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Production of endoglucanase by a novel strain *Streptomyces* sp. TKJ2 and its optimization under submerged fermentation

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ABSTRACT

A newly filamentous bacteria was recovered from Tikjda forest soil (Algeria) for its high endoglucanase activity. The nucleotide sequence of the 16S rRNA gene (1454 pb) of *Streptomyces* sp. TKJ2 showed a close similarity (99%) with other *Streptomyces* 16S rRNA genes. Endoglucanase production by *Streptomyces* sp. TKJ2 was optimized for varying culture conditions following one factor at time (OFAT) approach. An initial medium pH6 and incubation temperature 30°C were found to be optimal for endoglucanase production, after 7 days of incubation. Studies on nutritional factors revealed that the highest endoglucanase production was obtained in medium made up of 10 g l^{-1} carboxymethyl cellulose, 3 l^{-1} g yeast extract, 1 g l^{-1} NaCl, 2 g l^{-1} NH_4Cl, and 0.3 g l^{-1} $MgSO_4$. Among various carbon sources tested, the actinomycete secreted high level of endoglucanase on some waste such as wheat bran and sugar can.

KEYWORDS

Endoglucanase;
Optimization;
Phylogenetic tree;
Streptomyces sp. TKJ2.

INTRODUCTION

Cellulose is the major polysaccharide compound in plants and is the most abundant organic compound on earth. Degradation of the cellulosic materials can be achieved chemically, enzymatically, or by the combination of both chemical and enzymatic methods[1]. The enzymatic hydrolysis of cellulose by the cellulase enzyme is favoured rather than chemical means. Cellulases (endo-1,4-β-glucanase) are a group of hydrolytic enzymes which hydrolyze the glucosidic bonds of cellulose and related cello-oligosaccharide derivatives[2]. With the appearance of new frontiers in the field of biotechnology, the spectrum of cellulase has expanded into various industries, including food, textiles, laundry, pulp, paper, agriculture as well as in research and development[3].

The soil habitat presents an array of challenges to its microbial community. The actinomycetales are important members of this ecosystem and they have evolved complex morphological and physiological adaptations that enable them to thrive in this environment[4]. They are mycelial bacteria that resemble filamentous fungi in their apical growth, branching, and morphoge-

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netic development. These Gram-positive bacteria continue to be prolific source of new secondary metabolites with a range of biological activities that may ultimately find application as anti-infectious, anti-cancer agents or other pharmaceutical useful compounds[5-7]. By these metabolites, they are considered as major decomposers of complex polymers such as lignocelluloses and chitin, main components of agricultural and urban organic wastes[8].

Successful bioconversion of cellulosic materials depends mainly on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes[9]. Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and presence of inducers are important parameters for the optimized production of cellulase enzymes[10].

In previous studies performed in our laboratory, it was shown that *Streptomyces* SP. B-PNG23, a new actinomycete isolated in Béjaia in the north part of Algeria, showed high endoglucanase activity. Thus, this enzyme could be considered as a thermotolerant biocatalyst being interesting for biotechnological applications[11].

In the present work, we describe the isolation of a *Streptomyces* strain, labeled TKJ2, from a Tikjda soil sample, and its identification by molecular methods as well as the production and statistical optimization of the culture conditions employing one factor at a time approach (OFAT) under submerged fermentation conditions for enhanced endoglucanase production.

MATERIALS AND METHODS

Isolation, screening of endoglucanase producing actinomycetes and identification of potent isolate

For the isolation of new strains of actinomycetes that would have an endoglucanase activity, the soil samples were collected from Tikjda in Bouira north east of Algeria. The samples were then serially diluted and separated on actinomycete isolation agar containing Glucose-Yeast extract-Malt (GLM) (3 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 5 g l^{-1} peptone, 10 g l^{-1} glucose, 20 g l^{-1} agar, pH 7.2 and incubated at 28°C for 7 days.

The developed actinomycete colonies were purified by repeated transfer of cultures. The isolates were screened for endoglucanase production and selected on the basis of hydrolysis zone. For the identification of the potent endoglucanase producing actinomycete isolate TKJ2, a 1454 bp region of 16s rRNA gene was amplified using the universal primers 27f (52 -AGAGTTTGATCCTGGCTCAG-32) and 1492r (52 -GGTTACCTTGTTACGACTT-32). The PCR products were purified and sequenced as described earlier[12] and nucleotide sequence has been deposited in the GeneBank database (accession no. JX483709). The sequence data was analyzed for the homology with the similar existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using BLAST search.

Microorganism and its maintenance

Streptomyces sp.TKJ2 used in the present study is an aerobic filamentous actinomycete. The culture was grown and maintained on Williams and Kuster medium containing: 10 g l^{-1} starch, 0.3 g l^{-1} casein, 2 g l^{-1} KNO_3, 2 g l^{-1} NaCl, 2 g l^{-1} K_2HPO_4, 0.05 g l^{-1} $MgSO_4$ ($7H_2O$), 0.02 g l^{-1} $CaCO_3$, 0.01 g l^{-1} $FeSO_4$ (7H2O), 1 g l^{-1} glucose, 15 g l^{-1} agar, pH 7.2, incubated at 28°C[13].

Selection of basic medium for SmF

The production of crude enzyme was carried out in carboxymethyl cellulose containing: 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$ ($7H_2O$), 1 g l^{-1} NaCl, 0.01 g l^{-1} $FeSO_4$ ($7H_2O$), 0.01 g l^{-1} $MnSO_4$ (1 H_2O), 1 g l^{-1} NH_4Cl, 10 g l^{-1} CMC, 2 g l^{-1} yeast extract. The pH was adjusted to 7.0. Erlenmeyer flask (500 ml) containing 100 ml of carboxymethyl cellulose medium was inoculated with 3% of pre-culture.

Enzyme assay

Endoglucanase (CMCase, Endo-β-1,4- glucanase; E.C. 3.2.1.4) activity was determined according to[14]. A reaction mixture contained 2 ml of 2% (w/v) CMC in 50 mM sodium phosphate buffer, pH 7.0 and 1 ml of the crude enzyme sample. The reaction mixture was incubated in a water bath at 50°C for 30 min. The reaction was ended by adding 1.5 ml of dinitrosalicylic acid (DNS) reagent and by placing the reagent tubes in a water bath at 100°C for 5 min[15]. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the appropriate substrates per min per ml of crude filtrate under

assay condition.

Optimization of endoglucanase production

In order to screen the effective parameters for the optimization of endoglucanase production from *Streptomyces* sp. TKJ2, various process variables such as cultivation time (up to 10 days), initial pH (4.0–10.0), temperature (25–45 °C), carbon sources and concentrations of nutrients such as magnesium sulfate (0–0.6 g/l), NH4Cl (0–5 g/l), yeast extract (0–4 g/l), NaCl (0–5 g/l) have been investigated to enhance the production of endoglucanase. Each factor examined for optimization was incorporated further in the subsequent experiments. All other experiment conditions were kept constant otherwise it was stated.

Statistical analysis

One way analysis of variance (ANOVA) was done using statistical package for the determination of significant differences within different conditions, Tukey test was applied. Three replicates were determined for each condition. A significant difference was found when $p < 0.05$.

RESULTS AND DISCUSSION

The present study aimed to describe the isolation of actinomycetes strain which have endoglucanase activitiy from Tikjda in Bouira north east of Algeria (Figure 1). This ecosystem was selected for its abundance of flora and organics matters. The site is indeed particularly exposed to an exceptional snow accumulation due to the ferruginous mineral subsoil. Different process parameters influencing the cellulase production by *Streptomyces* sp. TKJ2 under submerged fermentation conditions were studied to maximize the production. The optimization was carried out using a one factor at a time approach (OFAT).

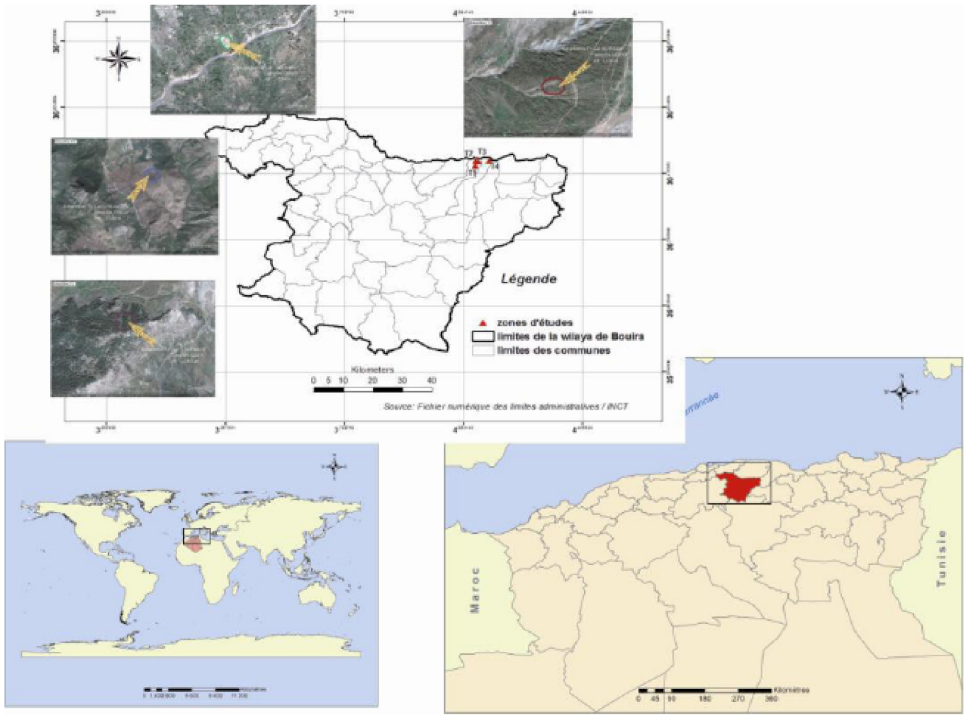

Figure 1 : Tikejda area

Molecular characterization

The identification of the actinomycete isolate TKJ2 which was done by 16S rRNA gene sequence, revealed that the isolate TKJ2 was closely related to the genus *Streptomyces*. TKJ2 16S rRNA gene had 99% similarity (E. value 0.0) with the 16S rRNA gene of *Streptomyces tricolor*, *Streptomyces cavourensis*, *Streptomyces flavovirens*, *Streptomyces praecox* and *Streptomyces microflavus*. Several researchers have reported the usefulness of 16S rRNA gene sequence as a tool to confirm the identity of actinomycetes[16,17]. The phylogenetic tree which is obtained by applying the neighbor joining method is illustrated in Figure 2. The organism was labled as Streptomyces sp. TKJ2.

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Figure 2 : Neighbor-joining tree based on 16S rDNA sequences showing the relations between strain TKJ2 and different species of *Streptomyces*

Effect of incubation period, pH and temperature

The production of cellulase by *streptomyces* sp. TKJ2 was optimized following one factor at a time approach (OFAT). The same technical approach was used by Deswal et al. (2011)[18] and Ketna et al. (2013)[19] to optimize cellulase production by the fungus, *Fomitopsis* sp. RCK2010 and *Aspergillus sydowii* respectively.

Time course of endoglucanase production by *Streptomyces* sp. TKJ2 showed the maximum endoglucanase production (0.89 IU/ml) after 7 days of incubation and thereafter it gradually declined ($p<0.05$) (Figure 3). In addition, prolonged incubation periods (7 days) were required to obtain maximum enzymatic production by streptomycetes and that agrees with Arunachalam et al. (2010)[20]. After 7 days, endoglucanase production was decreased, which may be due to substrate consumption, another reason is the catabolite repression caused by cellobiose[21]. It might also be due to the denaturation of the enzyme, resulting from variation in pH and cellular metabolism during fermentation[22].

Temperature and pH values were found to be important parameters that influenced enzyme production[23,24,11]. In this study, endoglucanase production in

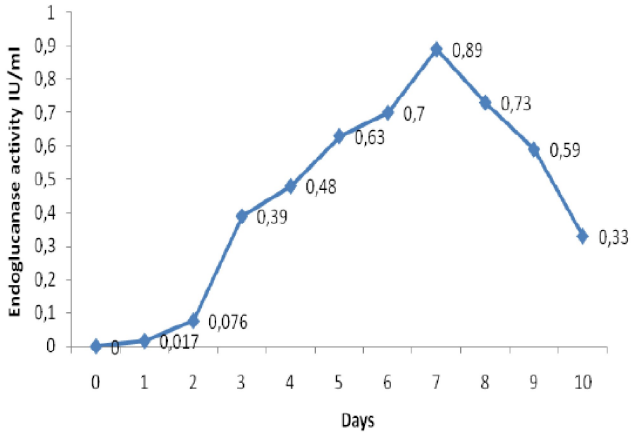

Figure 3 : Effect of incubation period en endoglucanase production

the culture was significantly different ($p<0.05$) with all values of pH and temperature. Growth medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane[25]. Our results also confirmed that growth medium pH was an important factor affecting the carboxymethyl cellulase production. The strain showed a greater cellulase production at pH 6 (Figure 4a). Jaradat et al. (2008)[26] found that CMCase enzyme from *Streptomyces* sp. (strain J2) was active over a pH range of 4-7 with maximum ac-

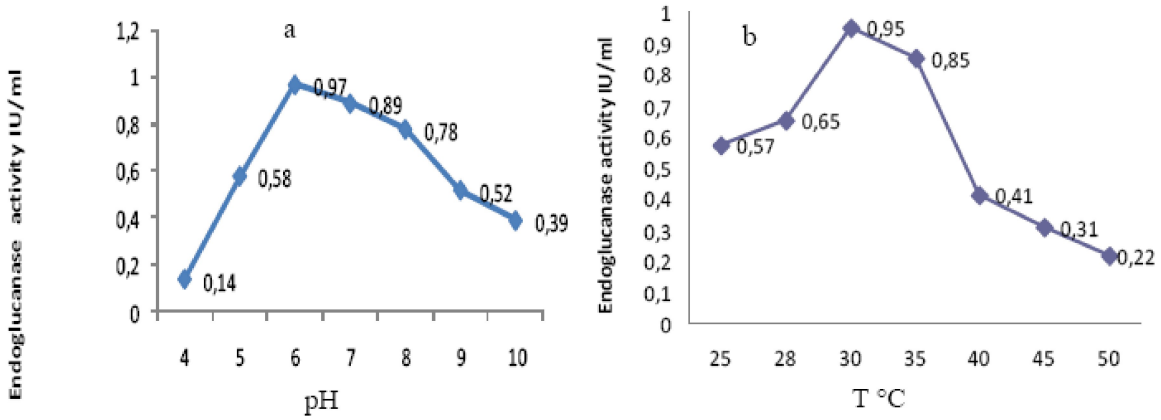

Figure 4 : Effect of initial medium pH (a), temperature (b) on endoglucanase production

tivity at pH 6. Furthermore, the endoglucanase obtained from *Cellulomonas*, *Bacillus* and *Micrococcus* sp., hydrolyzed substrate in the pH range of 4–9 with a maxima of pH 7.0[10]. Solingen et al. (2001)[27] studied the new alkaline *Streptomyces* species isolated from east African soda lakes which showed an optimal pH of 8.

As the temperature increases, endoglucanase production was also increased with an optimum of 30 °C (Figure 4b) which is similar to the findings of Goldbeck et al. (2013)[28]. After 30 °C, it was observed that there was a sharp decline in endoglucanase production. It may be due to the fact that at low temperature, the transport of substrate across the cell is suppressed and lower yield of product is obtained. On the other hand, at high temperature, the maintenance energy requirements for cellular growth is high, due to thermal denaturation of enzymes of metabolic path way resulting in minimum amount of product formation[29].

Effect of carbon source on cellulase production

Cellulase is an inducible enzyme and several carbon sources have been found to promote efficiently enzyme production[30]. Among these carbon sources: carboxymethyl cellulose, glucose, maltose, wheat bran, sugar cane, wheat straw and strach which were the best carbon sources for enzyme production with better production by carboxymethyl cellulose (0.96, 0.89, 0.74, 0.73, 0.69, 0.64 and 0.61 IU/ml respectively). It was observed that carboxymethyl cellulose increased significantly ($p<0.05$) the cellulase production when compared to other carbon sources (Figure 5). A major carbon source for the production of fungal CMCases by *Aspergillus* and *Trichoderma* species was reported to be wheat bran[31,32]. Although some aerobic *Bacillus* species have been shown to produce endoglucanases that can degrade amorphous cellulose, most of them cannot degrade crystalline cellulose efficiently[33]. *A. fumigatus* strain FBSPE-05 was found to produce maximal level of endoglucanase (0.35 U/ml) in presence of sugar cane under submerged culture[34].

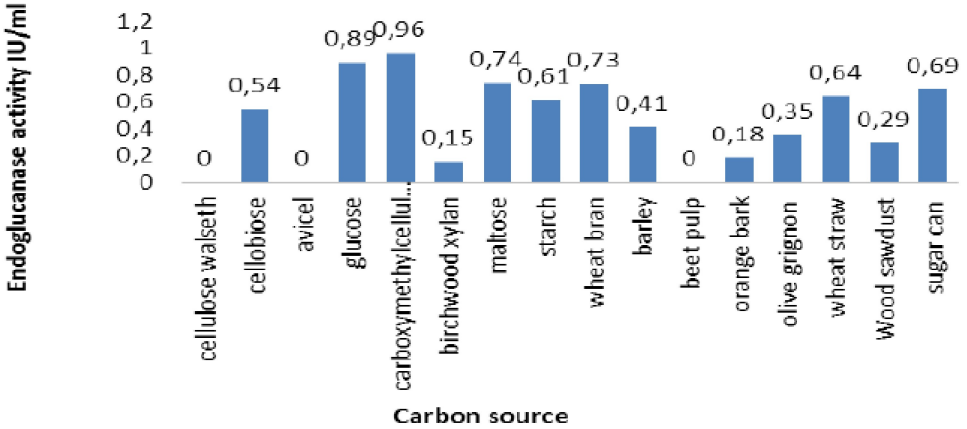

Figure 5 : Effect of carbon source on endoglucanase production

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Effect of culture medium

Nutrient concentrations in the growth medium influenced the production of cellulases by the bacterium. *Streptomyces* sp.TKJ2 showed significantly ($p<0.05$) a high production of cellulase when the growth medium was supplemented with yeast extract (3 g/l), 1 g l^{-1} sodium chloride, 2 g l^{-1} ammonium chloride and 0.3 g l^{-1} magnesium sulfate (Figure 6). Cellulase activity by A. *terreus* was enhanced with the addition of calcium chloride (3 mM) and magnesium sulfate (5 mM) in the medium[35].

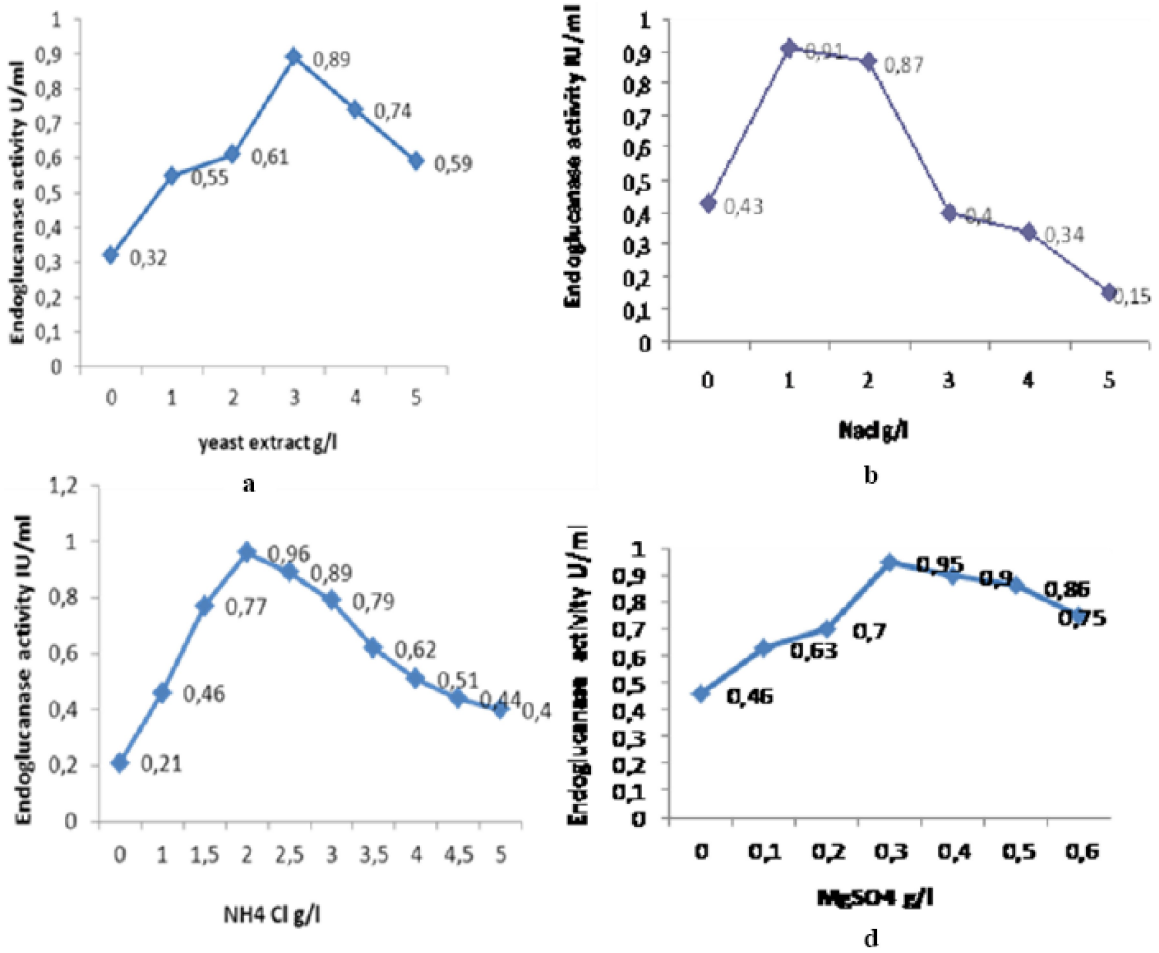

Figure 6 : Effect of yeast extract (a), sodium chloride (b), ammonium chloride (c) and magnesium sulphate (d) on the production of endoglucanase after 7 days of fermentation in shake flask culture containing 10 g carboxymetyl cellulose at 30 °C.

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