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Original Article

Influence of bioprocess variables on the production of extracellular chitinase under submerged fermentation by *Streptomyces pratensis* strain KLSL55

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ABSTRACT

Chitinases are the enzymes which are capable of hydrolyzing chitin to its monomer N-acetyl glucosamine (GlcNac). Present study emphasizes on the impact of critical process variables on the production of chitinase from *Streptomyces pratensis* strain KLSL55. Initially the isolate was noticed to produce 84.67 IU chitinase in basal production medium. At optimization of bioprocess variables, the physical parameters pH of 8.00, 40 °C of incubation temperature, agitation speed of 160 rpm and 1.25 mL of spore suspension were found optimum for improved production of chitinase. Further, formulated production medium with 1.5% colloidal chitin, 1.25% fructose greatly influenced the chitinase production. At all described optimum conditions with formulated production media, a total of 14.30-fold increment was achieved in the chitinase production with final activity of 1210.67 IU when compared to the initial fermentation conditions in basal production medium.

1. Introduction

Chitin is β-1-4 linked homopolymer of N-acetyl glucosamine and is the second most abundant biodegradable polymer next to cellulose. Chitin is found as a part of fungi, crustaceans, insects and arthropods [1]. In nature chitin accumulates as a waste product from seafood production and processing industries. The accumulation of these wastes results in environmental hazard to various seafood processing countries. Though traditional methods involve in the conversion of chitinous wastes to useful chitin and chitin oligomers using strong acids and bases. As chitin is crystalline in nature, hydrolysis of chitin is an economically expensive and ecologically harmful process thus necessitating the search for an alternative eco-friendly approach to this problem. The vast diversity and useful applications of micro organisms need to be understood as they serve as an enormous source of a wide range of enzymes. Chitinases are a group of enzymes that catalyze the hydrolysis of chitin into low-molecular weight products. Chitinases are reported to be found in the organisms such as bacteria, fungi, insects, higher plants and some vertebrates [2–8].

Microorganisms utilize chitin as an energy source and thereby recycle the carbon and nitrogen back into the ecosystem [9]. Chitinases are gaining the importance over the years as they serve as an eco-friendly and cost effective alternative in the fields such as agriculture, Medicine, biotechnology, industrial applications and waste water management [10–12].

Chitinase producing microbes are common in nature and they are important degraders of chitin. *Streptomyces* are said to produce a huge number of extracellular enzymes as a part of their saprophytic mode of life. The ability of *Streptomycetes* to produce enzymes as products of their primary metabolism could lead to the production of many proteins of industrial importance [13].

Streptomycetes are said to be the major chitinovorous microbial groups in soil due to their ability to degrade chitin. This has been long regarded as characteristic feature of a soil *Streptomycetes* [14]. The organisms which produce chitinase can be used directly in biological control of fungi, or indirectly by the use of purified protein or through the gene manipulation [15]. In production of biomolecules of microbial origin, optimization process variables and formulation of production media is very important in order to maximize the yield and productivity and thereby minimize the production cost [16]. Hence, the present piece research was aimed to optimize both physical and nutritional parameters for the enhanced production of chitinase by *Streptomyces pratensis* KLSL55 under submerged fermentation (SmF).

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2. Materials and methods

2.1. Chemicals and reagents

The chemicals and reagents used in this experimental design were procured from HiMedia Ltd Mumbai India.

2.2. Chitinolytic actinomycete

The actinomycete strain KLSL55 was isolated form soil sample of Kalaburagi region, Karnataka India. The strain was found to be the potential for the chitinase production [17]. Hence, the selected isolate was further subjected for characterization and identification.

2.3. Characterization and identification of actinobacterial strain KLSL55

The chitinolytic actinomycete strain KLSL55 was characterized with colony characters, pigmentation pattern, microscopic features, biochemical and physiological properties [18]. Further, the actinomycete was subjected for 16 S rRNA sequencing. The genomic DNA was isolated as per the standard kit method Biopure™ kits (Bioaxis DNA Research Centre) and it was amplified by using Universal Primers 27F and 1401R [19]. The polymerase chain reaction (PCR) was carried out under the following conditions: Initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 60 s, for annealing 53 °C for 45 s, 68 °C for 90 s and the final extension at 68 °C for 10 min. Both the forward and reverse sequencing reactions of PCR amplicon were carried out on ABI 3730XL sequencer to obtain the sequence. Thus obtained nucleotide sequences were compared with sequences deposited in available databases of NCBI GenBank using the software BLAST algorithm. Further, sequences were aligned and Phylogenetic tree was generated using Geneious software. The sequence obtained were submitted gene bank and accession number for the same were obtained.

2.4. Inoculum preparation

Spore suspension of *Streptomyces pratensis* strain KLSL55 was prepared from 5 days old culture grown on starch casein agar slant by adding 10 mL of sterile distilled water containing 0.1% of Teen 80 and suspended the spore with sterile loop [20]. This spore suspension (with 1×10^8 spores mL^{-1}) as used as preinoculum for fermentation studies.

2.5. Production media

100 mL of media containing (g/L): colloidal chitin, 10; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 1.36; $MgSO_4 \cdot 6H_2O$, 0.3; with pH 7.2 [17] was used as basal production media for chitinase production by *Streptomyces pratensis* strain KLSL55.

For the production of chitinase above mentioned 100 mL media was inoculated with 1 mL of preinoculum and kept for fermentation at 40 °C in shaking condition at 160 rpm. Further, at every 24 h chitinase titer was analyzed.

2.6. Enzyme extraction and chitinase assay

For enzyme chitinase extraction, 5 mL of fermenting media was aseptically withdrawn and subjected for centrifugation at 10,000 rpm for 10 min at 4 °C. Thus obtained supernatant was collected and used as crude enzyme for chitinase assay. The chitinase activity assay was performed as per the method Miller 1959 [21] wherein, 1 mL of crude enzyme was allowed to react with 1 mL of 0.5% colloidal chitin in 0.1 M citric acid buffer containing (g/L): citric acid, 19.21; sodium citrate, 29.41; of pH 7.0 [22] followed by incubating the reaction mixture at 40 °C in shaking water bath for 30 min. After incubation the reaction was terminated by the addition of 2 mL DNS reagent containing (g/L): Sodium hydroxide, 10; Sodium potassium tartarate, 192; Dinitrosalicylic acid, 10; Phenol, 2 and Sodium sulphite, 0.5; [21] followed by heating for 5 min in boiling water bath. The coloured solution after cooling was allowed to centrifuge at 10,000 rpm for 5 min at room temperature and the optical density of the supernatant was measured at 540 nm against the control (blank). Supernatant was used for the analysis of reducing sugar using the dinitrosalicylic acid (DNS) method [21]. One unit of chitinase was defined as the amount of enzyme that liberates 1 μmol of reducing sugar min^{-1} mL^{-1}.

2.7. Optimization of cultural conditions for chitinase production

The Erlenmeyer flask containing 100 mL production medium was inoculated with 1 mL spore suspension (1×10^8 spores mL^{-1}) and incubated in shaker incubator at 160 rpm for 120 h. Then chitinase activity was assayed daily by the DNS method as described above. During this study, once optimized particular parameter was kept constant for the optimization of subsequent parameter.

2.8. Optimization of physical parameters for chitinase production

The effect of various physical parameters on chitinase production was analyzed in 100 ml of basal production medium. For optimization of pH, the production medium was constituted with varying pH from 4.5 to 9 with 0.5 unit of interval and used for chitinase production. The optimum temperature was determined by incubating the production media at varying temperatures from 25° to 50° C with variation of 5 °C. Optimum agitation speed was determined by incubating the production media containing Erlenmeyer flasks at different agitation speed in shaker incubator ranging from 100 to 200 rpm with interval unit of 20 rpm. For assessing optimum fermentation period, production setup was subjected for enzyme titer at every 24 h. The influence of inoculum size was assessed by inoculating the flasks containing production with varying volume of inoculum (spore suspension, 1×10^8 spores mL^{-1}) ranging from 0.25 to 2.50 ml with increment of 0.25 ml. In all the above mentioned optimization procedures the enzyme assay was carried out at every 24 h.

2.9. Optimization of additional nutrients and chelators for chitinase production

2.9.1. Effect of substrate concentration on chitinase production

Effect Influence of additional nutrients on the production of chitinase by *Streptomyces pratensis* strain KLSL55 was assessed. Herein, effect of substrate concentration, different carbon sources, inorganic nitrogen source and metal ions (as mentioned in Table 1) were analyzed.

2.9.2. Effect of metal ions on chitinase production

Metal ions such as Cu^{++}, CO^{++}, Ca^{++}, Mg^{++}, Mn^{++}, Zn^{++} and Fe^{++} were used at a concentration of 0.05% to analyze their influence on chitinase production. Fermentation media with different metal ions were inoculated and kept for incubation. After incubation the chitinase activity was determined at every 24 h.

Table 1
Various nutrients, metal ions and their concentrations analyzed for chitinase production by *Streptomyces pratensis* strain KLSL55.

Sl. No.	Nutrient source		Range of concentration analyzed (%)	Unit interval (%)
1.	Colloidal Chitin		0.25–2.50	0.25
2.	Carbon Sources	Glucose	0.25–2.50	0.25
		Fructose	0.25–2.50	0.25
		Maltose	0.25–2.50	0.25
		Lactose	0.25–2.50	0.25
		Arabinose	0.25–2.50	0.25
		Mannitol	0.25–2.50	0.25
		Sucrose	0.25–2.50	0.25
		Starch	0.25–2.50	0.25
3.	Nitrogen Sources	KNO_3	0.1–0.5	0.1
		$(NH_4)_2SO_4$	0.1–0.5	0.1
		NH_4Cl	0.1–0.5	0.1
4.	Metal Ions	Cu^{++}	0.05	Fixed
		CO^{++}	0.05	Fixed
		Ca^{++}	0.05	Fixed
		Mg^{++}	0.05	Fixed
		Mn^{++}	0.05	Fixed
		Zn^{++}	0.05	Fixed
		Fe^{++}	0.05	Fixed

Potassium nitrate -KNO_3, Ammonium sulphate $(NH_4)_2SO_4$ Ammonium chloride NH_4Cl.

3. Results and discussion

3.1. Characterization and identification of actinobacterial strain KLSL55

The cultural, microscopic and physiological properties of the isolate KLSL-55 were summarized in Table 2. All the properties mentioned in the table confirm that, the isolate is an actinomycete especially is of genus *Streptomyces*. The morphological, biochemical and physiological properties of the members of genus *Streptomyces* have unique and crucial characters in their identification [18]. The strain found to be gram positive with branching mycelium. The aerial mycelium of the organism was gray and substrate mycelium being white without any diffusible pigment. The maximum physiological growth of KLSL55 was observed at pH 8 and temperature 40 °C revealing it as alkalophilic *Streptomyces*.

The homology of the partial 16S rRNA gene sequence of the isolate KLSL55 obtained by BLAST algorithm has been depicted in Fig. 1. The Phylogenetic analysis of the gene sequence from Geneious software (BLAST) revealed that, the isolate KLSL55's linear DNA has maximum of 100% similarity with *Streptomyces pratensis* strain ch24-1 (Fig. 1). The 16S rRNA sequence of the *Streptomyces pratensis* strain KLSL55 submitted to GenBank has received accession number of KY780930.

3.2. Optimization of process parameters for chitinase production

3.2.1. Effect of initial pH on chitinase production

The effect of different initial pH of the fermentation media on the yield of chitinase are presented in Fig. 2. The maximum chitinase production was found at pH 8.0 with the activity of 96.70 IU, there was the constant increase was noticed in the pH range of 6.5–8.0 and thereafter enzyme production was shrunken. The lowest chitinase activity (13.70 IU) was observed at pH 9.0 after 48 h of time course. The results obtained in this particular are quite comparable with the reports on the production of chitinase by *Bacillus laterosporus* [23], *Micrococcus* sp. AG84 [24], *Alcaligenes xylosoxydans* [25], *Serratia marcescens* XJ-01 [26], *Aeromonas* sp. JK1 [27] and *Bacillus pabuli* [28] wherein the maximum yield of chitinase was noted at alkaline conditions.

3.2.2. Effect of incubation temperature on chitinase production

Variation in the fermentation temperature brought reliable changes in the production of chitinase by *Streptomyces pratensis* strain KLSL55 and the results were presented in Fig. 3. In the temperature range of 25–40 °C, there was gradual increment in production of chitinase and the maximum productivity of 99.65 IU was found at 40 °C, higher than 40 °C declined the chitinase yield. Most of the microbes exhibited highest productivity of chitinase at

Table 2
Morphological, physiological and Biochemical characteristics of potential chitinolytic strain KLSL55.

Sl. No.	Test particulars	Observation	Sl. No.	Test particulars	Observation
1.	Gram's staining	Gram positive	15.	Cellulose hydrolysis	Positive
2.	Motility	Non-motile	16.	Casein hydrolysis	Positive
3.	Endospore staining	Spore former	17.	Starch	Positive
4.	Aerial mycelium	Gray	18.	Urea	Negative
5.	Substrate mycelium	White	19.	Sucrose	Positive
6.	Spore chain	Spiral	20.	Xylose	Positive
7.	Diffusible pigment	Negative	21.	Mannitol	Positive
8.	Growth at pH	6.5–8.5	22.	Galactose	Positive
9.	Growth at temperature (°C)	30–45 °C	23.	Fructose	Positive
10.	Oxidase	Positive	24.	Glucose	Positive
11.	Catalase	Positive	25.	Maltose	Positive
12.	Nitrate reduction	Positive	26.	Arabinose	Positive
13.	Growth in lysozyme	Positive	27.	Lactose	Positive
14.	Gelatin hydrolysis	Positive	28.	Raffinose	Positive

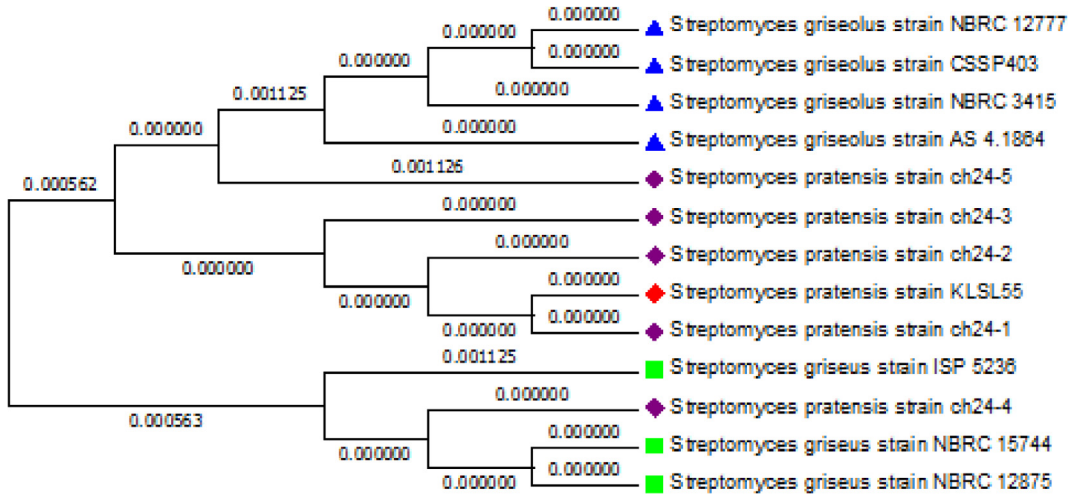

Fig. 1. Phylogenetic tree of *Streptomyces pratensis* strain KLSL55 showing similarity with *Streptomyces pratensis* strain ch24-1.

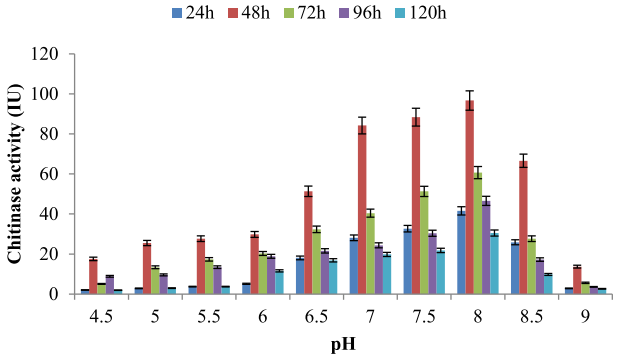

Fig. 2. Effect of initial pH of the fermentation media on chitinase production by *S. pratensis* strain KLSL55.

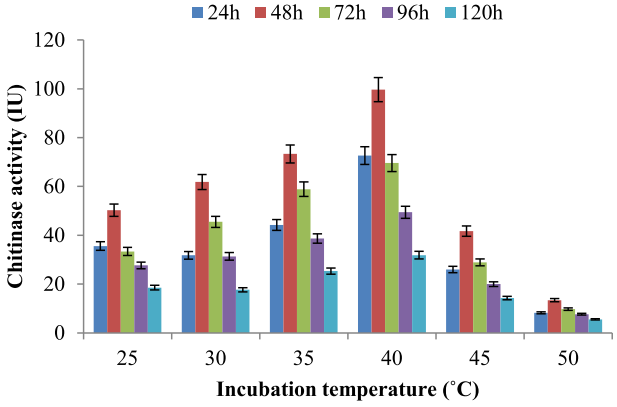

Fig. 3. Effect of incubation temperature on chitinase production by *S. pratensis* strain KLSL55.

40 °C. In this pipeline, Microbispora sp. V2 showed maximum chitinase production at 40 °C temperature [29]. Taechowisan et al. [30] reported that the synthesis of chitinase by *S.aureofaciens* is highest at temperature in between 30–40 °C.

3.2.3. Effect of agitation speed on chitinase production

The impact of agitation speed on the production of chitinase by *Streptomyces pratensis* strain KLSL55 under submerged fermentation was shown in Fig. 4. The maximum chitinase production

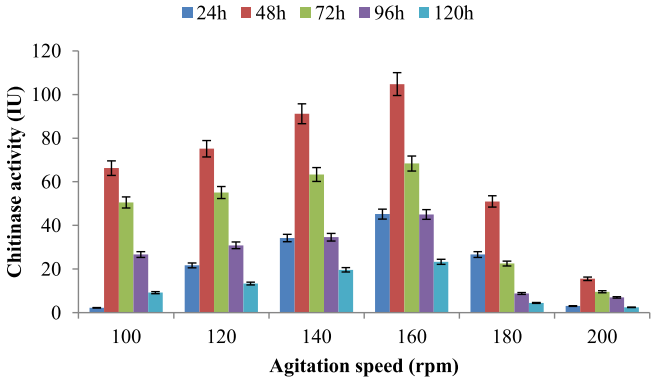

Fig. 4. Effect of agitation speed on chitinase production by *S. pratensis* strain KLSL55.

was found at an agitation speed of 160 rpm with 104.75 IU of chitinase yield after 48 h. The chitinase activity was increased as there is increase in the agitation speed from 100–160 rpm thereafter the chitinase yield was found to be decreased. Chitinase productivity by microbes is good at shaking condition than the static condition; especially the higher rpm rates will results in good titer of enzyme [31].

3.2.4. Effect of fermentation period on chitinase production

Results recorded in Fig. 5.shows effect of fermentation period on chitinase production by *Streptomyces pratensis* strain KLSL55. The chitinase production was initially found after 24 h of

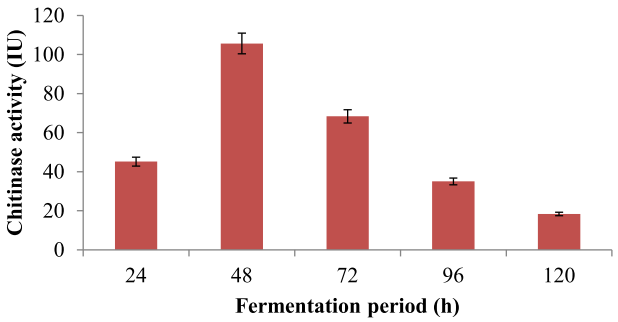

Fig. 5. Effect of fermentation period on chitinase production by *S. pratensis* strain KLSL55.

incubation and reached maximum levels at 48 h of fermentation. It could be seen that maximum enzyme production with activity of 105.65 IU was occurred at 48 h. The extended fermentation period decreased the enzyme productivity. This could be either due to the depletion nutrients or substrate in the production media. The optimum fermentation period for chitinase production do vary with the organism, its growth pattern and its nature. The optimum fermentation period for chitinase production by *Microbispora* sp. V2 was reported to be 48 h [29] whereas 120 h was found optimum in case of *Streptomyces* sp. NK1057 [32]. In another study, 72 h was found optimum for chitinase production by *Streptomyces halstedii*. Hence our report shows that the organism is a fast grower and it produces maximum amount of chitinase in short period of time.

3.2.5. Effect of inoculum size on chitinase production

It is evident from Fig. 6 that, inoculum size influenced the production of chitinase by *Streptomyces pratensis* strain KLSL55. There was a gradual increase in enzyme production as there is increase in inoculum size; the maximum enzyme production with 141.20 IU noticed when 1.25 ml of inoculum (1×10^8 spores mL^{-1}) was used, there after no significant increase in enzyme yield was noticed. Lower inoculum size results in lesser number of microbial cells in production medium and this require longer time grow in optimum number to utilize the substrate and form desired product. Generally increased inoculum size improves the growth and growth related activities of microbes up to certain level and with further increased inoculum size there could be reduction in microbial activity due to nutritional limitation.

3.2.6. Effect of colloidal chitin concentration on chitinase production

The chitinase productivity by *S. pratensis* KLSL55 at different colloidal chitin concentration is represented in Fig. 7. Since chitin is the substrate for chitinase, the addition to production media stimulates enzyme production. The increment in the colloidal chitin concentration from 0.25–1.5% resulted in the enhanced chitinase production there after the increment in CC concentration has decreased the enzyme yield. However the maximum chitinase production was obtained at1.5% of CC concentration with the activity of 157.54 IU and the least activity (19.29 IU) was observed at 2.5% of CC concentration. It was in conformity with the finding of Gupta et al. [33] wherein chitinase production by *S. viridificans* was maximum at 1.5% colloidal chitin. According to Taechowisan et al. [30] reported that the production of chitinase by *S. aureofaciens* CMU Ac 130 was optimal with 1% of colloidal chitin concentration.

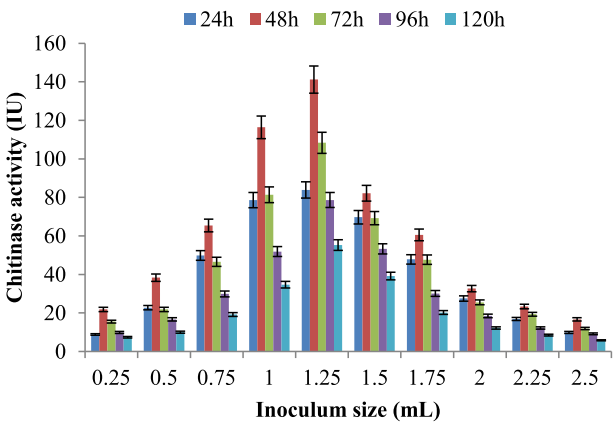

Fig. 6. Effect of inoculum size on chitinase production by *S. pratensis* strain KLSL55.

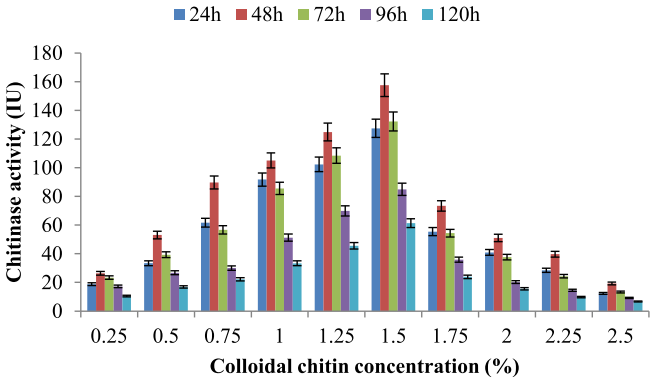

Fig. 7. Effect of colloidal chitin concentration on chitinase production by *S. pratensis* strain KLSL55.

3.2.7. Effect of carbon sources on chitinase production

Results on effect of supplementation of production medium with different carbon sources on enzyme production are shown in Fig. 8. Interestingly the carbon sources glucose, fructose, maltose and lactose have greatly enhanced the chitinase yield with enzyme activity of 768.33 IU, 904 IU, 690 IU and 762 IU respectively. The highest enzyme production was noticed when production media was supplemented with 1.25% of fructose, whereas, arabinose, mannitol and sucrose found to have negative effect on chitinase productivity at all concentration. Thus fructose at 1.25% concentration pretended as key ingredient in the enhanced production of chitinase with an enzyme activity of 904 IU. In similar type of studies, Saima et al. [34] reported that, fructose is the best carbon source after starch for enhanced production of chitinase by *A. hydrophila* HS4 and *A. punctata* HS6. Similar results were also reported with *Streptomyces aureofaciens* CMUAc130 [30], *S. marcescens* [35] and *Streptomyces* sp. ANU [36]. In contrast to these reports, the results of this investigation reveal that, fructose is one of the key carbon sources which may fetch considerable enhancement in enzyme titer in microbiological production of chitinase.

3.2.8. Effect of inorganic nitrogen sources on chitinase production

The results on the influence of additional inorganic nitrogen sources on chitinase production are presented in Fig. 9. Among the tested inorganic nitrogen sources only potassium nitrate found to increase the chitinase yield with an enzyme activity of 1016 IU at 0.5% concentration. Ammonium sulphate and ammonium chloride does not influence the enzyme productivity rather they found to decrease the chitinase yield. Similar results of declining of

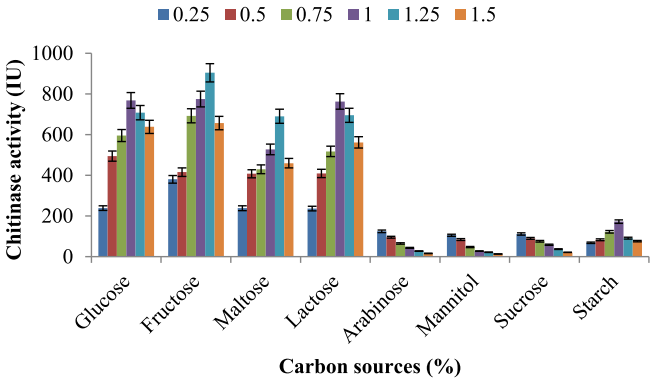

Fig. 8. Effect of different carbon sources on chitinase production by *S. pratensis* strain KLSL55.

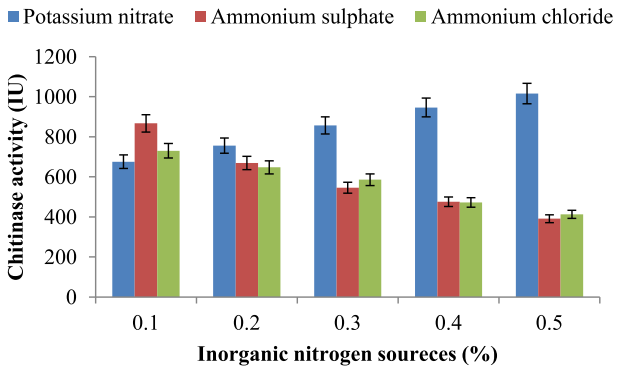

Fig. 9. Effect of different inorganic nitrogen sources on chitinase production by *S. pratensis* strain KLSL55.

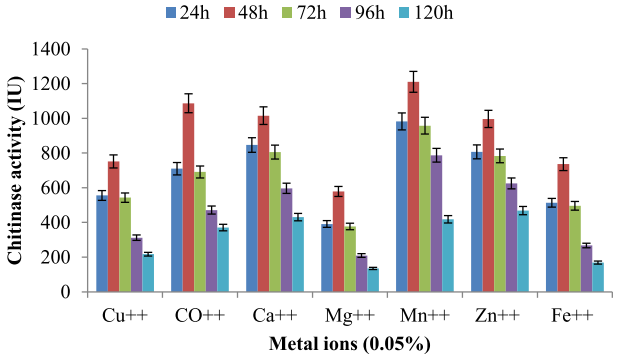

Fig. 10. Effect of different metal ions on chitinase production by *S. pratensis* strain KLSL55.

enzyme productivity by the supplementation of chitinase were reported by Farag and Al-Nusarie [37].

3.2.9. Effect of metal ions on chitinase production

Influences of different metal ions on chitinase production are represented in Fig. 10. The metals ions such as Mn^{++} and CO^{++} found to increase the chitinase yield with an enzyme activity of 1210.67 IU and 1086.67 IU at 0.05% concentration. Ca^{++} neither increased nor decreased the chitinase activity whereas; Cu^{++}, Zn^{++}, Mg^{++} and Fe^{++} were found to shrunken the chitinase yield. The present results are also supported by the previous reports where the chitinase production was enhanced wherein, Mn^{++} and CO^{++} served as best metal ions for the increased chitinase production [27,38] and [39].

4. Conclusion

Microbes are the most important sources to obtain any enzyme, because the produced enzymes are stable and production will be consistent than similar ones obtained from other sources. Microorganisms under restricted fermentation conditions can produce chitinase in considerable titer. SmF with formulated production medium and optimum conditions is promising tool in microbial technology for the production of microbial enzymes through inexpensive means. Based on the present study it is concluded that, *Streptomyces pratensis* strain KLSL55 offers scope for the production of chitinase under SmF. This report is a very rare among the few reports reported for chitinase production under SmF by actinomycetes. After optimization of process parameters and formulation of production medium, there was 14.30-fold increase in chitinase yield. Since information on the production of chitinase by actinomycetes under SmF is inadequate, results obtained here demand further in-depth study to exploit the strains of *Streptomyces pratensis* for industrial production of chitinase at reduced cost. Research output in that pipe line will make chitinase more attractive and feasible for agriculture and pharmaceutical industry. Therefore it is necessary to achieve complete understanding about the regulation, catalytic capacity, specificity and other aspects of optimization for its production at industrial scale.

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