

Green Catalysts and Secondary Metabolites Produced by *Paenibacillus alvei* NRC14 under Normal and Abiotic Stress Conditions

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Abstract

Production of enzymes and secondary metabolite in microorganisms is strongly influenced by nutritional factors and growth conditions. *Paenibacillus alvei* NRC14, a soil bacterial isolate, produces a variety of carbohydrate-active enzymes. The strain exhibits also multiple-adaptive response when exposed to abiotic-stress factors. *Paenibacillus alvei* NRC14 is a Gram-positive bacterium able to grow in a wide range of environmental conditions, does not require specific growth factors, and produces a variety of secondary metabolites such as polysaccharides, bioflocculants, and antibacterial agents. Chitosanase from strain NRC14 displays broad specificity towards chitin and chitosan with different degrees of deacetylation. Values of K_m and V_{max} for the extracted chitosanase towards chitosan are almost the same for hydrolyzing of chitosan. Chitosanases from strain NRC14 showed also broad specificity towards various substrates such as cellulose, CM-cellulose, and cellobiose.

Keywords: *Enzymes, secondary metabolites, Paenibacillus alvei, abiotic stress, response, bioflocculant, chitinase, chitosanase*

1. Introduction

Green biotechnology can be regarded as applied biocatalysis process with enzymes (biocatalysts) and microorganisms. In recent years, the emergence of environmental issues, “green biotechnology” or “white biotechnology” (in EUR) is designed to reduce environmental impact, use mild reaction conditions (physiological pH and temperature), compatible catalysts and solvent, combined with high activities in multifunctional molecules [1]. Biocatalysis processes are also shorter, generate less waste, and are, therefore, both environmentally and economically more attractive than conventional routes and has been hailed as promising technology for the 21st century [1]. Biocatalysis is becoming a favorable alternative to chemical processes and a vital part of green technology. Green biotechnology uses enzymes and microorganisms to make products in sectors such as chemistry, food and feed, paper and pulp, textiles and energy. Biocatalysts are superior challenge alternative to inorganic catalysts and have seen growing use for industrial production of fine chemicals. Bacterial enzymes are more attractive due to their high stability at extreme temperature and pH, unusual substrate specificity, tolerance to inhibitors, as well as low cost and high productivity [2].

Bacteria are exposed to changes in the environmental conditions which lead to morphological changes and protein-folding processes. The changes vary with the bacterial strain, abiotic stress, length of exposure to the antibiotic or abiotic stress, and formation of biofilm [3]. Bacteria utilize different strategies to adapt to varying environmental situations including exposures to the extreme in pH values, temperatures (high or low), nutrient starvation as well as high concentrations of salt and heavy metals [4-5].

The 16S rRNA gene has been usually used as a trustworthy molecular marker for phylogenetic identification of organisms. Recently, partial 16S rDNA sequence and rRNA gene restriction patterns have been used for the rapid identification or classification of *Bacillus* species and related genera, respectively [6]. In the second edition of Bergey's Manual of systematic Bacteriology [7], phylogenetic classification schemes landed the two most prominent types of endospore-forming bacteria, clostridia and bacilli, in two different Classes of Firmicutes. The genus *Bacillus* belongs to the order *Bacillales* and the Family *Bacillaceae*. In this family there are 37 new genera with *Bacillus*. Table 1 represents the recent important taxonomic relocation in the genus *Bacillus* from 1st edition to 2nd edition. In this regard, a bacterial strain identified *Bacillus alvei* NRC14, is relocated to be *Paenibacillus alvei* NRC14 Table 1. This strain produces a variety of carbohydrate-active enzymes and exhibits multiple-adaptive response when exposed to abiotic stress conditions. The strain produced an antimicrobial agents when exposed to 40°C at shaking culture condition [8]. When exposed to a heat-shock stress followed by cold shock, under static condition, the strain synthesizes a biofilm for protection of the cells and the supernatant was found to contain a highly viscous polysaccharide possesses a good flocculating properties [9]. This work: 1) represents unique properties of the strain *P. alvei* NRC14; 2) investigates its behavior when exposed to abiotic stresses under shaking or static incubation conditions; and 3) evaluates the repertory of enzymes and secondary metabolites produced under normal and abiotic stress conditions.

2. Materials and Methods

2.1 Bacterial strain and growth medium

The strain *P. alvei* NRC14, isolated from Egyptian soil as a potent chitosanase producer, was maintained on nutrient agar slants at 4°C, with monthly transfers using chitosan-containing slants to retain viability. Prior to use, cultures of the strain was grown in nutrient broth to the log phase and repeated for 3 rounds to enrich for further determinations. The strain was grown on a nutrient medium which contained: (% w/v), flaked chitosan, 0.5; NaCl, 0.3; (NH₄)₂SO₄, 0.2; K₂HPO₄, 0.3; and MgSO₄.7H₂O, 0.05 with adjusted pH to 6.0. Growth of the cells was monitored by spectrophotometer at 660nm. Using this medium, different modifications for growth and polysaccharide formation were tested such as different incubation times, different pH values as well as various carbon and nitrogen sources. Different carbon sources were tested include chitin, chitosan, starch, fungal mycelium as well as some agro-wastes. Nitrogen sources used were NaNO₃, NH₄Cl, NH₄NO₃, (NH₄)₂SO₄, as well as yeast extract, treptophan, and peptone. Media were distributed in 30 ml portions into 250 ml Erlenmeyer flasks and inoculated with 2 ml of a 24-hrs culture pre-grown in nutrient broth medium (O.D₆₆₀=2.5). In all experiments, the cultures were incubated at 30° and 40°C with or without shaking.

Table 1. Important Taxonomic Relocations in the Genus *Bacillus* from 1986 to 2009 [6].

1 st Edition (1986)	2 nd Edition (2009)	Reference
<i>Bacillus acidocaldarius</i>	<i>Alicyclobacillus acidocaldarius</i>	Wisotzjsey <i>et al.</i> (1992)
<i>Bacillus alvei</i>	<i>Paenibacillus alvei</i>	Ash <i>et al.</i> (1993)
<i>Bacillus macerans</i>	<i>Paenibacillus macerans</i>	Ash <i>et al.</i> (1993)
<i>Bacillus polymyxa</i>	<i>Paenibacillus polymyxa</i>	Ash <i>et al.</i> (1993)
<i>Bacillus agri</i>	<i>Brevibacillus agri</i>	Shida <i>et al.</i> (1996)
<i>Bacillus brevis</i>	<i>Brevibacillus brevis</i>	Shida <i>et al.</i> (1996)
<i>Bacillus larvae</i>	<i>Paenibacillus larvae</i>	Heyndrickx <i>et al.</i> (1996)
<i>Bacillus alginolyticus</i>	<i>Paenibacillus alginolyticus</i>	Shida <i>et al.</i> (1997a)
<i>Bacillus amylolyticus</i>	<i>Paenibacillus amylolyticus</i>	Shida <i>et al.</i> (1997b)
<i>Bacillus azotofixans</i>	<i>Paenibacillus azotofixans</i>	Logan <i>et al.</i> (1998)
<i>Bacillus lentimorbus</i>	<i>Paenibacillus lentimorbus</i>	Pettersson <i>et al.</i> (1999)
<i>Bacillus popilliae</i>	<i>Paenibacillus popilliae</i>	Pettersson <i>et al.</i> (1999)
<i>Bacillus globisporus</i>	<i>Sporosarcina globisporus</i>	Yoon <i>et al.</i> (2001)
<i>Bacillus pasteurii</i>	<i>Sporosarcina pasteurii</i>	Yoon <i>et al.</i> (2001)
<i>Bacillus psychrophilus</i>	<i>Sporosarcina psychrophila</i>	Yoon <i>et al.</i> (2001)
<i>Bacillus stearothermophilus</i>	<i>Geobacillus stearothermophilus</i>	Nazina <i>et al.</i> (2001)
<i>Bacillus thermodenitrificans</i>	<i>Geobacillus thermodenitrificans</i>	Nazina <i>et al.</i> (2001)

2.2 Preparation of substrates for enzyme assays

Colloidal chitin and soluble chitosan (at 1% concentration), as substrates for determination of enzyme activity, was prepared as described in our previous work [10].

2.3 Preparation of Chitosan

Chitosan with different degrees of acetylation (50, 70, and 99% deacetylation) were prepared by the methods of [11-12]. Estimation of K_m and V_{max} values were measured in presence of chitosan with degrees of deacetylation 50% and 99%. Kinetic studies were carried out at 40°C pH 5.6 (acetate buffer) under conditions corresponding to linear variation of activity.

2.4 Estimation of enzymes activities

Activity of β -1,3glucanase was estimated by the method of Wichitra *et al.* [13] using laminarin as substrate. The enzyme activity was defined as micromoles equivalents of glucose released /ml of culture/min. Activity of cellulase and β -1,4glucanase was estimated using carboxymethyl cellulose as the substrate [14]. Chitinase and chitosanase activity was determined as described previously [10]. Protease activity was estimated as reported in our previous work [15].

2.5 Partial purification of exopolysaccharide

The biopolymer flocculant of strain NRC14 was purified by the method of Shih *et al.* [16] as follows: the viscous culture broth (400 ml) was mixed with three volumes of cold distilled water and centrifuged at 7000xg for 20 min. The resultant supernatant was poured into two volumes of cold ethanol and allow to be kept overnight at 4°C to precipitate the bioflocculant. The precipitate was collected by centrifugation at 7000xg for 20 min, and kept at 4°C.

2.6 Flocculating properties

The flocculating activity of the biopolymer flocculant was measured according to the method reported by Kurane *et al.* [17] using a suspension of kaolin, soil, or starch (5g/L, in distilled water) as test materials. Briefly, 0.1 ml of biopolymer flocculant solution were added to 100ml of the test material suspended solutions. The mixture was vigorously stirred and poured into a cylinder and allowed to stand for 10 min. The optical density of the clarifying solution (*A*) was measured with a spectrophotometer at 550 nm [18]. A control experiment was prepared using the same method, except that the bioflocculant solution was replaced with distilled water (*B*). The flocculating activity was measured using the equation: Flocculating activity (%) = $[(B - A)/B] \times 100$;

where *A* is the absorbance of the sample experiment at 550 nm, and *B* is the absorbance of the control at 550 nm.

2.6 Analytical methods

The content of glucosamine was measured by the Elson-Morgan method [19] using glucosamine as a standard. Viscosity of the biopolymer flocculant solution was determined using a Brookfield viscometer type LV (at 50 rpm).

3. Results and Discussion

3.1 Kinetics of cell growth

3.1.1 Effect of nutrient

Enzymes production by the strain *P. alvei* NRC14 was extensively tested using different carbon sources, temperatures, and pH values. The strain generally possesses a great affinity for degrading hard substrates. Enzymes, secondary metabolites, and growth behavior of strain NRC-14 with chitin and chitosan as carbon and nitrogen sources are greatly differed:

1. When the strain was grown with colloidal or flaked chitin (pH 7/30°C), chitinase was hardly estimated, while at pH 7/40°C, the culture broth was completely free of enzymes but it showed inhibitory effects (Table 3 and Fig. 4). However, using the chitin monomer, *N*-acetylglucosamine, as a carbon source resulted in detection of high quantities of enzymes (Table 2).
2. Using soluble chitosan (pH 6/30°C), cell growth of the strain increased exponentially and highly amounts of chitosanase and glucosamine were detected. Abundant formation of glucosamine by strain NRC-14 suggesting presence of exo-hydrolases.
3. Growth of the strain at pH 6 and 40°C resulted in production of enzymes and antimicrobial compounds showed inhibitory effects (Table 3 and Fig. 5).
4. When flaked chitosan was used at pH 3.0 and 40°C, the culture broth contained chitinase, chitosanase, and protease, and exhibited antibacterial effect (Table 3 and Fig. 5).
5. When grown with flaked chitosan at pH 6 and 10°C, the strain synthesized a highly viscous polysaccharide possesses good flocculating activity (Fig. 5). The biopolymer flocculant showed a good flocculating activity against charcoal particles (Fig. 6).

Results of abiotic stress exposure and adaptation by strain NRC14 may suggest that, after a sudden exposure to temperature elevation, pH variation, heat shock, or cold shock, the strain may

respond by formation of a biofilm (exopolysaccharide) as a form of self-protection. Generally, sudden exposure to abiotic stress lead to expressing a specific set of genes. The heat-shock response comprises the expression of protein chaperones and proteases [20]. Bacteria encounter stresses in their natural environments. These stresses elicit a variety of specific and highly regulated adaptive responses that not only protect bacteria from the offending stress but also manifest changes in the cell [21]. Thus, exposure to nutrient starvation/limitation (nutrient stress), membrane damage (envelope stress), elevated temperature (heat stress), pH variation, or ribosome disruption (ribosomal stress), all impact bacterial growth through their initiation of stress responses that positively induce resistance determinants or promote physiological changes compromise antimicrobial activity [21].

Table 2. Enzymes and secondary metabolites produced by strain *P. alvei* NRC14 under normal conditions during growth on different carbon sources and pH values.

Carbon source	pH	T ^o C	Metabolites produced	Effect	Enzymes produced	Reference
1. Flaked chitosan	6.0	30	Biopolymer flocculant	Great flocculating activity (Fig. 4).	Chitosanase, chitinase, and β -1,4 glucanase.	[22]
2. Flaked chitosan + sugarcane bagasse	5.5	30	Chitosan-oligosaccharides	Show inhibitory effects (Fig. 4).	Chitosanase, cellulose, β -1,4 glucanase, and xylanase.	[23]
3. Fungal mycelium (<i>Aspergillus niger</i>)	7.0	30	–	The culture supernatant with enzymes showed inhibitory effect (Fig. 4)	Chitinase, chitosanase, β -1,3 glucanase, and protease	[10]
4. <i>N</i> -acetyl-glucosamine	7.0	30	–	–	Chitinase, chitosanase, and β -glucanases.	[10]

Table 3. Enzymes and secondary metabolites produced by strain *P. alvei* NRC14 during growth on different carbon sources at various temperatures and pH values.

Carbon source	pH	T ^o C	Metabolites produced	Effect	Enzymes produced	Reference
1. Flaked chitin	7.0	40	Antifungal compound	Show inhibitory effect (Fig. 5)	Culture broth was free of enzymes.	[10]
2. Flaked chitin + sugarcane molasses	6.5	40	Antimicrobial compound	Show inhibitory effects (Fig. 5)	Not detected	[24]
3. Flaked chitosan	6.0	40	Antifungal compound	Show inhibitory effect (Fig. 5)	chitinase, Chitosanase, and β -glucanase	[25]
4. Flaked chitosan	3.0	40	Antibacterial compound	Show inhibitory effect (Fig. 5)	Culture broth contained chitinase , chitosanase, and protease	[8]
5. Sucrose/Starch	6.0	10	Biopolymer flocculant	A great flocculating activity (Fig. 5)	Not detected	[9]

3.1.2 Cell growth Behavior under shaking and static conditions

Cell growth Behavior of strain *P. alvei* NRC14 under shaking and static conditions was determined using different carbon sources. Production of enzymes by the strain is greatly affected by the growth state condition; shaking or static culture. Chitosanase production by the strain is most favorable at either shaking or static growth conditions with carbon sources such as soluble chitosan, flaked chitosan, or fungal mycelium (Fig. 1), while chitosanase was highly produced with glucose, sucrose, or starch under only shaking growth conditions (Fig. 1). It is concluded that, aeration may be a limit factor for cell growth and production of enzymes, when glucose, sucrose, or starch is used as a carbon source, in comparison with decreased aeration under the static condition.

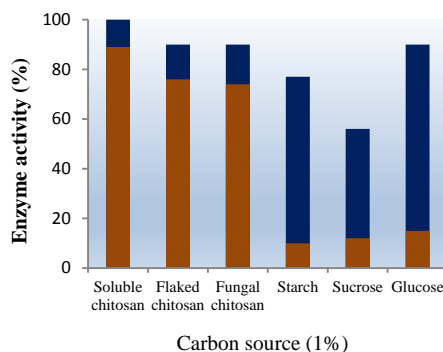


Fig. 1. Effects of shaking (■) or static (■) growth conditions on chitosanase production by *P. alvei* NRC14 grown with different carbon sources (1 %) at 30°C. Activity of enzyme with soluble chitosan was set as 100%.

3.1.3 Effect of temperature and pH values

Being the most important environmental factors for microbial life, temperature and pH effects were evaluated as they directly influence functional properties of the cellular components. Growth of the strain with flaked chitosan at different temperatures and pH values is obviously differed (Fig. 2). Under normal conditions, cell growth increased rapidly through 48 hrs (Fig. 2 A), with the formation of a biopolymer flocculant, where the cells and other impurities aggregated, the culture seemed clear, and the growth was sharply decreased (Fig. 2 A). On other hand, cell growth under heat shock (6.0/40°C) or pH/heat shock (3.0/40°C) stresses resulted in lower cell growth, higher viscosity, and increased (98%) flocculating activity (Fig. 2 B and C).

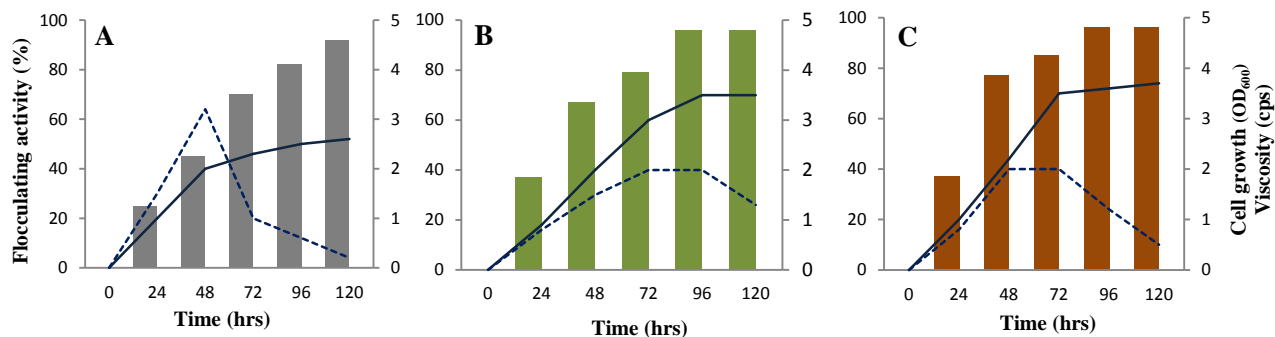


Fig. 2. Growth of the strain *P. alvei* NRC14 under different temperatures and pH values: (A), pH 6.0/30°C; (B), pH 6.0/40°C; and (C), pH 3.0/40°C. Symbols: cell growth (dashed line); viscosity (solid line); and flocculating activity of the bioflocculant (column).

3.1.4 Effect of acetyl content of chitosan

Effect of the acetyl content of chitosan on enzyme activity from *P. alvei* NRC14 was determined. The strain was found to be active with chitosan had different degrees of acetylation, i.e., 50, 70, and 99% (data not shown). The kinetic parameters, K_m and V_{max} values were determined from Lineweaver-Burke plot of chitosanase activity at 40°C using various concentrations of soluble chitosan 50 and 99% deacetylated as substrates. Values of K_m and V_{max} from velocities obtained with chitosan have different degrees of deacetylation were convergent (Fig. 3), suggesting efficiency and multi-specificity of the enzyme for hydrolyzing chitosan. The reciprocal plot of the initial velocity data deviates from linearity at high substrate concentration (Fig. 3), which is characteristic of a substrate-inhibited enzyme [26] (Pelletier and Sygusch 1990).

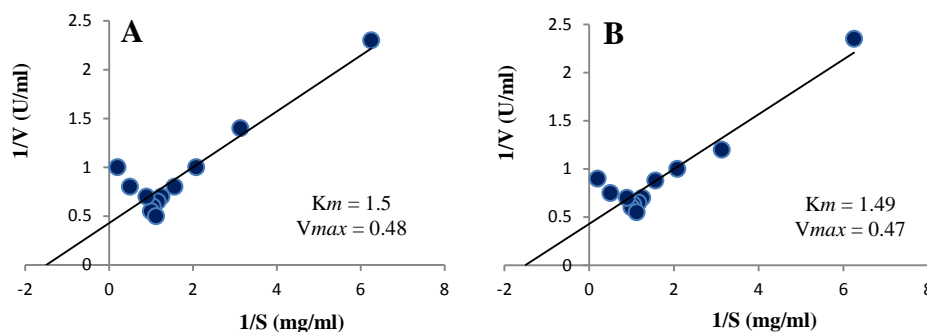


Fig. 3. Double reciprocal plot for determining the K_m and V_{max} values of chitosanase against: (A) soluble chitosan 50% deacetylated; or (B) soluble chitosan 99% deacetylated.

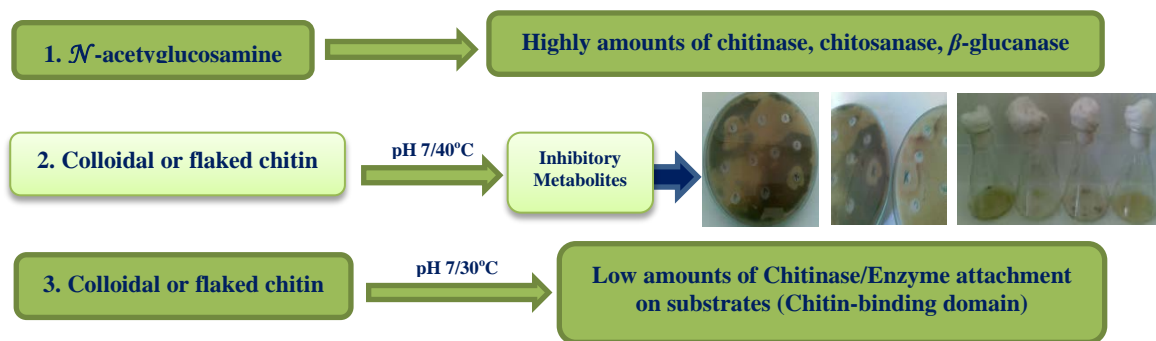


Fig. 4. Variation in enzymes and secondary metabolites produced by *P. alvei* NRC14 at different temperatures and pH values using chitin as carbon and nitrogen sources.

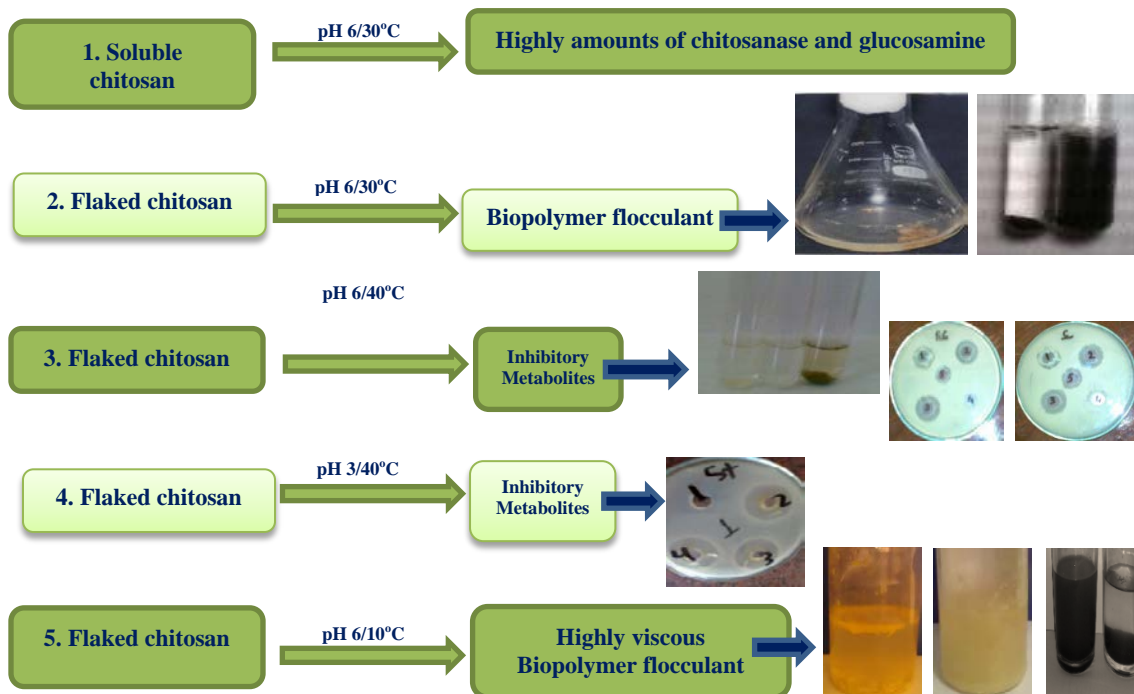


Fig. 5. Variation in enzymes and secondary metabolites produced by *P. alvei* NRC14 at different temperatures and pH values using chitosan as carbon and nitrogen sources.

3.2 Flocculating properties of the exopolysaccharide

Most of the exopolysaccharides produced by microorganisms possess flocculating properties. Biopolymers produced by strain *P. alvei* NRC14, at either normal or stress conditions, were tested for flocculating properties. Biopolymers produced by the strain exhibited potential flocculating properties against kaolin, soil, and starch solutions (Fig. 6). Biosynthesis and composition of biopolymers are a function of a variety of environmental factors. Specific culture conditions can

dramatically impact the yield and chemical composition of the polysaccharides.

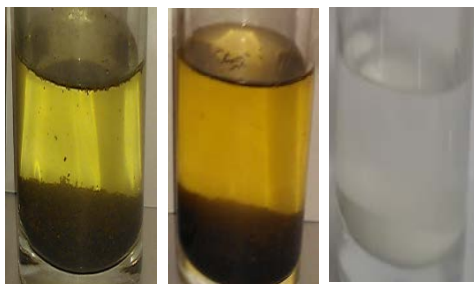


Fig. 6. Flocculating properties of the partially purified biopolymer flocculants produced by the strain *P. alvei* NRC-14 in aggregation and precipitation of kaolin (left image), Soil (middle image), or starch (right image) after 3-min of addition (at 0.1% (v/v)).

During studies on the chitinolytic and chitosanolytic system of *P. alvei* NRC14, it was found that, the strain produces low chitinase activity and abundantly chitosanase activity. This character leads us to study the adhesion of chitinase to insoluble polysaccharides; a mechanism by which the enzyme exhibit low activity [10-27]. Results revealed that chitinase strongly adhered to insoluble substrate after secretion by the strain. Moreover, to assess whether the binding of the enzyme to chitin is specific, the capacity of chitinase to bind to polysaccharides such as chitin, chitosan and avicel was evaluated. Results indicated that the chitinase produced by strain NRC-14 has a broad affinity to bind to insoluble polysaccharides [27]. During more than 20 years of work with strain NRC14 for identifying novel metabolites and enzymes, it was found to be a good producer for a broad spectrum of antimicrobial metabolites and a wide variety of novel compounds that possess many beneficial effects. Enzymes, antimicrobial metabolites, and exopolysaccharides produced by *P. alvei* NRC14 are expected to be used as a potent agent against bacteria and fungi and in biocontrol against plant pathogens. Effect of temperature, pH values, and carbon source on enzymes and metabolites produced by strain NRC14 revealed the variation in enzymes and secondary metabolites produced by the strain.

Mechanisms of abiotic stress adaptation by microorganisms are extensively studied. Such mechanisms may include: 1) Heat acclimation which is the result of the organism's ability to tolerate heat more effectively by developing and secreting exopolysaccharides; 2) The subsequent release of an antimicrobial compound may be another mechanism for complete protection by a bacteria strain; and 3) Formation of heat-shock proteins, that may also play an important role in cell protection. When a bacterial cell becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to hard conditions [28]. In fact, bacteria can utilize nutrients in the culture medium to synthesize high molecular-weight polymers internally within the cells under the action of specific enzymes, and these polymers can be exerted and exist in the medium or on the surface of the bacterium as capsules. Therefore, by the action of such bacteria, some substances in their environment can be converted into complex polymers [29]. Of interest is that, during the long term of working with strain NRC14, no cell lyses was observed and no contamination in their slants or flasks was occurred. The bioflocculant produced by the strain may provide a protective

function for the cells. Moreover, this novel strain can adapted with hard conditions and stress factors. Formation of biofloculants and antimicrobials by *P. alvei* NRC14 is proposed to promote survival in harsh environments to ensure carryover until external conditions become favorable. Such behavior is frequently occurred as a strategy for self-protection by the strain from environment challenges and toxic factors.

Conclusion

Bacteria have developed sensory systems that facilitate adaptation to changes in the environmental conditions. Enzymes and secondary metabolite production is strongly influenced by nutritional factors and growth conditions. The strain *P. alvei* NRC14 produces a variety of enzymes and biologically active compounds. In fact, this strain is particularly interesting because of: 1) large quantities and varieties of carbohydrate-active enzymes and extracellular metabolites, using a minimal medium; 2) capability of controlling its growth conditions with a variety of substrates for production of enzymes, antimicrobial agents, amino-sugars, and biofloculants with unique properties; 3) novel enzymes with physical and physiological characteristics like high productivity, specificity, stability at extreme temperature, pH, and low production costs; and 4) adaptation to abiotic-stress conditions by special metabolic pathways, to survive in extreme conditions. Such strain can potentially be applied in the biocontrol, medicinal, and pharmaceutical fields. Broad specificity for various substrates such as *N*-acetylglucosamine, chitobiose, chitin as well as chitosan with different degrees of deacetylation suggest that, strain NRC-14 may secrete an enzymatic system which makes it possible to degrade chitinous polymers with a wide range of acetylation.

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