Production of Chitinase and its Optimization from a Novel Isolate *Serratia marcescens* XJ-01

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Abstract Production of chitinase from bacteria has distinct advantages over fungi, due to the formation of mycelia of fungi in the later phase of fermentation. A novel chitinase-producing bacterial strain XJ-01 was isolated from the Yulu fishing field of Changsha, Hunan province, China, by enrichment and spread-plate technique, sequentially. Physicochemical characterization and 16S rRNA sequencing revealed that strain XJ-01 belongs to *Serratia marcescens*. By optimizing the fermentation condition based on $L_9(3^4)$ orthogonal experimental design, a maximal chitinase activity up to 15.36 U/ml was attained by that stain under the condition: 0.5% $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source, 0.75% colloidal chitin as the carbon source, temperature of 32°C, time of 32 h and pH 8.0.

Keywords *Serratia marcescens* · Chitinase · Isolation and characterization · Fermentation optimization

Introduction

Chitin, a linear polymer of β -1,4-N-acetylglucosamine units with abundance next to cellulose, is the most abundant natural amino polysaccharide [1]. It is widely distributed as a structural component of crustaceans, insects, as well as a component of the cell walls of most fungi and

some algae [2]. Its degraded products, viz. low molecular weight chitooligomers, chitooligosaccharides, monomers, have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties, showing the application potentials in food and nutrition, biotechnology, material and medical science, agriculture and environmental protection [3].

Chitin could be degraded by chemical, physical, or enzymatical way. Although chemical and physical methods have been industrialized, they are limited by their disadvantages of high cost, low yield and no specificity, even environmental pollution. To contrary, enzymatical degradation of chitin is much more eco-friendly but relatively slow in kinetics [4]. Therefore, enhancing performance of the enzymatical way has been the target for most chitin chemists and engineers, in which the most important thing is to find the high activity of chitinase and the chitinase-producing organisms.

Chitinase-producing microorganisms are reported normally eukaryotic fungi, and very few are bacteria. A brief account on chitinases from microorganisms is listed in Table 1. Bacteria, however, have distinct advantages in fermentation over the fungi, due to the formation of mycelia of fungi at the later phase of fermentation. Chitinases are glycosyl hydrolases (EC 3.2.1.14), which are present in a wide range of organisms including bacteria, fungi, insects, plants and animals [5]. Detection of chitin-degrading microorganisms from natural sources such as soil and sediments is useful in the isolation of strains that produce chitinase.

In this report, we isolated 21 strains of different chitinolytic microorganisms from soil and sediments. The best chitinase-producing strain was isolated and identified as *Serratia marcescens* XJ-01 according to its characteristics, and optimization of its fermentation condition for chitinase production was investigated.

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Table 1 Chitinase origins

Bacteria	<i>Serratia marcescens</i> , <i>Serratia liquefaciens</i> , <i>Aeromonas hydrophila</i> , <i>Alteromonas</i> strain 0–7, <i>Bacillus megaterium</i> , <i>Bacillus licheniformis</i> , <i>Bacillus circulans</i> , <i>Enterobacter agglomerans</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas maltophilia</i> , <i>Vibrio vulnificus</i> , <i>Vibrio fluvialis</i> , <i>Vibrio harveyi</i> , <i>Xanthomonas</i> spp., <i>Clostridium perfringens</i> , <i>Clostridium paraputrificum</i>
Actinomycetes	<i>Streptomyces lividans</i> , <i>Streptomyces olivaceoviridis</i> , <i>Streptomyces lavendulae</i> , <i>Streptomyces griseus</i> , <i>Streptomyces coelicolor</i>
Fungi	<i>Trichoderma</i> , <i>Saccharomyces cerevisiae</i> , <i>Candida albicans</i> , <i>Aspergillus nidulans</i> , <i>Beauveria</i> , <i>Rhizopus oligosporus</i> <i>saito</i>
Virus	<i>Chlorella Virus PBCV-1</i> , <i>Helicoverpa armigera nucleocapsid nucleopolyhedrovirus</i> (HaSNPV), <i>Cydia pomonella granulovirus</i> (CpGV), <i>Baculoviruses</i> , <i>Autographa californica multiple nuclear polyhedrosis virus</i> (AcMNPV)

Materials and Methods

Reagents

EZ-10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc.), *Taq* DNA polymerase (Fermentas), E.N.Z.A. Gel Extraction Kit (Omega Bio-Tek, Inc.), universal PCR primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGHTACCTTACGAC TT-3') were provided by Sunbiotech Co. Ltd., Beijing, China. Colloidal chitin was prepared from powdered chitin by the method of Xiao [6]. Other reagents were of analytical grade and double distilled water was used.

Culture Media

LB medium is a common medium used for identifying bacteria, and selective medium used below is for the sake of obtaining chitinase-producing microorganisms. Generally speaking, strain of *S. marcescens* will show red colony when grown on medium containing beef extract or peptone [7]. Selective medium contained 1% colloidal chitin (w/v), 0.5% (NH₄)₂SO₄ (w/v), 0.05% MgSO₄·7H₂O (w/v), 0.24% KH₂PO₄ (w/v), 0.06% K₂HPO₄ (w/v), pH 7.0. Solidified selective medium was obtained by adding 1.5% agar (w/v) into the selective medium. LB medium contained 1% beef extract (w/v), 1% peptone (w/v), 0.5% NaCl (w/v), pH 7.0. Solidified LB medium was from adding 1.5% of agar (w/v) into LB medium.

Enrichment and Isolation of Strain XJ-01

For enrichment of chitinase-producing strains, five samples from different locations in Changsha city region, Hunan province, China, were respectively added into 50 ml sterile selective medium in 150 ml Erlenmeyer flasks, followed by incubation under aerobic condition at 30°C for 24 h. After 3 generations of enrichment, the culture was gradually diluted to a suitable concentration, and then spread on the solidified selective medium, and incubated at 30°C for 3 days. Colonies showing zones of clearance against the creamy background were regarded as chitinase-producing and re-spread until pure cultures were obtained. The

isolated strains with an equal inoculum density were respectively subcultured in 100 ml of selective medium in 250 ml flasks shaken at 30°C and 180 rpm. After incubation for 28 h, the culture supernatant was collected for measurement of chitinase activity. Strain with highest chitinase activity present in its culture supernatant was maintained on colloidal chitin agar slant and used for further study. This isolate was named as strain XJ-01.

Cellular Morphology and Sub-Cellular Structure Observation

The morphology and motile behavior of the isolate strain XJ-01 were observed with optical microscope (Olympus CX-31). Surface features of the cells harvested from the exponential growth phase were examined by scanning electron microscope (SEM, JEOL JSM-6360 LV).

Physicochemical Characterization of Strain XJ-01

1 ml of the 24 h-old inoculum (about 1×10^8 cells) of strain XJ-01 was inoculated in 99 ml of LB medium, in which the growth curves under different temperature and pH were monitored by direct counting under optical microscope every 4 h. Utilization of typical substrates was investigated by monitoring the growth curves in a basal medium with 0.5% (NH₄)₂SO₄ (w/v), 0.05% MgSO₄·7H₂O (w/v), 0.24% KH₂PO₄ (w/v), 0.06% K₂HPO₄ (w/v), pH 7.0, and gradually supplemented with the various typical substrates. V–P test was used for detecting the ability of producing acetyl methyl carbinol by bacteria, and the test was performed by using 5% of α -naphthol and 40% KOH that were added to the medium to be tested [8].

Amplification, Sequencing and Phylogenetic Analysis of 16S rRNA Gene

The cells of strain XJ-01 were harvested after incubation in liquid LB medium for 24 h. After centrifugation at 10,000 rpm for 5 min, cells were collected and washed several times by Tris–HCl–EDTA (TE) (pH 8.0) buffer. Genomic DNA was extracted by EZ-10 Spin Column

Genomic DNA Minipreps Kit according to the operation instruction of the Kit.

Amplification of 16S rRNA gene of the isolate was carried out in PCR cyclyer (T-Gradient Thermoblock, Biometra) using universal primers 27F (5'-AGA GTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGG HTACCTTACGACTT-3'). Amplification reactions were performed in a total volume 50 µl. The reaction mixture contained 5 µl of 10× PCR buffer, 5 µl of 25 mmol/l MgCl₂, 5 µl of 2 mmol/l dNTPs mixture, 2 µl of primer 27F (62.5 µmol/l), 2 µl of primer 1492 R (62.5 µmol/l), 1 µl of *Taq* DNA polymerase (5 U/µL) and 2 µl of template DNA. Thermo cycling procedure was as follow: an initial keeping at 94°C for 3 min, followed by 30 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 90 s, then an extension in the last cycle at 72°C for 5 min. The PCR product was purified using E.N.Z.A Gel Extraction Kit, then sent to Sunbiotech Co. Ltd for sequencing [9].

Phylogenetic Analysis

Homology sequence alignment of the 16S rRNA sequence with other origin from Genebank was performed by using ClustalX 1.8 software, and phylogenetic tree was constructed with MEGA3.1 software based on sequence similarity analysis.

Determination of Chitinase Activity

Chitinase activity was determined by measuring the amount of the reducing end group, degraded from colloidal chitin, as described in the previous [10]. One unit of activity is defined as the amount of enzyme that liberated 1 µmol of reducing sugar per min.

Optimization of Fermentation Condition

For effective production of chitinase, 1 ml of the 24 h-old inoculum (about 1×10^8 cells) was inoculated in 99 ml of the liquid selective medium. Suitable carbon source, nitrogen source, temperature and pH for strain XJ-01 were investigated in terms of effects on chitinase activity. Further optimizing these four factors were performed based on L₉(3⁴) orthogonal experimental design, as shown in

Table 2. The chitinase activity under each set of design was tested, and based on which the factorial analysis was then performed to find the optimal fermentation condition.

Results and Discussion

Morphology and Micro-Structure of Strain XJ-01

The colony photographs of strain XJ-01 in LB and selective media, and the electron micrograph of strain XJ-01 were observed, respectively. It was found that ivory-white colonies, which were round, smooth, convex and had complete edges (Fig. 1a) were formed after 2 day's incubating of the isolate XJ-01 on the LB solidified agar medium, and clear zones (Fig. 1b) were appeared on solidified selective medium after cultivated for 3 days. Under optical microscope the cells of strain XJ-01 were short rod-shaped, Gram-negative, motile, and most occurring singly during exponential phase in liquid medium (data not shown). Under SEM observation the cells of strain XJ-01 had long peripherally flagella (Fig. 1c).

Physicochemical Properties

The growth curves of the isolate at different temperature and pH are shown in Fig. 2. It shows that the optimal temperature and pH are 32°C and 8, respectively. The results of utilization of typical substrates and V-P test are concluded in Table 3. It shows that strain XJ-01 has the basic characteristics of *S. marcescens*.

Analysis of 16S rRNA Gene Sequence

1.44 kb of 16S rRNA gene of strain XJ-01 was obtained by sequencing and the accession number in Genebank was FJ530951. The gel picture of amplified product and the phylogenetic tree is shown in Figs. 3 and 4, respectively. Figure 4 shows that strain XJ-01 shares the most homology with *S. marcescens* AU763 (99% similarity). In terms of the phenotype and physicochemical properties, strain XJ-01 could be classified as *S. marcescens*. *S. marcescens* is gram negative bacterium, classified in the large family of *Enterobacteriaceae*, occurs in water and soil, on plant, in insects and in man and animal. As one of the most successful microorganisms employed for degrading chitin, the chitinolytic machineries have been best characterized [11–13].

Optimal Condition for Chitinase Production

For effective production of chitinase, the effects of carbon source, nitrogen source, pH, temperature and time on the

Table 2 Factors and levels selected

Level	Factor			
	Chitin (%)	(NH ₄) ₂ SO ₄ (%)	Temperature (°C)	pH
1	0.50	0.25	30	7
2	0.75	0.50	32	8
3	1.00	0.75	35	9

Fig. 1 Colony photographs (**a** after 2 day's incubation in LB medium, **b** after 3 day's incubation in selective medium) and electron micrograph (**c**) of strain XJ-01

Fig. 2 Effects of pH (open square) and temperature (filled triangle) on growth of strain XJ-01

Table 3 Main physiological characteristics of strain XJ-01

Physiological characters	Results	Physiological characters	Results
V-P test	+	Utilization of sorbitol	–
Urease activity	–	Utilization of mannitol	–
Utilization of lactose	–	Utilization of Phenol Red	–
Utilization of maltose	+	Utilization of malonate	–
Utilization of sucrose	+	Utilization of amylum	–
Utilization of arabinose	–	Utilization of pectin	–
Utilization of rhamnose	–	Utilization of citrate	+
Utilization of fucose	+	Utilization of xylose	–

Where “+” denotes positive, “–” denotes negative

chitinase activity were investigated, and the results are shown in Figs. 5, 6, and 7, respectively. Figure 5 shows the effects of carbon source and nitrogen source on chitinase activity of strain XJ-01, and $(\text{NH}_4)_2\text{SO}_4$ and 0.75% colloidal chitin were as the best nitrogen source and carbon source, respectively. Then the effects of chitin and $(\text{NH}_4)_2\text{SO}_4$ concentrations on chitinase activity of strain

Fig. 3 Agarose gel electrophoresis of PCR-amplified 16S rRNA. Lane 1, marker (1 kb, Ferment Molecular Biochemicals). Lane 2, 16S rRNA of XJ-01. Lane 3, Blank control

XJ-01 were as shown in Fig. 6. Figure 7 reflects the effects of pH, temperature and time of fermentation on chitinase activity of strain XJ-01. Based on Figs. 5, 6, and 7, the optimal fermentation conditions for producing chitinase for these single factors are respectively as follows: temperature 32°C, time 32 h, pH 8, 0.5% $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source and 0.75% colloidal chitin as the carbon source.

Orthogonal Test

In order to get the interaction of the above factors on activities of chitinase, the orthogonal test in 3 levels, $L_9(3^4)$ was chosen for further optimization.

The levels of these factors are listed in Table 4. The range analysis in the orthogonal experiments are shown in Table 2, Mean 1, Mean 2, Mean 3 are the average of the responses corresponding to levels 1, 2, and 3, respectively, and Range (R) = max(Mean) – min(Mean). The larger the value of Mean, the better the condition, and the larger the value of R, the greater effect the factor. Based on this idea, according to the values of Mean in Table 4, the best

Fig. 4 Phylogenetic tree of strain XJ-01 **Fig. 5** Effects of carbon source and nitrogen source on chitinase activity of strain XJ-01 **Fig. 6** Effects of chitin (filled triangle) and (NH₄)₂SO₄ (open square) concentrations on chitinase activity of strain XJ-01

parameter condition for the highest chitinase activity is as follows: chitin 0.5%, (NH₄)₂SO₄ 0.5%, temperature 32°C, and pH 8.0.

Fig. 7 Effects of pH (open square), temperature (filled triangle) and time (filled circle) of fermentation on chitinase activity of strain XJ-01

When $F_{0.10} < F$, the response was recorded as insignificant. In variance analysis, $F_{0.10} = 9.00$ is taken as a reference value, and $F > 9.00$ means a significant effect of that factor [14]. According to the results of variance analysis in Table 5, there were no significant effects of the four factors (carbon source, nitrogen source, temperature and pH).

When cultured at optimal condition, the extracellular chitinase activity could reach its maximum (15.36 U/ml) at 32 h, which is much higher in much less time than many other typical chitinase producing wild type strains such as *Serratia* sp. PS-2 (0.68 U/ml), *S. marcescens* QMB1466 (3.66 U/ml), *Bacillus circulans* No. 4.1 (9.6 U/mg) and *Vibrio pacini* CY01 (9.97 U/ml) [15–18]. This result suggests that the isolate XJ-01 has a great potential industrial application in degradation of chitin and other related fields.

Table 4 Original values and experimental results of orthogonal experiment

No.: Factor:	1 Carbon source (%)	2 Nitrogen source (%)	3 Temperature	4 pH	Results Enzyme activity (U/ml)
1	0.50	0.25	30	7	12.42
2	0.50	0.50	32	8	15.36
3	0.50	0.75	35	9	8.46
4	0.75	0.25	32	9	13.20
5	0.75	0.50	35	8	12.30
6	0.75	0.75	30	7	13.50
7	1.00	0.25	35	8	10.32
8	1.00	0.50	30	9	11.76
9	1.00	0.75	32	7	12.30
Mean 1	12.08	11.98	12.56	12.34	–
Mean 2	13.00	13.14	13.62	13.06	–
Mean 3	11.46	11.42	10.36	11.14	–
Range	1.54	1.72	3.26	1.92	–

Table 5 Results of variance analysis

Factor	Errors square sum	DOF	Mean square deviation	F	F _{0.10}	Response
Carbon source (%)	3.60	2	1.80	1.00	9.00	Insignificant
Nitrogen source (%)	4.62	2	2.31	1.28	9.00	Insignificant
Temperature	16.59	2	8.30	4.61	9.00	Insignificant
pH	5.65	2	2.82	1.57	9.00	Insignificant
Error	3.60	2	1.80	–	–	–

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