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Full Length Research Paper

Comparative evaluation of rice and sunhemp root inhabiting *Pseudomonas fluorescens* for optimized glucanase production

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Exploitation of rhizosphere inhabiting *Pseudomonas fluorescens* for useful products like industrial enzymes remains largely less explored. Glucanase enzyme produced by *P. fluorescens* has wide range of application in food, textile and brewing industry, other than their antifungal activity for agricultural use. In our study, native isolates of *P. fluorescens* from rice and sunhemp rhizosphere varied in their preference to carbon and nitrogen source nutrients under *in vitro* conditions attributing to the possible difference in the metabolic profile of their respective ecological niche. The optimum conditions for maximum growth and glucanase enzyme production were a pH of 6.5 for both isolates and temperature of 37 and 40°C for rice and sunhemp isolates respectively. The rice rhizosphere isolate of the bacteria was a rapid producer of glucanase (24 h) compared to sunhemp isolate (48 h). Extracellularly secreted proteins were separated and partially purified by dialysis and sephadex column chromatography. Electrophoretic resolution of purified proteins in correlation with their enzyme activity revealed a 29 kDa isoform of glucanase in rice isolate and a 32 kDa isoform of the same in sunhemp isolate. In-gel activity assay was also performed to confirm the identity and activity of the purified protein.

Key words: Glucanase, *Pseudomonas fluorescens*, purification, rice, sun hemp.

INTRODUCTION

Glucanases are multi-utility enzymes which break the glucan chain of polysaccharide molecules. Glucanases find their application in starch production, production of baked goods and diet products, in textile industry as a bleaching agent, food additive to enhance digestibility of animal and poultry feed, in clarification process during wine production, and in beer brewing to remove glucans contained in barley. In addition to these industrial uses, they are also antifungal enzymes that have the capacity to hydrolyze the cell walls of fungi.

Fungi and bacteria are the most exploited microbial sources of glucanase, of which the enzyme produced by *Aspergillus*, *Bacillus* and *Trichoderma* are commercially available. These microbial derived glucanases have potential application in biotechnology such as cell fusion, transformation and protoplast preparation (Kitamoto et al., 1987) in brewing industries and during the clarification of slimy must (Dubourdieu et al., 1985). It enhances utilization of barley and other cereals in poultry (Almirall et al., 1995; Yu et al., 2002). β–Glucanase breaks the polymeric chain into smaller pieces and reduces the gut viscosity and hence improves the nutritive value of grains rich in non-starch polysaccharides. Glucanase

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supplementation improves the digestibility of nutrients in birds fed with barley-based diets. It enhances fermentation in the gastero-intestinal tract so that absorption of nutrients is increased (Choct, 2001). The enzyme also has utility in the process of producing yeast extract (Ryan and Ward, 1985) and in wine extract clarification (Villettaz et al., 1984; Pretorius and van der Westhuizen, 1991).

Commercial endo β–glucanase products currently used in animal feed are derived mainly from species such as *Trichoderma* and such glucanase are not initially developed specifically for use in animal feed hence may not be ideally suited for this application in terms of their physicochemical properties. β–Glucanases produced by several fungi and bacteria are one of the most potent enzymes for degrading fungal cell wall (Bodemann et al., 1985; Chet et al., 1998) used in agricultural biocontrol of diseases.

Fluorescent pseudomonads are unique group of beneficial bacteria whose potentials are being exploited biotechnologically for the benefit of mankind. The most beneficial strains of fluorescent pseudomonads have soil as their major habitat. Many hydrolytic enzymes are produced by these bacteria, among which, glucanase, in addition to chitinase, is being reported to be involved in plant disease control (Nandakumar et al., 2001, 2002; Vivekananthan et al., 2004).

With the long term objective to explore the utility of plant inhabiting bacteria as candidates for industrial production of glucanase, the present study has been taken up. Although many other microorganisms are already used in industry for mass production of glucanase, studies on characterization of glucanase from *Pseudomonas* spp. with an industrial perspective is limited. We report the isolation, optimized production, partial purification and characterization of glucanase from rice and sun hemp root inhabiting *Pseudomonas fluorescens*, which represents a preliminary study in identifying candidate strain with higher enzyme producing capabilities, before scaling-up of commercial production of the enzyme.

MATERIALS AND METHODS

Isolation of root inhabiting *Pseudomonas fluorescens*

Rhizosphere soil samples from the standing crop of rice (*Oryza sativa* L.), cultivar Co-43 and sunhemp (*Crotalaria juncea* L.), and cultivar Co-1 were collected from on-farm trial fields of Tamil Nadu Agricultural University, Coimbatore, India. Plants selected at random from the centre of field were uprooted and the soil clump removed. The root portion with thin film of soil was transferred to polythene bags and vigorously shaken to collect the rhizosphere soil. King' B selective media (peptone- 20 g; magnesium sulphate- 1.5 g; dipotassium hydrogen phosphate- 1.5 g; glycerol- 10 ml/L) was used to isolate *P. fluorescens* isolates by serial dilution method. Fluorescent single colonies were subcultured in King's B agar slants and maintained. Gram staining was performed to determine the Gram reaction of the isolated bacterial species as per the standard procedure. The biochemical tests *viz*., protease, lipase, catalase, IMViC, indole, methyl red, Voges Proskauer and citrate utilization tests were carried out and recorded as per the standard procedures.

Glucanase production

P. fluorescens isolates, each from rice and sunhemp rhizosphere, were inoculated onto glucanase production liquid media and incubated at 37°C in an orbital shaker at 100 rpm for 48 h. Modified King's B was used as production media (with 40 gL^{-1} refined barley powder). The effect of various carbon and nitrogen sources was determined by supplementing the glucanase production media with 5% of either of maltose, fructose, sucrose and glucose and with 5% of either of ammonium nitrate, ammonium sulphate, potassium nitrate and peptone. The effect of pH of growth media on glucanase production was investigated by using glucanase production media prepared with various pH *viz*., 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0. The bacteria were grown by incubating at different temperatures such as 4, 30, 37, 40, 45 and 50°C. The effect of incubation period of the culture on glucanase production was investigated by incubating and harvesting the enzyme every 12 h for four days.

Enzyme preparation and assay

Bacterial cells in the liquid culture were pelleted by centrifuging at 8000 g for 15 min at 4°C. The supernatant was subjected to precipitation by ammonium sulphate (65% saturation) overnight at 4°C. Protein was precipitated by centrifugation at 6000 g for 15 min at 4°C. The pellet was redissolved in 10 ml of 0.01 M sodium phosphate buffer (pH 7.0). To the protein sample, 7 ml of ice-cold acetone was added dropwise and incubated overnight at 4°C. The samples were centrifuged at 8000 g for 30 min at 4°C and the pellet obtained was dissolved in 1 ml of 0.1 M sodium phosphate buffer. Concentration of protein in crude / partially purified sample was determined by Lowry's method (Lowry et al., 1951) using BSA as standard.

Glucanase activity was assayed by laminarin-dihydro salicylic acid method (Pan et al., 1991). The reaction mixture consisted of 135 µl of 4% laminarin (Sigma, St Louis, USA) and 135 µl of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was stopped by adding750 µl of dinitro-salicylic acid and boiled for 5 min. About 4.5 ml of distilled water was added to the reaction mixture and the absorbance was read at 510 nm. The amount of reducing sugars released was calculated from a standard curve prepared with glucose, and the glucanase activity was expressed in units (µmol glucose equivalent / min).

Partial purification of glucanase

Based on the results obtained in the optimization experiments, the optimal conditions for maximum glucanase production was determined and used for the preparation of glucanase for purification. *P. fluorescens* isolates were cultured in optimized media and under optimized conditions for two days and the bacterial cells pelleted by centrifugation at 6000 g for 15 min at 4°C. The supernatant was used as crude enzyme source for partial purification following ascending steps in the order of: ammonium sulphate precipitation; acetone precipitation; dialysis; sephadex G-50 column chromatography. Ammonium sulphate and acetone precipitation was carried out as described earlier. The pre-treated

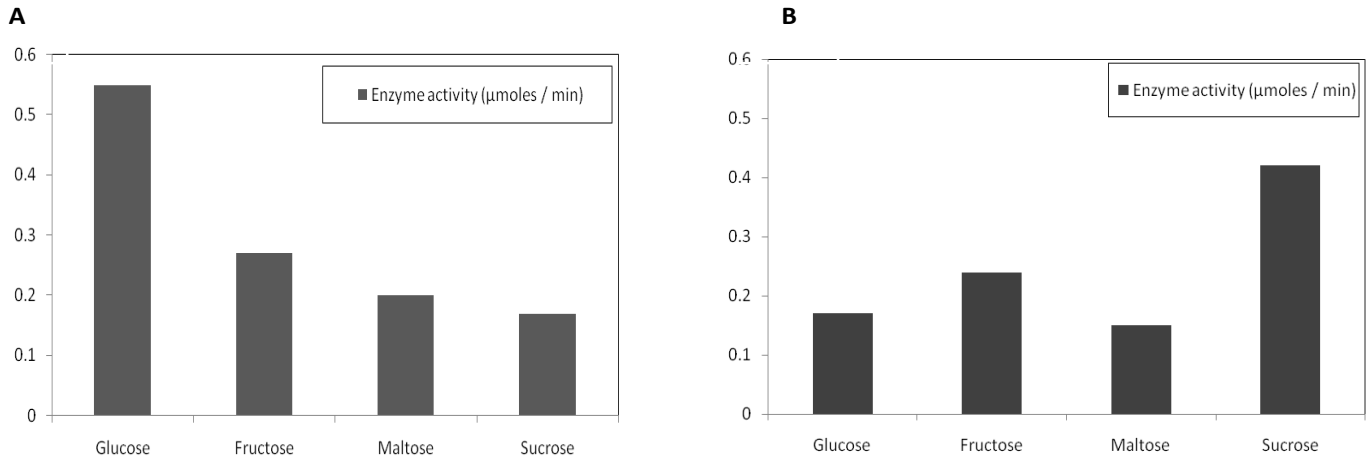

Figure 1. Effect of different carbon sources in growth media on the glucanase activity of *Pseudomonas fluorescens*; a) rice isolate and b) sunhemp isolate.

dialysis tube was filled with 5 ml of the sample preparation and dialysed against 0.01 M sodium phosphate buffer (pH 7.0), overnight at 4°C under constant stirring. The buffer was changed two times during dialysis. About 2 g of sephadax G-50 was soaked overnight in 20 ml of 0.2 M sodium phosphate buffer (pH 7.0) at 4°C. The column was packed and 0.1 M sodium phosphate buffer (pH 7.0) was used to pack the column. About 3 ml of the dialysate was loaded onto the column and with 0.5 M sodium phosphate buffer (pH 7.0) as elution buffer; fractions of 3 ml were collected at a flow rate of 180 ml h^{-1}. Proteins were precipitated from the fractions obtained from column chromatography, using ice cold acetone. Three volumes of ice cold acetone was added to the fractions, mixed well and incubated for 10 min at -80°C. Samples were centrifuged at 8000 g for 15 min at 4°C. The pellet was air dried and dissolved in 200 µl of 0.1 M sodium phosphate buffer (pH 7.0).

SDS-PAGE and in-gel activity analysis

SDS-PAGE analysis was done in vertical slab gel as described by Laemmli (1970). Crude, partially purified, and column purified protein samples were prepared by mixing 80 µl of sample with 20 µl of 4× sample buffer. Samples were heated in boiling water bath for 2 min and loaded on to the wells with protein molecular weight marker (Genei, Bangalore, India) in one lane. Electrophoresis was carried out at constant voltage of 100 V. The gel was stained with Coomassie brilliant blue R-250. In-gel activity analysis of purified glucanase was carried out in a native gel electrophoresis system followed by enzyme specific staining. Non-denaturing polyacrylamide gel electrophoresis without SDS was used. The column purified samples (fractions which showed glucanse activity) were analyzed and the electrophoresis was carried out at constant voltage of 50 V in cold room. The gel was overlayed with a filter paper impregnated with 50 mg ml^{-1} of refined barley powder. After 30 min at 37°C, the gel was stained with aniline blue (0.05% w/v in water) overnight and viewed under UV transilluminator.

RESULTS

The culture morphology including slimy growth and fluorescence in King's B media observed under UV light revealed the identity of the bacteria isolated. The bacterial species isolated in the present study were found to be Gram negative rods and the results are further confirmed by growing the isolates in McConkey agar media. The observation of zone of clearance on skim milk agar indicated the production of hydrolytic enzymes (data not shown). The isolates recorded positive to catalase, methyl red, citrate utilization, proteinase tests and negative to indole, lipase and Voges Proskauer tests.

In the present study, glucose was found to be the best carbon source in the media which supported better growth and enzyme production by isolate collected from rice rhizosphere. In contrast, sucrose supported better growth and glucanase production in sun hemp isolate (Figure 1). Similarly, the preference of nitrogen source also varied between the isolates (Figure 2). Rice isolate preferred potassium nitrate and sunhemp isolate preferred peptone. The optimum pH of the growth media which resulted in higher enzyme production and in turn the enzyme activity is 6.5 for both isolates (Figure 3). Rice isolate of *P. fluorescens* showed higher enzyme activity at a temperature of 37°C whereas sunhemp isolate was at 40°C (Figure 4). Rice isolate recorded maximum activity of glucanase enzyme at 24 h of incubation and furthering of the incubation time lowered the enzyme activity (Figure 5). Whereas, sunhemp isolate recorded maximum activity at 48 h of incubation.

After optimized media components and growth conditions for maximum glucanase production were determined, the bacterial isolates were grown in the optimized media and the extra-cellular protein was harvested and purified. At each step of purification, the protein yield was less indicating the enzyme purification from the total protein pool. The final purification step of

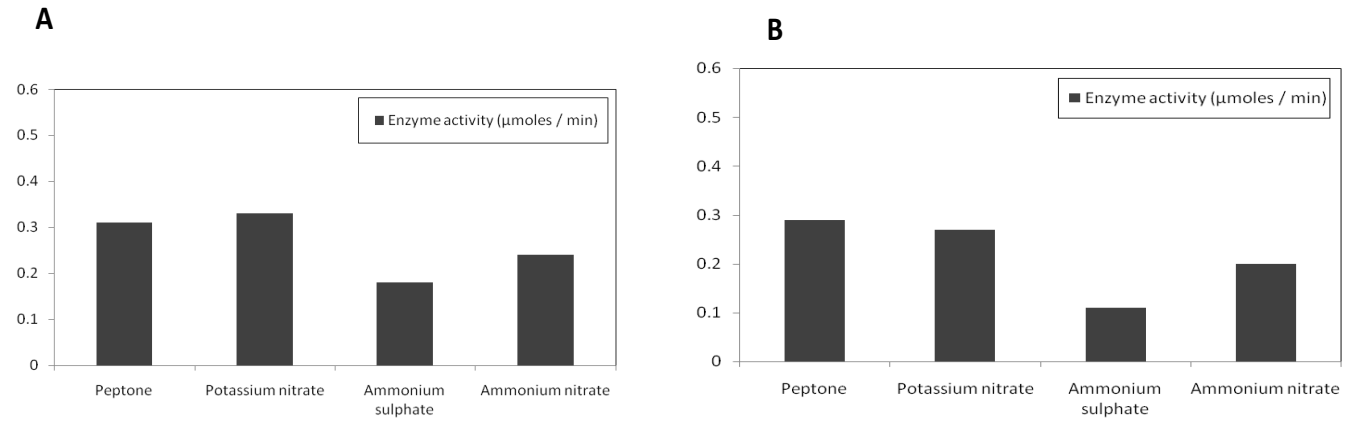

Figure 2. Effect of different nitrogen sources in growth media on the glucanase activity of *Pseudomonas fluorescens*; a) rice isolate and b) sunhemp isolate.

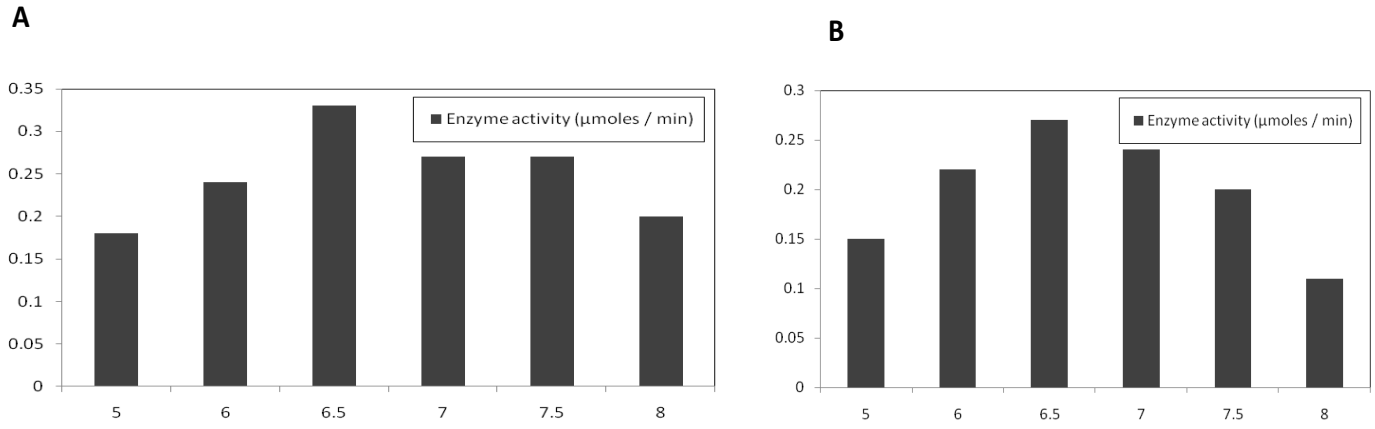

Figure 3. Effect of pH of the growth media on the glucanase activity of *Pseudomonas fluorescens*; a) rice isolate and b) sunhemp isolate.

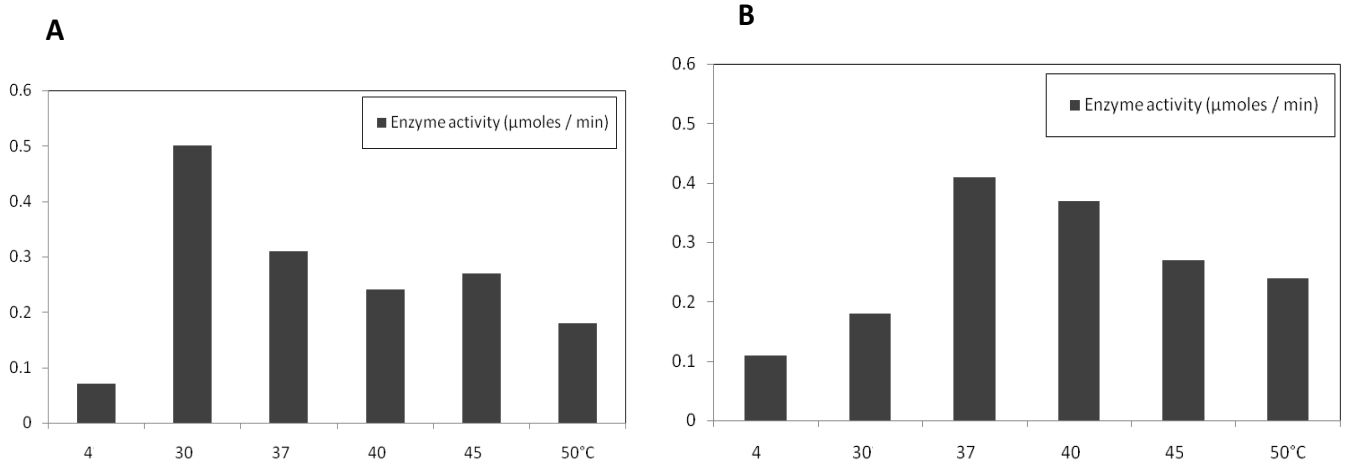

Figure 4. Effect of cultivation temperature on the glucanase activity of *Pseudomonas fluorescens; a)* rice isolate and b) sunhemp isolate.

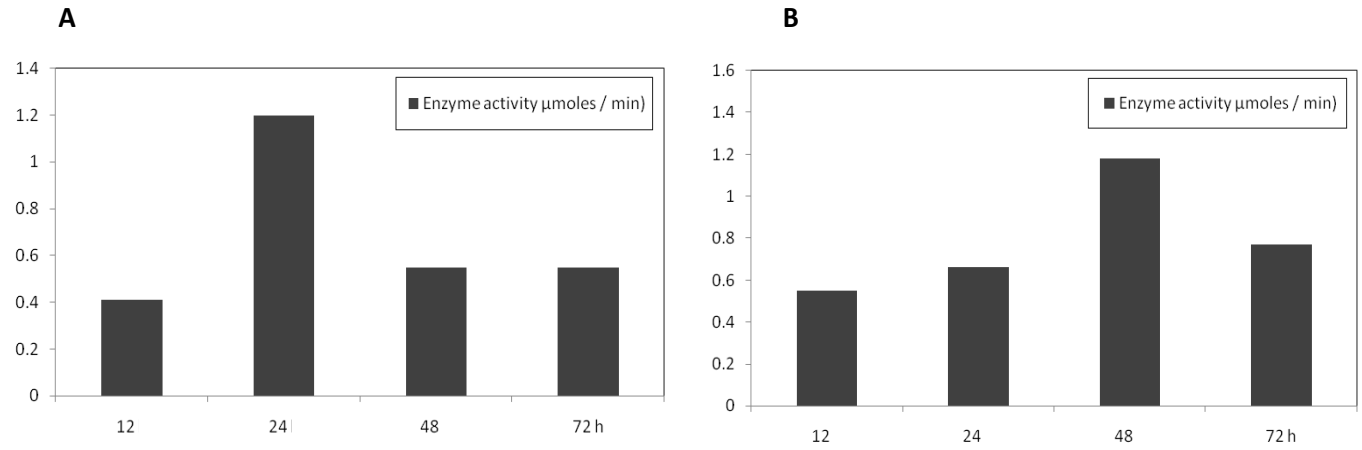

Figure 5. Effect of cultivation time on the glucanase activity of *Pseudomonas fluorescens*; a) rice isolate and b) sunhemp isolate.

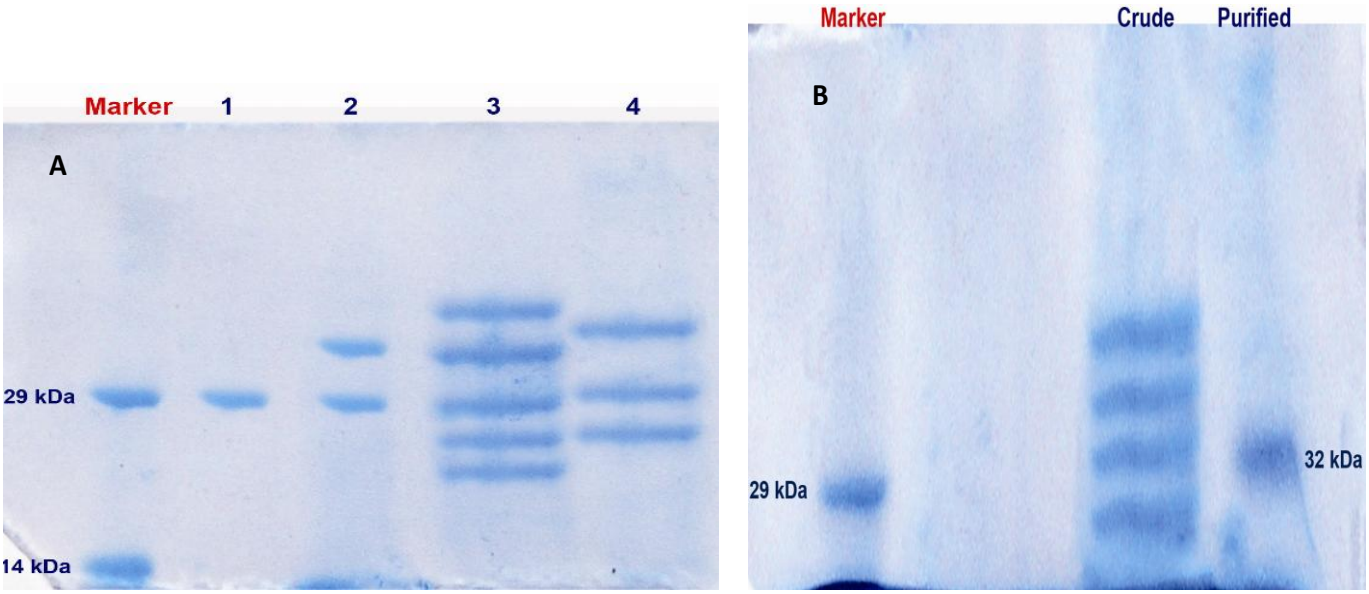

Figure 6. SDS-PAGE analysis of secreted protein fractions of *Pseudomonas fluorescens*; a) rice isolate. Lanes 1- after column chromatography purification, 2- dialysis, 3- ammonium sulphate precipitation and 4- acetone precipitation; b) sunhemp isolate. Lanes 1- no sample, 2- after ammonium sulphate precipitation, 3- column chromatography purification.

column chromatography resulted in collection of ten fractions of 3 ml each and a final washing of 20 ml. The assay of glucanase activity in the eluted fractions showed observable activity in the first three fractions. The fractions were pooled, precipitated and analyzed in SDS-PAGE along with crude preparations. The extra-cellular total proteins of both the rice and sunhemp isolates showed a profile of many proteins with molecular weights ranging from 20 to 43 kDa (Figure 6). The crude culture filtrate, total extra-cellular protein and the column purified rotein were analyzed for sunhemp isolate. The total protein profile showed many proteins of molecular weight ranging from 24 to 43 kDa. The column purified protein is of 32 kDa. For rice isolate, crude culture filtrate, total proteins after ammonium sulphate precipitation, partially purified proteins after dialysis and the column purified protein were analyzed. As like sunhemp isolate, rice isolate also had many proteins in the total protein profile and three proteins in the dialysate (Figure 6). Single protein band of molecular weight 29 kDa was obtained in

the column purified sample. The purified protein with glucanase activity from sunhemp isolate is a 32 kDa protein whereas of rice isolate, it is a 29 kDa protein.

Native PAGE analysis also revealed that the glucanase produced is a single isoform (data not shown).

DISCUSSION

Agriculturally important isolates of *P. fluorescens* are efficient and less exploited candidates for utilization in industry for commercial production of enzymes. Their strength as potential enzyme source is due to their active performance in producing hydrolytic enzymes in their ecological niche, when compared to isolates from barren soil or any other source. There are many previous reports on the isolation of *P. fluorescens* from rhizosphere of rice plants (Nandakumar et al., 2001; Nagarajkumar et al., 2004). However, the literature is deficient in reports from sunhemp rhizosphere.

Optimization of culture media is an important approach for improving growth and production of extracellular glucolytic enzymes (Szengyel et al., 2004). The selection of suitable carbon and nitrogen sources has particular importance in the process of extracellular production of enzymes by microbes (Saravanan et al., 2007). The difference observed in the preference to carbon and nitrogen nutrients by the bacteria isolates might be attributed to the difference in the nutrients of root exudates of rice and sunhemp. Rice belongs to graminae and sunhemp being pulse family member, are genetically and taxonomically different and therefore, the root exudates that harbor rhizosphere microbiota are likely to be different. Evaluation of the nutrient status of the root exudates from the rhizosphere of these plants may provide clues to understand and validate the aforesaid hypothesis of differential nutrient requirement by the isolates.

The average pH of the soil in most cultivable lands of India is reported to be 6.5. As the pH of the growth media was same as that found in the natural soil environment, it could have supported better growth as revealed from the pellet weight and OD value of bacteria as well as the enzyme produced and its activity. It indicates that the bacterial isolate could be actively producing glucanase enzyme in the rhizosphere of rice and sun hemp to combat the harmful fungal pathogens and other competitive microbes in the soil. The optimum pH for glucanase production in other organisms reported include 5.5 for *Trichoderma asperllum* (Bara et al., 2003), 7.5 for *Bacillus subtilis* (Leelasuphakul et al., 2006), 5.5 to 6.5 for *Ampullaria crossean* (Li et al., 2009), 6.0 for *Rhizoctonia solani* (Vijayendra and Kashiwagi, 2009), 4.6 for *Aspergillus* sp. (Abe et al., 1999) and 6.0 for *Pichia pastoris* (Xu et al., 2006). The difference in soil temperature of submerged rice fields and the garden land under direct sunlight (sunhemp) could have been the reasons for the differential optimal temperature required for glucanase production by the isolates, as observed in our study. According to Bara et al. (2003), the optimum temperature for maximum production of glucanase by *T. asperllum* is 55°C. The various other temperature regimes reported for glucanase production are 50°C for *B. subtilis* (Leelasuphakul et al., 2006), 50 to 55°C for *A. crossean* (Li et al., 2009) and 40°C for *R. solani* (Vijayendra and Kashiwagi, 2009). These observations lead to our understanding that the isolates differ in their glucanase production abilities and substrate utilization capabilities.

Cell free protein extraction and profiling resulted in many proteins of varying molecular weight in the SDS-PAGE. It is obvious that many other proteins are produced by the bacteria during growth in the enzyme production media along with the actual enzyme induced which could be either the isoforms of glucanase or other carbohydrate breaking supporting enzymes. This observation led us to further purification of the extra-cellular proteins and determination of glucanse activity in every purified fraction. The purified protein with glucanase activity was a 32 kDa protein in sunhemp isolate and 29 kDa protein in rice isolate. The molecular weight reported for glucanase varies with the microbial source. *R. solai* glucanse of 62 kDa was reported (Vijayendra and Kashiwagi, 2009). Glucanase of *T. asperellum* was 83.1 kDa (Bara et al., 2003), whereas a 47 kDa glucanase was reported for *P. pastoris* (Xu et al., 2006). Several authors have reported that glucanase consists of isoenzymes with different molecular weights. For example, different strains of the same species of *B. circulans* was reported to produce glucanse of molecular weights of 28, 42, 72, 87 and 91 kDa, whereas the protein from *B. clausii* was 71 kDa (Leelasuphakul et al., 2006). In contrast, the glucanase activity of purified enzyme from *B. subtilis* was shown to be a single active isoform by native PAGE analysis. Our study also revealed a single active isoform of glucanase in the native PAGE.

P. fluorescens is widely used as a biocontrol agent in agriculture for its ability to produce hydrolytic enzymes like chitinase and glucanase. Glucanase produced by bacteria hydrolyse the cell walls of fungal pathogens in the crop rhizosphere. However, there are not much attempts to commercialize the root inhabiting *P. fluorescens* strains for producing industrially important glucanse. Our study represents the *in vitro* production and purification of glucanse under optimized growth conditions. The results of our study are preliminary, which warrants further investigation for exploiting agriculturally important *P. fluorescens* strains for the mass production of glucanase enzyme and utilization in the industry. We suggest the root inhabiting *P. fluorescens* could be the best alternatives to existing microbes for higher

production of glucanse in large scale.

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