Research Article **Bio Chemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

UTILIZATION OF PRAWN SHELL POWDER FOR THE PRODUCTION OF CHITINASE BY KURTHIA GIBSONII MB126

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ABSTRACT

239 bacterial isolates producing extracellular chitinase were selected from coastal environments of Kochi using a solid medium containing prawn shell powder. Among them 23 isolates which produced clear zone with diameter >1.5cm were tested for production of chitinase in a medium containing prawn shell powder. The bacterium designated as Mb 126 was the highest yielding and was selected for further studies. It was identified as *Kurthia gibsonii*. 0.6% of prawn shell powder, pH 6.5, temperature of incubation 35^0 C and agitation rate 100 rpm were the conditions optimum for chitinase production. This is the first report of chitinase production by *kurthia gibsonii*.

KEYWORDS : Chitinase, Prawn shell waste, *Kurthia gibsonii* Mb 126, Optimization.

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INTRODUCTION

A large number of shell fish processing units exist in marine environments of Kochi. The chitinous waste generated from these units creates serious environmental problems. Chitin is the 2nd most abundant renewable natural resource [1]. It consists of a linear chain of β 1-4 N acetyl glucosamine [2]. It is present in the cell wall of higher fungi, exoskeletons of insect and shells of crustaceans. Chitin is degraded by chitinase (E.C.3.2.1.14,Poly {1,4-N acetyl D-glucosaminide } glucanohydrolase) to N acetyl glucosamine (GlcNac). Chitinase has received increased attention because of their wide applications such as production of single cell protein [3], mosquito control [4], biocontrol agent of fungal pathogens [5,6], isolation of fungal protoplasts [7,8], and preparation of oligosaccharides and N acetyl glucosamine [9]. Chitinases are produced by higher plants [10], fungi[11], several bacteria [12,13] and actinomycetes [14].

In India alone 60,000 to 80,000 tons of chitinous wastes are produced annually, from which a lot of chitin can be recovered[15]. The waste and effluents produced by shell fish processing industry cause serious environmental problems if it is not treated well. Enzymatic degradation of chitinous waste by microbial chitinase is more ecofriendly and economic than any other chemical method for the treatment of chitinous waste. The objectives of the present study were to isolate a novel strain of bacterium for chitinase production and to optimize the conditions for the production.

MATERIALS AND METHODS

Isolation of chitinase producing bacteria

Soil samples were collected from the premises of prawn peeling units in the coastal areas of Kochi in Kerala. Five grams of soil was added to 250 ml flask containing 1% of colloidal chitin prepared from prawn shell waste as described by Roberts and Selitrennikoff [16]. After incubation for two days at 37^0C the suspension was plated onto a screening medium (pH 6.5) containing (%,w/v): prawn shell powder,1g and agar, 2g. The plates were incubated at 37^0C for 2 days. Chitinase producing bacteria were isolated based on the zone of clearance around the colonies. The colonies with surrounding clear zone >1.5cm were selected subcultured and maintained in nutrient agar slants at 4^0C.

Assay of Chitinase

The bacterial suspension was inoculated into 100 ml of the 1% suspension of prawn shell powder (pH 6.5) and incubated at 37^0 C for 48 hours in an incubator shaker agitated at 100 rpm .The culture was centrifuged at 8000 rpm for 20 minutes and the supernatant was collected to estimate the chitinase activity. 1ml of the supernatant was mixed with 1 ml of 1% of colloidal chitin in 0.05 M phosphate buffer (pH 7) prepared according to[16] and incubated for 1 hour at 40^0 C. N acetyl Glucosamine was assayed by DNSA method[17]. One unit of chitinase was defined as the amount of enzyme that liberated one micromole of N acetyl glucosamine per ml per minute under the experimental condition.

Identification of bacterium

The cultural, morphological and physiological biochemical properties of of the bacterium Mb126 were studied. As per the *Bergey's Manual of Systematic Bacteriology* [18] the bacterium was identified as *kurthia*. The result was confirmed by 16s r RNA gene sequencing. Isolation of genomic DNA and PCR amplification was done according to Sambrook *et al* [19]. A similarity search for the nucleotide sequence of 16s rRNA gene of the test isolate was carried out using a BLAST search at NCBI[20]. Primers used for PCR were

10-30 F - (Forward Primer) 5'-GAG TTT GAT CCT GGC TCA G-3'

530R (Reverse Primer) 5'-G(A/T)A TTA CCG CGG CGG CTG-3'.

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Optimization of culture condition

The factors were studied in a sequential manner. One factor was optimized at a time. The optimal level of this factor was incorporated in the next step.

Effect of growth phase & incubation period.

The culture was inoculated into fermentation medium containing prawn shell powder and incubated for different periods of time and the enzyme production was assayed.

Agitation

The production of chitinase was monitored varying the agitation rate (50-250 rpm) of the incubator shaker.

Substrate Concentration

Different concentrations of prawn shell powder were incorporated into the fermentation medium. Fermentation was performed for 3 days at 37^0C and the enzyme was assayed.

pH and temperature

The optimum temperature and pH for enzyme production were selected by varying the pH of the medium (3-8) and temperature of incubation (20-60^0C) .

Metal ions and surfactants

To evaluate the effect of metal ions and surfactants the bacterium was grown in the presence of various metal ions (5mM) such as $MnCl_2$, $CaCl_2$, $HgCl_2$, $CuSO_4$, $ZnCl_2$ and $MgCl_2$ and surfactants (1%v/v) such as Tween 20, Triton X –100 and Tween -80.

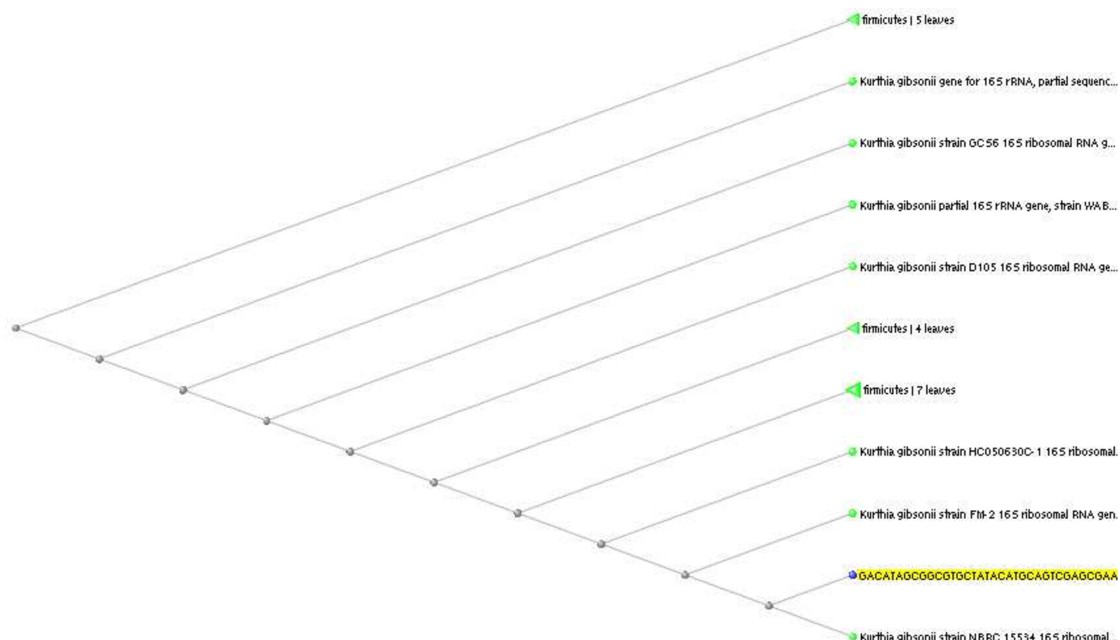
RESULTS AND DISCUSSION

Among the 239 bacteria isolated from coastal environments of Kochi, 23 produced clear zones above the diameter 1.5 cm. Chitinase producton by these bacteria ranged from 17 to 27 U/ml. The bacterium designated as Mb 126 showed highest production and was selected for further studies. The physiochemical characteristics of Mb126 are given in Table1. Results indicated that the organism was belonging to *Kurthia.*

Table 1
Morphological and physiochemical properties shown by Kurthia gibsonii Mb126

Test	Result
Gram Staining	+ve
Shape	Rod
Arrangement	Single
Spore	-ve
Motility	+ve
Mac Conkey	No growth
Indole Production	-ve
Methyl red	-ve
Voges Proskauer	+ve
Citrate Utilization	-ve
H_2S Production	-ve
Nitrate Reduction	+ve
Pigment	Pale yellow
Catalase	+ve
Gas production	-ve
Bird's feather growth on nutrient gelatin slants	+ve
N acetyl β D Glucosamine Utilization	-ve
γ Hydroxy butyric acid Utilization	-ve
D - Fructose	-ve
D - Mannose	-ve
Uridine 5-monophosphate	+ve

16S rRNA gene sequence of the isolate is given below
GACATAGCGGCGTGCTATACATGCAGTCGAGCGAATGACGAGAAGCTTGCTTCTCTGATTTAGC
GGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTACAGATCGGGATAACTCAGGGAAACCT
GGGCTAATACCGGATAATCCTTCGAATCACATGTTTTGAAGTTGAAAGGCGCTTCGGCGTCACT
GTAGGATGGGCCCGCGGTGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCAACGATGC
ATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGA
TGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGTTAGGAAATGAACGTA
CCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCCGCCGCGGTAATTCCAA
The partial 16S r RNA gene sequence of the isolate has been deposited in the GenBank data base (No. JN637370). A BLAST search of the 16srDNA sequence against NCBI nucleotde database revealed 99% identity with *Kurthia gibsonii*. Hence the isolated strain was identified and designated as *Kurthia gibsonii* Mb126. Phylogenetic tree is given below.



Various physical and chemical factors were found to be influencing the production of chitinase by this bacterium. Bacterial growth and chitinase production by *Kurthia gibsonii* Mb126 during cultivation are illustrated in Fig.1. The isolate could produce chitinase from the early exponential phase of growth. A steady increase in enzyme production was observed with the progress in bacterial growth. The maximum production was obtained after 60-72 hours of incubaton; thereafter the enzyme production reduced

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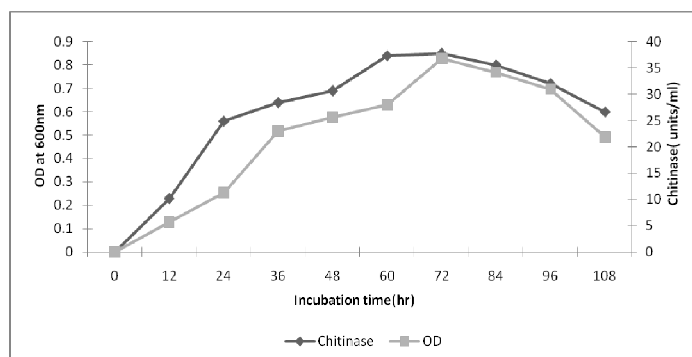

Figure 1

Effect of Growth phase and incubation period on chitinase production by Kurthia gibsonii Mb126

Agitation of culture at 100 rpm resulted in maximum enzyme production (Table 2). There was a sharp decline in the production on increasing the agitation rate to 200 and above. The reason for this is to be investigated further.

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Table.2
Effect of agitation of culture on chitinase production by Kurthia gibsonii Mb126

Shaking speed(rpm)	Chitinase (units/ml)
Control	20.6
50	21
100	35.6
150	23.4
200	3.4
250	1.9

Prawn shell powder was used as a sole carbon and nitrogen source for the production. Effect of various concentrations of prawn shell powder in the medium on chitinase production is shown in figure 2. The maximum chitinase production was found in medium with 0.6% prawn shell powder while from 0.7 % the production slightly declined. It can be assumed that the higher substrate concentration may be increasing the medium viscosity resulting in oxygen limitation for bacterial growth. Madhavan and Nair (1975) have reported that the prawn waste was containing 39.74 % protein, 23.08% chitin, 5.04 % fat and 21.13% ash[21]. This nutrient rich nature of prawn shell powder may be the reason for its suitability for use in the medium both as a nitrogen and carbon source. Chitinase production could not be observed in nutrient broth not containing any chitinous substrate (data not shown), this reveals the inducible nature of chitinase.

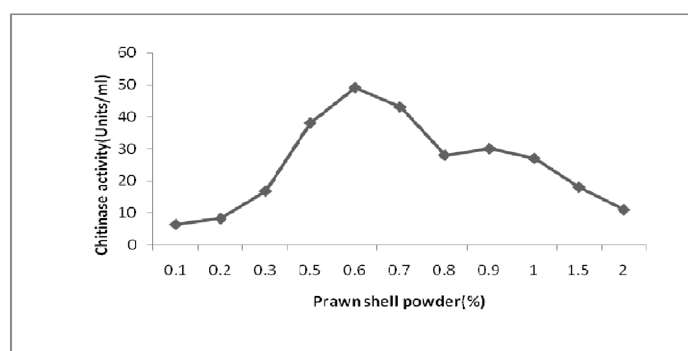

Figure 2
.***Effect of various concentrations of prawn shell powder in the medium on chitinase production by Kurthia gibsonii Mb126***

Temperature of incubation significantly affected the enzyme production. *Kurthia gibsonii* Mb126 produced maximum chitinase at 35^{0}C . Effect of temperature of incubation on chitinase production by *Kurthia gibsonii* Mb 126 is shown in Fig.3. Chitinase production was almost steady between 30-50 ^{0}C Keeping the fermentation medium in this mesophilic range require less energy cost since the ambient temperature in a tropical country like India is more or less in the same range. Bacterial growth and chitinase production were absent at 60°C. The optimum temperature for chitinase production were 40^{0}C in the case of *Microbispora sp*.V2 [22], 35^{0}C in the case of *Massilia timonae* [23] and 30^{0}C in the case of *Serratia marcescens* [24].*Pseudomonas aeruginosa* K-187 recorded an optimum temperature of 45°C [25] while the optimum temperature of *Streptomyces lydicus* WYEC108 for chitinase production was 25 – 30°C [26].

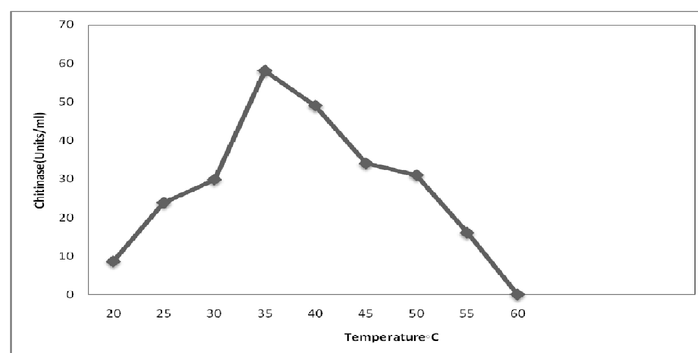

Figure 3
Effect of temperature of incubation on chitinase production by Kurthia gibsonii Mb 126

Addition of $Mg2^{+}$ slightly increased the Chitinase production, while $Hg2^{+}$,$Mn2^{+}$,$Zn2^{+}$,$Cu2^{+}$ inhibited the production (Table 3). Metals influence the development of cells and have a considerable influence on enzymatic activity. Enzymatic inhibition by $Hg2^{+}$,$Mn2^{+}$,$Zn2^{+}$,$Cu2^{+}$ may be by masking the catalytically active subunits of the enzyme or by degrading proteins or by changing the conformation of the enzyme. In the case of $Mg2^{+}$,it may be providing proper ionic strength for enzyme activity.

Table 3
Effect of Metals on chitinase production by Kurthia gibsonii Mb126

Metal	Chitnase(units/ml)
Control	59
$MnCl_2$	22
$CaCl_2$	41.7
$ZnCl_2$	32
$MgCl_2$	65
$HgCl_2$	22.5
$CuSO_4$	20.4

The chitnase production was maximum at pH 6.5 (Figure 4). Between 4.5 and 7.5 chitinase production was relatively stable. The similar pH optima has been reported in the case of *Aeromonas* sp. [27] and *Massilia timonae* [23]. Taechowisan *et al.* (2003), reported that the production of chitinase by *Streptomyces aureofaciens* was optimal at pH 6.5-7 [28]. pH 7 in the case of *Microbispora* sp.V2 [22], 6 in the case of *Streptomyces* sp. ANU 6277 [29] and pH 8 in the case of *Serratia marcescens* [24] have been reported as optimum for chitinase production

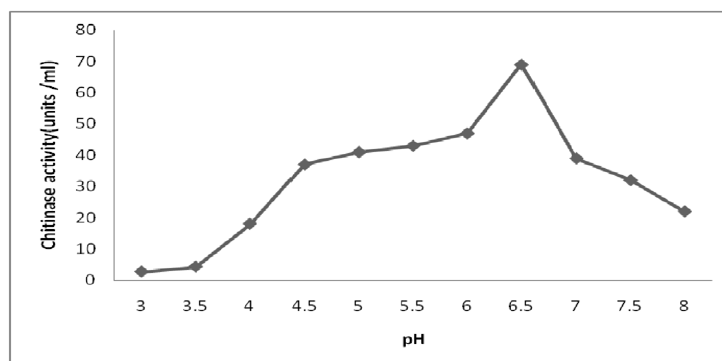

Figure 4
Effect of pH on chitinase production by Kurthia gibsonii Mb 126

Addition of surfactants did not affect the enzyme production. It has been proposed that detergents enhance enzyme secretion by increasing cell membrane permeability [30]. However there is no significant variations in enzyme producton with Tween 20,Tween 80 or Triton x-100 (data not shown).A similar result was reported in the case of *Aspergillus carneus* [31] .

CONCLUSION

As a result of optimization of conditions the production of chitinase by *Kurthia gibsonii* Mb126 could be increased from 27 to 65 units/ml. The bacterium was able to produce chitinase in a medium containing prawn shell powder without any external carbon and nitrogen sources. To the best of our knowledge this is the first report on the production of chitinase by *Kurthia gibsonii*. Hence further studies are in progress with the objective of purification and characterization of the enzyme

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