



## Optimization of cellulase and chitinase enzymes production by plant growth promoting rhizobacteria

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### Abstract

The current study aimed to optimize the production of cellulase and chitinase enzymes by plant growth promoting rhizobacteria (PGPR). Bacteria were pre-isolated from soil and identified as; *Pseudomonas fluorescens* NBRC (KW1), *Serratia liquefaciens* ATCC 27592 (EW1), *Bacillus subtilis* SBMP4 (EF1) and *Bacillus megaterium* NBRC 15308 (TF2). The effect of different growth parameters including; pH, temperature, carbon and nitrogen sources, and incubation periods were optimized for production of cellulase and chitinase enzymes by the selected bacterial strains. Three strains mainly, *B. subtilis* SBMP4, *S. liquefaciens* ATCC 27592 and *P. fluorescens* NBRC) recorded positive results for cellulase production. *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1) strains demonstrated significant ability to produce cellulase at 40°C, while *P. fluorescens* NBRC (KW1) strain showed the maximum enzyme production at 30°C. Carboxymethyl cellulose gave the highest cellulase production compared to the other carbon sources. Chitinase enzyme was optimally produced by *B. subtilis* SBMP4 and *S. liquefaciens* ATCC 27592 strains under primary screening. *B. subtilis* SBMP4 had the strongest ability to produce chitinase at 40°C, while *S. liquefaciens* ATCC 27592 at 30°C. The optimum pH observed was at pH 6.0 to get the maximum chitinase production by *S. liquefaciens* ATCC 27592. Potassium nitrate (KNO<sub>3</sub>) as an inorganic nitrogen source presented the highest chitinase production by *B. subtilis* SBMP4, whereas yeast extract demonstrated significant chitinase production by *S. liquefaciens* ATCC 27592.

**Keywords:** *Pseudomonas* sp., *Serratia* sp., *Bacillus* sp., Chitinase, Cellulase

### 1. Introduction

Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental-friendly industrial sectors. Many microorganisms produce a wide variety of hydrolytic enzymes against different substrate, such as cellulose

and chitin. Cellulose makes up the cell walls of most plants and it is the most abundant biological polymer on the earth. Zhang and Lynd, (2004); Bai *et al.*, (2012) reported that it is the primary product of terrestrial ecosystem photosynthesis. Jonas and Farah,

(1998) documented that cellulose is a strong fibrous crystalline polysaccharide that resists hydrolysis, and is water insoluble.

According to Venkata *et al.*, (2013); Vijayaraghavan *et al.*, (2016), cellulose consist of thousands of D-glucose units that are linked together with  $\beta$ -1, 4-glycosidic linkage. It is generally degraded by Cellulase, which can hydrolyze the  $\beta$  1,4 glycosidic linkage to release glucose (Perez *et al.*, 2002; Abou-Taleb *et al.*, 2009 ).

Previous studies of Sang-Mok and Koo, (2001); Kirk *et al.*, (2002) pointed that cellulases are inducible enzymes produced by several microorganisms, mainly by bacteria and fungi. Moreover, Immanuel *et al.*, (2006); Kasana *et al.*, (2008) added that cellulases have various potential industrial and biotechnological applications, and hence are in high demand. Several studies over many years were interested in the isolation and characterization of cellulose degrading bacteria more than fungi, because bacteria have elevated growth rate and more resistance to an extreme environment, as compared with fungi (Lynd *et al.*, 2002; Odeniyi *et al.*, 2009; Rasul *et al.*, 2015). Bacteria belonging to several genera including; *Cellulomonas*, *Cellulovibrio*, *Pseudomonas*, *Bacillus*, *Bacteroides*, *Acetovibrio*, *Streptomyces* and *Micrococcus*, produce different types of cellulase (Immanuel *et al.*, 2006; Kurniawan *et al.*, 2018).

Patil *et al.*, (2000) demonstrated that chitin is one of the most common biodegradable polymer after cellulose, which exists naturally in the biosphere as a structural polysaccharide of  $\beta$ -1,4-N-acetyl-D-glucosamin. Zarei *et al.*, (2012) added that this component is present in the fungal cell walls and in the exoskeletons of arthropods. Previous study of Chang *et al.*, (2007) reported that chitin and its derivatives have versatile biological activities and agrochemical applications, thus they have high economic values.

Chitinases are receiving an increased attention due to their broad range of applications. Sahai and Monacha, (1993) demonstrated that chitinases are

enzymes that play an important role in biological hydrolysis of chitin. Hartl *et al.*, (2012); Garcia-Longori *et al.*, (2014); Cheba *et al.*, (2016) reported that these enzymes are produced by several microorganisms including bacteria (i.e. *Bacillus*, *Pseudomonas*, *Aeromonas*, *Vibrio*, *Serratia*, *Enterobacter*), yeasts, actinomycetes, fungi, plants and animals. Many of these chitinase producing microorganisms are recently used as important biocontrol agents against fungal phytopathogens, by degrading the chitin component of the fungal cell wall (Nguyen *et al.*, 2015), and also control insect pests (Rathore and Gupta, 2015). In addition, they act as plant growth-promoting, as they promote nutrients absorption and self-defense of the plants (Sahai and Monacha, 1993). Many chitinolytic bacteria including; *S. marcescens* and *Enterobacter agglomerans* have the potential for the biocontrol of many phytopathogenic fungi (Downing and Thomson, 2000). Moreover, Shanmugaiah *et al.*, (2008) added that various species of *Bacillus* have the ability to produce chitinase such as; *B. licheniformis*, *B. circulans*, *B. laterosporus*, *B. amyloliquefaciens*, *B. pabuli*, *B. magaterium*, *B. stearothermophilus*, *B. subtilis* and *B. thuringiensis*.

The objective of the current study was to optimize the growth conditions of several bacterial strains (i.e. *P. fluorescens* NBRC, *S. liquefaciens* ATCC 27592 and *B. subtilis* SBMP4), for production of high levels of the cellulase and chitinase enzymes.

## 2. Material and methods

### 2.1. Bacterial strains

Four strains of plant growth promoting rhizobacteria (PGPR) were pre-isolated from soil and identified by Ghanem, (2017) as; *P. fluorescens* NBRC (KW1), *S. liquefaciens* ATCC 27592 (EW1), *B. subtilis* SBMP4 (EF1) and *B. megaterium* NBRC 15308 (TF2).

### 2.2. Screening for cellulase producing bacteria

Determining of the ability to produce cellulase enzymes were carried out by growing the 4 bacterial

strains individually on basal media with following composition: 0.01% MgSO<sub>4</sub>, 0.1% yeast extract, 0.2% KH<sub>2</sub>PO<sub>4</sub>, 0.7% K<sub>2</sub>HPO<sub>4</sub>, 0.05% Sodium citrate, supplemented with 1% carboxymethyl cellulose (CMC) as carbon source, and then incubating the plates at 30°C for 48h. After incubation, the plates were flooded by 1 % Congo red and then replaced with 1 mol/ l of NaCl. Positive results were detected by the formation of clear halo zones around the bacterial colonies, according to Zhou and Li, (2004).

### 2.3. Detection of cellulase activity

The strains that showed clear zones were cultured in basal broth medium supplemented with CMC, at 37 °C for 24h. After incubation, the cultures were centrifuged for 15 min. at 8000 rpm, and then the supernatant was used as a source of the crude enzyme solution for measurement of the enzyme activity. Cellulase activity was measured through a spectrophotometer (SE6300 UV-VIS) at 540 nm, using the DNSA (3,5-dinitrosalicylic acid) method according to Lynd *et al.*, (2002). Cellulase activity was estimated through detection of the amount of free reducing sugars produced by hydrolysis of the CMC. One unit of enzyme activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µmol of glucose within 1 min. of the reaction (Shanmugapriya *et al.*, 2012).

### 2.4. Optimization of different culture parameters for cellulase production

The selected bacterial strains were grown in CMC broth at various culture conditions including; temperature, pH, substrate concentration, incubation time and carbon sources, to determine the effects of all these factors on cellulase production. The enzyme activity was measured at different pH levels ranging from 5-11, at various growth temperatures ranging from 20°C-45°C, and different incubation periods varying from 1-5 d at 30°C. The effect of different carbon sources on cellulase production was studied by replacing carboxymethyl cellulose in CMC broth

medium with several carbon sources individually such as; xylose, starch, lactose and cellobiose.

### 2.5. Chitinase production

For the primary screening of chitinase production, the bacterial strains were grown on Colloidal chitin agar prepared in reference to Wiwat *et al.*, (1999), and then plates were incubated at 30°C for 7 d. The formations of clear halo zones around growth of the strains indicate that they can hydrolyze chitin.

### 2.6. Measurement of chitinase enzyme activity

Strains which showed the ability to hydrolyze chitin were selected, and then chitinase activity was measured according to the method proposed by Chakraborty *et al.*, (2012). Through this method, the positive strains were inoculated and incubated for 5 d in colloidal chitin broth medium. After incubation, the cultures were centrifuged at 12,000 g for 20 min. to obtain culture supernatants. About 1 ml of each culture supernatants was added individually to 1 ml of 1% (w/v) colloidal chitin prepared in a phosphate buffer pH 6, and then incubated at 50°C for 30 min. After incubation, the enzyme activity was terminated by placing the mixtures in boiling water bath for 3 min. The solutions were centrifuged at 2,600 g for 10 min., and then the amount of reducing sugars produced in the supernatant was determined by Dinitrosalicylic acid (DNSA) according to the method of Tsujibo *et al.*, (1991); using N-acetyl glucosamine as a reference compound (Miller, 1959). Finally, the absorbance was measured at 585 nm using a spectrophotometer. One unit of enzyme was defined as the amount of enzyme that released 1 µmol of N-acetyl-D-glucosamine per minute.

### 2.7. Optimization of different culture parameters for chitinase production

The selected bacterial strains were grown in colloidal chitin broth at different culture conditions such as; temperature, pH, substrate concentration, incubation time and nitrogen sources, to determine the

optimum conditions for chitinase production. The enzyme activity was measured at different pH levels ranging from 5-11, and at various incubation temperatures ranging from 20°C- 45°C, and different incubation periods ranging from 1-7 d at 37°C. The effect of various nitrogen sources on chitinase production was studied by replacing the yeast extract in colloidal chitin broth medium with different nitrogen sources such as; ammonium, urea, KNO<sub>3</sub>, NH<sub>4</sub>Cl and peptone.

## 2.8. Statistical analysis

Collected data were statistically analyzed using the appropriate analysis of variance (ANOVA), according to Steel and Torrie, (1981). The experiments were carried out in completely randomized design, using three replicates. Computer program software CoStat version 6.311 was used to analyze the data of the experiments. Least significant difference (LSD) at 5% level was used separately to evaluate the response of each character.

## 3. Results and Discussion

### 3.1. Screening of cellulase producing bacteria

Out of the four tested bacteria only 3 strains namely; *P. fluorescens* NBRC, *S. liquefaciens* ATCC 27592 and *B. subtilis* SBMP4, demonstrated positive results through producing clear halo zones on CMC agar plates after Congo-red staining.

### 3.2. Effect of different culture conditions on cellulase production

#### 3.2.1. Effect of culture temperature

Evaluating the cellulase enzyme activity at different incubation temperatures revealed that *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1) had the strongest ability to produce cellulase at 40°C (Fig.1). On the other hand, *P. fluorescens* NBRC (KW1) strain expressed maximum enzyme production at 30°C. These results were similar to previous studies of Jansová *et al.*, (1993); Sethi *et*

*al.*, (2013) that showed maximum cellulase activity of *B. subtilis* at 40°C.

#### 3.2.2. Effect of pH on cellulase production

The effect of different pH ranges (5.0-11.0) on the cellulase production was studied. Currently, the optimum pH was observed at neutral pH 7.0, giving the maximum cellulase production by the three tested strains; however, the ability of producing this enzyme was decreased significantly below and above pH 7 (Fig. 2), in agreement with the previous results of Sethi *et al.*, (2013).

#### 3.2.3. Effect of different carbon sources

Various carbon sources including; xylose, starch, lactose, and cellobiose were used instead of CMC in the CMC broth medium. Results showed that CMC was the optimum carbon source for highest cellulase production, followed by lactose and celliobiose (Fig. 3).

#### 3.2.4. Effect of incubation periods

Cellulase production by all the three tested strains reached its maximum after incubation for 4 d, below and after this period its production gradually declined (Fig. 4).

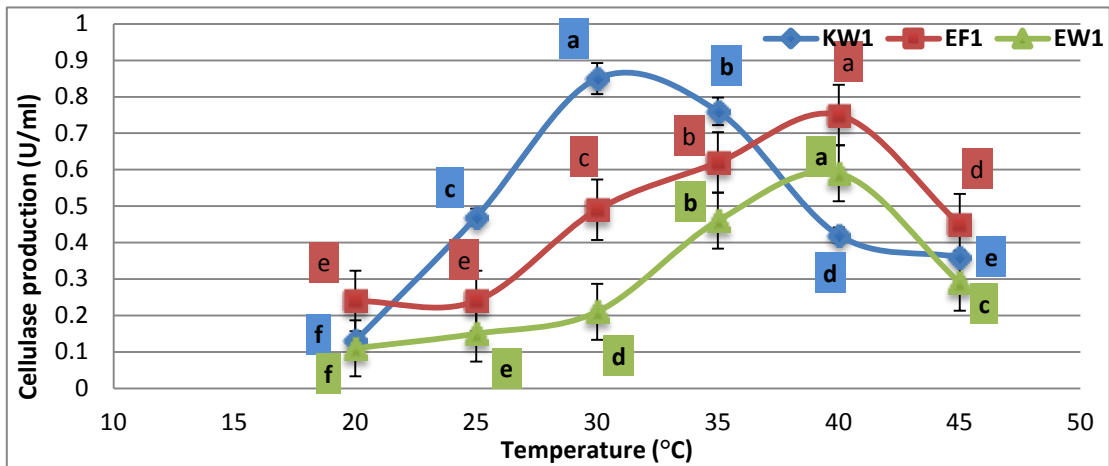
### 3.3. Screening for chitinase producing bacteria

Out of the 4 bacterial strains, only two bacterial strains namely; *B. subtilis* SBMP4 and *S. liquefaciens* ATCC 27592 are selected as chitin degraders, recognized through the formation of clear halo zones around the bacterial colony on the colloidal chitin agar medium.

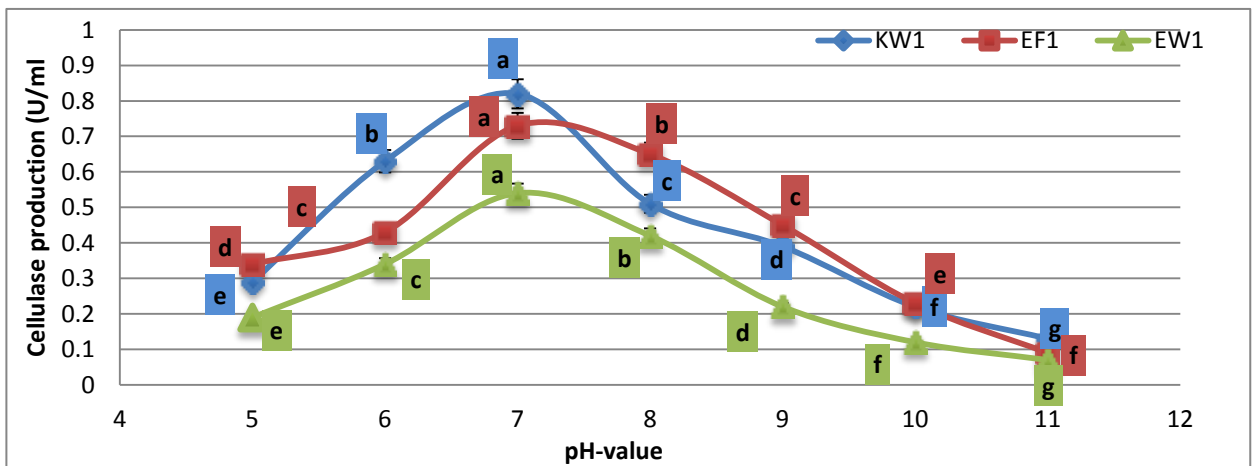
### 3.4. Effect of different culture conditions on chitinase production

#### 3.4.1. Effect of incubation temperature on chitinase production

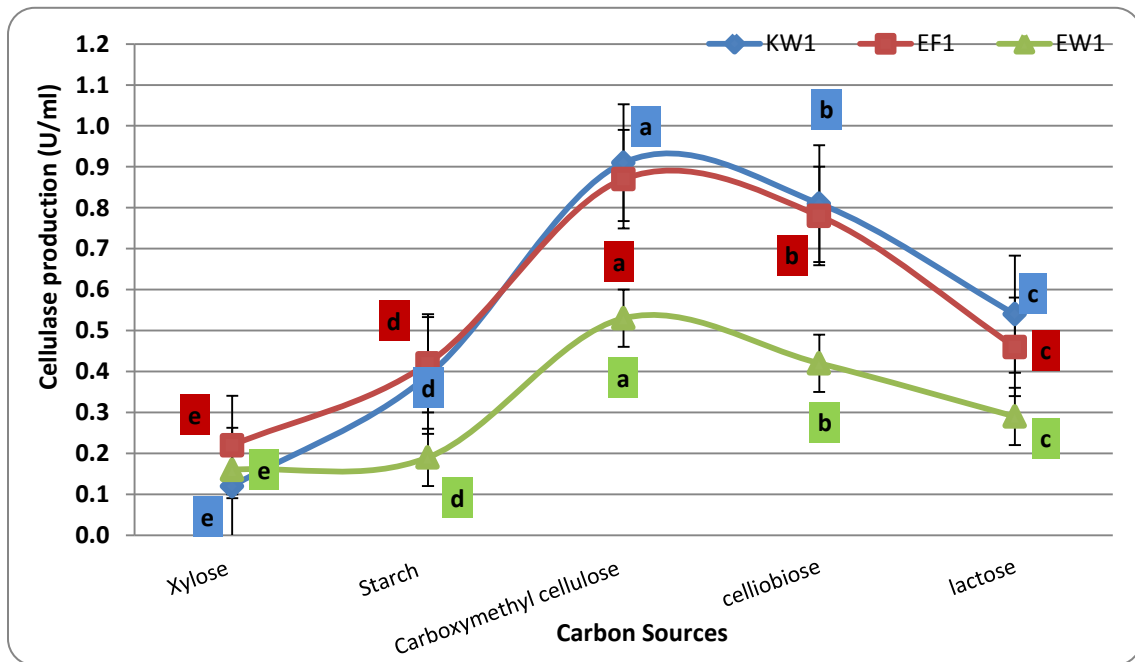
Results obtained presented that *B. subtilis* SBMP4 has the strongest ability to produce chitinase at 40°C



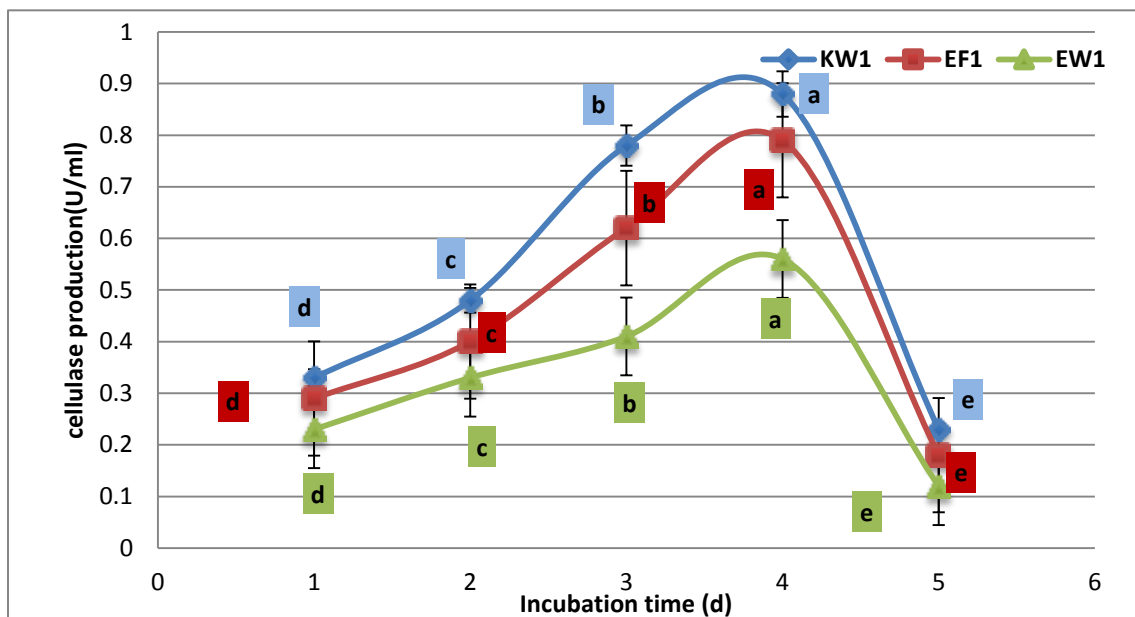
**Fig. 1:** Effect of different incubation temperatures on cellulase production by *P. fluorescens* NBRC (KW1), *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1). Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.



**Fig. 2:** Effect of the different pH-values on the cellulase production by *P. fluorescens* NBRC (KW1), *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1). Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.



**Fig. 3:** Effect of different carbon sources on cellulase production by *P. fluorescens* NBRC (KW1), *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1). Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.



**Fig. 4:** Effect of the incubation periods on the cellulase production by *P. fluorescens* NBRC (KW1), *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1). Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.

followed by 35°C, while *S. liquefaciens* ATCC 27592 optimum temperatures is at 30°C (Fig. 5). Similarly, previous study of Bhushan and Hoondal, (1998) recorded an optimum activity of chitinase enzyme by *Bacillus* sp. at 40-55°C. Current results are closed those of Frandberg and Schnurer, (1994) who reported that the optimum temperature for the activity of chitinase produced by *B. pabuli* was at 40°C. Furthermore, Shanmugaiah *et al.*, (2008) recently demonstrated that the best temperature for chitinase production by *B. laterosporus* was at 35°C. On the other hand, Lamine *et al.*, (2012) reported that the optimum temperature for chitinase production by *S. marcescens* was at 30°C.

### 3.4.2. Effect of different pH on chitinase production

The effect of different pH on the activities of chitinase was studied. The optimum pH observed is at 6.0 to get the maximum chitinase activity by *S. liquefaciens* ATCC 27592, while the ability of producing this enzyme decreased significantly below and above pH 6.0 (Fig. 6). In addition, the recorded results showed that the initial pH of 7.0 is optimum to get maximum chitinase production by *B. subtilis* SBMP4. However, previous study reported broad range of pH-values of 4.5- 7.5 for chitinase production by *B. cereus* (Pleban *et al.*, 1997). In another study of Frändberg and Schnürer, (1994), pH of 8.0 was recorded as optimal for chitinase production by *B. pabuli*.

### 3.4.3. Effect of various nitrogen sources on chitinase production

Among the various nitrogen sources tested, results showed that KNO<sub>3</sub> gave the highest chitinase production by *B. subtilis* SBMP4, while yeast extract expressed significant chitinase production by *S. liquefaciens* ATCC 27592 (Fig. 7). Lowest chitinase production by *B. subtilis* SBMP4 is recorded in presence of NH<sub>4</sub>Cl, and *S. liquefaciens* ATCC 27592 (Fig. 7).

### 3.4.4. Effect of different incubation periods on chitinase production

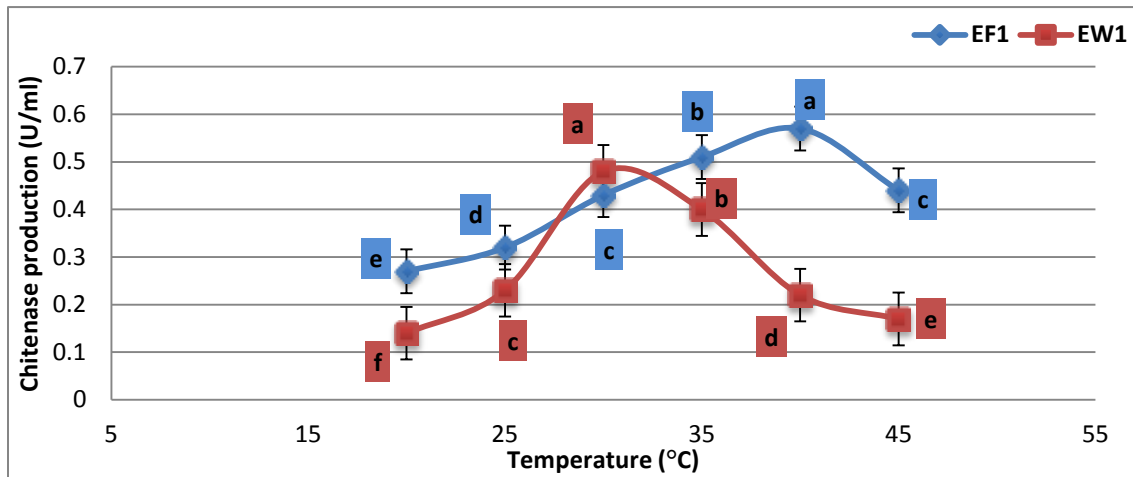
Chitinase production by *S. liquefaciens* ATCC 27592 reached its maximum level after the 4<sup>th</sup> day of incubation, after that its production decreased gradually upon incubation for longer periods. On the other hand, maximum level of enzyme production is recorded at the 5<sup>th</sup> day by *B. subtilis* SBMP4, and then decreased by further incubation (Fig. 8).

## Conclusion

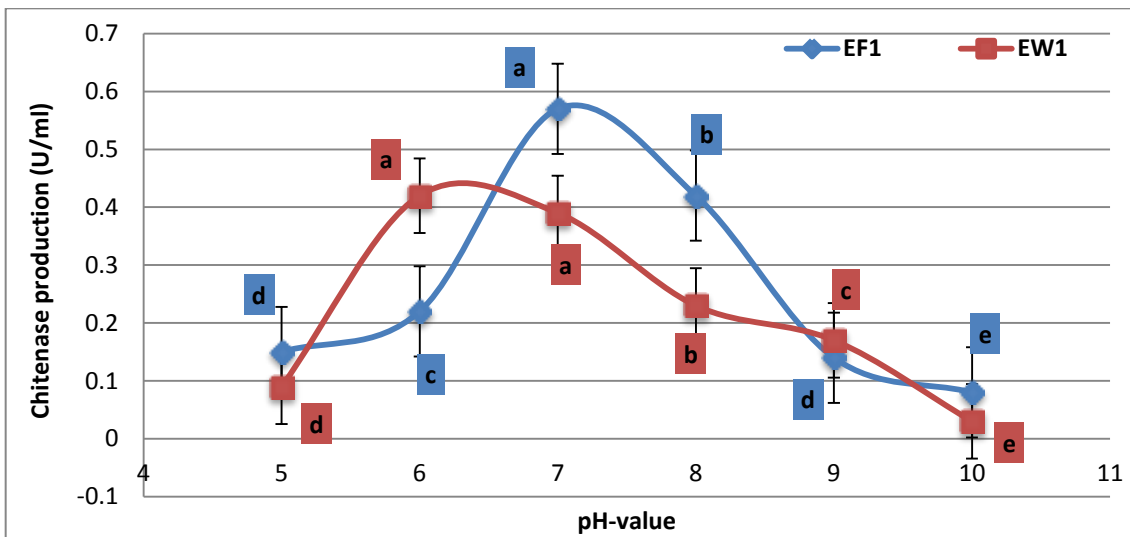
This study provides the optimum incubation temperatures for maximum cellulase production by *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1) at 40°C, while *P. fluorescens* NBRC (KW1) is at 30°C, in a basal media of pH 7 containing CMC recorded as the most suitable carbon source. On the other hand, the optimum temperature and pH value for maximum chitinase production by *S. liquefaciens* ATCC 27592 is at 30°C and pH 6, whereas the best nitrogen source recorded is yeast extract. For *B. subtilis* SBMP4, the optimum temperature and pH determined are at 40°C and pH 7, and the best recorded nitrogen source for producing the chitinase enzyme is KNO<sub>3</sub>.

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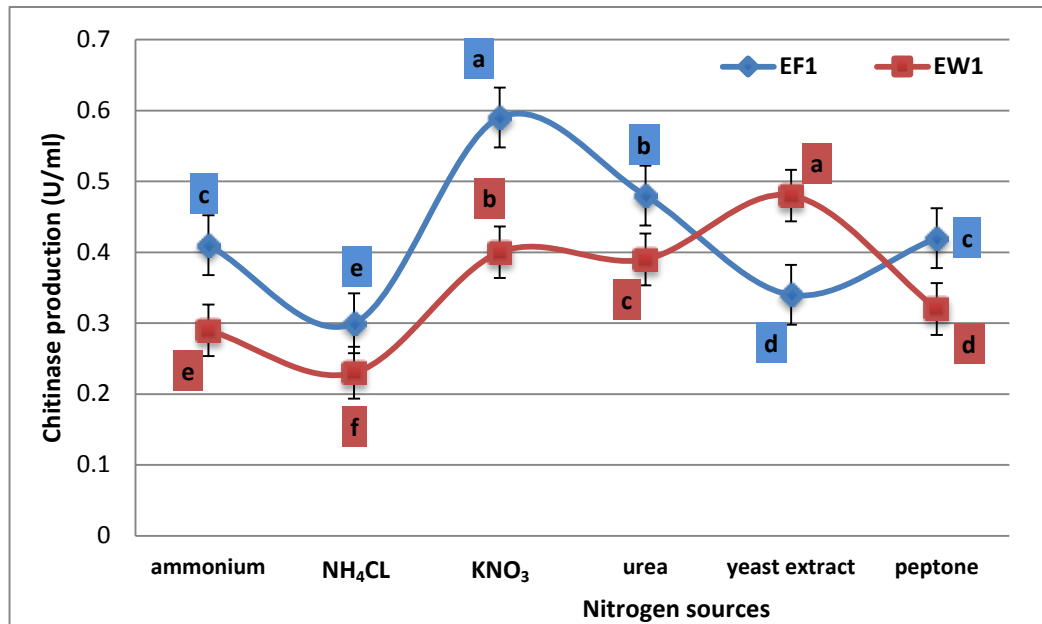


**Fig. 5:** Effect of different incubation temperatures on chitinase production by *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1), on the colloidal chitin medium. Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.

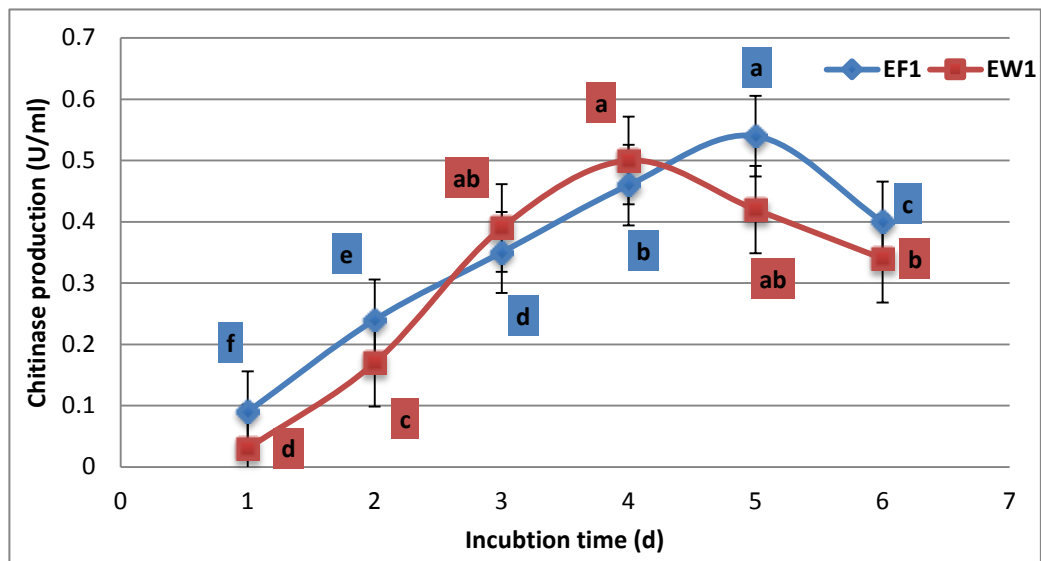


**Fig. 6:** Effect of different pH-values on chitinase production by *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1), on the colloidal chitin medium. Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.





**Fig. 7:** Effect of several nitrogen sources on chitinase production by *B. subtilis* SBMP4 (EF1) and *S. liquefaciens* ATCC 27592 (EW1), on the colloidal chitin medium. Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.



**Fig. 8:** Effect of different incubation periods on the chitinase production by *B. subtilis* SBMP4 (EF1), and *S. liquefaciens* ATCC 27592 (EW1), on the colloidal chitin medium. Error bars represent standard deviations. Values followed by the same letter do not represent significant difference.

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