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Bio Technology

Elixir Bio Tech. 55 (2013) 13116-13119

ISSN: 2229-712X

Morphological, biochemical and molecular characterization of anon chitinase protease producing bacteria, its biodegrading effect on shell fish waste and its enzyme kinetics

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ARTICLE INFO

Article history:
Received: 17 December 2012;
Received in revised form:
13 February 2013;
Accepted: 19 February 2013;

Keywords
Protease,
Bacteria,
Fish,
Biochemical.

ABSTRACT

Soil samples from dump yards in Thiruvanthapuram were collected for isolation of bacteria. Isolates obtained were screened for non chitinase and protease activity, by culturing on skimmed milk and colloidal chitin agar plates respectively. The organism which did not degrade chitin but degraded protein was selected and identified by simple staining, gram staining, IMViC, and with further molecular identification by 16SrRNA analysis, it was confirmed that the isolate was *Serratia marcescens.* The economical application of *Serratia marcescens* was analyzed by quantifying the enzyme production and its degrading capacity on shell fish waste. The enzyme kinetics was studied after its purification and characterization using column chromatography and SDS-PAGE respectively. The optimum temperature and pH for maximum enzyme activity was identified and studied for future applications on a broad scale.

1. Introduction

Sea food exports associated industries shares a major economic back bone of coastal regions in south India. The waste product of crustacean especially shrimp's are considered to have a huge impact on ecology of these regions owing to difficulty in processing and safer removal. Shrimp's Shells are considered as the excellent source of chitin but the incompetency of chemical processing limits, wide spread use and applications. Microbial deproteinization can have a significant impact in the production of value added products from shrimp shell in a more eco-friendly manner, limiting the bio-wastes to minimum. Chitinase deficient microorganism with abundant protease production can be preferred its biological conversion of chitin in a more eco-friendly manner.

2. Materials and methods

2.1 Isolation of Microorganism

Soil samples were collected in sterile containers from dump yards of Thiruvananthapuram and transferred to the lab aseptically. The microorganisms present in the collected sample were isolated by serial dilution method. The isolated microorganism was screened for protease and chitinaseactivity.

2.1.1 Screening of Protease Activity (Coolbear*et al.*, 1991)

The microorganisms obtained from natural sources were grown on skimmed milk agar medium plates for 24hrs. Efficient proteolytic organisms which produced clear hydrolytic zone around the culture were cultured in protease production medium and tested for protease activity by Anson M.L. method.

2.1.2 Screening of Chitinase Activity (Roberts & Selitrennikoff, 1988)

The organisms producing clear hydrolytic zone on skimmed milk agar medium plates were then plated on to colloidal chitin agar plates and were incubated at 30ºC for up to 10 days. After 10 days 0.1% Congo red was added to the plate. Organisms not having chitinase activity were selected for further studies.

2.1.3 Identification of Microorganism

The organism was identified by primary staining method, gram staining, followed by biochemical tests (IMVC Tests), and Molecular identification by 16SrRNA analysis.

2.2Enzyme activity of *Serratia marcescens*on shrimp shell wastes.

2.2.1 Partial purification of shrimp shell waste (Muralidhara,1981)

Theexoskeletons, which have been peeled from shrimp, and the exoskeleton materials was then mixed with 70% saline solution.This was then filtered using muslin cloth and was kept for drying under vaccum condition. The dried shrimp shells were then powdered using blender.

2.2.2 Deproteinization of partially purified shrimp shell waste (Hall,G.M.,C.L.Reid and Z.Zakaria, 1994) To 100ml protease production media , 3g shrimp shell powder was added as the chitin source and was inoculated with the isolate.Fermentation was carried out at 37^0**C** at pH-7.The Protease enzyme activity, amount of chitin and protein in the sample were analysed in alternative days(3rd,5th,7th and 9th day).

2.2.3 Demineralization (Percot*et al.*,2003)

1g of microbiallydeproteinized shell powder (MDS) was mixed with 50ml 6N HCL in a tightly closed conical flask and placed in a water bath for 2-3hrs at 100^0C followed by keeping for evaporation at 40-50^0C in hot air oven, until it was fully evaporated and precipitate is formed in the bottom of conical flask. The precipitate was diluted in distilled water. The amount of chitin in the sample was analysed.

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2.2.4 Estimation of chitin (George.C.Chen and Bruce.R.Johnson.1983)

The chitin was assayed colorimetrically by George.C.Chen and Bruce.R.Johnson method and a standard graph was drawn and the amount of chitin in the sample was calculated.

2.2.5 Estimation of protein content from microbially and chemically deproteinized sample (Lowry *et al.*,1951)

sample were treated for the estimation of protein content by using Lowry's method. The color developed on addition ofFolin's Reagent was measured in a spectrophotometer at 670nm.

2.3 Purification and characterization of enzyme produced bySerratia marcescens

2.3.1Purification and Molecular Weight Determination of Protease Enzyme (Nilson.B.H.K*et al*., 1993)

The culture supernatant of the organism was collected from 24 hours old culture and was subjected to salting out with ammonium sulphate (enzyme grade, SRL) at a final saturation of 80% (w/v). The precipitate was recovered by centrifugation at 10,000 rpm for 15 minutes at 4^0C. The pelleted proteins were collected. The pellet was dialyzed against phosphate buffer (pH 7.4, 0.02M) using dialysis membrane (Dialysis membrane 60, Himedia).

2.3.2 Purification of Protease by Column Chromatography

A graph was plotted with concentration of sodium chloride along x-axis and optical density along y-axis**.** From the graph, which fraction have maximum protease activity was obtained.

2.3.2.1 Molecular Weight Determination of Protease by SDS-Polyacrylamide Gel Electrophoresis (SDS-Page) (Sambrook*et al*., 1989)

The purified protein were mixed with equal volume of SDS-Gel loading buffer and loaded to on to a 12.5% Polyacrylamide gel. The electrophoresis was developed at the protein band were analyzed by Coomassive brilliant blue staining. The molecular weight was determined by using GelEval 1.22 software.

2.4 Study of Enzyme Kinetics of Proteolytic Enzymes from Serratia marcescens

The enzyme kinetics was studied to optimize pH,temperature required for the maximum activity of the enzyme.

2.4.1 Optimum pH for enzyme activity:

The effect of pH was determined by varying the pH 3, 4 5, 6, 7, 8, 9, 10 and 11 of phosphate buffers, by the modified method of Anson.M.L.*et al*,1938.

A graph was plotted with different pH values along X-axis and optical density at 660nm along Y-axis. From the graph optimum pH of protease was obtained.

2.4.2 Optimum temperature for enzyme activity:

The effect of temperature on enzyme activity was determined by incubating the medium at different temperatures (30°C, 45°C, 50^0C, 55^0C & 60^0C) by the modified method of Anson.M.L.*et al*.

A graph was plotted with different temperature values along X-axis and optical density at 660nm along Y-axis. From the graph optimum temperature of protease having peak value was obtained.

3. Results and discussion

3.1 Isolation of chitinase and protease producing microorganism

3.1.1 Isolation of Microorganisms

Following the procedure outlined in materials and method altogether ten microbial colonies were isolated from serially diluted soil samples, which was further screened for enzyme activity.

3.1.2 Screening of Protease Activity (Coolbearetal. 1991)

On screening a single colony was found to produce a clear hydrolytic zone of 15mm diameter. This colony was further cultured in protease production medium and incubated at 37˚C for 24hrs from which supernatant was taken as the crude enzyme source and tested for protease activity by Anson.M.L.method. It was observed that the microorganism has the proteolytic activity 3.75×10^{-2}µmol/ml/min enzyme unit.

3.1.3 Screening of Chitinase Activity (Roberts & Selitrennikoff, 1988)

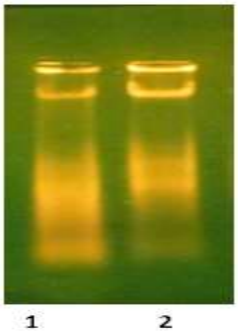
The microorganisms which produced prominent hydrolytic zone on skimmed milk agar plates failed to produce zone on the colloidal chitin agar plate showed the absence of chitinase activity. Therefore, the microorganism isolated from the soil sample appeared to be promising candidate for deproteinization of shrimp shell. Their chitinase activity was determined by Miller method. The chitinase activity of the microorganism estimated was negligible to produce any effect on the chitin present on the shrimp shell waste during its deproteinization.

3.2 Identification of Microorganism

EXPERIMENT	RESULT
CULTURE MORPHOLOGY	Reddish Pink coloration on nutrient agar plate.
SIMPLE STAINING	Short rod shaped cells
GRAM STAINING	Gram –ve
INDOLE	+ve
METYL RED	-ve
VOGUES PRAUSKER	-ve
CITRATE	+ve

Table 3.2.1: Identification of Microorganism

3.3 Molecular characterization serratia marcescens using 16SrRNA



Lane 1: Genomic DNA
Lane 2: Genomic DNA
FIG 3.3.1 Genomic DNA isolation

3.3.2 Polymerase Chain Reaction (PCR)

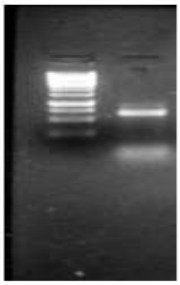


FIG 3.3.2: LANE 1: 1kb DNA LADDER (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp)
LANE 2: AMPLIFIED PRODUCT OF 16s rRNA GENE AT48°C

3.3.3 16s rRNA gene amplification

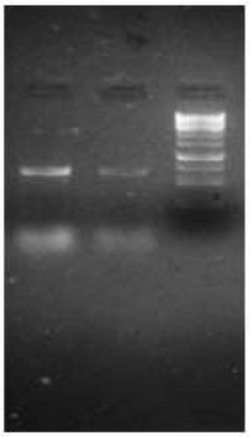


FIG 3.3.3: 16s rRNA gene amplification

LANE 1: AMPLIFIED PRODUCT OF 16s rRNA GENE AT50°C

LANE 2: AMPLIFIED PRODUCT OF 16s rRNA GENE AT50°C

LANE 3: 1kb DNA LADDER (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp)

>OciSeq_SER_Bac16srRNA-F_072.ab1
ACACCAAATTGCCCAAGGTTCGAGGCGGGGTAAGCAC
AGGGGAGCTTGCTCCCCGGGTGACGAGCGGCGGACGG
GTGAGT
AATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTA
CTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGA
CCAAAG
AGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCA
GATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCT
AGGCG
ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGT
GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC
CATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAA
GCACTT
TCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCAT
CAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACT
CCGTGC
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTG
TTAAGT
CAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA
TTTGAAACTGGCAAGCTGGAGTCTCGTAGAGGGGGGT
AGAATT
CTGAG

Fig 3.3.3.1: Sequence

3.4 Deproteinization of shrimp shell wastes using *Serratia marcescens*

Days	% of chitin	% reduction of protein content
3rd day	12%	45.45%
5th day	15%	72.7%
7th day	**23%**	**77.2%**
9th day	22%	77.2%

Table 3.4.1 Deproteinization of shrimp shells BY *Serratia marcescens*

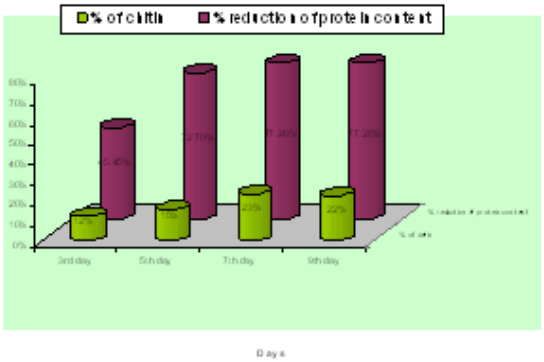


Fig 3.4.2 % of chitin content & % of reduction of protein content on deproteinization

3.5 Protease Activity of Deproteinization

Intervals	Protease activity (µmol/ml/min)
3rd day	2.31×10^{-2}
5th day	4.04×10^{-2}
7th day	**4.33×10^{-2}**
9th day	4×10^{-2}

3.6.Molecular Weight Determination of Protease by SDS-Polyacrylamide Gel Electrophoresis (SDS-Page) (Sambrook*et al.*, 1989)

The molecular weight of components of purified protease was determined by measuring the relative mobility of proteins using SDS-PAGE. It was observed that there was a thick band in the purified enzyme lane, which was suggested to be protease and has molecular weight, was determined by GelEval1.22 software as 80KDA.

3.7 Study of Enzyme Kinetics of Proteolytic Enzymes from *Serratia marcescens*

3.7.1* Optimum pH for Protease Activity *Serratia marcescens

The effect of pH on protease activity was checked by quantifying the amount of tyrosine liberated by Anson method (Anson.M.L*et al*., 1938). According to the graph, the protease activity was observed to be high at a pH of 7 in the supernatant of Serratia marcescens. It can be suggested that the protease produced by Serratia marcescens is a neutral protease

3.7.2 Optimum Temperature for Protease Activity of *Serratia marcescens*

The effect of temperature on protease activity was checked by quantifying the amount of tyrosine liberated by Anson method (Anson.M.L*et al*., 1938). From the graph, the protease activity was found to be higher at a temperature of 37^{o}C in the supernatant of *Serratia marcescens.*

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