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Original Research Article

Production of an alkali tolerant extracellular xylanase from *Bacillus pumilus* VLK-1 in solid state fermentation and its use in tomato juice clarification

Lalit Kumar[A*], Sushil Nagar[A], Davender Kumar[A], Neelam Garg[B], and Vijay Kumar Gupta[A]

[A]Department of Biochemistry, Kurukshetra University, Kurukshetra- 136 119, India
[B]Department of Microbiology, Kurukshetra University, Kurukshetra- 136 119, India

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Abstract

Xylanase production from Bacillus pumilus VLK-1 in solid state fermentation was enhanced through optimization of the process using one variable approach. The enzyme production was highest (42324±786 IU/g) upon 96h of incubation at 37°C using wheat bran as a substrate moistened with mineral salt solution (pH 9.0) containing 0.1% folic acid, 0.2% riboflavin and 6.0% yeast extract in the ratio of 1:2.5. Addition of Tween 80 to the extraction buffer increased the enzyme yield. An optimization of the fermentation conditions increased the 5.7-fold xylanase production. The augmentation of enzyme production using cheap and abundantly available wheat bran is likely to reduce the cost of enzyme for its industrial application. The purified xylanase showed its optimum efficiency in tomato juice enrichment on treatment of fruit pulp with enzyme (20 IU/g) at 40ºC for 30 min. Treatment of tomato pulp with purified xylanase increased juice yield (68%), clarity (7%), reducing sugars (192%), titratable acidity (19%), and filterability (8%) whereas reduced the viscosity by (8%) indicating an improvement in physico-chemical characteristics of the juice. The results showed that purified B. pumilus VLK-1 xylanase could be exploited for fruit juice enrichment.

Keywords: *Xylanase, Bacillus pumilus, solid state fermentation, tomato and wheat bran.*

1. Introduction

Enzymatic processes in industry are preferred as they occur under mild conditions of pH, temperature and pressure. Moreover, they are specific, involve high reaction rates, can be easily controlled, and reduce the environmental pollution. Some industrial enzymes can be produced using lignocellulosic materials as substrate, which comprise of 38-50% cellulose, 23-32% hemicelluloses and 15-25% lignin. A major component of hemicelluloses found in plant cell walls is xylan, which consists of a backbone of 1, 4-linked-β-D-xylopyranose residues substituted with acetyl groups, arabinose, and 4-O-methylglucuronic acid residues. The demand for industrial enzymes, particularly of microbial origin is ever increasing owing to their applications in a wide variety of processes. Xylanase (endo-1, 4-β-D-xylan xylanohydrolase; EC 3.2.1.8) is an industrially important enzyme that catalyzes the depolymerization of xylan backbone by cleaving β-1, 4-glycosidic bonds resulting in the production of xylose and xylo-oligosaccharides of various lengths (2-5 xylose residues). Xylanase has a broad range of industrial applications, which include biobleaching of pulp, textile industry, improvement in animal feed digestibility, xylo-oligosaccharide production, extraction and clarification of fruit juices, texture improvement of bakery products, conversion of lignocellulosic biomass into valuable products, wastewater treatment, and so on (Polizeli et al., 2005; Subramaniyan and Prema, 2002). Xylanase and cellulase, together with pectinases, account for 20% of the world enzyme market (Polizeli et al., 2005).

Xylanases for use in industry may be obtained from bacteria, fungi and actinomycetes. Generally, large scale cultivation of fungi and actinomycetes is often difficult because of their slow generation time, co-production of highly viscous copolymer and poor oxygen transfer (Mtui, 2012). Xylanase production from bacteria may be advantageous due to higher enzyme production resulting from their high growth rate. Among the bacteria, *Bacillus* species are used to produce xylanase because they yield robust enzyme (Nagar et al., 2010, Sharma et al., 2013). Xylanase production from *Bacillus* spp. may be carried out either in solid state (SSF) or submerged fermentation. In SSF, microorganisms are grown on moist solids in the absence of free flowing water. The solid substrates act as source of carbon, nitrogen, minerals and growth factors and have the capacity to absorb water in order to provide natural habitat and growth requirements of microbes. Enzyme production in SSF is usually much higher than that of submerged fermentation (Haltrich et al., 1996). Enzyme production in SSF may offer several economical and practical advantages over submerged cultivation such as need of simpler equipment, a simple fermentation medium, requirement of less energy, higher product yield, reduced waste water output, lower capital and operational

*Corresponding author**: Lalit Kumar**

costs, low catabolic repression, and does not require a rigorous control of fermentation parameters (Krishna, 2005; Pandey et al., 1999).

Abundantly available and low cost agricultural waste materials such as wheat bran, wheat straw, rice straw, sugarcane bagasse, corn cobs, apple pomace, and forest residues can be used for cost-effective production of xylanase. There are several reports of bacterial xylanase production using agricultural wastes as substrates in submerged fermentation (Azeri et al., 2010; Kumar et al., 2013; Sa-Pereira et al., 2002; Nagar et al., 2012); however, studies on xylanase production from bacteria in SSF using agricultural residues as substrates are limited (Bajaj and Singh, 2010; Nagar et al., 2011; Sanghi et al., 2008; Sharma et al., 2013). Xylanase production through SSF is of interest for countries like India with abundant biomass and agro-residues.

The production of fruit juices is important both from the human health and commercial standpoints (Bhat, 2000). Raw fruit juices are cloudy, viscous and turbid due to the presence of polysaccharides (starch, pectin, cellulose, and hemicelluloses) and lignin (Uhlig, 1998; Lee et al., 2006). Such juices have low yield, high viscosity, low filterability, and less consumer acceptability. Macerating enzymes i.e. pectinases, cellulases and hemicellulases are used in the extraction and clarification of fruit juices (Galante et al., 1998b; Grassin and Fauquembergue, 1996a). The literature pertaining to the use of xylanase in juice extraction and clarification is limited. So, it would be pertinent to investigate the potential of xylanases in fruit and vegetable juice enrichment.

In the present study, xylanase production in SSF was carried out from *Bacillus pumilus* VLK-1, an alkaliphilic xylanolytic strain isolated in our laboratory, using wheat bran as the substrate and fermentation process was optimized for maximum production of xylanase. Further, the enzyme produced was purified to study its potential in enhancing the extraction and clarification of tomato juice.

2. Materials and Methods

2.1 Raw materials and chemicals

Lignocellulosic substrates (wheat bran) were obtained from the local market, washed and dried. The chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO), Hi-Media Laboratories India (Mumbai, India), and Merck Laboratories, India.

2.2 Microorganism, maintenance and inoculum production

Bacillus pumilus VLK-1 (Gene bank accession no JQ350583), an alkaliphilic xylanolytic bacterial strain isolated in our laboratory from soil, was cultured on xylan-agar medium which contained (g/L): peptone 5.0, beef extract 3.0, xylan 1.0 and agar 20.0 (pH 7.0) followed by incubation at 37 °C for 24 h. The culture was stored at 4 °C in the form of slant and sub-cultured fortnightly. Inoculum was developed by seeding the autoclaved nutrient broth contained in a conical flask with a loop full of the overnight grown culture of *B. pumilus* VLK-1 followed by incubation at 37°C at 200 rpm for 18 h. One ml of the primary inoculum was transferred to 50 ml of nutrient broth aseptically and incubated at 37°C at 200 rpm for 8 h. This secondary inoculum was used for inoculation.

2.3 Production of xylanase in SSF and xylanase extraction

Xylanase was initially produced from *B. pumilus* VLK-1 in SSF under unoptimized conditions. Erlenmeyer flasks (500 ml), each containing 10 g wheat bran moistened with 20.0 ml of the mineral salt solution ($MgSO_4$, 0.5 g/L and K_2HPO_4, 1.5 g/L; pH 8.0), were autoclaved at 1.05 kg/cm^2 for 45 min, cooled to room temperature, inoculated with 10% (v/w) of 8 h old inoculum of *B. pumilus* VLK-1 and kept in an incubator at 37°C which was humidified with sterile water. The flasks were gently tapped intermittently for mixing of their contents. After 72 h of incubation, the flasks were removed. Xylanase was extracted from the fermented wheat bran by adding 100 ml of 0.05 M potassium phosphate buffer (pH 7.0) to each flask along with shaking in an orbital shaker at 100 rpm for 1 h. The enzyme was collected by squeezing the fermented wheat bran through a wet muslin cloth followed by its centrifugation at 10,000 x g for 20 min at 4ºC. The clear supernatant thus obtained was used for xylanase assay. All the production experiments were conducted in triplicates and standard deviation was calculated.

2.4 Assay of xylanase activity

Xylanase was assayed by measuring the reducing sugars (xylose equivalent) released from birchwood xylan using 3, 5-dinitrosalicylic acid (Miller, 1959). The reaction mixture containing 490 µl of 1 % birch wood xylan and 10 µl of appropriately diluted enzyme extract was incubated at 60 °C for 10 min. The reaction was then terminated by adding 1.5 ml of 3, 5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all of the reagents but the reaction was terminated prior to the addition of enzyme. The reaction mixture was boiled in a water bath for 10 min followed by cooling in ice-cold water. Absorbance of the resulting color was measured against the control at 540 nm in a spectrophotometer. The amount of xylose produced was measured from a standard curve prepared using its different concentrations. One international unit (IU) of xylanase activity was defined as the amount of enzyme required to release 1µmol of reducing sugar as xylose equivalent per minute under the specified assay conditions.

2.5 Optimization of xylanase production in SSF

Xylanase production in SSF was optimized by one variable at a time approach. The fermentation was carried

out in Erlenmeyer flasks (500 ml) each of which contained 10 g wheat bran moistened with 20.0 ml of the mineral salt solution ($MgSO_4$, 0.5 g/L and K_2HPO_4, 1.5 g/L; pH 8.0). The flasks were autoclaved, cooled and inoculated with 8 h old inoculum of *B. pumilus* VLK-1. After incubation for 72 h at 37°C in an incubator, xylanase was extracted from the fermented wheat bran as described above. The enzyme yield was optimized with respect to various parameters i.e. inoculum age and size, cultivation time, temperature, pH, ratio of solid substrate to mineral salt solution, agro-residues, nitrogen source, folic acid, and riboflavin by varying one parameter at a time. All the experiments perform in triplicate and the average values have been reported.

2.5.1 Effect of inoculum age and size

Flasks containing 10 g of wheat bran moistened with 20 ml of mineral salt solution were autoclaved and inoculated with 5, 10, 15 and 20% (v/w) of 8 h old inoculum. The flasks were then incubated at 37ºC for 72 h followed by extraction and assay of xylanase as described in section 2.3.

2.5.2 Optimization of cultivation time, temperature and pH

Wheat bran (10 g) taken in each Erlenmeyer flask (500 ml) was moistened with 20 ml of mineral salt solution, autoclaved, cooled, inoculated with 8 h old inoculum of *B. pumilus* VLK-1 and incubated at 37°C in an incubator for different time period (0-120 h). A set of three flasks was kept for each incubation time. Following incubation for a defined period, xylanase was extracted from the fermented wheat bran and assayed for its activity as described in section 2.3. The influence of cultivation temperature on xylanase production was studied by incubating the flasks containing inoculated wheat bran (10 g) at different temperatures (37-50ºC) for 72 h. The optimum pH for xylanase production was determined by varying pH of the mineral salt solution from 6.0 to 11.0 used for moistening of wheat bran.

2.5.3 Optimization of moisture level (ratio of wheat bran to moistening agent)

The effect of varying the ratio of solid substrate (wheat bran) to the moistening agent (basal salt solution) from 1:1 to 1:4 (w/v) was examined on xylanase production by *B. pumilus* VLK-1.

2.5.4 Optimization of agro-residue and nitrogen source

The effect of different agro-residues such as wheat bran, wheat straw, rice straw, saw dust, sugarcane bagasse, and corn cob as the sole substrate, was determined on xylanase production in SSF. These substrates were not pre-treated before use. Nitrogen source was optimized by supplementing the wheat bran with 1% (w/w) of different organic (peptone, yeast extract, beef extract, and casein) and inorganic nitrogen (KNO_3, $NaNO_3$, $Ca(NO_3)_2$ and NH_4Cl) nitrogen sources. The optimum concentration of the best nitrogen source was also determined.

2.5.5 Effect of folic acid and riboflavin

The effect of folic acid and riboflavin on xylanase production by *B. pumilus* VLK-1 in SSF was evaluated by supplementing the moistened wheat bran with these additives in separate sets of flasks at a concentration of 0.05 to 0.2 % (w/w) of the substrate. The enzyme was extracted and assayed from the flasks following an incubation of at 37 °C for 72 h.

2.6 Effect of surfactants on xylanase extraction

The different surfactants namely Tween 20, Tween 40, and Tween 80 were added to the extraction buffer at a final concentration of 0.1% (v/v) to examine their effect on the extraction of xylanase from the fermented wheat bran.

2.7 Preparation of tomato pulp and optimization of conditions for xylanase treatment

Tomatoes (*Lycopersicon esculentum*) were purchased from the local market, washed thoroughly with water, and macerated using a blender to form a smooth textured pulp. It was filtered through two layers of muslin cloth and then centrifuged to separate juice from the pulp.

Tomato pulp was treated with soluble xylanase purified from *B. pumilus* VLK-1 for enrichment of juice. The conditions for treatment of tomato pulp with xylanase viz. enzyme dose, incubation temperature and incubation period were optimized so as to improve the physico-chemical characteristics of juice. To optimize the enzyme dose, 20 g of tomato pulp was treated with different doses of purified *B. pumilus* VLK-1 xylanase (0, 10, 20, 30 and 40 IU/g pulp) at 30ºC for 30 min. The enzyme-untreated pulp sample was kept as control. After incubation, the enzyme was inactivated by heating the suspension in a boiling water bath for 5 min. The pulp was then cooled and filtered through two layers of muslin cloth. The filtrate was centrifuged at 10,000xg for 15 min to separate juice from the pulp. The supernatant (juice) was used for analysis of yield, reducing sugars and clarity. The results were calculated as percent of control.

The optimum time of enzymatic treatment was determined by treating 20 g of tomato pulp with purified xylanase (20 IU/g pulp) at 30ºC for different time periods (0, 15, 30, 45 and 60 min) keeping the zero time pulp as control. The resulting juice was analyzed for yield, reducing sugars, and clarity. To optimize the temperature for xylanase treatment, tomato pulp (20 g) was treated with purified xylanase (20 IU/g pulp) at different temperatures (30, 40, 50 and 60ºC) in a water bath for 30 min. The fruit pulp at 30ºC was kept as control. The resulting juice was analyzed for yield, reducing sugars, and clarity. The results were expressed as percent of control.

2.8 Treatment of tomato pulp with xylanase under optimized conditions

Tomato pulp (20 g) was treated with purified xylanase (20 IU/g pulp) under optimized conditions at 40°C for 30 min keeping enzyme untreated pulp as control. The juice was separated from both the untreated and xylanase-treated pulps as mentioned above and analyzed for yield, clarity, reducing sugars, viscosity, titratable acidity, and filterability.

2.9 Determination of physico-chemical characteristics of tomato juice

Tomato juice (untreated and xylanase-treated) was analyzed for yield, clarity, reducing sugar content, viscosity, titratable acidity, and filterability. The juice yield was determined by measuring volume of the supernatant obtained after centrifugation of the pulp filtered through muslin cloth and it was expressed as % (volume of juice per 100 g of pulp). The clarity of juice was determined by measuring the percent transmittance (%T) at 650 nm against distilled water using a double beam UV-Vis spectrophotometer (Systronics-2203, India). Percent transmittance was considered a measure of juice clarity. Reducing sugars released after the xylanase treatment were measured in the tomato juice by using 3,5-dinitrosalicylic acid (Miller, 1959) and quantified from a standard curve of xylose. The filterability (per min) of juice was determined from the reverse of the time taken to filter 50 ml of xylanase-treated juice through Whatman No. 1 filter under vacuum. Total titratable acidity (% citric acid) and viscosity (Pa.s) of the juice were determined as described earlier (Nagar et al., 2012).

3. Results

3.1 Optimization of xylanase production in SSF

Under unoptimized SSF conditions *B. pumilus* VLK-1 produced 7414 IU/g of extracellular xylanase, which was lower than previously reported values. So, the process of SSF was optimized through one variable approach so as to enhance the enzyme production. The results obtained from optimization experiments are described below.

3.1.1 Inoculum age and size

The inoculation of moistened wheat bran (10 g) with 10% (v/w) inoculum of different age (2-12 h old) revealed the highest enzyme production (24158 IU/g) during the log phase at 8 h of incubation at 37°C. On further increase in inoculum age, a decline in enzyme titer was recorded (Fig.1).

The profile of xylanase activity as a function of increasing inoculum size showed the highest activity (24,952 IU/g) on inoculation of wheat bran with 5% (v/w) inoculum. A further increase in inoculum size led to a decline in enzyme titer. The activity of xylanase was at 15, 20 and 25% inoculum was recorded as 24024, 23512 and 20,988 IU/g, respectively (Fig. 2). The results of this study indicated an optimum age of inoculum as 8h old and size as 5%.

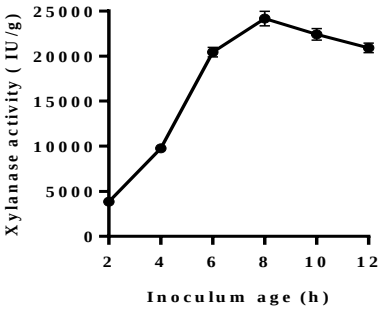

Fig. 1 Effect of inoculum age on xylanase production by *Bacillus pumilus* VLK-1 in SSF using wheat bran moistened with basal salt solution (1:2) inoculated using 10% (v/w) inoculum of different age followed by incubation at 37 °C for 72 h

3.1.2 Cultivation time, temperature and pH

The activity of xylanase registered a progressive increase with rise in cultivation time reaching a peak (29318 IU/g) at 96 h and remained more or less constant thereafter (Fig. 3)

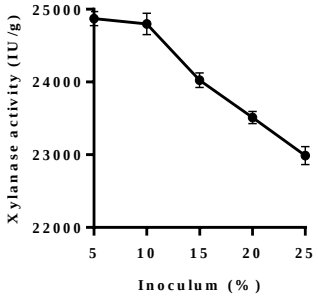

Fig. 2 Effect of inoculum size on xylanase production by *B. pumilus* VLK-1 in SSF using wheat bran moistened with basal salt solution (1:2) inoculated with different concentrations of 8 h old inoculum and incubated at 37 °C for 72 h

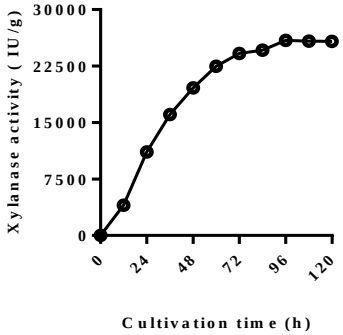

Fig. 3 Effect of cultivation time on xylanase production by *B. pumilus* VLK-1 in SSF using wheat bran as substrate

The profile of xylanase production from *B. pumilus* VLK-1 in SSF activity at different temperatures (37-50ºC) revealed the highest enzyme titer (25016 IU/g) at 37°C

(Fig. 4). As compared to 37°C, the enzyme production was slightly lower (22922 IU/g) at 40°C, declined drastically at 45°C and was negligible at 50°C. Xylanase production is markedly influenced by pH since it determines growth rate of the microorganism used for fermentation. An increase in pH from 6.0 to 11.0 of the mineral salt solution used for moistening of wheat bran enhanced the xylanase production by *B. pumilus* VLK-1 in SSF exhibiting the highest titer (24826 IU/g) at pH 9.0 (Fig. 5). The activity of xylanase at pH 8.0 and 10.0 (97.1% and 97.9% respectively) was close to that recorded at pH 9.0, indicating an alkaliphilic nature of the bacterial strain.

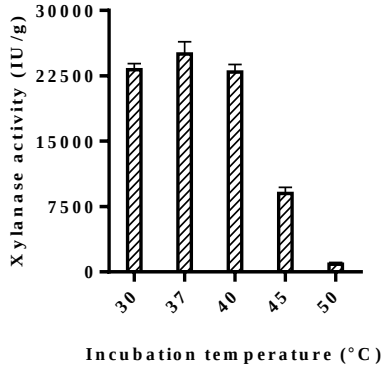

Fig. 4 Effect of incubation temperature on xylanase production by *B. pumilus* VLK-1 in SSF using wheat bran as substrate

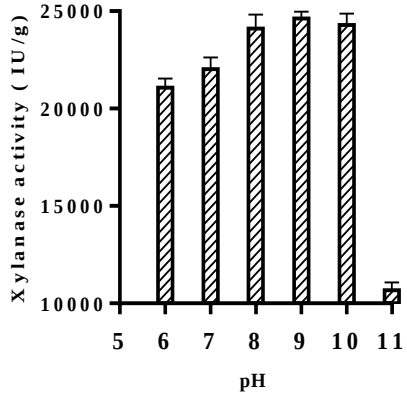

Fig. 5 Effect of pH of the moistening agent on xylanase production by *B. pumilus* VLK-1 in SSF using wheat bran as substrate

3.1.3 Optimization of the moisture level

Moisture level of the solid substrate would affect the enzyme production. The moisture level was altered by varying the ratio of wheat bran to the mineral salt solution used as the moistening agent from 1:1–1:4 (w/v). The results on the effect of different moisture levels revealed an enhancement in xylanase production by *B. pumilus* VLK-1 with maximum (27,287 IU/g) at a ratio of 1:2.5 (Fig. 6). The enzyme activity was nearly the same at a moisture level of 1:3. At higher ratios of wheat bran to the moistening agent, the enzyme titer was very less.

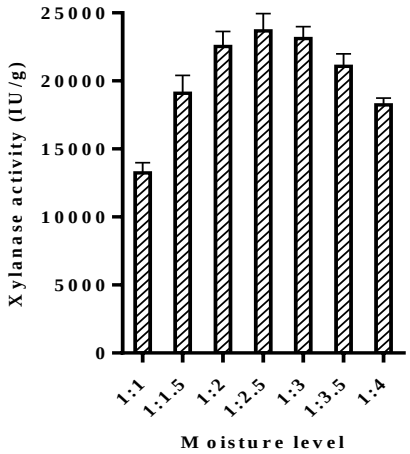

Fig. 6 Effect of varying moisture level (ratio of wheat bran to basal salt solution) on the production of xylanase by B. pumilus VLK-1 in SSF

3.1.4 Effect of different agro-residues and nitrogen source

Selection of an appropriate agro-residue (to be used as substrate) is a crucial step for SSF as it affects the final cost of the product. In this study, various cost-effective and abundantly available agro-residues were evaluated as substrates for xylanase production by *B. pumilus* VLK-1. Among the agro-residues used for SSF, wheat bran induced the highest xylanase production (23,994 IU/g). Enzyme titer was much lower in the presence of agro-residues such as wheat straw, paddy straw, and sugarcane bagasse, and was negligible in the presence of sawdust and gram bran (Fig. 7).

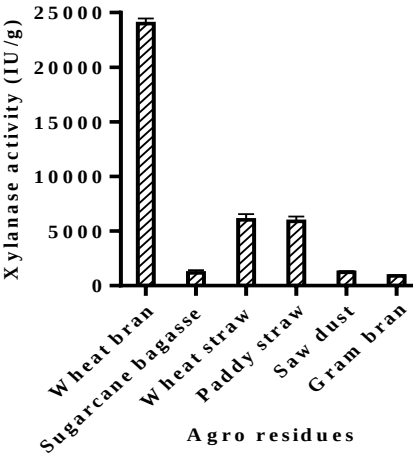

Fig. 7 Effect of different agro-residues on xylanase production by *B. pumilus* VLK-1 in SSF

Among the different organic and inorganic nitrogen sources added to wheat bran at a final concentration of 5% (w/w), yeast extract led to maximum xylanase production (24,924 IU/g) by *B. pumilus* VLK-1 followed by beef extract (24048 IU/g), casein (23156 IU/g) and peptone (24322 IU/g). In control (wheat bran devoid of nitrogen source), the enzyme titer was much lower as compared to that of the yeast extract (Fig. 8).

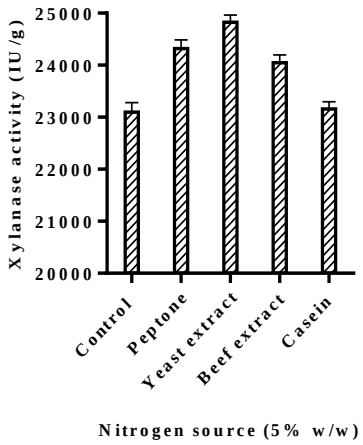

Fig. 8 Effect of different nitrogen sources on xylanase production by *B. pumilus* VLK-1 in SSF using wheat bran as substrate

On increasing the concentration of yeast extract in SSF, there was a progressive enhancement in xylanase production reaching the highest level (25495 IU/g) at a concentration of 6% followed by a decline (Fig. 9).

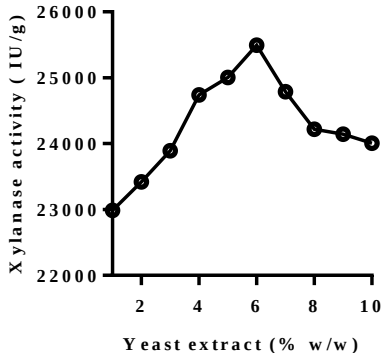

Fig. 9 Effect of different concentrations of yeast extract on xylanase production in SSF

3.1.5 Effect of folic acid and riboflavin

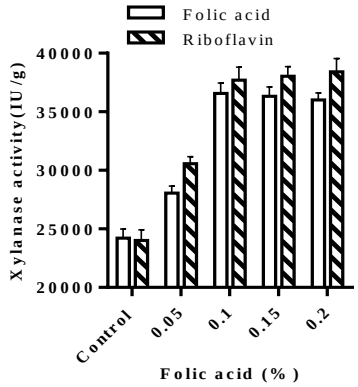

Fig. 10 Effect of folic acid and riboflavin on xylanase production by *B. pumilus* VLK-1 in SSF

Addition of folic acid to wheat bran for SSF enhanced the production of xylanase and its optimum concentration was found to be 0.1%. Similar to folic acid, riboflavin also augmented the production of xylanase which was highest at a concentration of 0.2% (Fig. 10).

3.2 Effect of surfactants on extraction of xylanase

The results of the addition of the surfactants Tween 20, Tween 40, Tween 60, and Tween 80 to the buffer used for extraction of xylanase from the fermented wheat bran showed that Tween 80 increased the enzyme yield whereas other additives didn't have any significant effect (Table 1).

Table 1 Effect of various surfactants on the extraction of xylanase from the fermented wheat bran

Surfactants (0.1%)	Xylanase activity (IU/g)
Control	36,640 ±388
Tween 20	36,817 ±295
Tween 40	35,914 ±917
Tween 60	35734 ±1256
Tween 80	40,264 ±1108

3.3 Xylanase production under optimized SSF conditions

The optimum fermentation conditions for xylanase production determined from various experiments conducted in this study included wheat bran (10 g) as solid substrate moistened with 25 ml of mineral salt solution (0.5 g/L $MgSO_4$ and 1.5 g/L K_2HPO_4; pH 9.0) containing 6% yeast extract, 0.1% folic acid, and 0.2% riboflavin and inoculated with 5% of 8 h old secondary inoculum of *B. pumilus* VLK-1 incubated at 37°C for 96 h. Xylanase titer obtained under the optimized conditions was found to be 42324 ± 786 IU/g of wheat bran which was 5.7-fold higher than obtained under unoptimized conditions.

3.4 Purification of xylanase produced by B. pumilus VLK-1 in SSF

Xylanase was purified to homogeneity from *B. pumilus* VLK-1 by 15.4-fold with a recovery of 88.3% in a single step using CM-Sephadex C-50 chromatography. On applying a continuous gradient of 0-1.0M KCl to elute the bound enzyme, a single peak of enzyme activity was obtained. The most active enzyme fractions were pooled, dialyzed and concentrated using Millipore Amicon Ultra-15 Centrifugal filter unit (10 kDa cut off). The homogeneity was ascertained from a single protein band in 12% SDS-PAGE gel (data not shown). The potential of the purified enzyme was evaluated in enrichment of tomato juice.

3.5 Optimization of conditions for treatment of tomato pulp with xylanase

Enzyme dose, incubation period and temperature for xylanase treatment of tomato pulp were optimized by examining the increase in juice yield, clarity and reducing sugars after treatment with enzyme. On treatment of tomato pulp with different concentrations of purified

xylanase at 30 °C for 30 min, maximum increase in yield, clarity and reducing sugars of juice was observed with an enzyme dose of 20 IU/g pulp as compared to control (Fig.11a). At enzyme doses lower or higher than 20 IU/g pulp, the values of the above parameters decreased indicating that 20 IU/g was the optimum dose of xylanase for treatment of tomato pulp. The profile of juice yield, reducing sugars and clarity on treatment of tomato pulp with xylanase (20 IU/g) at 30°C for different time periods (15-90 min) revealed the highest values of these parameters after an incubation for 30 min (Fig. 11b) which was, therefore, taken as the optimum time. The effect of xylanase treatment (20 IU/g) of tomato pulp at different temperatures (30-60°C) for 30 min showed an optimum temperature of 40°C as the increase in juice yield, clarity and reducing sugar were maximum at this temperature (Fig.11c).

3.6 Effect of xylanase treatment under optimized conditions on tomato juice enrichment

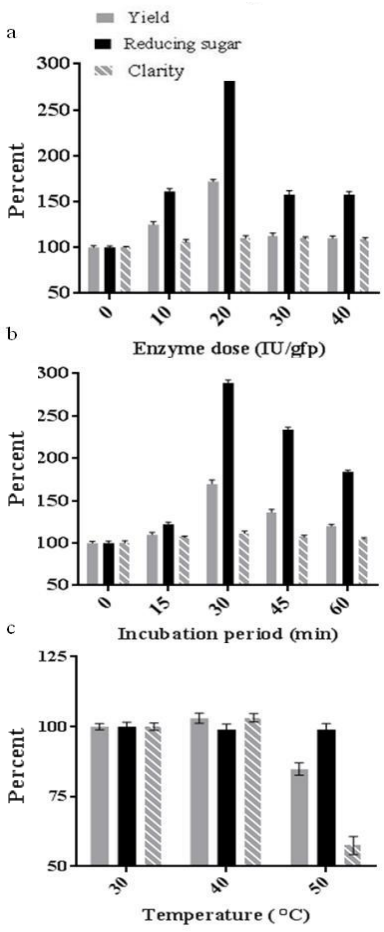

Fig. 11 Optimization of enzyme dose (a), incubation period (b) and temperature (c) for treatment of tomato pulp with purified *B. pumilus* VLK-1 xylanase

Enrichment of tomato juice after treatment with purified *B. pumilus* VLK-1 xylanase under optimized conditions (20 IU/g at 40°C for 30 min) was measured in terms of its yield, clarity, reducing sugars, viscosity, acidity, and filterability. Treatment of tomato pulp with purified xylanase increased the juice yield, clarity (measured in terms of % transmittance at 650 nm), and reducing sugars by 68%, 7%, and 192% respectively whereas reduced the viscosity by 8% as compared to the control. Titratable acidity was 19% higher in the juice obtained from enzyme-treated tomato pulp as compared to control. The filterability of the juice registered an increase (8%) on treatment of tomato pulp with xylanase (Fig. 12).

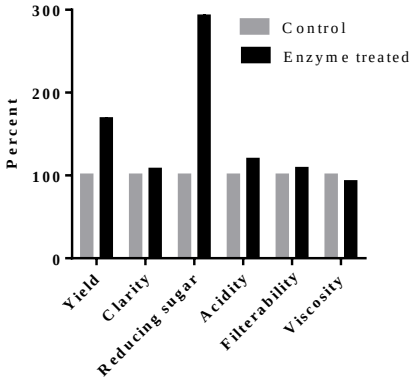

Fig. 12 Changes in physico-chemical characteristics of tomato juice after xylanase treatment

4. Discussion

In this study, production of extracellular xylanase from *B. pumilus* VLK-1 in SSF using a cost-effective agro-residue as substrate was optimized so as to enhance its titer and to reduce its cost.

The optimum size of 8h old inoculum of *B. pumilus* VLK-1 for SSF was found to be 5% (v/w) which was lower than that documented by other workers. A lower inoculum level would be beneficial for scale up of xylanase production. The optimum inoculum size reported by other researchers for xylanase production from most *Bacillus* spp. was 10% (Poorna and Prema, 2007; Sindhu et al., 2006; Virupakshi et al., 2005) or 15% (Archana and Satyanarayana, 1997; Battan et al., 2007; Nagar et al., 2011; Sanghi et al., 2008). The incubation period required for enzyme production may vary depending upon the growth rate of the microorganism. The optimum time for extracellular xylanase production by *B. pumilus* VLK-1 in SSF was found to be 96h which is similar to *B. megaterium* (Sindhu et al., 2006). The highest enzyme titer from other *Bacillus* spp. was recorded at 48h (Nagar et al., 2011) or 72h (Kambly and Jadhav, 2012; Sanghi et al., 2008; Virupakshi et al., 2005).

The optimum cultivation temperature of *B. pumilus* VLK-1 for xylanase production was 37°C which is in accordance with other reports (Battan et al., 2007; Heck et al., 2005; Poorna and Prema, 2007; Sanghi et al., 2008). Several *Bacillus* species were reported to exhibit maximum enzyme production in SSF at cultivation temperature of 30°C (Nagar et al., 2011), 40°C (Sindhu et al., 2006; Kapilan and Arasaratnam, 2011) and 55°C (Kiddinamoorthy et al., 2008). The optimum cultivation

temperature of a bacterial strain may be its inherent characteristic. The decline in enzyme titer above the optimum temperature might be due to evaporation of water which in turn would possibly affect the bacterial growth and hence the enzyme production. The highest enzyme titer at alkaline pH values indicated alkaliphilic nature of the bacterial strain. The optimum moisture level for xylanase production in this study ranged from 1:2.5-1:3, which is in agreement with earlier reports (Battan et al., 2007; Nagar et al., 2011; Sanghi et al., 2008). The decline in enzyme activity at lower moisture levels might be due to decreased solubility of nutrients present in wheat bran whereas at higher moisture levels oxygen transfer might be affected due to reduction in porosity of wheat bran as suggested by Ramesh and Lonsane (1990).

In SSF, solid substrates are used for enzyme production. Since the use of pure xylan would be uneconomical for xylanase production, various agro-residues could be exploited for its cost-effective production. In India, agro-residues are available in abundance at low cost. Different agro-residues such as wheat bran, wheat straw, sorghum straw, corn cobs, rice straw, soya meal, husk, paddy husk rice bran, sugarcane bagasse, sawdust, have been utilized as substrates for xylanase production in SSF (Archana and Satyanarayana, 1997; Garg et al., 2011; Kapilan and Arasaratnam et al., 2011; Nagar et al., 2011; Poorna and Prema, 2007; Sonia et al., 2005). Most researchers have reported wheat bran as the best substrate for xylanase production (Sanghi et al., 2008; Nagar et al., 2011). In the present investigation also, wheat bran was found to result in maximum production of xylanase. This was probably due to the presence of substantial amounts (40%) of xylan in wheat bran (Thiago and Kellaway, 1982) which is the substrate for xylanase. Wheat bran is considered as the universal substrate among agro-residues for xylanase production because it is rich in nutrients for growth of microorganisms and remains loose even under moist conditions providing a large surface area (Archana and Satyanarayana, 1997).

Xylanase production from *B. pumilus* VLK-1 under the optimized conditions using one variable approach was found to be 42324 ± 786 IU/g of wheat bran, which is much higher than that reported several other *Bacillus* spp. The highest xylanase production using wheat straw was found to be 25103 IU/g (Garg et al., 2011) and using wheat bran as 294 U/g (Abo-State et al., 2013).

The enzyme was purified and used for improving the yield and other characteristics of tomato juice. The optimization of conditions for enzymatic treatment of tomato pulp was necessary for maximum improvement in juice characteristics. The optimized treatment conditions are likely to depend on the type of fruit pulp. The optimum enzyme dose was ascertained as 20 IU/g because at this concentration there was maximum increase in tomato juice yield, clarity and reducing sugars. A similar increase in these parameters has been recorded by several researchers but the magnitude of increase may vary depending on the type of fruit pulp and treatment conditions (Nagar et al., 2012; Dhiman et al., 2011). The optimum time for treatment of tomato fruit pulp with *B. pumilus* VLK-1 xylanase was recorded as 30 min. Prolonged incubation with the enzyme could result in the formation of haze particles consisting of protein-carbohydrate and protein-protein complexes (Sin et al., 2006). Previously, the optimum time for xylanase treatment was found to be in the range of 30-90 min. The effect of temperature showed maximum increase in juice yield, clarity and reducing sugars after incubation of tomato pulp with xylanase at 40°C, which is close to 37°C for citrus juice (Dhiman et al., 2011) and same as for apple, pineapple, and tomato juice (Nagar et al., 2012).

The use of *B. pumilus* VLK-1 xylanase for treatment of tomato pulp improved the physico-chemical characteristics of the resulting juice. An increase in juice yield and clarity has been reported by several workers after enzymatic treatment of pulp with xylanase alone or in combination with other enzymes such as pectinase and cellulase (Ahmad et al., 2009; Bhushan et al., 2013; Nagar et al., 2012; Olfa et al., 2007; Pal and Khanum, 2011; Shah, 2007). The increase in juice yield and clarity may be due to hydrolysis of xylan of tomato fruit cell wall resulting in a decline in its water holding capacity consequently releasing water (Grassin and Fauquembergue, 1996a). Tajchakvit et al. (2001) suggested the involvement of various factors including liquefaction of fruit, stabilization of fruit pulp, reduction of viscosity and degradation of hemicelluloses in enhancing the juice yield. Sin et al. (2006) suggested that the increase in the clarity of juice might be due to aggregation and settling down of the cloud particles. Treatment with xylanase also enriched the reducing sugar level of tomato juice which was possibly due to hydrolysis of xylan by the enzyme resulting in the formation of xylose and short chain xylooligosaccharides. Similarly, other researchers also recorded an increase in reducing sugar content of different fruit juices (Bajaj and Manhas, 2012; Dhiman et al., 2011; Nagar et al., 2012; Sharma et al., 2012).

The viscosity of tomato juice obtained after xylanase treatment was lower than the control. Since a juice of high viscosity may cause clogging of filtration apparatus due to the presence of cloud particles (Jacob et al., 2008), a decline in viscosity of juice following xylanase treatment is likely to be advantageous. A reduction in viscosity of different juices after enzymatic treatment was observed by various researchers (Bhushan et al., 2013; Nagar et al., 2012; Shah, 2007). Xylan, a hemicellulose, is one of the components responsible for turbidity and viscosity of fruit juices (Lee et al., 2006). Therefore, its degradation by xylanase would be expected to reduce the viscosity of juice. An increase in titratable acidity would increase shelf life of juice (Baker and Bruemmer, 1972). An increase in filterability of tomato juice on treatment of fruit pulp with xylanase could be the result of xylan hydrolysis by the enzyme. Other researchers have also reported an increase in filterability of juice after treatment

with xylanase (Dhiman et al., 2011).

In summary, a high level of extracellular xylanase was produced in SSF by *B. pumilus* VLK-1, an alkaliphilic strain isolated in our laboratory, using wheat bran which is a cheap and abundant agro-residue. This is likely to result in cost effective enzyme production for industrial use. The purified enzyme exhibited potential in enrichment of tomato juice and hence could be exploited in food industry.

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