STUDIES ON CHITOSAN ACTING ENZYME OF *RHODOTORULA GRACILIS*

A Thesis Submitted to the University of Mysore
for the Degree of

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

BY

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CERTIFICATE

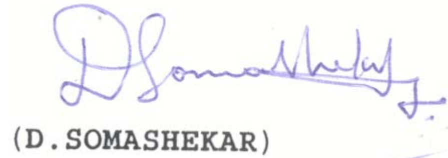
I hereby certify that the thesis entitled "**STUDIES ON CHITOSAN ACTING ENZYME OF RHODOTORULA GRACILIS**", submitted by **Mr. D.Somashekar** for the degree of **Doctor of Philosophy** to the University of Mysore, Mysore, is the result of the research work carried out by him in the Department of Microbiology and Bioengineering, C.F.T.R.I., Mysore, under my guidance during the period 1987 to 1994.

(RICHARD JOSEPH)
Guide

DECLARATION

I hereby declare that this thesis entitled "**STUDIES ON CHITOSAN ACTING ENZYME OF RHODOTORULA GRACILIS**" which is submitted herewith to the University of Mysore, Mysore, for the degree of Doctor of Philosophy is the result of the research work carried out by me in the Department of Microbiology and Bioengineering, Central Food Technological Research Institute, Mysore, India, under the guidance of **Dr. Richard Joseph** during the period 1987 to 1994.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.



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c) *Mucor janssení*
d) *Cunninghamella verticillata*
e) *Absidia glauca*
f) *Mucor circinelloides*

LIST OF ABBREVIATIONS

A.	Absorbance at
BSA	Bovine serum albumin
^{o}C	Degree centigrade
cm	Centimeter
CMC	Carboxymethylcellulose
DNS	Dinitrosalicylic acid
etc.	And other things of the same kind
EDTA	Ethylenediaminetetraecetic acid
EDAC	1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide
Fig.	Figure(s)
g	Gram(s)
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
h	Hour(s)
K_m	Michaelis-Menten constant
L	Litre(s)
M	Molar
m	Milli
min.	Minute(s)
ml	Millilitre(s)
mol	Moles
Mr	Relative molecular mass
ug	Microgram(s)
ul	Microlitre(s)
um	Micrometre(s)
umol	Micromole(s)
MSM	Minimal salts medium
MS+P	Minimal salts + peptone
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
nm	Nanometer
NBS	N-bromosuccinimide

O.D.	Optical density
PCR	Polymerase chain reaction
pNp	Para-nitrophenyl
PAGE	Polyacrylamide gel electrophoresis
PDM	Potato dextrose medium
pp.	Page
per se	In itself
pCMB	Para-chloromercuribenzoate
%	Per cent
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
sp./spp.	Species
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
U	Units
UDP	Uridine diphosphate
V_{max}	Velocity maximum
Vis-a-Vis	As compared with
viz.	Namely
i.e.	That is
v/v	Volume/volume
W/V	Weight/volume

SYNOPSIS

SYNOPSIS

The present investigation has been undertaken to characterise a chitosan hydrolysing enzyme produced extracellularly by *Rhodotorula gracilis* CFR-1 and to understand its biological role.

During these studies it was revealed that the chitosan acting enzyme is a novel hydrolase which has not hitherto been reported in literature. Thus, the studies were focussed on characterisation of this enzyme and the conditions of culture under which this enzyme is produced extracellularly by the yeast.

The thesis has been presented in four broad chapters, namely, 1: Introduction, review of literature and scope of the study; 2: Materials and methods; 3: Results and discussion; and 4: Summary and conclusions. The chapter on Results and discussion has two sections, one dealing with the characterization of the novel chitosanolytic enzyme and the other on its biological role in *Rhodotorula gracilis*.

A brief outline of the thesis is as follows.

In the **Introduction**, the subject matter of the thesis is briefly introduced indicating the academic interest on a newly found chitosanolytic enzyme and its biological role. This is followed by a concise review of literature, wherein the nature and occurence of the substrate, chitosan and its

biodegradation have been discussed. Chitosan is a polymer of glucosamine which has been reported to exist in the cell walls of fungi belonging to the class *Zygomycetes*. Chitosan may also arise resulting from enzymatic deacetylation of chitin (polymer of N-acetyl glucosamine), the latter occuring widely in crustaceans and fungi. The review also contains information on chitosanolytic enzymes reported in literature as found in several microorganisms such as *Bacillus sp., Bacterium sp., Pseudomonas sp., Enterobacter sp., Streptomyces sp., Myxobacter sp., Nocardia orientalis, Fusarium sp., Penicillium islandicum* and in a few plants such as *Cu cumis sativus* (cucumber), *Allium porrum* (leek), *Allium cepa* (onion), *Pisum sativum* (pea). The review brings out the fact that the properties of the chitosanolytic enzymes from various sources have not been studied in adequate depth. Concerning the biological role of these enzymes in microorganisms, it is generally believed that they may be involved in nutrition of those microorganisms which thrive on decaying organic matter rich in chitin and chitosan. Some reports are mentioned about a possible defense role for these enzymes in the plants and microorganisms. The scope of the present investigation has been shown to have a strong justification owing to the enzyme studied being a novel chitosanolytic enzyme.

In the second chapter of **Materials and Methods**, details of the standard methods employed in this work have been listed with relevant references. For the characterisation of the products of chitosan hydrolysis catalysed by the enzyme, some new approaches in the procedure of high voltage electrophoresis on cellulose acetate, cathodic polyacrylamide gel electrophoresis (PAGE), ultracentrifugation, gel permeation

and calcofluor white dye binding assay have been adopted. These innovations were necessitated because of the high basicity of the substrate chitosan and its product. of degradation.

The third chapter of **Results and Discussion** has two sections as indicated. In the first section, data obtained on the characterisation of the chitosanolytic enzyme secreted by Rhodotorula gracilis CFR-1 have been presented. The organism is a locally isolated strain which is being studied for its lipid overproducing property. The chitosanolytic enzyme is apparently produced constitutively by this organism and secreted into the culture media. After separation of the cells, the culture broth was used as the crude enzyme preparation. The enzyme was purified by conventional methods of salting out by ammonium sulphate, gel permeation, ion exchange and affinity chromatographies, and by chromatofocussing. In a typical protocol standardized finally, two steps of purification procedure were adopted which yielded a partially purified enzyme preparation having three-fold purification and 34% recovery of the enzyme. There was a limitation to the procedure adopted for the assay of this enzyme, only by measuring reduction in the viscosity of the chitosan solution, because the enzyme catalysed hydrolysis of chitosan did not yield detectable amount of glucosamine or generate reducing groups. Almost all the chitosanase assay procedures reported in literature are based on the measurement of these products of hydrolysis. In the present work a calcofluor white dye binding procedure for assay of this enzyme was also employed as a new method.

The enzyme was found to be optimally active at pH 4.5 and at 45°C. It was most stable at 4°C and at pH 5.0. Manganese chloride was found to have a slight stimulatory effect on enzyme activity, while mercuric chloride and copper sulphate were inhibitory at 1 mM level tested. Among the other effectors, the tryptophan reagent, N-bromosuccinimide and the thiol reagent para-chloromercuribenzoate inhibited the enzyme. The enzyme was specific for chitosan. It did not hydrolyse chitin, glycol chitosan, xylan, soluble starch, carboxymethylcellulose or pectin. The apparent K_m of the enzyme was 0.83 mg chitosan/ml and its V_{max} 142 units/min/mg.

That the present enzyme is a novel one came to light when the products of chitosan hydrolysis was carefully analysed. Ultracentrifugation, polyacrylamide gel electrophoresis and gel permeation studies suggested that chitosan of average molecular mass 36,000 employed in the reaction was reduced by the enzymic catalysis to one fourth this size (9,500 daltons) without further hydrolysis of this product. In the analytical ultracentrifugation, the hydrolysed chitosan sedimented at 2.15 as against 3.545 for the unhydrolysed sample. Electrophoretic separation of the chitosan hydrolysis products was performed by the procedure of cathodic disc PAGE which allows for the migration of charged molecules from anode towards cathode. This was done because of the highly basic nature of chitosan. Chitosan and the hydrolysis products were visualised by staining with Procion brilliant red. The hydrolysis product was seen as a single band.

In the second section of the **Results and Discussion**, data obtained on studies carried out to understand the

biological role of this enzyme are presented and discussed. The organism was grown in media containing different carbon and nitrogen sources. The growth of the organism and enzyme production were high in yeast extract, peptone, dextrose broth (YEPD) compared to the minimal salts medium containing different carbon sources like glucose, sucrose, xylose and mannitol. The organism did not grow in the medium having lactose, cellulose, starch or chitosan. The influence of different nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, urea, peptone and individual amino acids) on enzyme production was tested. The growth and enzyme production by the organism was high in the minimal salts medium containing peptone as nitrogen source. Excepting tryptophan, the rest of 19 amino acids were found to be stimulatory for enzyme production.

The enzyme production was highest at 48 h of growth in YEPD and at 30 h in potato dextrose broth (PDB). In the PDB, unlike in YEPD there was gradual decrease in enzyme level after 36 h.

Mutants deficient in the chitosanolytic enzyme were isolated by chemical mutagenesis using N-methyl-N' -nitro-N-nitrosoguanidine. This was done to see if the mutants deficient in the chitosanolytic enzyme will be affected in budding process. Different mutants were obtained which were showing morphological variations and producing low levels of the enzyme compared to the wild type strain of *Rh. gracilis* CFR-1. It was however not possible to ascertain if the budding process was affected in these mutants.

Forty zygomycetes cultures were tested for antagonism by *Rh. gracilis* by the plate culture method. Eleven of these cultures were apparently antagonised by *Rh. gracilis*.

Based on the above findings alone, a precise biological role to the chitosanolytic enzyme produced by *Rh. gracilis* could not be assigned. Since *Rh. gracilis* was inhibited by the presence of chitosan in the growth medium, a nutritional role for this enzyme could be ruled out.

Based on the data obtained on antagonism of fungi, it appears more likely that the present enzyme is involved in the defence mechanism in *Rh. gracilis* CFR-1.

The thesis has a final chapter on **Summary and Conclusions**. In this chapter, all the data obtained are summarised and the major conclusions drawn are listed.