present observations, PL production in SSF was maximal after an incubation period of 3 days by *Bacillus* VITSG-1 and *B. clausii* (Gopinath and Suneetha 2012; Li et al. 2012) and after 5 days by *G. stearothermophillus* Ah22 (Demir et al. 2011), indicating a slower rate of PL synthesis in *B. subtilis* SAV-21.

Effect of moisture content

Moisture level is one of the most critical parameters in SSF because it determines the swelling, water surface tension, solubility and availability of nutrients to microbes for their growth, thereby affecting enzyme production (Lonsane et al. 1992). Moisture content of the fermentation medium determines the success of the process. Water uptake in the biomass and evaporation are localized at the surface of the solid substrate particles. Hence, the optimum humidity allows the entry of nutrients easily through the cell membrane, which favors maximum enzyme production. In the current study, an initial moisture content of 60% resulted in maximum production of both pectinase (929.92 ± 50.31 U/gds) and PL (6.12 ± 0.43 U/gds), respectively by *B. subtilis* SAV-21 (Table 2). A lower enzyme production at suboptimal moisture levels could be the result of reduced growth of the microbe due to a decrease in solubility of nutrients present in the substrate together with low degree of substrate swelling (Murthy et al. 1999). The decline in enzyme production at 90% moisture content might be due to the fact that high moisture content influences O_2 transfer rates by affecting the bed depth and leads to particle agglomeration of the moist fermenting solids. The present findings are in agreement with the production of pectinase by *A. niger* (Akhter et al. 2011) and PG by *Bacillus* sp. (Rehman et al. 2012). However, *B. megatherium* exhibited enhanced pectinase production at 40% moisture content (El-Shishtawy et al. 2014). Maximum pectinase production by *B. subtilis* and *B. pumilus* dcsr1 in SSF occurred at 1:2.5 (*w*/*v*) moisture levels (Ahlawat et al. 2008; Sharma and Satyanarayana 2012) whereas a moisture content of 90% was reported as optimum for pectinase production by *Bacillus sp*. MG-cp-2 (Kapoor et al. 2000). On the other hand, maximum pectinase production by some fungi was reported at moisture contents of 70% (Rao et al. 2014) and 75–90% (Bai et al. 2004). The optimal moisture level required for PL production by *B. subtilis* SAV-21 was identical to that reported for *B. pumilus* (P9), *Bacillus* VITSG-1 and *B. borstelenesis* (P35), which was found to be 67% (Nadaroglu et al. 2010; Gopinath and Suneetha 2012; Demir et al. 2014).

Effect of metal salts

Addition of metal salts viz. $CaCl_2{\cdot}2H_2O$, KCl, $ZnCl_2$, NaCl, $MgSO_4$, H_3BO_3 and $CoCl_2$ on the production of pectinase

Table 2 Effect of moisture content on pectinase and pectin lyase production by *B. subtilis* SAV-21

Moisture content (%)	Pectinase activity (U/gds)	Pectin lyase activity (U/gds)
30	763.54 ± 55.86	2.76 ± 0.21
40	783.30 ± 61.22	3.53 ± 0.29
50	818.39 ± 66.83	4.32 ± 0.31
60	929.92 ± 50.31	6.12 ± 0.51
70	820.11 ± 73.38	4.78 ± 0.44
80	785.83 ± 31.11	4.05 ± 0.22
90	748.59 ± 22.49	2.54 ± 0.19
100	672.62 ± 28.23	1.76 ± 0.11

revealed a slightly higher production in the presence of $CaCl_2 \cdot 2H_2O$ (940.95 ± 65.43 U/gds) and KCl (904.66 ± 81.41 U/gds) as compared to control (820.11 ± 73.38 U/gds), which was devoid of any metal salt. In the presence of other metal salts, enzyme titer was almost the same as in the control. Similar to these findings, an increase in pectinase production has been reported by Kashyap et al. (2003). In contrast, Khan et al. (2012) reported maximum pectinase production in a control to which no metal salt was added.

On the other hand, PL production was maximum in the presence of NaCl (6.23 ± 0.55 U/gds) followed by $CaCl_2$ (5.73 ± 0.39 U/gds) and KCl (5.25 ± 0.26 U/gds) as compared to the control (4.78 ± 0.44 U/gds). Other metal ions did not affect the production of PL. On the contrary, Chiliveri et al. (2016) did not observe the stimulatory effect of any metal ion on pectate lyase and PG production in SSF by *B. tequilensis* SV11.

Conclusion

This study revealed that a mixture of OP and CF (4:1) was the most promising substrate for producing pectinase and PL in SSF by *B. subtilis* SAV-21. Parametric optimization enhanced the production of pectinase and PL by 5.4- and 5.15-fold, respectively. To our knowledge, this is the first report of the production pectin-degrading enzymes by *B. subtilis* SAV-21 in SSF using CF. These enzymes may be exploited in industry.

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Compliance with ethical standards

Conflict of interest The authors state that there are no conflicts of interest regarding the publication of this article.

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