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Optimised production of chitinase from a novel mangrove isolate, *Bacillus pumilus* MCB-7 using response surface methodology

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ABSTRACT

Chitinases – the primary chitin lytic enzyme has gained great attention due to its industrial, environmental, agriculture, medicinal and biotechnological applications. However, the industrial production of this enzyme is still at its infancy due to low yielding strains and high cost of production. The current paper targets the cumulative optimisation of chitinase production from low cost substrate chitin using high yielding novel chitinase producing strain. Bacterial chitinase producers were isolated from the potential mangrove ecosystem of Valanthakad, Cochin, Kerala. Among the various isolates, the best chitinase producer MCB-7 identified as *Bacillus pumilus* exhibited an inherent high chitinase activity of 3.36 U/mL. Using Plackett–Burman design the factors such as chitin concentration, peptone and pH were found to be significant. Further optimisation with a multivariate statistical approach using Box–Behnken design resulted in a 6.9 fold increase of chitinase activity to a final yield of 23.19 U/mL. The use of cheap and consistent nature of the chitin as carbon source along with a high chitinase yielding isolate would be beneficial in providing consistent yields of the enzyme. To the best of our knowledge, this is the first report on high chitinase yielding *B. pumilus* strains from mangroves. The current isolate of *B. pumilus* MCB-7 is also unique in its halotolerance, pH tolerance and thermostability – which could widen its spectrum of applications.

1. Introduction

A wide range of microorganisms of different ecosystems participate in the chitinase mediated chitin degradation in their life-cycle to obtain nutrition, exhibit antagonism and combat parasites. The diversity and extremity of various environments also contribute to special attributes or high yields of their inherent chitinase enzymes. Mangroves unique for its high biological diversity, varying salinity and organic matter content often prompt its autochthonous microbial population to develop a highly active chitinase system. Exploration of such environment could be good sources of novel chitinase producing microbial strains. Microbial degradation of chitin occurs via two steps: first the hydrolysis by chitinase poly (1.4 (N-acetyl-β-D-glucosaminide) glycanohydrolase; EC3.2.1.14) to oligomers mainly dimers, followed by their degradation to free N-acetyl-glucoseaminidase EC3.2.1.30) (Farag and Al-Nusarie, 2014).

The realm of chitinase applications extends to vast areas including environmental remediation, alcohol fermentation, biomedicine, pest control etc. Almost 1–100 billion metric tonnes of chitinous waste are produced annually from aquatic life forms (Rattanakit et al., 2002). Chitin is the second abundant renewable natural resource obtained from crustaceans and insects. Aquatic products such as shrimps, crabs, squids, oyster, and cuttlefish (Gohel et al., 2006) have almost 10–55% chitinous material. Most of the chitinous waste is disposed off through ocean dumping, incineration, and land filling. Thus there is an imperative need of economical and commercially feasible methods for managing the enormous waste which may otherwise lead to economic loss, wastage of natural resource, and the problem of environmental pollution. Utilisation of these chitinous waste by microbes offers the best solution to the problem leading to recycling of nutrients in the environment (Gohel et al., 2007).

The incorporation of chitinase producing *Mucor circinelloides* strains along with colloidal chitin as carbon source was found to dramatically increase bioethanol production (Inokuma et al., 2013). The recent interest in chitinous material based membranes and scaffolds for drug delivery and tissue engineering greatly increases the need of purified chitinase enzymes (Anitha et al., 2014). Chitinases are also used as biocontrol agents against fungal phytopathogens and pests as chitin is an essential component of fungal cell wall and cuticle of insects (Maisuria et al., 2008;

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Mendonsa et al., 1996). Therefore, the applications of bacterial and fungal chitinases offer a potential alternative to the chemical fungicides (Bhushan and Hoondal, 1999; Ghanem et al., 2011; Huang and Chen, 2008). Many bacterial and fungal chitinase also have the ability to lyse many of the plant pathogens and thereby capable of eradicating fungal pathogens and enhance the agricultural production in the world.

Considering the enormous application of chitinase in various fields, the commercial production and scale up is of critical importance. The objective of the present work was to evaluate and identify the significant factors in the chitinase production, model designing to study the interaction between significant variables and to choose the optimum value for the maximum desirable response using statistical designs of Plackett–Burman and Box–Behnken design of response surface methodology.

2. Materials and methods

2.1. Sample collection and selection of bacterial strain

Soil samples were collected from the mangrove island of Valanthakad backwaters of Cochin, Kerala, India; situated on the eastern side of the Vembanad ecosystem [9°55′10. 24″ N latitude and 76°20′ 01. 23″ E longitude]. A total of 51 different soil samples were aseptically collected from different regions, serially diluted and screened for the chitinase enzymatic assay.

2.2. Preparation of colloidal chitin and media

Colloidal chitin was prepared from pure chitin (Himedia, Mumbai) by the method of Wiwat et al. (1999). Briefly commercial chitin was hydrolysed by concentrated hydrochloric acid by stirring at 1 h at 4 °C. The hydrolysed chitin was further washed several times with distilled water to remove the acid completely and the pH was brought into the range of 6–7. The colloidal chitin was thereafter filtered using Whatman filter paper, collected and stored in the form of a paste at 4 °C.

Chitin agar medium amended with 0.5% colloidal chitin was used as a selective agar for the isolation of prominent chitinase producing bacteria after enrichment. Colonies with surrounding clear zones (halo) were selected and grown in 100 mL of Luria-Bertani (LB) medium with 2% chitin powder at 30 °C for 72 h, and the chitinase released into the medium was quantitatively measured (Miller et al., 1998).

2.3. 16 S rDNA based molecular identification of high chitinase yielding bacteria

Among the different bacterial isolates, MCB-7 with highest chitinase production potential was selected and identified based on its morphological, biochemical characteristics and genotypic characteristics by 16 S rDNA sequence analysis using forward primer 8F: 5′-AGAGTTTGATCMTGG-3′ and 16 S reverse primer 1492R: 5′-ACCTTGTTACGACTT-3′ in Agilent Sure Thermal cycler. The resultant PCR amplicon was purified by QIAamp DNA Purification Kit (Qiagen) and sequenced at Eurofins Genomics Pvt. Ltd., Bengaluru, India. The sequence was analysed by BLAST algorithm at www.ncbi.nlm.nih.gov.in and deposited in the NCBI Genbank.

2.4. Chitinase activity assay

The extent of chitinase activity using colloidal chitin as substrate was evaluated by estimating the quantity of N-acetyl-D-glucosamine released, as a result of chitinase action. The assay mixture composed of 0.5 mL of 1% (w/v) colloidal chitin and 0.5 mL enzyme solution was incubated at 45 °C for one hour. The enzymatic reaction was further stopped by supplementing 3 mL 3,5-dinitrosalicylic acid reagent followed by heating at 100 °C for 5 min. The centrifuged supernatant was assayed for reducing sugars at 530 nm using UV spectrophotometer with slight modifications of Miller et al. (1998). The concentration of N-acetylglucosamine released was analysed against values of N-acetylglucosamine standard. One unit [U] of the chitinase activity was defined as amount of enzyme required to release 1 mM of N-acetyl D-glucosamine [as a standard] from chitin/min. Biomass of the bacteria was calculated and protein concentration was measured against bovine serum albumin as standard (Lowry et al., 1951).

The utility of pNp-GlcNAc as substrate was measured by the release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide (Miller et al., 1998). The assay mixture composed of 0.4 mL of 25 mM sodium phosphate buffer [pH 7.0], 0.5 mL of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide and 0.1 mL enzyme solution was incubated at 37 °C for 10 min and the optimal density was measured at 410 nm. One unit of enzyme activity was defined as amount of enzyme required to release 1 μmole of p-nitrophenol per minute from p-nitrophenyl-N-acetyl-β-D-glucosaminide. All experiments were conducted in triplicates and values were expressed as mean ± SD.

2.5. Design of experiment

Primary experiments of chitinase production was performed in a fermentation medium containing 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ at pH 7.0 and incubated at 37 °C in a shaker incubator at 150 rpm. A one percent [v/v] cell suspension (with optical density 1) was used as primary inoculums throughout the study. The inoculated culture flasks were incubated at different operational conditions and the extent of chitinase action was evaluated as described above. Optimisation of medium constituents to improve chitinase activity of MCB-7 was carried out in initially using Plackett–Burman design considering nine variables viz, chitin (1–20 g/L); peptone (0.5–8 g/L); $MnSO_4$ (0.1–3 g/L); $FeSO_4$ (0.1–3 g/L); KH_2PO_4 (0.1–3 g/L); yeast extract (0.5–5 g/L); pH (3–10); incubation time (12–120 h) and temperature (20–80 °C) as depicted in Table 1. The variables having the most relevant effect on chitinase yield were identified using a 2-level Plackett–Burman design and their levels were further optimised for enhanced chitinase production by employing a Box–Behnken design. Multiple trials were conducted as suggested by the model and all retrieved data was analysed for variance by ANOVA. The experiment was carried out in triplicate to estimate the experimental errors and to test for lack-of-fit of the data using the second degree polynomial model.

Table 1
Variables chosen for analysis by Plackett Burman design.

Sl. no	Nutrients code	Nutrient	Low value coded as (−1) (g/L)	High value coded as (1) (g/L)
1	A	Chitin	1	20
2	B	Peptone	0.5	8
3	C	$MnSO_4 \cdot H_2O$	0.1	3
4	D	$FeSO_4 \cdot 7H_2O$	0.1	3
5	E	KH_2PO_4	0.1	3
6	F	Yeast extract	0.5	5
7	G	pH	3	10
8	H	Incubation time (h)	12	120
9	I	Temperature (°C)	20	80