Process Optimization on Chitinase Production by Locally Isolated *Enterobacter* sp. and *Zymomonas* sp.

Lisa G. A. Ong, Hoi Khei Lam, Mei Yin Lim, and Tian Xiang Tan

***Abstract*—Twenty three isolates which were collected from soil samples around UTAR, Kampar, Perak and Pantai Bagan Nakhoda Omar, Sabak, Selangor were successfully isolated using chitinase selective medium supplemented with colloidal chitin and bromocresol purple as indicator. Based on their enzyme production, two most potent isolates were selected for identification. *The* isolates were tentatively identified as *Enterobacter* sp. and *Zymomonas* sp. based on their morphological and biochemical test. The preliminary study showed that both *Enterobacter* sp. and *Zymomonas* sp. produced the highest chitinase at 48 hours. The process optimization was carried out using one-factor-at-a-time and the parameters involved were the percentage of colloidal chitin, incubation temperature and pH. The optimum parameters for *Enterobacter* sp. to produce chitinase were 5 % colloidal chitin, at 30 °C and pH 7 with 2.92 U/mL/day of productivity. As for *Zymomonas* sp., the chitinase productivity (2.54 U/mL/day) was obtained from the optimum conditions i.e. 15 % colloidal chitin at 30 °C and pH 6.**

***Index Terms*—Bromocresol purple, colloidal chitin, *Enterobacter, Zymomonas*.**

I. Introduction

Chitinase is a glycosyl hydrolase enzyme which hydrolyzes chitin that can be found in bacteria, fungi, plants, insects, mammals and viruses. The chitinase produced by these organisms have various roles, i.e. chitin metabolism in growing hyphae, defense mechanisms in response to pathogens and abiotic stress, in nutrition and parasitism. Other than that, chitinase has been used as biopesticides and inhibitors such as phytopathogen inhibition to strength plant defenses [1]. Bacterial chitinase are widely distributed in the genus of Serratia [2], Bacillus [3], Vibro [4], *Aeromonas* [5] and etc.

The applications of chitinase include environmental remediation, alcohol fermentation, biomedicine, pest control [3]-[6] etc. Chitin is the most abundant renewable biomass after cellulose. Chitin is mainly obtained from crustaceans and insect and about 1 – 100 billion metric tons of these wastes are obtained from aquatic life [7]. Most of these wastes are disposed of through land filling, incineration and/or ocean dumping. Thus an economical and commercially feasible method is needed to manage this huge amount of chitinous waste, which may cause environmental pollution and wastage of natural resources [3]. These can be overcome by recycling the nutrients from the chitinous waste to the environment by microorganisms [8]. Due to the industrial applications of chitinase, numerous studies had been done to optimize fermentation conditions to maximize the production of chitinase from different microbial sources [3], [9], [10]. The objectives of this present work were to isolate prominent chitin degrading bacteria from soil samples and optimize the fermentation parameters to maximize its production.

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II. Materials and Methods

A. Soil Samples Collection

Soil samples were collected aseptically in a sterile sample collection bottle from Westlake hostel, lakeside near to UTAR block E and UTAR entrance, as well as Pantai Bagan Nakhoda Oman, Sabak Bernam, Selangor.

B. Preparation of Colloidal Chitin

The modified method of [5] was used to prepare the colloidal chitin using chitin powder from crab shells (Malacai tesque, Japan), where 5 g of the chitin powder was treated with 75 mL of concentrated HCl and vigorous stirred at 30 °C for 60 min. The mixture was then treated with 2 L of cold distilled water (~4 °C) and centrifugation at 12,000 rpm for 25 min to obtain precipitated colloidal chitin. The colloidal chitin was washed with approximate 1 L of sterile distilled water and centrifuged again at 12,000 rpm for 25 min to collect the white precipitate. The precipitate was washed with distilled water until it reached pH 7 [11]. The colloidal chitin was then pressed between filter paper to remove additional moisture and will be used as substrate.

C. Isolation and Screening of Chitinase Producing Bacteria

The collected soil samples (2 g) was added with 18 mL of sterile distilled water and mixed thoroughly. A 10-fold serial dilution was carried out and the diluted sample (0.1 mL) was transferred onto chitin agar medium which consist of (g/L): $MgSO_4 \cdot 7H_2O$, 0.3; $(NH_4)_2SO_4$, 3; KH_2PO_4, 2; citric acid monohydrate, 1; agar, 15; colloidal chitin, 4.5; bromocresol purple, 0.15; Tween-80, 200 µL and adjusted the pH to 4.7 [12]. Colonies showing purple zone after incubation of 24 h at 30 °C were considered as chitinase producing bacteria.

D. Characterization of Chitin Lytic Bacterium

The identification of Isolates SH 17 and SPBNO 1 was carried out according to the methods as describe in Bergey's Manual of Determinative Bacteriology [13]. The tests carried were: gram stain, arginine dihydrolase, lysine decarboxylase,

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citrate utilization, catalase, hydrogen sulfide, indole, triple sugar iron, starch hydrolysis, gelatin hydrolysis, oxidase, mobility, oxidation fermentation, methyl red and Voges Proskauer.

E. Optimization of Chitinase Production

The optimization was carried based on the one-factor-at-a-time where Lauria Bertaini (LB) broth (g/L: tryptone, 10; yeast extract, 5; NaCl, 10 amended with colloidal chitin 5, pH 7) was used for the chitinase production. To determine the optimum time for chitinase production, the bacteria (10%) was inoculated and incubated at 30 °C, 200 rpm for 7 days. Sampling was carried out every 24 h, where the cultures were centrifuged at 10,000 rpm for 14 min and the supernatant was used for chitinase assay. Effect of different concentration of colloidal chitin (5 – 20 g/L) was carried to determine the best substrate concentration. After that, with the optimized substrate concentration, different incubation temperature (28 – 30 °C) will be investigated and follow by different pH (4 – 7).

F. Chitinase Assay

Chitinase assay was performed based on the release of reducing sugar from colloidal chitin according to [14] with slight modification. Crude enzyme (0.5 mL) was added with equal volume of 1% (w/v) colloidal chitin in phosphate buffer (0.1 M, pH 7). After incubation at 37°C for 2 hours, the reaction mixture was added with 1 mL of DNS reagent and boiled for 5 min. The reaction mixture was centrifuged at 9,000 rpm for 5 min and the supernatant was measured at 540 nm. One unit (U) of chitinase was defined as the amount of enzyme which releases one μmol of N-acetyl-D-glucosamine (GlcNac) from 1% (w/v) colloidal chitin per hour under given conditions.

III. Results and Discussion

A. Isolation and Screening for Chitinolytic Bacterium

A total of 23 isolates were obtained from the soil samples based on their ability to degrade the colloidal chitin and with the purple zone on the plate (Fig. 1). By using this technique, it was easier to observe as compare to the conventional method without additional of dye [12]. Isolates SH 17 and SPBNO1 were selected for further study based on their chitinase production (data not shown).

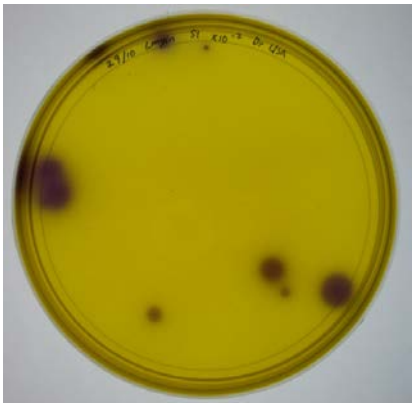

Fig. 1. Purple hydrolyzing zone produced by bacteria colony on the colloidal chitin agar supplemented with bromocresol purple.

B. Identification of Potential Isolates Based on Their Biochemical Properties

The identification of Isolates SH 17 and SPBNO 1 was carried out based on their morphological, biochemical and physiological characteristics according to the methods described in Bergey's Manual of Determinative Bacteriology [13]. The isolates were appeared in bacillus shape, gram negative, and non-spore forming (Table I). Based on all the results obtained with regards to the physiological and biochemical properties, Isolates SH 17 and SPBNO 1 were belonging to *Enterobacter* genus and *Zymomonas* genus, respectively. Further identification of the isolates till species level need to be carried out via molecular approached.

TABLE I: Characterization Of Isolates SH 17 and SPBNO 1

Characteristic	SH 17	SPBNO 1
Colony shape	Circular	Circular
Elevation	Convex	Convex
Margin	Entire edge	Entire edge
Surface	Smooth	Smooth
Color	Creamy white	Creamy
Gram reaction	Negative	Negative
Cellular morphology	Rod	Rod
Endospore formation	Negative	Negative
Oxidase	Negative	Negative
Indole production	Negative	Negative
Methyl red	Negative	Negative
Voges-Poskauer reaction	Positive	Negative
Citrate utilization	Positive	Negative
Hydrogen sulfide production	Negative	Negative
Lysine decarboxylase	Negative	Negative
Arginine dihydrolase	Negative	Negative
Motility	Negative	Negative
Gelatin hydrolysis	Positive	Negative
Starch hydrolysis	Negative	Negative
Catalase production	Positive	Positive
Oxidation-fermentation	Fermentative	Fermentative
Triple ion sugar	Slant agar: positive, Blunt agar: negative	Slant agar: negative, Blunt agar: negative

C. Optimization of Chitinase Production

The optimization was carried out based on one-factor-at-a-time. Incubation time was first carried out to determine the optimum chitinase production for a period of 7 days. The results obtained in Fig. 2 showed the highest production was obtained after 24 h for both *Enterobacter* sp. (6.70 ± 0.15 U/mL) and *Zymomonas* sp (1.68 ± 0.14 U/mL). From Fig. 2, two chitinase production peaks can be observed for both bacteria. This might due to the production of toxic chemicals or inhibitors in the medium [9], [15]. Besides, different bacteria strain has different incubation time for high production of chitinase. After 24 h of fermentation, [5] found out that highest chitinase could be obtained by *Aeromonas hydrophila*. However *Aeromonas punctata* produced the highest chitinase production after 48 h [5]. For examples, [16] reported that after 48 h of incubation highest chitinase production was obtained from *Bacillus cereus*, *B. alvei* and *B. sphaericus*. The same pattern was observed by [17] with *Microbispora* sp.

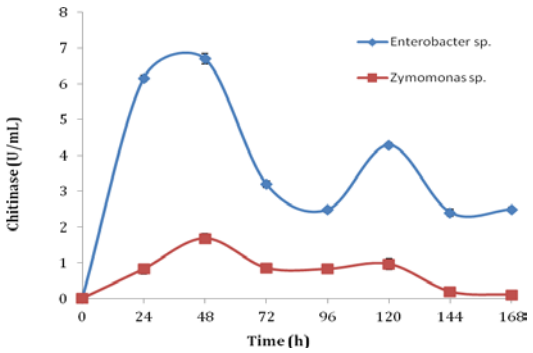

Fig. 2. Effect of incubation time on chitinase production.

Different concentrations of colloidal chitin ranging from 5 g/L to 20 g/L were investigated on the production. The incubation temperature, pH and agitation speed were maintained at 30 °C, 7 and 200 rpm, respectively. At 48 hours the chitinase produced by *Enterobacter* sp. and *Zymomonas* sp. were shown in Fig. 3. The optimum substrate concentration for both bacterial was different, where *Enterobacter* sp. gave highest chitinase production (6.7 ± 0.14 U/mL) at lower concentration of colloidal chitin, which was 5 g/L. Whereas *Zymomonas* sp. produced highest chitinase (2.78 ± 0.21 U/mL) at 15 g/L of colloidal chitin. The results suggested substrate inhibition or end product inhibition which was N-acetylglucosamine [18] occurred and influenced the production of chitinase by *Enterobacter* sp. This indicates that different strain of bacteria able to tolerate different concentration of colloidal chitin for the production of chitinase. These were proved by different researchers [3], [19], [20] i.e. [21] reported the maximum chitinase production was at 3 g/L of colloidal chitin.

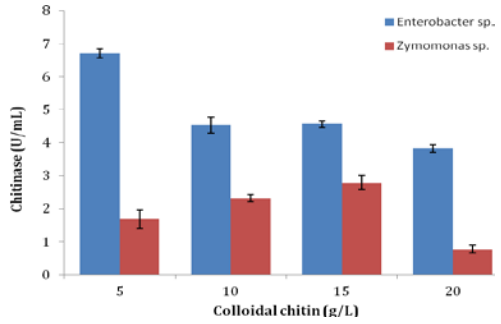

Fig. 3. Effect of colloidal chitin concentrations on chitinase production.

The effect of temperature on the production of chitinase by *Enterobacter* sp. and *Zymomonas* sp. were carried out based on the optimum colloidal chitin which was 5 g/L and 15 g/L, respectively. To investigate the optimum incubation temperature for chitinase production, both bacteria were grown at 28 – 37 °C. This range of temperature was chosen is due to the isolates are mesophilic bacteria, which obtained from environmental. This is due to temperate plays an important role in various biological processes. Hence the growth of bacteria and enzyme production are affected with a slight change of the temperature. The optimum temperatures for both bacteria were at 30 °C (Fig. 4). Different microorganism has different optimum temperature for its growth and chitinase production [5], [22], [23].

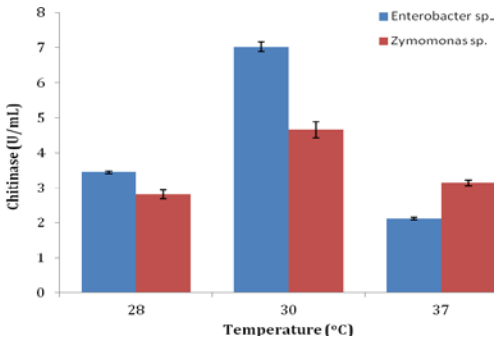

Fig. 4. Effect of temperature on chitinase production.

Based on Fig. 5, *Enterobacter* sp. growth well at neutral pH and has the highest chitinase production which was 5.58 ± 0.15 U/mL, while Zymomonas sp. growth well under slight acidic condition that was at pH 6 with the chitinase production of 5.08 ± 0.17 U/mL. Some microorganisms are able to produce high amount of chitinase under alkaline condition [3], [5], [20], while some microorganism preferable acidic condition for chitinase production [22], [24]. This can conclude that pH plays an important role production of chitinase.

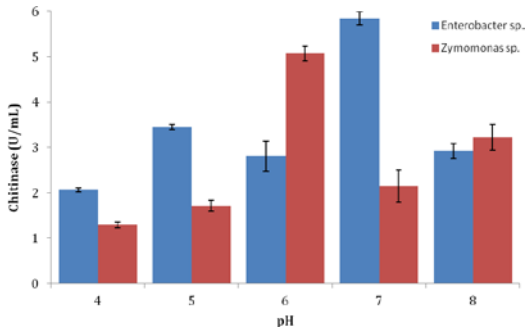

Fig. 5. Effect of pH on chitinase production.

IV. Conclusion

Twenty three potential chitinase producing bacteria have been successfully isolated from environmental soil samples. Two potential isolates have tentatively identified as *Enterobacter* sp. and *Zymomonas* sp. and their production parameters have been optimized. *Zymomonas* sp. favorable acidic condition and produced acidic chitinase, while the chitinase obtained from Enterobacter sp. was at neutral pH. Both isolates grown well at 30 °C. *Enterobacter* sp. preferable low concentration of colloidal chitin, while *Zymomonas* sp. more desirable at higher concentration of colloidal chitin. To further identify these two isolates till the species level, 16S rDNA need to be carried out in future study.

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