# STUDIES ON PRODUCTION, PURIFICATION AND CHARACTERIZATION OF γ-BHC DEGRADING ENZYME FROM *GEOTRICHUM CANDIDUM* NCDC - 228


THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

## DOCTOR OF PHILOSOPHY
## IN
## DAIRYING
## (DAIRY MICROBIOLOGY)

*BY*

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NATIONAL DAIRY RESEARCH INSTITUTE
( I. C. A. R. )
KARNAL - 132001 (HARYANA), INDIA

**2002**

Regn. No. 1019802

*Dedicated to*

*My*

*Loving Parents,*

*Life Partner*

*'n'*

*Family*

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Approved by

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# CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON PRODUCTION, PURIFICATION AND CHARACTERIZATION OF γ-BHC DEGRADING ENZYME FROM *GEOTRICHUM CANDIDUM* NCDC-228"** submitted by **Ms. ANSHU CHOUDHARI** in partial fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY in DAIRYING (DAIRY MICROBIOLOGY)** of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

**(P.K. AGGARWAL)**
MAJOR ADVISOR & CHAIRMAN
(GUIDE)

# Acknowledgements

*Guru sua jahi panth dikhava,*
*Bin Guru jagat ko nirgun pava !*

– Jayasi

*The words of the great Sufi saint have rightly described my mentor, Dr.P.K.Aggarwal who has been instrumental in my coming of age as now. He not only suggested to me this topic of current importance for research but also keenly developed the whole programme inspiring me with a burning zeal, being an embodiment of erudition and enlightenment and maintaining his cool did not let me flagger in my studies and also sincerely stood by me to develop my all round faculties. I am highly obliged to him and feel wordless to reimburse his contribution.*

*Thanks are due to the Director, National Dairy Research Institute, Karnal for providing me necessary facilities and financial help in the shape of Senior NDRI Fellowship to carry out the investigation.*

*I would fail in my duties if I do not acknowledge the gratitude of Dr.V.K. Batish, the present Head, Dairy Microbiology Division, Dr. Harish Chander, Dr.Rattan Chand, Dr. S.M. Dutta, Dr. K.K. Singhal, Dr. H.K.L. Tandon, Dr.(Mrs.) B.K. Wadhwa, and the members of my Advisory Committee, Dr. Y.S. Rajput, Dr. B.R. Yadav, Dr. (Mrs.) Bimlesh Mann and the other well wishing teachers whose intellectual, material and critical help enabled me to complete the work satisfactorily.*

*I owe much to my friends, Atul, D.P. Mishra and my juniors Vijay Paul, Pawas, Pragya, Mandal, Kunal who in a friendly capacity directed my studies and rendered their valuable assistance in achieving this goal.*

*Thanks are also due to Mr. Chamela Ram, Mr. Parveen, Mrs. Sheela Ji for their co-operation with kindness. I am also thankful to Mr. Lahri Singh for his support and help.*

*I am also thankful to Mr. Devinder Kumar of TRADS Computers for timely help.*

*The perseverance of my beloved parents who took pains to show me light of the world, bring me up and enable me to reach this level of education cannot be reciprocated. I will remain indebted to them for whole of my life. The immense love and affection of my sisters (Anu, Disha) and brother (Siddharth), who yearned for my attaining this status, can also not be matched by any words.*

*No words can express my feeling of gratitude, respect and adoration for my life partner, Shri Parveen Chaudhary, who lent his full support in seeing this endeavour of mine come to logical conclusion. The generosity of my parents-in-law and brothers-in-law can also not get unacknowledged as they have been looking forward to my reaching this level.*

*In the last, I am highly indebted to the authors whose work has made the basis of this manuscript. I acknowledge my sincere gratitude to them.*

*I bow my head to the Almighty God who confined in me and empowered me to perform the work successfully.*

Choudhari

**(ANSHU CHOUDHARI)**

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# ABSTRACT

Incidence of pesticides in human food including milk has become ubiquitous for their widespread application. It is imperative to remove these pesticide residues from milk for good consumer health and sustained dairy industry. *Geotrichum candidum* NCDC-228 and its enzyme being economical and ecofriendly are effective tools in bioremediation of γ-BHC. The mould could optimally produce the enzyme at 25°C in 7 d under static conditions in potato dextrose broth of initial pH 6. Salting of mould culture filtrate with 60 to 70 per cent ammonium sulphate effected 10.82 fold purification and Sephadex G-75 gel filtration caused 18.01 purification fold. Dialysis and SDS-PAGE indicated the enzyme to be about 19.5 kD large. Optimum activity of the enzyme in buffer occurred at 25°C and around pH 6.5. An Enzyme : substrate (γ-BHC) concentration ratio of 1 : 4 gave a linear ascending relationship. The enzyme was stable on holding at 20 to 25°C for 10 min and pH 6.0 for 60 min. $Na^+$, $Mg^{2+}$, $Ca^{2+}$ enhanced the enzyme activity but $Zn^{2+}$ and $Fe^{3+}$ inhibited it. The enzyme had a $K_m$ value of 29.41 μM and $V_{max}$ 1.45 μM at 25°C. Organoleptic, TTC, MBRT and acid production tests indicated that the enzyme was highly active in milk and γ-BHC was degraded by the same extent as was in buffer. It was fully active even at 37°C in milk. The use of this non-toxic protein in immobilized form is advocated to be more economical.

जिओट्राईकम कॅन्डीडम– एन.सी.डी.सी. 228 के गामा बी.हेच.सी. अवघटनकारी किण्वक का उत्पादन, संशोधन एवं गुण संधान

## सारांश

कीटानाशको के विस्तृत प्रयोग के कारण उनका दूध सहित मानव भोजन में पाया जाना सार्वभौमिक हो गया है। उपभोक्ता के अच्छे स्वास्थ्य तथा डेरी उद्योग के स्थायित्व के लिए दूध में से कीटनाशकों के अवशिष्ठों का निष्कासन अत्यावश्यक है। जिओट्राईकाम कॅन्डीडम एन.सी.डी.सी. 228 एवं उसका किण्वक गामा–बी.हेच.सी. का जैविकीय निराकरण करने वाला कम खर्च तथा पर्यावरण परक होने के कारण प्रभावी साधन है। फंफूद द्वारा इस किण्वक के उत्पादन के लिए उत्तम परिस्थितियां थी 25° से. पर 7 दिन तक, प्रारंभिक पी.हेच. 6 वाले आलू शर्करा (ग्ल्यूकोज) के शोरबा में अविचल संवर्धन करना। साठ–सत्तर प्रतिशत अमोनियम सल्फेट में लवणीकरण से वृद्धिरस में 10.82 गुणा विशुद्धीकरण हुआ और सेफाडेक्स जी–75 जैल ह्ननन से 18.01 गुणा विशुद्धीकरण हुआ। डायलिसिस और एस.डी.एस. पेज से किण्वक, लगभग 19,500 डालटन पाया गया। प्रतिरोधक में किण्वक की उत्तम क्रिया 25°से.पर वह लगभग 6.5 पी.हेच. पर पाई गई। किण्वक व गामा बी.हेच.सी. के 1:4 परिमाण में उठती हुई सरल रेखा का संबंध पाया गया। किण्वक 20-25°से.पर 10 मिनट तक और 6 पी.हेच. पर 60 मिनट अथवा 6 पी.हेच. पर 10–60 मिनट तक स्थिर रहा। सोडियम, मैग्नीशियम व कैल्शियम से किण्वक क्रिया में बढ़ोत्तरी तथा जस्त तथा लोहे से इसमें ह्ननन हुआ। किण्वक की 25° से. पर के.एम. 29.41 माइक्रो मोल और वी.मैक्स 1.45 माइक्रो मोल थी। ज्ञानेन्द्रियप्रभावक, टी.टी.सी., नीला मिथाइलीन अवघटन समय और अम्ल उत्पादन परीक्षणों ने संकेत दिया कि किण्वक दूध में अति सक्रिय था और इसने अशुद्ध किण्वक (वृद्धिरस) द्वारा प्रतिरोधक में गामा बी.हेच.सी. के समान ही दूध में कीटनाशक का अवघटन किया। यह दूध में 37 से. पर भी पूर्णरूपेण क्रियाशील था। इसे और भी कम खर्चीला बनाने के लिए इस विषहीन प्रोटीन का अविचलीकृत दशा में उपयोग करने का परामर्श दिया जाता है।

# CHAPTER 1

# Introduction

# 1. INTRODUCTION

India has made impeccable strides in all walks of life in the post independence era. From carrying begging bowl around the world our motherland has attained not only self-sufficiency but she has also achieved the status of having surplus grains and other foods. A number of factors have contributed to this state of enormity. Among these, the role of pesticides can not be minisculed in bringing about these 'green revolution' and 'white revolution'. Paul Mueller discovered in 1939 that DDT synthesized 65 years ago by Ziedler possessed insecticidal properties. This information attracted the workers over the globe and thus ushered the era of use of chlorinated hydrocarbons and subsequently the related chemicals as insecticides etc. These man-made synthetic chemicals proved highly successful for the purpose these were aimed at.

Pesticides along with many other synthetic chemicals are covered under a broad term 'xenobiotics'. International Dairy Federation (1997) have categorised 'pesticides' as any substance intended for preventing, destroying, attracting, repelling or controlling any pest including unwanted species of plants or animals during the production, storage, transport, distribution and processing of food, agricultural commodities or animal feeds or which may be administered to animals for the control of ectoparasites. These include insecticides, herbicides, fungicides, rodenticides, nematicides, chemosterilents, molluscicides, plant growth regulators, defoliants, desiccants, attractants and repellants. The term normally excludes fertilizers, plants and animal nutrients, food additives and animal drugs.

The crop losses by pests are common all over the world. In India, the crop losses range from 10 to 30 per cent under normal ecological situation. The maximum losses occur in oilseeds (25%), followed by cotton (22%), rice (18.6%),

sugarcane (15%), wheat (11.4%), sorghum (10%) and pulses (7%). The losses have been estimated to be of the order of 29 billion rupees annually.

The consumption of pesticides in the country is obviously the maximum among the South-Asian nations. During the last few decades this has increased by 500 times from 154 tonnes in 1953 to 75,000 tonnes in 1993-94. Out of this huge quantity of pesticides 66.5 per cent is used on cotton and rice crops only. The three commonly used pesticides in India, viz., hexachlorocyclohexane (γ-BHC), DDT and malathion account for 70 per cent of the total pesticide consumption for various purposes. These pesticides are still being preferred by small farmers because these are cost effective, usually easily available and display a wide spectrum of antibioactivity.

Being true to the universal dictum that every worldly thing has both the aspects – good and bad, the xenobiotics started showing their ill effects in the nineteen fifties. Their boon of being persistent started becoming bane to humanity. Among the synthetic organic insecticides which are small molecules of 200 to 400 daltons, are organochlorines (OC), organophosphates (OP) and organocarbamates (OCM) and other groups. The foremost compounds comprise 38 per cent and are the are most persistent and bioaccumulative because of their very slow metabolic removal while OP and OCM have acute toxicity but lesser persistence. The OC pesticides are not only persistent but also lipophilic and, therefore, accumulate in the plant and animal tissues (Stan, 1990). HCH and DDT can remain stable for years in the temperate and very cold undynamic soil conditions. Their occurrence in foods and feeds is undesirable and hence is termed 'contamination'. According to IDF (1997), 'pesticide residues' means any specified substance in food, agricultural commodities or animal feed resulting from the use of pesticides. The term includes any derivatives of pesticides, such as conversion products, metabolites, reaction products and impurities considered to be of toxicological significance.

Residues of organochlorine insecticides including γ-BHC and DDT enter the food chain through various sources and get stored in fatty tissues. Lactating animals get rid themselves of these residues by secreting them in milk besides normal metabolic excretory channels. Having natural affinity for lipoid material, their occurrence in milk is obvious and in this way they affect the growth and biological activity of beneficial dairy microorganisms (IDF, 1990). In milk and milk products, which form a major chunk of total intake of food, γ-BHC has exceeded the permitted maximum residue limits in dairy products in recent years. The excretion of these insecticides in Indian milk and milk products ranges from up to about 6 ppm in infant foods to 61 ppm BHC in bovine milk and from up to 4 ppm to 216 ppm DDT, respectively. Human milk is no exception with 28 ppm of BHC and 102 ppm DDT residues (Wadhwa, 2000).

These residues cause allergies and gastrointestinal problems etc. Pesticide residue problems have assumed terrifying proportions as is evident from plethora of reports of serious dangers these pose, especially to young children, neonates and suckling ones who are more frequently exposed and are in vulnerable physiological conditions. The deleterious effect of the insecticide on useful microorganisms also is of no less magnitude. Dairy starter cultures are adversely affected as non-target organisms. This impact not only affects product quality but also disturbs factory schedule, thus causing huge nutrient and economic losses. Therefore, it is necessary to remove these residues from food and feeds for better human and veterinary health and sustenance of dairy industry. Lindane, named after its discoverer, is the γ-isomer of HCH. It is a chlorination product of benzene and also is an important broad spectrum pesticide which has been showing increased residues. It is, therefore, one of the pesticides the use of which has been restricted in India with effect from 31.12.1992. Consequently, the use of lindane formulations generating smoke for indoor use has been prohibited, provided that it may be continued to be produced in the country for use in controlling the insect

pests of field crops and subject to modification in the Certificate of Registration to this effect.

Many bacteria and fungi like species of *Aerobacter* (i.e. *Enterobacter*), *Bacillus, Citrobacter, Clostridium, Escherichia, Lactobacillus, Pseudomonas, Aspergillus, Geotrichum, Saccharomyces, Trichoderma* etc. have been reported to be potential degraders of γ-BHC (Heritage and MacRae, 1977; Ohisa *et al.*, 1980; Lal and Saxena, 1982; Manivannan, 1997; Mittal, 1999). In this direction, work done at NDRI, Karnal has revealed that among these organisms, a milk cream isolate strain No. NCDC-228 of the popularly known milk mould *Geotrichum candidum* held a great promise for effectively reducing γ-BHC levels in milk.

The studies further showed that some extracellular enzyme produced by this organism actively degraded lindane. This potentiality opens new avenues for exploring the use of either the organism or its enzyme on an industrial scale for decontamination of foods from BHC. However, so far no systematic study has been reported on the production, characterization and utilization of such dairy microbial enzymes useful for decontamination of food including milk and milk products from γ-BHC.

Keeping in line with this existing information, the present study was undertaken to produce, purify and characterize the extracellular enzyme from *G.candidum* NCDC-228. The following objectives were, therefore, envisaged.

1. Laboratory scale production and purification of γ-BHC degrading enzyme from *Geotrichum candidum* NCDC-228.
2. Characterization of the purified enzyme preparation.
3. Evaluation of the purified enzyme in reducing γ-BHC levels in milk.

# CHAPTER 2

# *Review of Literature*

# 2. REVIEW OF LITERATURE

The xenobiotic era started during the early 1940s and the abusive implications of use of these chemicals started occurring during the 1950s. Their consistent presence in foods (i.e. contamination) has become a matter of concern and has attracted not only the scientists but also the public at large.

## 2.1 SOURCE OF CONTAMINATION

Organochlorine pesticides (OCP) being lipophilic in character tend to accumulate in fatty tissues of milch animals and occur in their milk because of its high fat content. Their residues reach the lactating mammary glands from various sources such as fodder (pasture, forage and supplement), environment (water, air and soil), insect control practices and accidents (IDF, 1990). The possibilities of contamination of milk and milk products with BHC (all the four isomers, viz., $\alpha$, $\beta$, $\gamma$ and $\delta$) are mainly through fodder and feed contaminants. HCH and DDT contents were found in most of samples of soil, fodder and feed ingredients, the levels were as high as 47 ppm for HCH and 0.46 ppm for DDT in gram chuni (Sandhu, 1980).

A large quantity of HCH is used on rice crop for controlling insect pests which persist on straw and grain till harvest through storage and consumption. When milch animals are fed on this contaminated straw, the insecticide residues (OCPR) get accumulated in adipose tissues and also pass into milk in case of lactating animals. Rice bran which, thus, becomes a rich source of HCH residues is used as an ingredient in the ready-made feed concentrates. Contaminated feed concentrate passes these BHC residues to milch animals (Agnihotri, 1999). Feeding cows with BHC treated straw and rice has been reported to result in highly contaminated milk (Battu *et al.*, 1989).

In a study, out of 137 milk samples analysed from Orissa University of Agriculture and Technology (OUAT), Bhubaneswar, all except ten were found to be contaminated with residues of HCH (mean values of residues being < 0.01–0.568 mg/kg. OUAT also observed that in case of feed concentrates, most of the 377 samples analysed contained HCH (mean value < 0.01 to 2.27 mg/kg) milk (Agnihotri, 1999). The amount of residues of HCH present in straw and feed in almost all samples from different countries were above the prescribed limits. Thus, it was assumed that the straw was a potential source of HCH residues in milk. Many other studies also confirmed that the contamination with OCPRs in milk was mainly through feed (Vandenhoek *et al.*, 1975; Verman *et al.*, 1977).

Pesticide residues have been detected in extensively cultivated layers of arable soils (Marth, 1965; Edward, 1966; Gunter, 1980). Soil is considered to be one of the principle reservoirs of pesticides due to direct application of HCH, aldrin etc. to soil for control of termites and other soil borne pests. Aerial spray of pesticides on crops and farm yard manure applied to soil can also be one of the sources of pesticides' entry into the soil. Irrigation water, too, adds to their accumulation in soil. Based on the type of pesticide OCPRs accumulate and persist in soil for very long time. Technical grade aldrin, chlordane, endrin, heptachlor, hexachlorocyclohexane and toxaphane were observed to reach 40, 40, 41, 16, 10 and 45 ppm, respectively, over a period of 14 years. However, accumulation of purified aldrin and commercial grade of corresponding pesticides are less than that of technical grade (Nash and Woolson, 1967).

The concentration of β- and γ-isomers of BHC was more in whole grain as compared to deoiled rice bran and oil meals because of their more accumulation in the lipid fractions of feed. It was further observed that the levels of β- and γ-isomers were significantly lower in the forages (Prasad and Chhabra, 2000). Bhattacharjee (1996) reported very high content of OCPRs (HCH – 488.78, DDT – 93.20, endosulphan – 34.81, heptachlor epoxide – 46.64 and aldrin –162.26 ppm

on fat basis) in the concentrate cattle feeds. Less HCH residues were detected in pigeon pea fodder fed to cow in Gujarat, but the high levels in their milk indicated that the contamination also occurred from other sources of feed such as vegetable by-products (Raj *et al.*, 1989). Appearance of OCPs in milk may also have been due to grazing of animals on contaminated pastures. Kapoor and Kalra (1988, 1993) reported that in lactating buffaloes the intake of DDT ranged from 0.1 to 2.5 mg/day whereas that of total HCH ranged from 0.3 to 3.2 mg/day when fed on normal green fodder and concentrate diet.

All the feed ingredients analyzed were contaminated with OCPRs in spite of the fact that the use of predominant organochlorine pesticides such as BHC and DDT has been banned in India. In general, the order of contamination was BHC > endosulphan > heptachlor > DDT > aldrin. The per cent incidence of BHC in feeds was in the order of concentrate mixture > maize > wheat bran > rice bran > cottonseed cake > barley > mustard cake. Among the roughages the higher content of BHC was in maize fodder followed by oats, sorghum, berseem, wheat straw, lucerne and paddy straw in the decreasing order (Prasad, 1998).

Concentrated mixtures revealed high contents of BHC because of high level of contamination of their formulating ingredients, viz. maize, groundnut cake and wheat bran (SRS/NDRI, 1996). Higher content of BHC in rice bran and groundnut cake has also been reported earlier (Battu *et al.*, 1989; Ramesh *et al.*, 1989, 1990, 1991). Sandhu (1980) also has observed that most of the ingredients of cattle feeds were contaminated with BHC. More than 80 per cent pesticides consumed in India are being used on four major crops, viz., wheat, rice, cotton and jowar. Therefore, higher content of BHC in wheat bran and rice bran might be due to excessive use of pesticides on the above crops. The contamination with BHC was because of its intensive use obviously due to its low cost, long persistence and broad spectrum action on pests. Not only the agricultural, but also the health, sanitary and industrial applications are responsible for the residues of

pesticides to occur in milk. Contamination of milk with pesticides may arise from the use of pesticides for control of insects as mosquito repellant, airborne spraying and fumigation in malaria eradication programmes, airborne dust from pesticide factories causing direct atmospheric exposure of animals to these pesticides (Kapoor *et al.*, 1980; Breylona *et al.*, 1986; Kapoor and Kalra, 1988; Battu *et al.*, 1989; Kaushik *et al.*, 1991). The interaction of pesticides with the environment and consequently the various sources of contamination of milk and milk products and other foods with the residues are summarized in Fig. 2.1, under the caption 'the vicious pesticide cycle'.

## 2.2 CARRY OVER AND METABOLISM

After absorption of organochlorine pesticides (such as BHC, DDT) either through fodder, soil or cutaneous contact, these lead to the liver directly or indirectly where these are slowly metabolized. After passing through liver they again pass into circulatory system. Due to lipophilic nature, they are stored in the fat cells of animals. The organochlorine pesticides get deposited in internal organs and notably in the adipose tissues. Studies on the tissues of dairy cattle, sheep, pig, rat, mouse and poultry have indicated the occurrence of pesticide deposits in their body tissues. Lindane is rapidly metabolized to a number of polar compounds by cows, mice and rats. Dechlorination and hydrolysis of HCH to water soluble trichlorophenols occurs by the action of enzyme S-acyl-glutathion transferase in warm blooded animals (Bridges, 1960; Fries *et al.*, 1969; IDF, 1990).

## 2.3 EXCRETION IN MILK

Organochlorine pesticide residues are lipophilic in nature and relatively stable. Most of the OCPRs and their metabolites are readily excreted in the milk fat. Lactating females excrete pesticide residues by the natural excretory systems obviously more rapidly than their males. The rate of excretion of OCPRs into milk depends on the stage of lactation, the quantity of milk fat produced, breed of

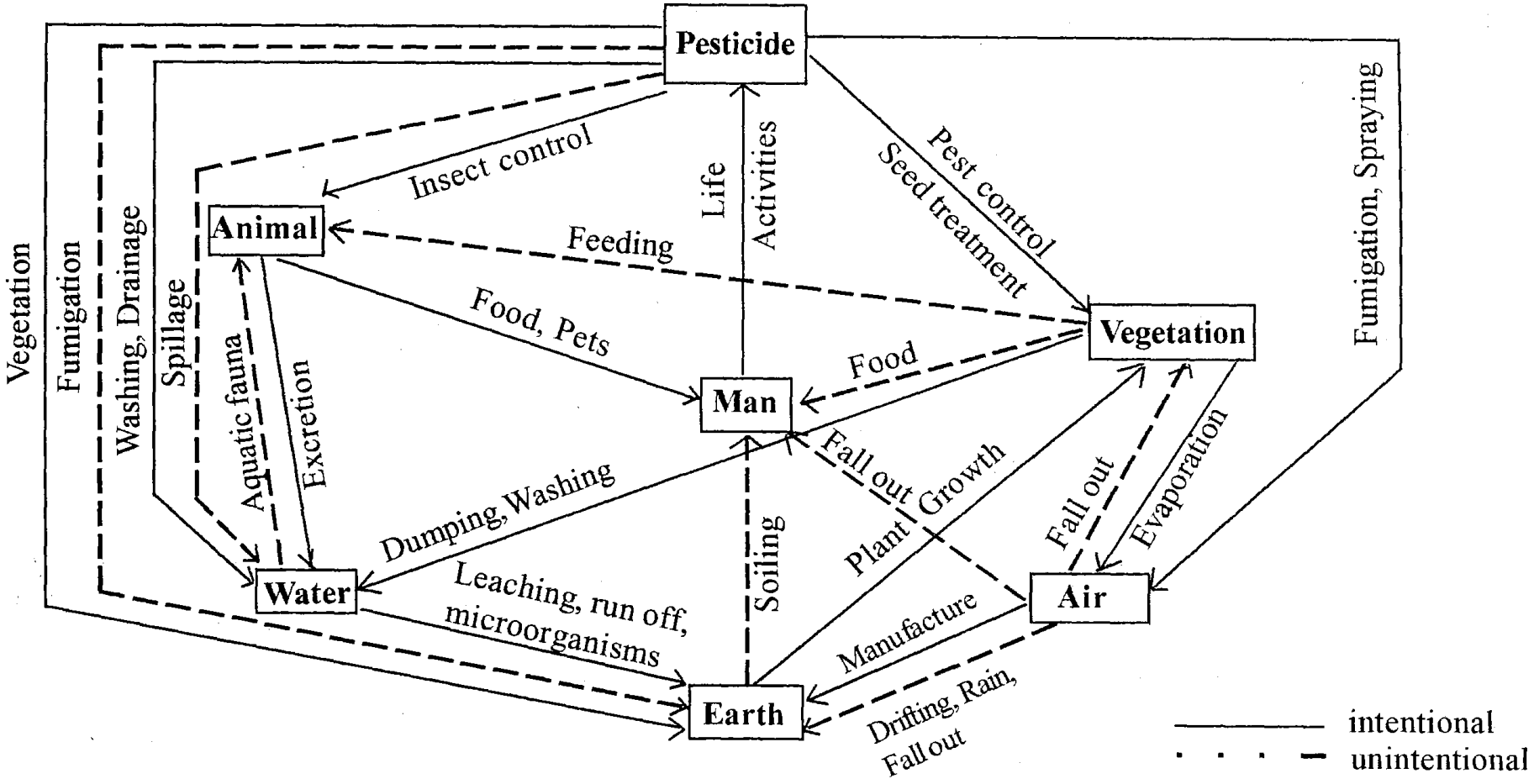



**Fig.1 THE VICIOUS PESTICIDE CYCLE**

the animal, nature of the pesticide, its amount consumed, the duration of exposure and previous history of exposure to pesticides (Bruce *et al.*, 1965; Demott *et al.*, 1966; Verman *et al.*, 1977).

## 2.4 RESIDUES IN MILK AND MILK PRODUCTS

The status of residues of organochlorine pesticides (OCPR), as surveyed in European countries has shown that the contamination of milk in general was very low than the MRL (IDF,1990). Lindane and DDT were detected in 47 to 82 per cent of cow milk in Turkey (Konar, 1982). The result of an Austrian programme of 1985-1988 to monitor pesticide residues in milk revealed the universal presence of HCH, mostly α-HCH and γ-HCH (Puchwein *et al.*, 1990). Bellisai *et al.* (1988) observed that food samples of animal origin, mainly cow milk, exceeded Italian legal limits for organochlorine content in these. Lindane in Egyptian buffalo milk was found to be in the range of 0.01 to 0.03 ppm (Sania *et al.*, 1983; AbdAlla *et al.*, 1991), while Breyl *et al.* (1990) reported that Czechoslovakian milk, butter, cream, ewe milk and cheese contained 0 to 1.66 mg lindane per kg fat. Garrido *et al.* (1994) observed that 90 per cent of the Spanish sterilized milk samples were contaminated with OCPRs. They found that 89.9 per cent of the samples were contaminaed by one or more HCH isomers.

In India also the situation is alarming as reported by various workers. A monitoring survey conducted in Gujarat, India revealed that all samples of butter and ghee were contaminated to some extent with isomers of HCH and DDT. Out of 52 ghee samples, 6 exceeded the permitted maximum residue limit (MRL) of 0.2 fg/g of HCH (Shah *et al.*, 1992). Residues of HCH were measured in 100 samples of milk (50 bovine, 50 human) and milk products (butter and baby food milk powder) collected from Hisar. Among these, 15 bovine milk samples were contaminated at a higher rate (i.e. above MRL). The contamination of baby milk powder was very high. Mukherjee and Gopal (1993) reported that DDT and HCH residues in milk and milk products in Delhi often exceeded the limit prescribed by

FAO/WHO Expert Committee, the levels ranging from 0.022 to 0.166 μg/g for DDT. Kumar *et al.* (1991) found that out of five commercial brands of baby milk formula, three showed DDT and all had HCH residues which were above the safe limits. Gupta *et al.* (1997) examined 9 bovine milk and 6 commercial dried milk samples, out of which five milk samples contained α- or δ-HCH residues above MRL. Other milk products – milk powder, dairy whitener, butter, ghee, cheese etc. were also not free from OCPRs (Awasthi and Ahuja, 1995). In India, the work done since 1972-to-date on the incidence of organochlorine pesticide residues in milk and milk products has revealed the presence of DDT and BHC in most of samples above maximum residue limit (MRL) and has been depicted in Table 1.

**Table 1. Residue levels of two important pesticides in milk and milk products in India**

| Product | DDT (ppm fat basis) | BHC (ppm fat basis) |
|---|---|---|
| Market milk | 0.01 – 216 | 0.02 – 61 |
| Human milk | 1.4 – 102.02 | 1.25 – 27.52 |
| Butter | 1.6 – 11.36 | 0.55 – 1.51 |
| Ghee | 0.25 – 7.24 | 0.30 – 23.80 |
| Infant formula | 0.17 – 4.30 | 0.12 – 5.70 |
| Skim milk powder | 0.80 – 1.24 | 0.43 – 0.78 |

## 2.5 ISOLATION AND ESTIMATION METHODS OF OCPRs IN MILK AND MILK PRODUCTS

Before branding a milk or milk product to be unacceptable with regards to the pesticide content in it, it has to be analysed for the same. The isolation of OCPRs from milk and milk products involves primarily the (i) extraction of fat containing fat soluble OCPRs and (ii) enrichment and clean up by removal of fat

and other interfering substances. The detection quantities of residues are expressed as μg/g or mg/kg (ppm) and ng/g or μg/kg (ppb).

### 2.5.1 EXTRACTION

Extraction of pesticides from foods into appropriate solvent is the first step in the analysis of pesticide residues and it is essential for their maximum quantification. The solvent selection depends on the polarity of the insecticide, nature of food samples etc. IDF (1991) have described soxhlet extraction for all milk products and AOAC method for milk and liquid products. Muccio (1998) developed a rapid procedure that allowed a single step selective extraction of organochlorine pesticides by means of acetonitrile saturated light petroleum. Praparontol and Stevenson (1991) used one step solvent extraction of milk with ethylacetate-acetone-methanol by ultrasoniccation. A technique was developed for estimation of HCH isomers and DDT with the help of petroleum ether and acetonitrile (Romanian Standard, 1987). Jagannath (1996) and Manivannan (1997) have used petroleum ether for extraction.

### 2.5.2 CLEAN UP

Clean up involves the removal of impurities from the desirable OCPR content. As a result, the insecticides get concentrated (enriched) also in the final test portion. Two different approaches have been documented for clean up of the extracted OCP residues–column chromatography clean up and acid clean up. Column chromatography methods reported include liquid-liquid partitioning with ether acetonitrile and clean up on a florisil column (Horwltz, 1975), or with dimethyl formamide (DMF) and clean up on alumina or florisil column (DeFaubert Maunder *et al.*, 1964; Specht, 1974). Some other columns used include aluminium oxide (Telling *et al.*, 1977; Greve and Grevenstuk, 1975), partially deactivated silica gel (Steinwandter, 1980) along with gel permeation chromatography. All these methods have been recommended by IDF (1991).

These column chromatographic clean up methods have some limitations like consumption of large volume of costly solvents and adsorbents, large time taken for evaporation of these solvents. As an alternative, Veierov and Aharonson (1978) described a simple acid clean-up procedure using concentrated sulphuric acid for enrichment of OCPRs. The same workers in 1980 further developed a process to extract fat and also to clean up the OCPRs from the extracted fat simultaneously (Veierov and Aharonson, 1980).

### 2.5.3 ESTIMATION

To assess the effects of a pesticide on living systems, the compound has first to be identified and its residue quantified in the incriminated food. The methods used to analyse and estimate such residues range from relatively less precise conventional methods to the modern techniques like spectrophotometric methods, thin layer chromatography (TLC), gas liquid chromatography (GLC), high pressure liquid chromatography (HPLC), gas chromatography mass spectrometery (GCMS) and liquid chromatography mass spectrometery.

Stiff and Castillo (1945) colorimetric method for OCPRs has been utilized for residue analysis more frequently. AOAC (1970) have described a colorimetric method for BHC analysis in which BHC was separated and extracted with carbon tetrachloride. It was finally converted into m-dinitrobenzene which gave magenta colour with butanone and alkali which could be measured at 565 mμ. But this method lacked sensitivity.

Thomson (1970) described a method of TLC using silver nitrate incorporated alumina G coated glass plates with 1 per cent n-hexane. Vishweshwaraiah and Jayaram (1973) developed a micro TLC method for BHC using tolidine. High performance thin layer chromatography with automated multiple development (AMD-HPTLC) was described by Burger (1984).

Wessel (1967) performed a collaborative study for the analysis of multiple residues of OCP and OPP in non-fatty and fatty foods. Gas chromatography (GC) with electron capture detector (GC-ECD) and GC-potassium chloride thermionic detector (KClTD) was used for analysis of OCPRs and OPPRs, respectively. Luke *et al.* (1975) developed a multi-residue method for isolating organophosphates and organocarbamates which were quantified using KClTD. Luke *et al.* (1981) simplified and shortened their earlier method of 1975 and they used Hall electrolytic conductivity detector (ElCD) which could be used in halogen and nitrogen modes for the determination of OC and OCM compounds. IDF (1991) recommended the GLC for separation, identification and estimation of OCPRs in milk and milk products. Holstege *et al.* (1994) developed a multi-residue method for 17 OC, 43 OP and 11 OCM residues. OCPRs were analysed by GC–ECD after florisil clean up. Lehotay and Eller (1995) developed a multi-residue method for analysis of 46 pesticides (11 OC, 21 OP, 3 OCM, 3 pyrethroids and 8 others) in fruit and vegetables by GC ion trap mass spectrometry (GC/ITMS).

The principle of GLC involves use of high temperature to volatilize the pesticides or its derivatives so that separation can be performed. HPLC scores over GLC in that high temperature is not required since mobile phase is liquid and solubility is the limiting factor instead of volatility. Sieber (1974) used HPLC for analysis of DDT, DDE, DDD, dieldrin, heptachlor epoxide with MeOH : water as solvent system. Brinkman *et al.* (1976) and Dolphin *et al.* (1976) analyzed a number of organochlorines like o,p' DDE, p,p'DDE, DDD, DDT, aldrin, heptachlor, BHC ($\alpha$, $\beta$, $\gamma$ and $\delta$), HCB, dieldrin by HPLC using hexane as mobile phase. Aitzet-Mueller (1995) have also used HPLC for detection of such organochlorines. Application of HPLC in separation of pesticides has been reviewed by Lawrence and Turton (1978) and Sharma (1995).

## 2.6 BIOLOGICAL ACTIVITY OF OCPs

The presence of extraneous substances always affects even the non-target organisms selectively or inadvertantly in a given ecosystem, may be, adversely or beneficially. Xenobiotics, in general, and pesticides, in particular, are no exception to this rule. Hence, pesticides affect both prokaryotes and eukaryotes or plants and animals although the quantum and nature of the effect may vary.

### 2.6.1 EFFECT ON ANIMAL SYSTEM

World was attracted to the pesticide residue problems because of the initial ill effects on man. The inherent toxicity of a pesticide to man is the same regardless of its use, either in agriculture or vector control practices and mode of application and hence internalization, assimilation and acquisition by topical contact, inhalation or when it is consumed as a residue in food. The difference in untoward effect might occur due to the difference of route of its acquisition and extent of exposure, which influence the dose absorbed that is most decisive in producing an ill effect. Organochlorine pesticides (γ-HCH, DDT etc.) are more persistent in the environment than other synthetic organic pesticides. These are efficiently absorbed by the gastrointestinal tract, but some are also absorbed through intact skin on dermal exposure. Although their mechanism of action is not yet fully understood, the major toxicity of this group of chemicals is thought to be related to the nervous system, both central and peripheral – they act by altering the electrophysiological properties of all membranes (particularly nerve axons) disturbing sodium and potassium ion exchange through membrane (MacRae *et al.*, 1993). The acute toxic effects of chlorinated insecticides become apparent minutes to hours after the relevant dose has been absorbed / assimilated. Prominent among those showing acute poisoning symptoms are neurological phenomena such as vomiting, paraesthesia, disturbance of equilibrium, generalized toxic chronic cramps. Sub-chronic and chronic toxicity shown in

warm blooded animals includes marked histopathological changes in vesiculated organs like liver, kidney and brain. Long term effects like damage to peripheral nerves and blindness as well as tumorogenic, gonadotropic, teratogenic and developmental may also occur (IDF, 1990). HCH and DDT at higher doses have been described as potential carcinogens (Levin *et al.*, 1974; Epstein, 1975; Bhatt, 1983). Nagasaki *et al.* (1972) reported that BHC also produced liver tumors in rodents. Alteration in oestrous cycle, reduction in the number of pregnancies and other impairments resulting in poor reproductive performance, decrease in litter size or even sterility has been reported in mice and rats exposed to various chlorinated pesticides like HCH, aldrin, dieldrin and chlordane. These have now been reported to cause imbalance in human sex ratio in that the young males of below 20 years on consistent exposure to these pesticides not only may lose fertility but also become incapable of producing males.

### 2.6.2 EFFECT ON ENVIRONMENTAL MICROORGANISMS

World attention was also drawn to the residual pesticidal effects on certain desirable microorganisms. The nature and mode of action of a pesticide on microorganisms depends upon the nature of pesticide, its concentration, its interaction with other chemicals in the environment, the type of microorganisms and the microcosm. Several workers have studied the toxicities of organochlorine insecticides towards microorganisms and it has generally been reported that these chemicals restricted the microbial growth. Lal and Saxena (1982) in their review have observed that organochloro pesticides in general reduced the microbial growth although some dissenting reports are also on record. Richardson and Foster (1966) demonstrated that γ-BHC, which has a relatively high water solubility (6 to 7 ppm) was most toxic at supersaturation (25 ppm) to *Rhizoctonia solani* in plate cultures. BHC at concentrations as low as 0.001 to 0.5 and up to 100 ppm decreased the cell number of *Tetrahymena pyriformis* significantly (Geike and Prasher, 1976). A number of other organisms including soil process bacteria,

yeasts, moulds, algae etc. are ill affected in this range of the insecticide. However, in contradiction, some such organisms have not been shown to be affected at all (Lal and Saxena, 1982). In an earlier study, Kumar (1972), Kumar *et al.* (1973) and Aggarwal *et al.* (1973) had observed that different xenobiotics affected soil microbiological processes and milk standardization tests differently. Albene *et al.* (1972) found that DDT at 100 ppm in agar consistently reduced counts of both aerobic and anaerobic bacteria cultured from mud. In loams, DDT reduced the number of bacteria whereas the number of fungi was increased indicating that bacteria are more susceptible to the ill-effects of these pesticides (Stojanvic *et al.*, 1972). The general inhibitory range of DDT was 0.001 – 425 ppm for different organisms, whereas some organisms were not affected even at higher concentration (Lal and Saxena, 1982). With regard to cytological and biochemical effects on microorganisms, organochlorine insecticides affect one or more sites depending upon the type of microorganisms. The major targets which are susceptible to DDT and related insecticides are cell membranes, enzymes and nucleic acids. Lethal action of DDT in *B. subtilis* was related to binding of DDT to membrane (Hicks and Corner, 1973) by disturbing its lipid composition. Organochlorine pesticides were found to alter the structural integrity of the membrane in such a way that it became more permeable to certain amino acids while inhibiting the transport of many others. Organochlorine insecticides also affected the synthesis of nucleic acids. In *Crithidia fasciculata*, DDT (425 ppm) inhibited the uptake of [$^3$H] thymidine and [$^3$H] uridine, thereby inhibiting the synthesis of both DNA and RNA by interference with complex regulation of transport of the precursors (Anonymous, 1976). In photosynthetic algae, *Dunaliella binoculata* and *Amphidinium carteris*, γ-BHC inhibited cell division and the synthesis of deoxyribonucleic acid and ribonucleic acid (Jeanne-Levain, 1979). BHC at 0.001 ppm did not affect the activity of δ-aminolevulinate dehydrogenase, hexokinase and pyruvate kinase, whereas it stimulated the activity of glutamic dehydrogenase, isocitrate dehydrogenase and malate dehydrogenase

(Geike and Prasher, 1978). Increasing its concentration to 0.1 ppm increased the activity of almost all of the enzymes except alanine aminotransferase. BHC at 1 ppm concentration inhibited the activity of almost all of the enzymes.

### 2.6.3 EFFECT OF BHC ON LACTIC ACID BACTERIA

BHC directly and adversely affected the growth and activity of *Streptococcus lactis* (*Lactococcus lactis*) in both cow and buffalo milk at all levels of concentration, *S. diacetylactis* (*L. lactis* subspecies *diacetylactis*) was more sensitive than *S. lactis* (Sharma, 1994). She also noticed that *S. thermophilus* was most resistant organism to BHC residues in both cow and buffalo milk and the growth of the starter culture was in general not adversely affected by lower pesticide concentration indicating that the organism was not suitable for TTC test to detect low levels of BHC. She further noticed that maximum adversely affected organism with respect to acid production was *S. diacetylactis*, whereas *S. thermophilus* was the least affected. Abdou *et al.* (1983) noted that > 2 ppm of BHC inhibited the growth of *S. lactis*, *S. cremoris* (*L. lactis* subsp. *cremoris*) and acid production by them. Sania *et al.* (1983) found that lindane at a strength of 0.01 to 0.03 ppm delayed milk coagulation for cheese making.

## 2.7 EXCLUSION OF XENOBIOTICS FROM FOODS

Man strives for not only producing sufficient food but also for good, wholesome and healthy food. Pesticides, on the one hand, have helped man immensely to produce large quantity of food but, on the other hand, these have become a great concern because of the deleterious effects of their residues. It, therefore, has become imperative to free the foods from such chemicals if the latter have gained access into the former. A number of biotic and abiotic natural and artificial methods have been devised to attain this goal.

### 2.7.1 NON-BIOLOGICAL DEGRADATION OF LINDANE

Li *et al.* (1970) found that direct steam injection vacuum treatment and sterilization of milk resulted in 90 and 22 per cent reduction in level of BHC but pasteurization and boiling of milk caused no such reduction. The latter workers also concluded that there was no effect of fermentation on reduction of lindane. Processing of cream to butter had no effect on BHC residues (SRS /NDRI, 1996). Rachev *et al.* (1974) found a high level of reduction, to the extent of 43.7 per cent, in BHC residues in milk on treatment with hydrogen peroxide at room temperature for 15 minutes. But Nath *et al.* (1997) found no effect of hydrogen peroxide treatment and pasteurization of milk on its BHC residues. Prasad and Chhabra (2000) observed a reduction in pesticide excretion in milk following the incorporation of charcoal in the diet of lactating animals at the rate of 1 g/kg body weight. They found that excretion of HCH, DDT, endosulphan, heptachlor and aldrin through milk was reduced to the level of 40.93, 21.77, 33.43, 50.04 and 31.12 per cent, respectively after the addition of charcoal in the animal diet.

### 2.7.2 MICROBIOLOGICAL DEGRADATION OF LINDANE

Gamma-HCH biodegradation has been widely reported in the literature. Its rapid disappearance has been documented from soil, aquatic environments and in the laboratory. This has been attributed to its susceptibility to degradation by microorganisms (Meksongsee and Guthrie, 1965; Yule *et al.*, 1967; MacRae *et al.*, 1969). Meksongsee and Guthrie (1965) have reported the degradation of lindane by *Aerobacter aerogenes, Bacillus cereus* and *B. megatherium* while such capability of *E. coli* has been reported by Vonk and Quirjns (1979) and Manivannan, 1997). Jagnow *et al.* (1977) screened different bacterial species like *Bacillus alvei, B. brevis, B. cereus, B. circulans, B. coagulans, B. laterosporus, B. lentus, B. licheniformis, B. macerans, B. polymyxa, Citrobacter freundii, Clostridium butyricum, C. pasteurianum, Enterobacter aerogenes, E. cloacae, Escherichia coli, Lactobacillus brevis, L. casei, L. plantarum, Leuconostoc*

*dextranicum, L. mesenteroides, Propionibacterium shermanii, Proteus mirabilis, P. vulgaris* and *Serratia marcescens,* for their ability in γ-BHC degradation. They found that members of Bacillaceae, *Clostridium* species and Enterobacteriaceae actively degraded γ-HCH, while representatives of Lactobacillaeceae and *Propionibacterium* were inactive. *Bacillus* and *Pseudomonas* sp. were able to degrade γ-BHC (Tu, 1975) while cell free extracts of *C. rectum* degraded lindane in presence of DTT. Microbial degradation of hexachlorocyclohexane (γ-HCH) isomers has been observed to occur more rapidly in flooded than in upland soils (MacRae *et al.*, 1967; Yoshida and Castro, 1970; Kohnen *et al.*, 1975; Siddaramappa and Sethunathan, 1975). Among different microorganisms, emphasis has mostly been on *Clostridium* and *Pseudomonas* and the species of the latter genus have been extensively studied. *P. fragi, P. paucimobilis* and *P. putida* strains have been reported to degrade BHC alongwith DDT to varying degrees (Matsumura *et al.*, 1976; Imai *et al.*, 1992; Singh and Kahlon, 1992; Manivannan, 1997). A *Pseudomonas sp* isolated from sugarcane rhizosphere soil, readily metabolized alpha, beta and gamma isomers of hexachlorocyclohexane added at an initial concentration of 28 μM to mineral salts medium as the sole source of carbon. γ-HCH disappeared completely within 28 h after inoculation of the bacterium into the medium under aerobic conditions, and there was a concomitant release of chloride ions almost in stoichiometric amounts which was measured colorimetrically by the method of Bergmann and Sanik, 1957; Sahu *et al.*, 1990). Among the other organisms, anaerobic *Clostridium* species have also attracted the workers as many have reported these bacteria to breakdown BHC. Certain members of this group, e.g., *C. sphenoides* (Heritage and MacRae, 1977) and *C. rectum* (Ohisa *et al.*, 1980) and some unspecified *Clostridium* (Ohisa and Yamaguchi, 1978) isolated from paddy fields etc., have been prominent. Katayama(1993) found that culture filtrate of *Trichoderma harzianum* could degrade BHC. Singh and Kahlon (1992) made an attempt to immobilize *P. putida* P1 cells in alginate and agar for effective degradation of lindane compared with

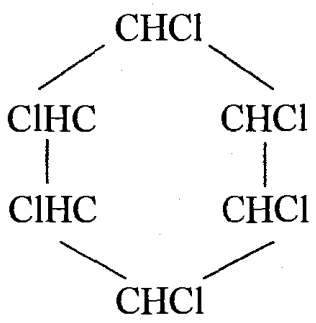
that obtained by use of free cells. The organism utilized lindane as sole source of carbon and energy and resulted in the release of chloride ions and accumulation of degradation products in the medium. The phenomena were faster under anaerobic conditions in a fermenter than the use of immobilized and free cells in a batch reactor. Later on, Kahlon *et al.* (1996) reviewed the use of immobilized cells of yeast, bacteria and algae to exclude lindane from water and observed that the process could result in 29 to 57 per cent loss of lindane.

### 2.7.3 REDUCTION BY DAIRY RELATED ORGANISMS

A number of dairy related organisms (bacteria, yeasts and moulds) also have been observed to degrade BHC, e.g., *Saccharomyces* species (Prusova *et al.*, 1976), *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Geotrichum* sp. Shaker *et al.* (1988) noted a whopping up to 92 per cent degradation of lindane after 96 h incubation at 110 ppm level in broth by yoghurt culture. However, Zidan *et al.* (1990) could observe only 2 per cent lessening of lindane in yoghurt. γ-BHC residues were brought down by 15 per cent in milk containing *Lactobacillus delbrueckii* subsp. *bulgaricus* grown for 24 h. There was a substantial decrease of 55 per cent when culture was further incubated for 48 h. In milk, the BHC level was reduced by 49 and 77 per cent after 24 and 48 h, respectively. *Pseudomonas fragi* caused 61 per cent loss in incidental BHC level of milk after 24 h (Jagannath, 1996). Kaushal (1998) observed that *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp *thermophilus* did not degrade BHC. Manivannan in 1997 found that a milk cream mould isolate *Geotrichum candidum* NCDC-228 was effective in BHC reduction in milk by 85 to 90 per cent. Mittal in 1999 observed that *Geotrichum candidum* NCDC-228 effected a noticeable reduction in BHC content from broth. Ledford and Chen in 1969 observed degradation of DDT and DDE by *Geotrichum candidum*. Out of the two strains, i.e., *G. candidum* 16 AAU and *G. candimum* ATCC-12784, former was more effective in reducing the levels of DDT and DDE.

### 2.7.4 ENZYMATIC DEGRADATION

As a prelude to the degradation studies of any compound, its chemical nature should be known. To relate the enzymic degradation of γ-BHC with the microbial activity *vis a vis* the degradation products obtained the typical chemical nature of the compound needs to be mentioned. In general, chlorinated insecticides are cyclic or condensed organic compounds in which chlorine averages 60 per cent of the molecular weight. Benzene hexachloride (BHC), the chlorination product of benzene is a mixture of various stereoisomers of 1,2, 3, 4, 5, 6 hexachlorocyclo-hexane (HCH).




**Structure of 1, 2, 3, 4, 5, 6 hexachlorocyclo-hexane**

Heritage and MacRae (1977) first reported enzymatic dechlorination of γ-BHC to γ-TCCH (3,4,5,6-tetra-chloro-1-cyclohexane) in *Clostridium sphenoides* in presence of glutathione. The enzyme responsible for dechlorination was associated with membrane fraction and showed more rapid metabolism compared to the soluble fraction. It indicated that the enzyme was intracellular The organism was grown anaerobically at 30°C for 42 h. The membrane preparation showed a more rapid conversion of γ-BHC to γ-TCCH than the cell free extract. The time course of metabolism of γ-BHC revealed that the enzyme involved was fairly stable. But the workers made no attempt to isolate and characterize the enzyme. Ohisa *et al.* (1980) found that cell free extract of *Clostridium rectum* strain S-17 isolated from paddy soil after incubation at 35°C for 9 to 12 h degraded lindane. During degradation of lindane, its products, i.e., γ-TCCH and γ-PCCH

(γ-1,3,4,5,6-penta-chlorocyclohexane) got converted into dichlorocyclodienes which further spontaneously changed into water soluble monochlorobenzene. While studying the degradation of γ-hexachlorocyclohexane by the insecticide-assimilating *Pseudomonas paucimobilis* UT26, Imai *et al.* (1989) found that the initial step of the process by this organism was dehydrochlorination of γ -BHC to γ -PCCH which was further degraded to an unidentified metabolite which the workers named as compound D.

## 2.8 PURIFICATION OF ENZYME

There have been some isolated attempts on production and purification of the enzymes effective in degrading the two important representatives of OCPs. However, the use of microorganisms for the purpose does not find mention in the literature barring only one systematic preliminary study carried on in the NDRI. Mittal (1999) could partially purify the γ-BHC degrading enzyme from *Geotrichum candidum* NCDC-228 using $(NH_4)_2SO_4$ precipitation. The crude enzyme (i.e. culture filtrate) degraded BHC by 24, 37.2 and 37.3 per cent in 4, 8 and 12 h, respectively. After precipitation, the activity of the preparation increased to 69 per cent in 4 h at 25°C. There was no activity at 37°C.

Ishida (1968) and Ishida and Dahm (1965) studied the γ-BHC degrading enzyme isolated from common housefly. He used Sephadex G-25 and G-200 gel filtration for purification of the enzymes which could metabolize a number of chlorine containing compounds like α- and γ-1,2,3,4,5,6-hexachlorocyclohexane, γ-and δ-2,3,4,5,6-pentachlorocyclo-hex-1-ene,2,3,4,5,6-pentachlorbenzyl chloride, 1-chloro-2,4-dinitrobenzene, 2,3,4,5,6-pentachloro-β-nitrostyrene and 1,1,1-tri-chloro-2,2-di-(p-chlorophenyl)-ethene. These enzymes were indistinguishable from one another in their molecular size estimated at 36,000 to 38,000 by using reference proteins. CM-cellulose column chromatography could separate these house-fly enzymes and also those obtained from rat liver homogenate.

## 2.9 GENETIC STUDY OF γ-BHC DEGRADING MICROORGANISMS

*Pseudomonas paucimobilis* UT-26 was capable of growing on γ-HCH and it was very effective in causing degradation of γ-HCH in soil. A genomic library of *P. paucimobilis* UT-26 was constructed in *P. putida* by using the broad-host-range cosmid vector pK513. Out of 2300 clones, 3 clones showed γ-HCH degradation. A 5-kb fragment from one of the cosmid clones was subcloned into PUC118 and subsequent analysis showed that a fragment of ca 500 bp was responsible for conversion of γ-HCH to 1,2,4-trichlorobenzene. Nucleotide analysis revealed an open reading frame (linA) of 465 bp within the fragment. The product of the linA gene was 16.5 kDa as determined by SDS electrophoresis (Imai *et al.*, 1991).

The environmental pollution because of residual xenobiotics is now a universal phenomenon. To ensure ample and good food, the exclusion of these residues has become essential. The inquisitiveness of man makes him constantly devise better, cost effective, convenient and risk free methods to achieve this goal. With this background, use of microbial enzymes is being explored and the present study was proposed as a modest effort to degrade γ-BHC residues from milk (and possibly milk products). As an initial step to this attainment, the production and characterization of the enzyme obtained from an otherwise harmless organism, i.e., *G. candidum* was envisaged in the proposed study.

# CHAPTER 3

# *Materials and Methods*

# 3. MATERIALS AND METHODS

To achieve the objectives underlining the present study, well established procedures, general methodologies, standard cultures, recommended media, quality chemicals, labware and equipment were used. The details of these various aspects are given in the following sections.

## 3.1 GLASS AND PLASTIC WARE

The general laboratory and other glasswares like gel chromatography column (with or without sintered glass discs), separting funnels etc. used were made from borosilicate glass and were procured from standard suppliers/fabricators. Wherever sintered glass columns were not available, glasswool ('Reidal') was used as the supporting material.

Membrane filtration assembly used in the present study was obtained from 'Sartorius' (glass) or 'mdi' (plastic). Similarly, the lab plasticware used in the study was also of standard makes.

The rubber bungs used in dye reduction test were made from good quality material.

Adjustable micropipettes of 20, 50, 250, 500 and 1,000 µl of 'Tarsons' make were used.

Dialysis tubes of cut-off molecular size of 10,000 Dalton (Sigma) were used for enzyme isolation from crude preparations.

The dialyzed enzyme preparation was prefiltered through 'mdi' microglass filters of GFS or $GF_2$ (1.2 – 1.6 µm) grade. Nylon membrane filters of 0.45 µm pore size and 13 and 47 mm dia and 'HNN' or 'SY' type obtained from 'mdi' (M/s. Advanced Microdevices Pvt. Ltd., Ambala Cantt.-133 001).

## 3.2 EQUIPMENT

Apart from general laboratory equipment, other special equipment used in the present study included the following:

(a) Electronic balance

(b) UV-visible spectrophotometer (Spectronic–21D 'Milton Roy')

(c) Refrigerated Centrifuge

(d) Shaker (Omni and Wrist shaker)

(e) Electrophoresis unit ('Biometra' Power Pack P25)

(f) High Pressure Liquid Chromatography HPLC ('Shimadzu' make with accessories)

(g) HPLC accessories

- Liquid chromatograph : 'Shimazdu' LC–6A solvent delivery module with a single plunger reciprocating pump.
- Column : 'Shimpack' CLC–ODS(M) 4.6 mm id x 20 cm long (silica particles, 5 μm particle dia, 100 A° pore size, Shimadzu Corporation, Japan.
- Detector : UV–Vis Spectrophotometer
- Data processor : 'Chromatopac' CR, 6A Recorder, 'Shimadzu'.
- Sample injector : 'Hamilton' syringe (25 μl)

## 3.3 CHEMICALS

Analytical and HPLC grade chemicals from reputed manufacturers were used in the present study. Sephadex G-75 used in gel filtration (particle size 10 to 40 μ) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Double glass distilled water was used for general purposes like preparation of general reagents, media, buffers etc. as follows:

### 3.3.1 REAGENTS FOR ELECTROPHORESIS (LAEMMLI, 1970)

**(i) Stock acrylamide/bisacrylamide (30%)**

Acrylamide 29.2 g and bis acrylamide 0.8 g were dissolved in distilled water and total volume was made up to 100 ml. The solution was filtered and stored at 4°C in amber coloured bottles.

**(ii) Sodium dodecyl/lauryl sulphate (SDS 10%)**

Ten g SDS was dissolved in water and total volume was made up to 100 ml.

**(iii) Stacking gel (4%)** : It was prepared by mixing following.

| | | |
|---|---|---|
| Distilled water | – | 6.1 ml |
| Acrylamide solution (30%) | – | 1.3 ml |
| Tris-HCl 0.5 M, pH 6.8 | – | 2.5 ml |
| SDS (10%) | – | 0.1 ml |
| Ammonium persulphate (10%) | – | 0.05 ml |
| N,N,N',N'-tetramethyl ethylene diamine(TEMED) | – | 0.01 ml |

The last two ingredients were added only at the time of use of the gel to prevent setting of the gel before use.

**(iv) Separating gel (15%)** It was prepared by mixing the following:

| | | |
|---|---|---|
| Distilled water | – | 2.3 ml |
| Acrylamide solution (30%) | – | 5.0 ml |
| Tris-HCl 1.5 M, pH. 8.8 | – | 2.5 ml |
| SDS (10%) | – | 0.1 ml |
| Ammonium sulphate (10%) | – | 0.1 ml |
| TEMED | – | 0.004 ml |

**(v) Ammoniacal silver staining solution**

One hundred ml of this solution was prepared as follows : Ammonium hydroxide (conc. ammonia) was added to 100 ml of distilled water. One hundred ninety microlitres of 10 N NaOH was added to this. The mixture was vortexed and 1 ml of freshly prepared $AgNO_3$ solution (0.8 g of $AgNO_3$ in 1 ml distilled water) was added drop-wise. Initially, a precipitate appeared which dissolved slowly.

**(vi) Developer solution**

It consisted of 0.005 per cent citric acid and 0.019 per cent formaldehyde (diluted from commercial 37 per cent solution).

### 3.3.2 PROTEIN ESTIMATION REAGENT (LOWRY *et al.*, 1951)

Reagent A : Copper sulphate 1% : One gram anhydrous copper sulphate crystals were dissolved in 100 ml of double glass distilled water.

Reagent B : Sodium-potassium tartarate solution 2% : Two g sodium-potassium tartarate were dissolved in 100 ml of distilled water.

Reagent C : 0.2 M NaOH : 0.8 g sodium hydroxide was dissolved in 100 ml of distilled water.

Reagent D : Sodium carbonate 4% solution: Four g sodium carbonate were dissolved in 100 ml distilled water.

Reagent E : To 49 ml reagent C, 49 ml of reagent D was added. To this was added 1 ml of reagent A followed by 1 ml of reagent B to get copper alkali solution. It was prepared afresh whenever needed.

Folin-Ciocalteau reagent : Folin-Ciocalteau reagent was diluted 1 : 1 with double glass distilled water.

### 3.3.3 TEST SOLUTIONS OF METAL SALTS

Salt solutions ($10^{-4}$ M) of $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Fe^{3+}$ were prepared by dissolving a required quantity of the salt in distilled water.

### 3.3.4 Buffers

**(i) Tris-HCl buffer (0.05 M, pH 7.2)**

**Stock solution A** : Tris (0.2 M) was prepared by dissolving 24.23 g salt in one litre distilled water.

**Stock solution B**: HCl (0.2 M concentrated HCl, 35.4 per cent, sp. gr. 1.18), 17.48 ml in one litre.

Mixed 500 ml of sol A + required vol of sol B, diluted to 800 ml.

**(ii) Tris-HCl (1.5 M, pH 8.8) (Laemmli, 1970)**

Tris (hydroxymethyl aminomethane) (18.15 g) was dissolved in 80 ml distilled water, pH adjusted to 8.8 with 1 N HCl and total volume was made up to 100 ml and stored at 4°C.

**(iii) Tris-HCl (0.5 M, pH 6.8) (Laemmli, 1970)**

Tris (6 g ) was dissolved in 80 ml distilled water and pH was adjusted to 6.8 with 1 N HCl. Total volume was made up to 100 ml with distilled water and stored at 4°C.

**(iv) Phosphate buffer (0.05 M)**

**Stock solution A** : 0.2 M solution of monobasic sodium phosphate prepared by dissolving 27.8 g salt in 100 ml distilled water.

**Stock solution B** : 0.2 M solution of dibasic sodium phosphate prepared by dissolving 53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in one litre

Solutions A and B were mixed according to the required concentration and pH.

Mixed x ml of A + y ml of B as directed and diluted to a total of 800 ml.

**(v) Citrate buffer (0.05 M)**

**Stock solution A** : 0.1 M solution of citric acid (21.0 g in 1000 ml)

**Stock solution B** : 0.1 M solution of sodium citrate (29.49 g $C_6H_5O_7Na_3.2H_2O$ in 1000 ml).

Mixed x ml of A + y ml of B as directed and diluted to 200 ml.

**(vi) Electrode (Reservoir) Buffer (5X, pH 8.3)**

Dissolved the following chemicals in distilled water :

| | | |
|---|---|---|
| Tris | – | 9.0 g |
| Glycine | – | 43.2 g |
| SDS | – | 3.0 g |

pH thus obtained was 8.3 ± 0.2

The buffer was diluted 5 times before use.

**(vii) Sample Buffer**

Sample buffer was prepared by mixing the following solutions :

| | | |
|---|---|---|
| Glycerol (20%) | – | 1.6 ml |
| SDS (10%) | – | 3.2 ml |
| β-Mercaptoethanol (10%) | – | 0.8 ml |
| Tris-HCl, 0.5 M, pH 6.8 | – | 1.0 ml |

Bromophenol blue was used as tracking dye.

### 3.3.5 REFERENCE INSECTICIDE

Technical grade lindane (γ-BHC) was procured from Sigma Chemicals. For comparison puproses, a sample was kindly provided by Pesticide Research Laboratory, Office of the Joint Director of Agriculture, Department of Agriculture, Government of Haryana, Karnal.

## 3.4 ORGANISMS USED

The mould strain *Geotrichum candidum* NCDC-228 isolated by earlier workers in our labs was used in the present study for enzyme production.

Mixed dahi culture obtained from National Collection of Dairy Cultures (NCDC), Dairy Microbiology Division, National Dairy Research Institute (NDRI), Karnal-132 001 was used for quality assurance experiments.

*Streptococcus salivarius* subsp. *thermophilus* NCDC-74 obtained from NCDC, NDRI was used for TTC test for presence of inhibitory substances in milk.

### Maintenance

The mould culture was maintained on potato dextrose agar (PDA; BIS:1969) slants. It was subcultured once in a fortnight and after incubation at 25°C for 48 h, stored in refrigerator. It was propagated in potato dextrose broth. The culture was checked for purity by microscopic examination before use. The two bacterial cultures were similarly grown in litmus milk at appropriate incubation temperature, viz., 30°C for dahi and 37°C for *Salivarius* subsp. *thermophilus* and were maintained in the refrigerator.

### Media used

Potato dextrose broth (PDB) and potato dextrose agar (PDA) were used for growth experiments with the mould and litmus milk was used for the two bacterial tests cultures.

## 3.5 INSECTICIDE TOLERANCE OF THE MOULD

The reference mould culture *G. candidum* NCDC-228 was evaluated for its ability to grow in the presence of the insecticide, i.e., γ-BHC incorporated into the growth media, viz., PDB and PDA. The mould propagated in PDB was plated on PDA and the colonies thus developed after 48 h at 25°C were counted based on which the original PDB suspension was adjusted to $1 \times 10^4$

cfu/ml. Ten ml aliquots of PD media fortified with 2.5, 5.0 and 10.0 ppm γ-BHC were inoculated with the mould (1 x $10^4$ cfu/ml) and incubated at 25°C for 2, 3, 5 and 7 d. The extent of growth in the broth was observed visually and the colonies developing on the PDA were enumerated.

## 3.6 FACTORS AFFECTING THE PRODUCTION OF γ-BHC DEGRADING ENZYME FROM *G. CANDIDUM*

Innumerable environmental factors may influence the production and activity of γ-BHC degrading enzyme. Incubation temperature, incubation period, initial growth medium pH and air supply to the growing mould were the factors studied in the instant investigation.

### 3.6.1 INCUBATION TEMPERATURE

Basal broth was inoculated with the young mould culture and the flasks were incubated at different temperatures, viz., 10, 15, 25 and 30°C for 5 d. After 5 d, the growth menstruum was separated from the culture initially by centrifugation at 10,000 rpm for 10 min followed by microglass filtration. Its γ-BHC degradation activity was tested by supplementing it with known concentration of γ-BHC solution using HPLC estimation.

### 3.6.2 INITIAL pH OF MEDIUM

Basal medium aliquots were adjusted to different pH values, viz., 3, 5, 7 and 9. These were then inoculated with active young *G. candidum* culture and incubated at 25°C for 5 d. Culture filtrate was separated by centrifugation and filtration and its γ-BHC degrading activity was estimated.

### 3.6.3 PERIOD OF INCUBATION

Potato dextrose broth was inoculated with the mould culture and incubated at 25°C for 3, 5, 7 and 9 d, after which the culture filtrate was obtained as usual in each case and checked for its γ-BHC degrading activity.

### 3.6.4 AERATION

Broth inoculated with *G. candidum* was kept under static and aerated (both rotary and wrist 120 rpm or oscillations/min shaking as the case might have been) conditions at 25°C. After 5 d of incubation, the culture filtrate preparations were checked for their γ-BHC degrading activity.

## 3.7 DEGRADATION ABILITY OF CULTURE FILTRATE OF *G. CANDIDUM* NCDC-228

Mould was grown under optimum conditions and culture filtrate was tested for its ability to degrade γ-BHC upto 12 h.

## 3.8 PREPARATION OF ENZYME

Following steps were involved in the preparation of γ-BHC degrading enzyme from *G. candidum* NCDC-208.

### 3.8.1 PREPARATION OF CULTURE FILTRATE

The mould was inoculated aseptically in PDB (pH 6.0) and incubated at 25°C for 7 d. The cell free culture filtrate was obtained by refrigerated centrifugation of broth culture at 10,000 rpm for 10 min followed by pre-filtration using GFS or GF2 grade microglass filter and then membrane filtration using sterile 0.45 μm pore size, 47 mm dia nylon membranes.

### 3.8.2 SALT (AMMONIUM SULPHATE) PRECIPITATION

To 100 ml of culture supernatant, calculated amount of ammonium sulphate was added slowly with constant shaking on magnetic stirrer to achieve 50 per cent saturation. The mixture was then kept stirring overnight in the refrigerator. Precipitates formed were separated by refrigerated centrifugation at 10,000 rpm for 10 min at 4°C and were dissolved in Tris-HCl buffer (pH 7.0). The fluid obtained from centrifugation was further fortified with a 10 per cent increment of $(NH_4)_2SO_4$ to make the latter to 60 per cent saturation and was centrifuged. The same process was repeated for 70 per cent $(NH_4)_2SO_4$

saturation. The buffered solution thus obtained after each ammonium sulphate precipitation step was dialyzed using 10,000 molecular weight cut off tubes. For this purpose, a dialysis tubing of suitable length was soaked and boiled in a solution of 2 per cent sodium bicarbonate and 0.05 per cent EDTA and cooled. The dialysis tubing was again boiled twice for 10 min each in distilled water and then used for further study. Dialysis was done for about 12 h with intermittent change of the buffer. The specific activity and recovery of the crude enzyme from dialyzed solution were calculated from the degrading protein content and total activity units of the γ-BHC degrading enzyme solution.

### 3.8.3 GEL FILTRATION

The crude enzyme preparation after ammonium sulphate precipitation followed by dialysis was further purified by gel filtration chromatography using sephadex G-75. It was soaked in sterile distilled water overnight at room temperature undisturbed and fine particles floating over the water layer were slowly drained off. This procedure was repeated twice. The slurry was then kept in boiling water bath for about 3 h. The beaker containing the slurry was covered with aluminium foil in which holes were made. The swelling procedure was done to degas the slurry which was then brought to room temperature. Excessive water was decanted off and the slurry was then suspended in 0.05 M Tris-HCl buffer (pH 7.02) for 1 h. The slurry was then packed in a column (50 cm x 2.5 cm i.d.) and void volume of packed column was calculated by using blue dextran B-2000. The column was calibrated with 0.05 M Tris-HCl (pH 7.2) by flushing 2 to 3 volumes of buffer through it. The crude enzyme preparation was carefully applied drop-wise over the surface of the gel with the help of sterile glass pipette. After soaking the enzyme into gel, the buffer was allowed to pass. Flow rate was set to 30 ml per h (0.5 ml/min). Five ml fractions were collected and analysed for protein content and γ-BHC degrading activity. Protein content of the fractions was monitored by

measuring the absorbance at 280 nm with spectronic 21D spectrophotometer. The fractions showing the γ-BHC degrading activity were pooled.

### 3.8.4 DETERMINATION OF PROTEIN

Protein content of the culture filtrate and the pooled enzyme fractions was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma Chemicals) as standard protein.

### 3.8.5 UNIT OF ACTIVITY

The γ-BHC degrading activity of the enzyme was expressed in terms of units. An enzyme unit was defined as the amount of protein required to degrade the μmole of γ-BHC per hour at 25°C.

### 3.8.6 DETERMINATION OF SPECIFIC ACTIVITY

Specific activity of the enzymic preparation was defined as "activity units of the enzyme per milligram of the protein" and was calculated using the formula :

$$\text{Specific activity} = \frac{\text{Total units of activity in fraction}}{\text{Total amount of protein in fraction}}$$

### 3.8.7 DETERMINATION OF LEVEL OF PURIFICATION

The quantum of purification of the fraction was calculated using the formula :

$$\text{Increase in purification} = \frac{\text{Specific activity of enzyme fraction}}{\text{Specific activity of original sample (culture supernatant)}}$$

### 3.8.8 DETERMINATION OF ACTIVITY RECOVERED

The activity recovery or yield was calculated using the formula :

$$\text{Activity recovery or yield} = \frac{\text{Total units of activity in fraction}}{\text{Total units of activity in culture}}$$

## 3.9 ESTIMATION OF γ-BHC REDUCTION ABILITY OF CULTURE FILTRATE AND ENZYME PREPARATION

Known amounts of the culture filtrate or the purified enzyme were added into tris-HCl buffer (0.05 M, pH 7.2) containing definite amounts of γ-BHC. The mixtures were incubated at 25°C for 4 h. Appropriate control and blank were also run alongwith. Residual insecticide was determined as given in the next section.

## 3.10 DETERMINATION OF γ-BHC

The residual content of γ-BHC in the above tris solution, PDB and milk was determined using HPLC system. The test samples were treated first for extraction of the insecticide from broth and milk and then for acid clean up as follows :

### 3.10.1 EXTRACTION AND CLEAN UP OF γ-BHC RESIDUES FROM BUFFER AND BROTH (KAPOOR *et al.*, 1981)

Ten ml of 'test' solution as such or one which pesticide solution or culture filtrate was added was treated with 10 ml each of acetate and n-hexane and mixed for 2 min. After transferring the homogenate into a tube, it was centrifuged for 10 min at 2,000 rpm. The upper organic phase was removed and passed through 5 g layer of anhydrous $Na_2SO_4$ into a 500 ml standard joint round-bottom flask. The lower layer was extracted twice with 100 ml portions of n-hexane and collected as before. Evaporation of the combined n-hexane phases to near dryness was done using a Buchi type rotary vacuum evaporator. After dissolving the residue in petroleum ether it was transferred into a glass separating funnel to which concentrated $H_2SO_4$ was added drop-wise at a rate of 4 ml/min. Lower $H_2SO_4$ layer containing digested fat, if any, was continuously removed from the funnel. The upper petroleum ether phase was washed with distilled water until neutral to litmus. This residue was concentrated over water bath (60–70°C), the contents were finally transferred

to a graduated tube (1 ml) and dried completely in a nitrogen atmosphere under reduced pressure.

### 3.10.2 EXTRACTION AND CLEAN UP OF γ-BHC RESIDUES FROM MILK

Method of Rose-Gottlieb (BIS: 1981) was used to extract the concentrated γ-BHC residues from milk fat. Test milk (i.e. milk as such or milk to which pesticide sol. and/or culture filtrate was added) was transferred to clean and dried separating funnels. One ml of ammonia sol. followed by 10 ml of ethyl alcohol was added and the contents were thoroughly mixed. To this were added 25 ml each of diethyl ether and petroleum ether. The contents were vigorously shaken (with occasional release of pressure by opening the stop cock, keeping the funnel inverted) for 5 min and rested for 30 min. The ethereal layers were collected in a standard joint round bottom flask. The extractions were repeated twice with 5 ml of ether aliquots. The ether was evaporated in a 70 to 80°C water bath and the fat was dissolved in 15 ml petroleum ether for further clean up and enrichment of pesticide residues. The extracted γ-BHC residue in petroleum ether was taken into clean and dried separating funnel to which sulphuric acid was added drop-wise and the contents were mixed with a gentle swirling. Approximately 25 to 40 ml of concentrated $H_2SO_4$ was added till both the petroleum ether and acid layers became colourless on standing for sometime. The lower reddish brown layer of digested materials was continuously discarded. The organic solvent layer was washed with distilled water till neutral to blue litmus.

### 3.10.3 LIQUID CHROMATOGRAPHIC ANALYSIS FOR γ-BHC

Ten mg of reference standard (γ-BHC) was dissolved in 10 ml of HPLC grade methanol to obtain stock solution of 1000 ppm. This stock solution was diluted 1:10 in methanol to get the working sol. of 100 ppm. For further dilutions, 100 ppm solution was diluted in methanol as and when required.

**(i) Running the HPLC**

Gamma-BHC residues obtaind after extraction, acid clean up, dehydration and concentration were dissolved in HPLC grade methanol. The contents were filtered through membrane filter and 20 µl doses were injected into HPLC under the following specified test conditions.

**(ii) HPLC test conditions**

| | | |
|---|---|---|
| Column | : | Shimpack CLC-ODS (M) |
| Phase | : | Reverse |
| Detector | : | UV-254 |
| Solvent system | : | Methanol water (70 : 30) |
| Flow rate | : | 1 ml/min |
| Maximum pressure | : | 250 kgf/cm$^2$ |
| Pressure maintained | : | 200 kgf/cm$^2$ and 50 kgf/cm$^2$ |
| Solvent filters | : | 0.45 µm pore size nylon membrane 47 mm dia |
| Sample filters | : | 0.45 µm pore size nylon membrane, 13 mm dia |

**(iii) Derivation of γ-BHC from HPLC chromatograms**

Amount of γ-BHC from test samples was calculated by comparison with retention time (RT) of standard γ-BHC with the help of following formula :

$$\text{Concentration of pesticide} = \frac{C \times a_2 \times V_1}{a_1 \times V_2 \times V}$$

where,

C = concentration of reference standard (µg/ml),

$a_1$ = area of peak from reference standard,

$a_2$ = area of peak from test sample extract,

$V_1$ = total volume µl (extract + solvent),

$V_2$ = volume injected, i.e., 20 µl, and

V = volume of test sample (buffer, milk, etc.)

## 3.11 CHARACTERIZATION OF THE ENZYME

### 3.11.1 MOLECULAR WEIGHT DETERMINATION BY SDS-PAGE

SDS-PAGE (Sodium dodecyl sulphate–polyacrylamide gel electrophoresis) in slab gels was performed by the method described by Laemmli (1970).

**(i) Gel preparation**

Glass plates were thoroughly cleansed with acetone swab and air dried. An appropriate quantity of separating gel (section 3.3.1) was poured between the plates. A layer of water was placed in the space above the separating gel. In 20 to 30 min, when a very sharp gel interface appeared indicating the completion of polymerization of the gel, water was carefully drained off. The stacking gel solution (section 3.3.1) was directly poured onto the polymerized separating gel and appropriate comb inserted immediately into the gel solution (being careful not to trap any bubbles). The gel was allowed to polymerize for 10 min. The comb was gently removed and the gel plates were transferred to the electrophoresis apparatus.

**(ii) Sample preparation**

Before loading of the sample on to the gel, the sample solution was mixed with twice its volume the sample buffer (section 3.3.3). The resultant mixture was heated in a boiling water bath for 3 min.

**(iii) Electrophoresis**

The diluted reservoir buffer (section 3.3.3) was poured into electrophoresis chamber. Heat treated samples were applied into the wells in the gel. The reservoir buffer was carefully layered on the sample. Electrophoresis was carried out at constant current of 75 mA till the sample crossed the stacking gel after which the current was raised to 100 mA till the marker dye reached the lower end of the gel. The temperature during

electrophoretic run was kept low by circulating water in the electrophoresis assembly.

At the end of electrophoresis, the gels were removed from the electrophoresis unit and subjected to silver staining.

**(iv) Silver staining**

The protocol followed for ammoniacal silver staining was the same as given by Harlow and Lane (1988).

1. After running SDS-PAGE, the gel was placed in five times the gel volume a solution of 50 per cent ethanol and 10 per cent acetic acid for overnight with occasional shaking at room temperature. The solution was decanted and gel was rinsed with water.

2. The gel was placed in five times the gel volume of 20 per cent ethanol for 30 min with occasional manual shaking at room temperature. Repeated this step.

3. The ethanol solution was removed. The gel was incubated with five times the gel volume of 5 per cent glutaraldehyde prepared in deionized water at room temperature for 30 min with shaking.

4. Glutaraldehyde solution was removed and the gel was rinsed with deionized water. Five times the gel volume of 20 per cent ethanol was added to the gel and the gel was kept in this solution for 20 min with shaking at room temperature.

5. After the last ethanol wash, the gel was rinsed with deionized water and incubated in five times the gel volume of water for 10 min at room temperature with shaking.

6. After the water was removed, four times the gel volume of a freshly prepared ammonical silver solution (section 3.3.1) was added.

7. The gel was incubated for 30 min at room temperature with gentle shaking.

8. After the ammonical silver solution was removed, gel was rinsed 5 to 6 times with deionized water over a period of 20 min.

9. After the gels were washed, five times the gel volume of freshly prepared developer (section 3.3.1) was added. The developer was removed when the bands appeared and the reaction was stopped by transferring the gel in a solution containing 10 per cent acetic acid and 20 per cent ethanol. It was maintained at room temperature.

**(v) Molecular weight from silver stained gel**

The relative mobility, Rf, of protein of interest (i.e. the enzyme) was measured with reference to marker protein and to the tracking dye (section 3.3.4, sample buffer).

$$Rf = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

A standard curve was constructed by plotting $\log_{10}$ values of molecular mass of the marker protein versus their relative mobility (Rf) revealing a straight line relationship from which the molecular mass of the γ-BHC degrading enzyme was calculated based on its relative mobility.

### 3.11.2 OPTIMUM TEMPERATURE

Optimum temperature for γ-BHC degradation activity of the purified enzyme was determined by incubating the reaction mixture for 4 h at different degrees of temperature, viz. 4°, 15°, 25° and 30°C as per section 3.10.

### 3.11.3 OPTIMUM pH

Gamma-BHC degradative activity of purified enzyme was determined as per section 3.10 at different pH values ranging from 3.0 to 9.0 during 4 h at 25°C. The buffers of different pH values used were 0.05 M citrate buffer (pH

range 3.0 to 6.0), 0.05 M phosphate buffer (pH range 6.0 to 7.5) and 0.05 M Tris-HCl buffer (pH range 7.5 to 9.0).

### 3.11.4 HEAT STABILITY

Small portions of purified enzyme were preincubated for 10 min at different temperatures, viz., 25°, 37°, 50° and 72°C after which these were immediately chilled in an ice cold water bath for a few minutes and added to substrate buffer mixture. Enzyme activity for γ-BHC degradation of these heat treated portions of the enzyme substrate buffer mixture was measured in each case.

### 3.11.5 pH stability

Purified enzyme preparation was preincubated in different buffers viz. 0.05 M citric acid-sodium citrate (for a pH range of 3.0 to 6.0), 0.05 M phosphate buffer (pH range 6.0 to 7.0), 0.05 M tris-maleate-NaOH buffer (pH range 7.5 to 9.0) for 1 h at 25°C. After incubation, the preparation was chilled in water bath. These samples were brought to pH 7.0 after which γ-BHC degrading activity was measured.

### 3.11.6 TIME FOR OPTIMUM DEGRADATION POTENTIAL

Reaction mixture was kept at 25°C at different time periods starting from 1 h to 6 h at pH 6.5 and then after each time interval, the γ-BHC degrading activity was measured as above.

### 3.11.7 EFFECT OF METAL IONS

The influence of different metals like $Na^{+}$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Fe^{3+}$ was determined on γ-BHC degrading activity of the purified enzyme preparation. It was studied by adding test solution of the appropriate metal salts in the reaction mixture followed by determination of γ-BHC degrading activity of the enzyme.

### 3.11.8 EFFECT OF SUBSTRATE CONCENTRATION

The relation between substrate concentration and γ-BHC degrading activity was established by determining reaction rate at various substrate concentrations ranging from 1 to 10 ppm. Michaelis constant of purified enzyme was calculated by double reciprocal plot method of Lineweaver and Burk (1934).

## 3.12 APPLICATION IN DAIRYING

The enzyme preparation was put to test for its efficacy in depolluting milk and its effect on commodity's quality as follows :

### 3.12.1 LESSENING OF γ-BHC LEVELS IN MILK

The purified enzyme was evaluated for γ-BHC reduction in cow and buffalo milk. For this, milk was taken instead of buffer to which γ-BHC solution and enzyme solution were added. Milk tube as such and one with added γ-BHC were run along with as negative (blank) and positive control.

Gamma-BHC reduction was measured after 4 h incubation at the optimum temperature of the enzyme activity as found in section 3.11.2 by the usual method as described earlier (section 3.10).

### 3.12.2 SENSORY EVALUATION

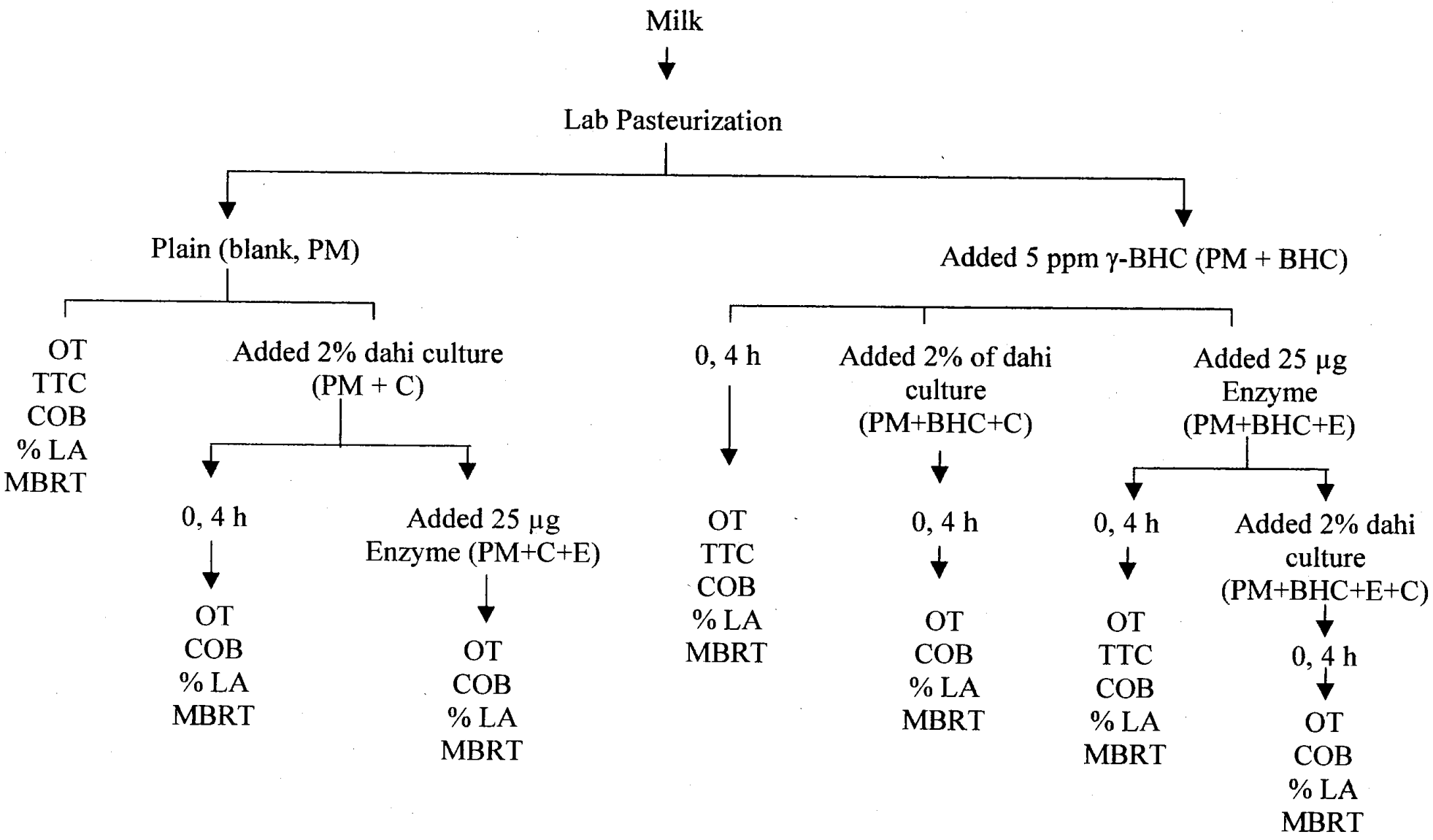
To ascertain the utility of the enzyme treatment of milk for its processing and consumption ability /safety, certain quality assurance tests were performed. These included some organoleptic tests like change in appearance, taste, flavour and some quality control tests like test for inhibitory substances (TTC), COB, acidity and dye reduction (MBRT). All these tests were performed as per standard procedures laid down in relevant Indian Standards

(BIS: 1977; BIS: 1981). The scheme for all these QA tests has been given in Fig. 2.

## 3.13 STATISTICAL TREATMENT

The significance of the results of the study was determined by ANOVA method as per Snedecor and Cochran (1966).

**FIG. 2 SCHEME FOR QUALITY ASSURANCE TESTS**



# CHAPTER 4

# *Results and Discussion*

# 4. RESULTS AND DISCUSSION

In pursuit of the ultimate objective of making our environment free from γ-BHC residues through microbial intervention as natural scavengers, the potentials of *G. candidum* NCDC-228 were harnessed. The findings of the present investigation have been recorded in the form of Tables 2 to17 and Figs. 3 to 26.

## 4.1 TOLERANCE OF *G. CANDIDUM* NCDC-228 TO γ-BHC

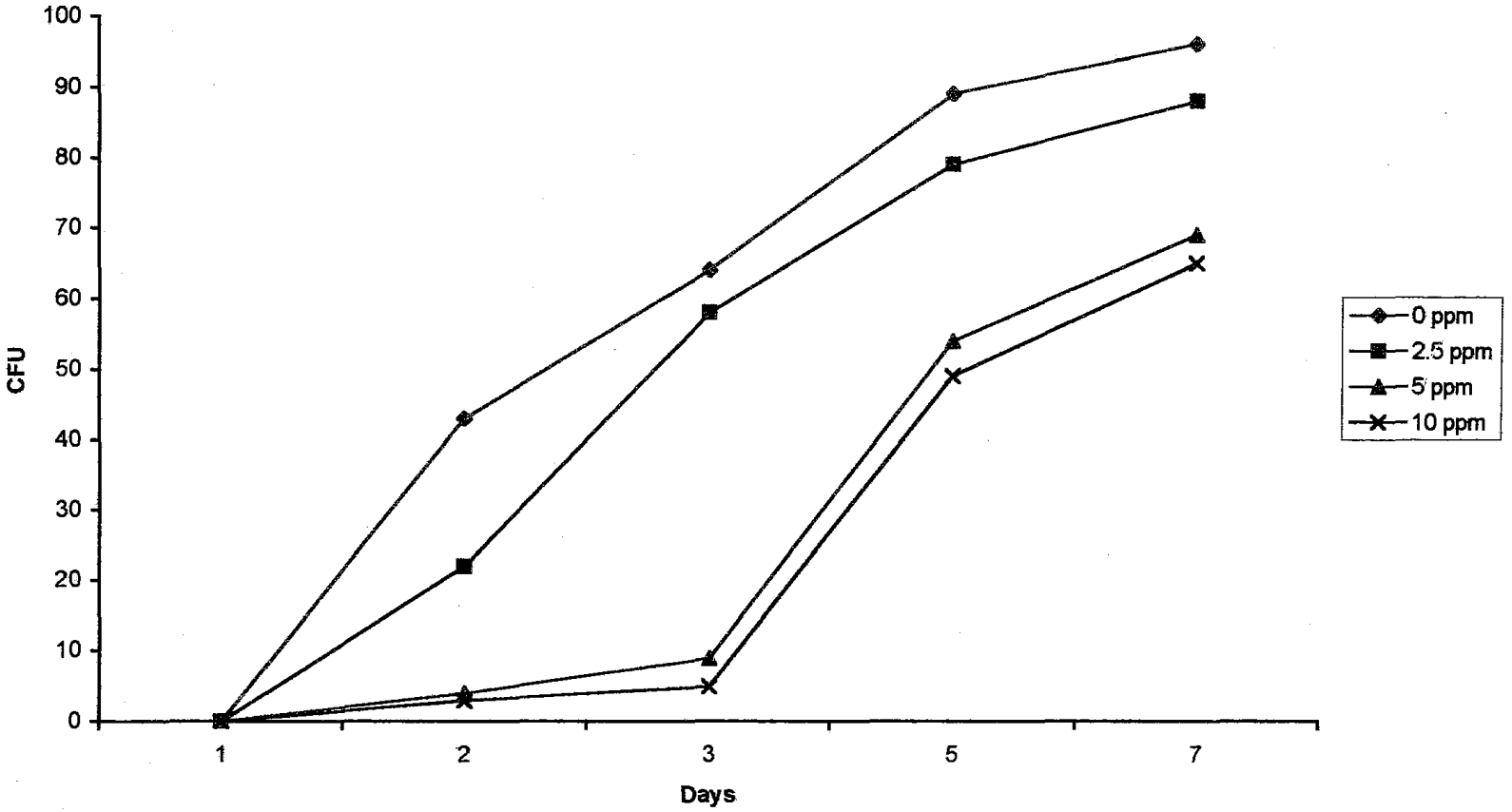
Before the production of γ-BHC degrading enzyme by *G. candidum* NCDC-228 was taken up, the mould was tested for its ability to grow in the presence of added insecticide in the growth medium the results of which have been compiled in Table 2. It did not show perceptible growth in PDA for the first day of incubation at 25°C. A good number of mould colonies started coming up by the second day but in a progressively decreasing order in the presence of increasing BHC concentration. The counts increased with the period of incubation. The mould developed a good number of colonies in the presence of even 10 ppm insecticide by the end of 7 d. In the PD broth, a similar trend in the overall density of growth was visually noticed.

The trend of growth of the organism as is clear from Fig. 3 indicated that the mould was not only able to withstand the restriction by the pesticide but it also gradually overcame it and started growing normally so much so that the gap between growth at 0 ppm and 10 ppm insecticide seemed to be lessening. It was, therefore, inferred that the mould either developed resistance to the insecticide or it might have started utilizing the chemical as a nutrient or a cometabolite.

**Table 2. Growth of *G. candidum* NCDC-228 on PDA supplemented with γ-BHC**

| Incubation days at 25°C | Colony forming units ($1x10^4$ /plate) on PDA supplemented with γ-BHC | | | |
|---|---|---|---|---|
| | 0 ppm | 2.5 ppm | 5.0 ppm | 10.0 ppm |
| 1 | 0 | 0 | 0 | 0 |
| 2 | 43 | 22 | 4 | 3 |
| 3 | 64 | 58 | 9 | 5 |
| 5 | 89 | 79 | 54 | 49 |
| 7 | 96 | 88 | 69 | 65 |

**Fig. 3 Growth pattern of *G. candidum* on PDA supplemeted with γ-BHC**



## 4.2 OPTIMIZATION OF CONDITIONS FOR PRODUCTION OF γ-BHC DEGRADING ENZYME FROM *G. CANDIDUM* NCDC 228

The optimum cultural conditions for growth of *G. candidum* NCDC 228 for production of the enzyme capable of degrading γ-BHC in solution were determined with respect to incubation temperature, initial pH of the medium, incubation period and aeration. The earlier preliminary work done in the Institute (Ann. .Rep. 2000-2001) on *G. candidum* had indicated that the organism's culture filtrate obtained from its growth at different degrees of temperature and pH between 5 and 6 possessed varying γ-BHC degrading ability. When grown at 25°C, it exhibited the maximum activity. Therefore, in the present study the enzyme activity was considered as the criterion for estimating its production by the mould.

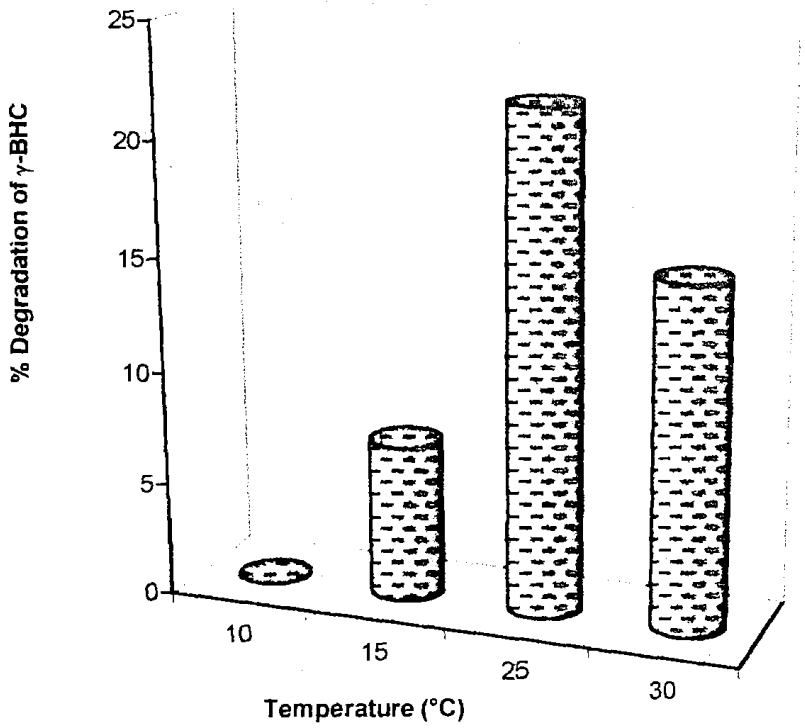
### 4.2.1 EFFECT OF INCUBATION TEMPERATURE

The enzyme producing mould strain under reference was grown in PDB at different incubation temperature, viz. 10°, 15°, 25° and 30°C. The results obtained have been presented in the Table 3 and Fig. 4. The insecticide recovery in the 0 h reading was taken to be 100 per cent for the purpose of computing the values obtained at 4 h with different degrees of temperature. The growth of the organism was quite slow at 10°C compared to the other temperature conditions. It was thus found that the production of the biomass and enzyme increased with temperature, attaining maximum when the mould was grown at 25°C ($P < 0.01$). However, the rate of enzyme production decreased with increase in growth temperature to 30°C. After incubating at 25°C, γ-BHC content of the mixture was reduced by 21.88 per cent (Fig. 4), while after incubation of mould at 30°C the reduction was 15.13 per cent. Consequently, the enzyme activity units were also maximum at 25°C. These findings corroborate with those obtained by the earlier workers (Mittal, 1999; Ann. Rep., 2000-2001). The difference in γ-BHC degradation at various levels

**Table 3. Effect of temperature of incubation on production of the enzyme in PD broth pH 5.5 during 5 days under static conditions**

| Tube No. | Incubation Temperature(°C) of Culture Broth | Reaction Time(h) | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|---|
| | | | ppm | % | |
| 1 | 25 | 0 | - | - | – |
| 2 | 25 | 0 | 4.89 | 100 | – |
| 3 | 10 | 4 | 5.16 | 100 | – |
| 4 | 15 | 4 | 4.55 | 93.05 | 1.16 |
| 5 | 25 | 4 | 3.82 | 78.12 | 3.68 |
| 6 | 30 | 4 | 4.15 | 84.87 | 2.54 |

Fig. 4 Effect of temperature of incubation on production of γ-BHC degrading enzyme of G. candidum NCDC-228



of incubation temperature indicated the possibility of 25°C being optimum for the enzyme production or its activity or both.

### 4.2.2 EFFECT OF INITIAL pH OF THE MEDIUM

Enzyme production during growth of the organism in potato dextrose broth adjusted to different initial pH values, viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 prior to inoculation is depicted in results computed in Table 4 and Fig. 5. It was found that at pH 3.0, 4.0, 7.0, 8.0 and 9.0 there was less growth as well as production of γ-BHC degrading enzyme. Maximum production of the enzyme occurred at initial medium reaction of pH 6.0 followed closely by its production at pH 5.0. The culture filtrate from mould growth at pH 6.0 caused a 27.7 per cent reduction (Fig.5) in level of γ-BHC showing enzyme activity units of 4.6. Deviating the pH of medium to either side from 5 and 6.0 caused a rapid decline ($P < 0.01$) in the production of γ-BHC degrading enzyme in terms of its activity. These results are in close agreement with those of Mittal (1999) who found that initial medium pH of 5.6 ± 0.2 at 25°C caused 23.9 per cent degradation in 4 h. In an analogous study on DDT, Ledford and Chen (1969) had observed that *Geotrichum candidum* 16AA4 caused maximum reduction in the insecticide concentration on growing in a medium having an initial pH 8.5 and on incubation at 37°C. No other report of a similar study on BHC could be found in the literature. The findings of the present study implied that either the enzyme was not produced at the extreme pH values or it was not active in these ranges of pH as the production of the enzyme was measured in terms of its activity.

### 4.2.3 EFFECT OF INCUBATION PERIOD

The findings of the γ-BHC degrading enzyme activity of culture filtrate of *Geotrichum candidum* NCDC 228 inoculated in potato dextrose broth having an initial pH 6.0 and at 25°C for 3, 5, 7 and 9 days have been recorded in Table 5 and Fig. 6. It was seen that the preparation obtained after 7 d of incubation had the maximum γ-BHC reducing enzyme activity and it caused

**Table 4. Effect of initial pH of PD broth on production of the enzyme at 25°C for 5 days under static conditions**

| Tube No. | Initial pH of Medium | Reaction Time (h) | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|---|
| | | | ppm | % | |
| 1 | 5.5 | 0 | - | - | – |
| 2 | 5.5 | 0 | 4.84 | 100 | – |
| 3 | 3 | 4 | 4.57 | 94.42 | 0.92 |
| 4 | 4 | 4 | 4.50 | 92.98 | 1.16 |
| 5 | 5 | 4 | 3.91 | 80.79 | 3.19 |
| 6 | 6 | 4 | 3.50 | 72.31 | 4.60 |
| 7 | 7 | 4 | 4.49 | 92.77 | 1.20 |
| 8 | 8 | 4 | 4.59 | 94.83 | 0.86 |
| 9 | 9 | 4 | 4.66 | 96.28 | 0.61 |

**Fig. 5 Effect of pH of medium on production of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

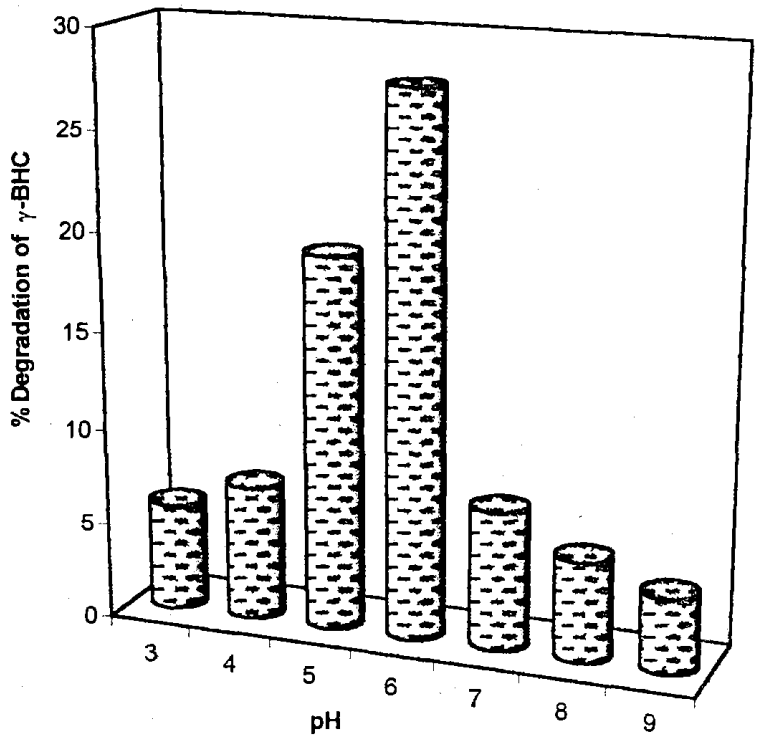


**Table 5. Effect of duration of incubation on production of γ-BHC degrading enzyme, medium pH 6.0 at 25°C under static conditions**

| Tube No. | Incubation days of culture broth | Reaction Time (h) | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|---|
| | | | ppm | % | |
| 1 | 0 | 0 | - | - | - |
| 2 | 0 | 0 | 4.80 | 100.00 | - |
| 3 | 3 | 4 | 4.16 | 86.67 | 2.20 |
| 4 | 5 | 4 | 3.74 | 77.92 | 3.64 |
| 5 | 7 | 4 | 3.34 | 69.58 | 5.02 |
| 6 | 9 | 4 | 3.46 | 72.08 | 4.60 |

30.42 per cent degradation with a resultant enzyme activity of 5.02 units/ml. According to our findings, when incubation period was increased from 3 to 9 d, there was a concomitant increase in enzyme production up to 7 d ($P < 0.01$) with a marginal decrease later on. Ledford and Chen (1969) inoculated the mould for 10 d and then analysed DDT degradation which was as high as 84 per cent. However, they could not get degradation of lindane by any of their test organisms. The lack of reports on this aspect prohibit the present workers to evaluate their findings.

### 4.2.4 EFFECT OF AERATION

Aeration by way of rotary or intermittent shaking of the culture vessels did not improve the production of the γ-BHC degrading enzyme by *G. candidum* NCDC 228. It rather subdued the phenomenon as there was some discernible decrease in enzyme production by about 7 per cent (Table 6, Fig. 7) in terms of enzyme activity (3.92 units/ml) compared to that obtained under static growth conditions (enzyme activity 5.02 units/ml). It was, therefore, implied that aeration was not much favourable for this enzyme production by the aerobic mould under the test conditions.

## 4.3 GAMMA-BHC DEGRADATION ABILITY OF CULTURE FILTRATE OF *G. CANDIDUM* NCDC-228

Cell free culture menstruum was evaluated for degrading γ-BHC. The results of the effect of this culture filtrate (CF) on γ-BHC have been recorded in Table 7 and Fig. 8. It was observed that the CF reduced γ-BHC level by 3 ppm out of the 5 ppm taken initially. Encouraged with the results, the incubation of the reaction mixture was extended up to 12 h. But the rate of reduction was much lower as only 3.23 and 3.33 ppm insecticide was degraded on prolonging the reaction time to 8 and 12 h, respectively. This trend was more elaborately shown by the Fig.8 whee the slope of the curves representing per cent degradation and per cent recovery of γ-BHC tended to become parallel to the

**Fig. 6 Effect of duration on production of γ-BHC degrading enzyme of *G.candidum* NCDC-228**

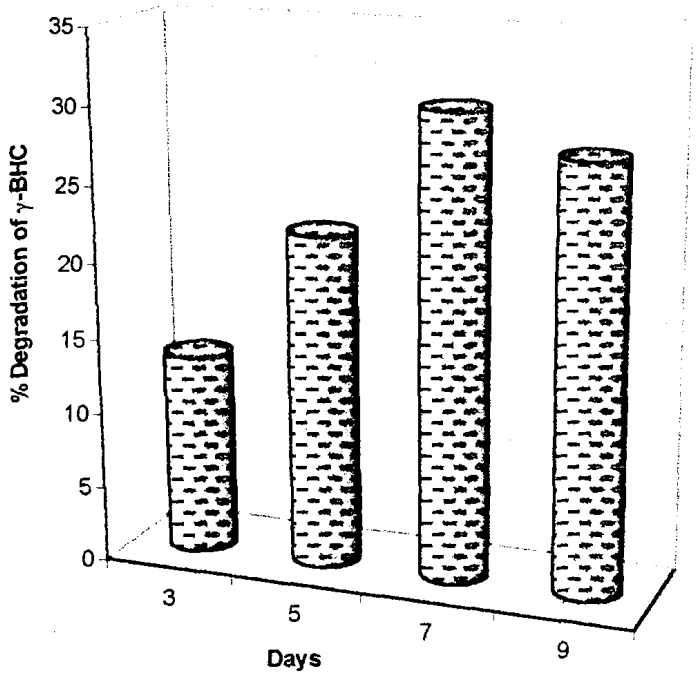


**Table 6. Effect of aeration of culture on production of γ-BHC degrading enzyme medium pH 6.0 at 25°C, after 7 days of incubation**

| Tube No. | Condition of culture broth | Reaction Time(h) | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|---|
| | | | ppm | % | |
| 1 | Static | 0 | - | - | – |
| 2 | Static | 0 | 4.82 | 100 | – |
| 3 | Aerated | 4 | 3.68 | 76.35 | 3.92 |
| 4 | Static | 4 | 3.29 | 68.26 | 5.02 |

**Table 7. Degradation of γ-BHC by culture filtrate of *G. candidum* NCDC-228**

| Tube number | Reaction time (h) | γ-BHC recovered (ppm) | γ-BHC degraded (ppm) |
|---|---|---|---|
| Control | 0 | 4.72 | – |
| 1 | 4 | 1.72 | 3.00 |
| 2 | 8 | 1.49 | 3.23 |
| 3 | 12 | 1.39 | 3.33 |

**Fig. 7 Effect of aeration on production of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

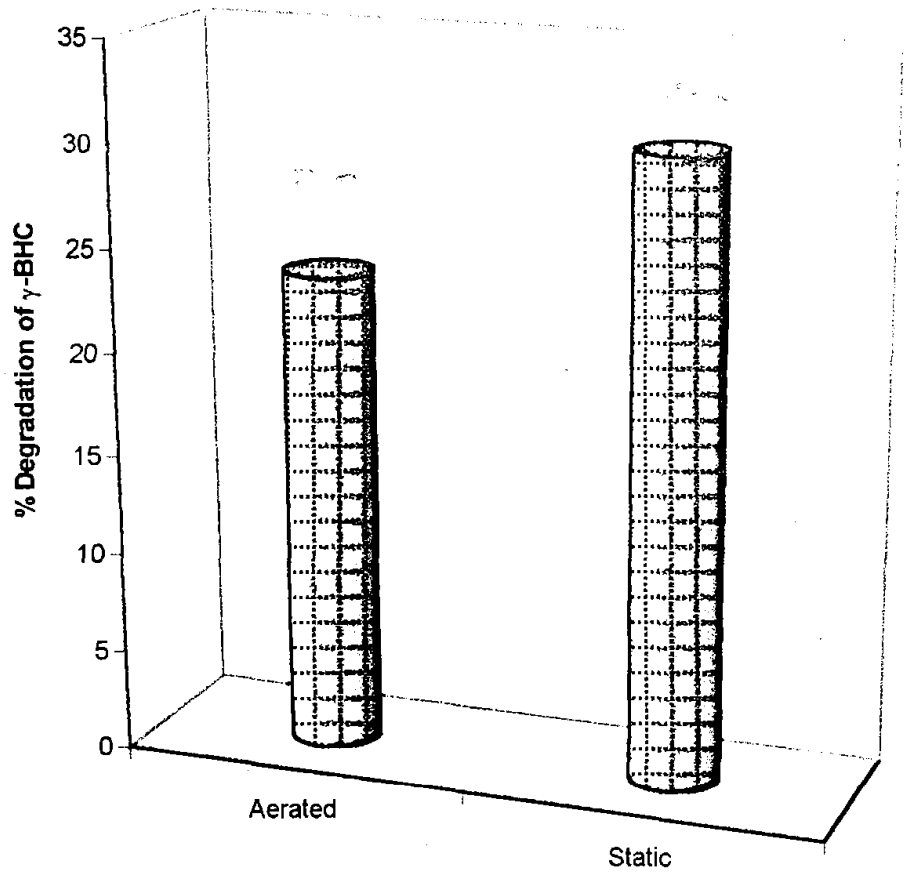
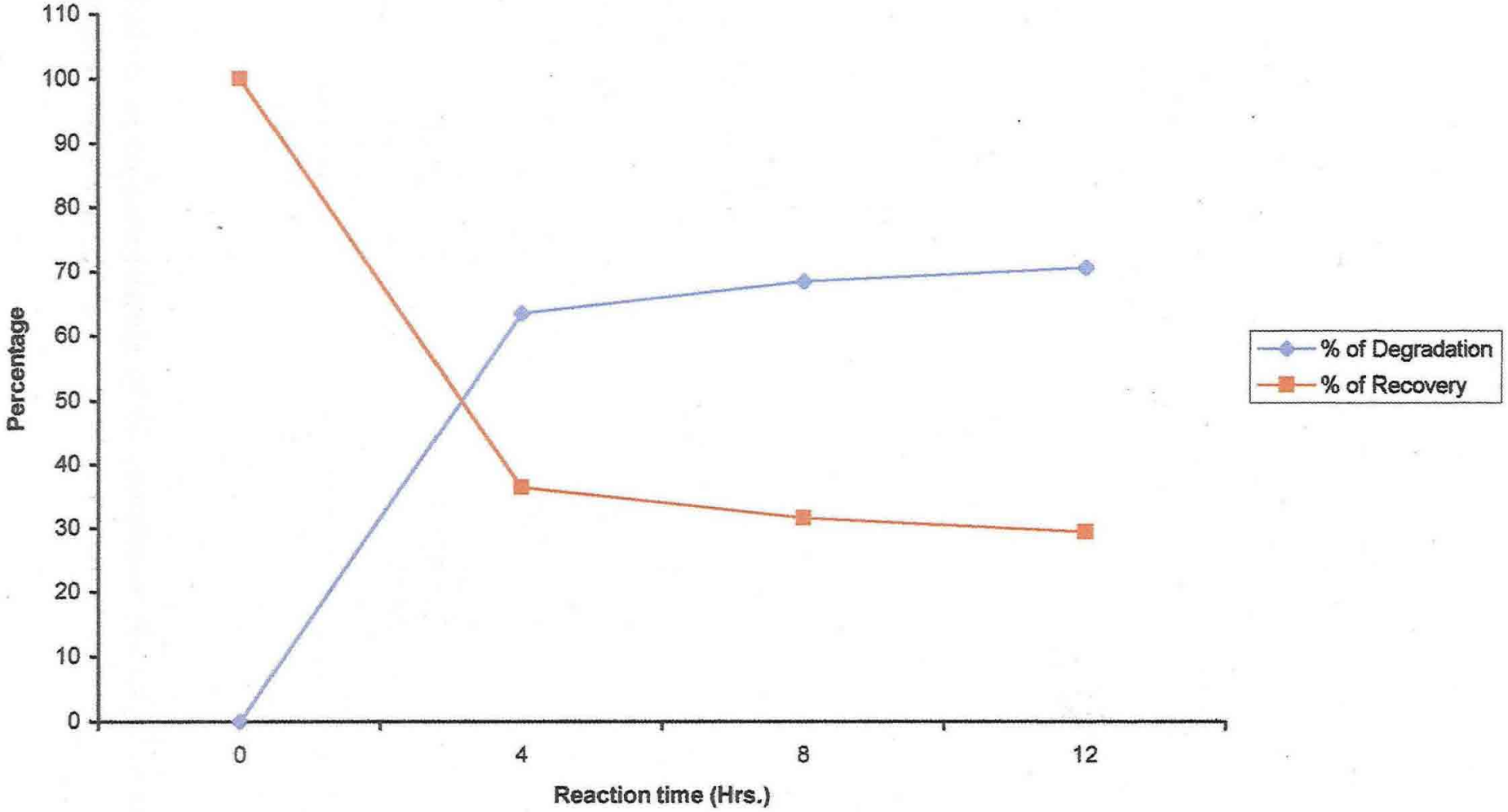
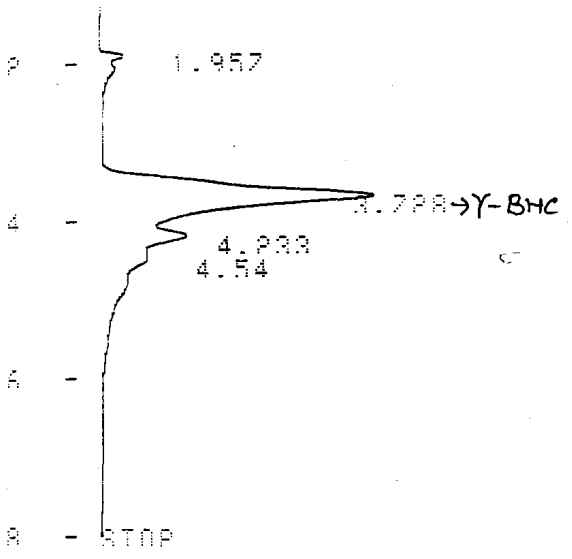


**Fig. 8 Loss of γ-BHC in presence of *G. candidum* NCDC-228 culture filtrate .**



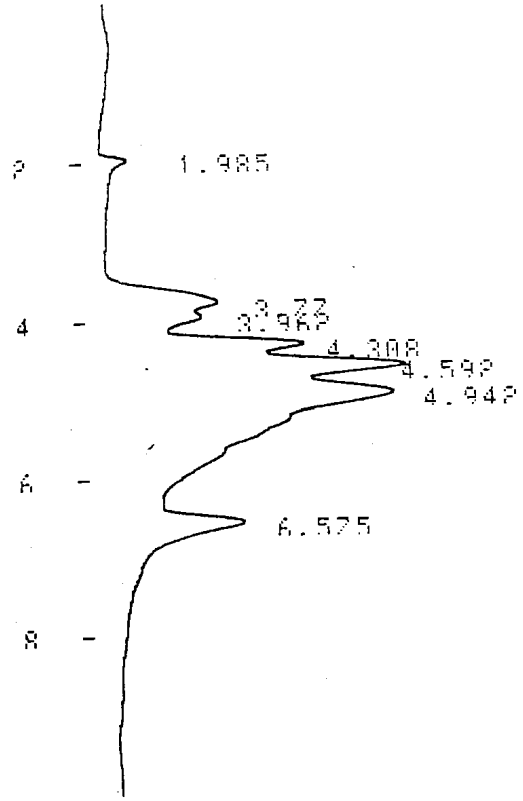



CHROMATOGRAM 1 MEMORIZED

CHROMATOPAC C-R6A FILE 5
SAMPLE NO 6 METHOD 441
REPORT NO P3P3

| PKNO | TIME | AREA | MK | TYPE | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 1.957 | 159 | | | 1.3618 | |
| 2 | 3.728 | 9406 | | | 80.5567 | |
| 3 | 4.233 | 1601 | V | | 13.7155 | |
| 4 | 4.54 | 516 | V | | 4.366 | |
| | TOTAL | 11676 | | | 100 | |

Fig. 9 Effect of culture filtrate of *G. candidum* NCDC 228 on γ-BHC in 0 h at 25°C



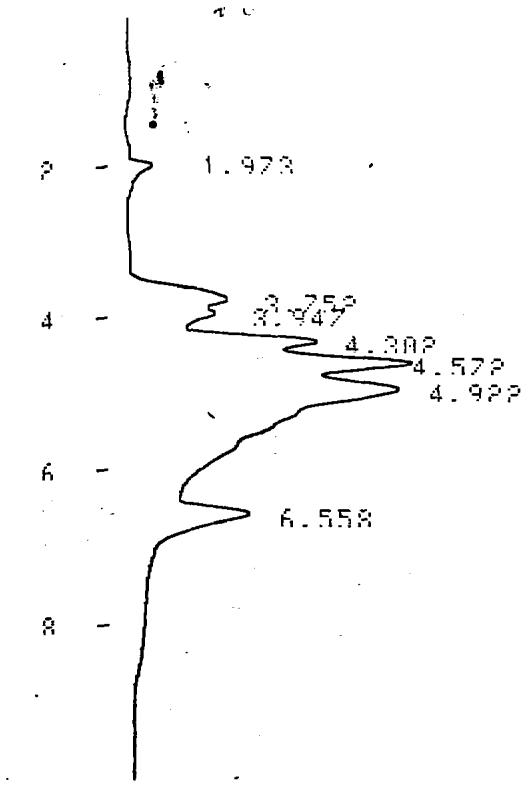

CHROMATOGRAM 1 MEMORIZED

CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 2300

FILE 5
METHOD 441

| PKNO | TIME | AREA | MK | IDNO | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 1.985 | 277 | | | 0.5935 | |
| 2 | 3.77 | 3249 | | | 6.959 | |
| 3 | 3.962 | 2012 | V | | 4.3105 | |
| 4 | 4.308 | 4334 | V | | 9.2825 | |
| 5 | 4.592 | 8326 | V | | 17.8347 | |
| 6 | 4.942 | 22931 | V | | 49.1183 | |
| 7 | 6.575 | 5556 | V | | 11.9015 | |
| | TOTAL | 46685 | | | 100 | |

Fig. 10 Reduction (63.55%) of γ-BHC by culturate filtrate (0.90 mg protein) of *G. candidum* NCDC 228 in 4 h at 25°C




CHROMATOGRAM 1 MEMORIZED

CHROMATOPAC C-R6A FILE 5
SAMPLE NO A METHOD 441
REPORT NO 2302

| PKNO | TIME | AREA | MK | TONO | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 1.973 | 381 | | | 0.8941 | |
| 2 | 3.752 | 2817 | | | 6.6197 | |
| 3 | 3.947 | 1834 | V | | 4.3086 | |
| 4 | 4.302 | 4141 | V | | 9.7311 | |
| 5 | 4.572 | 8019 | V | | 18.8429 | |
| 6 | 4.922 | 21520 | V | | 50.5646 | |
| 7 | 6.558 | 3847 | V | | 9.039 | |
| | TOTAL | 42559 | | | 100 | |

**Fig. 11 Reduction (68.44%) of γ-BHC by culturate filtrate (0.90 mg protein) of *G. candidum* NCDC 228 in 8 h at 25°C**

X-axis. Therefore, a reaction time of 4 h was decided upon for further tests in the study.

## 4.4 PURIFICATION

### 4.4.1 AMMONIUM SULPHATE PRECIPITATION

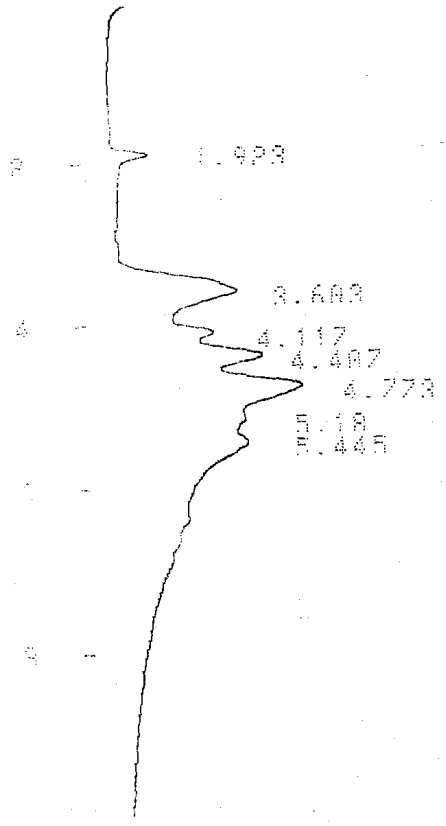
The mould culture filtrate (i.e. crude enzyme) of *G. candidum* NCDC-228 could be purified manifolds with salt precipitation using 60 to 70 per cent concentration of ammonium sulphate. This partially purified preparation exhibited an 10.82 fold increased γ-BHC degrading activity of the enzyme over that of cell free culture filtrate with a consequential activity recovery of 62.50 per cent and a corresponding specific activity of 24.03 unit/mg protein (Table 8). In a similar earlier study in our lab, 60 to 70 per cent $(NH_4)_2SO_4$ salt treatment had resulted in a two-fold purification of γ-BHC degrading enzyme obtained from the same mould, viz. *G. candidum* NCDC-228 (Mittal, 1999; Ann. Rep., 2000-2001). Earlier analogous attempts with 30 to 60 per cent $(NH_4)_2SO_4$ precipitation resulted in 105 per cent yield of recovery (corresponding to 128 unit/mg N) of γ-BHC degrading enzyme obtained from common housefly (Ishida and Dahm, 1965). However, the earlier workers have not clearly defined the enzyme unit. The difference in the results would also emanate from different sources of enzyme and the treatments given for its preparation and purification. No more work on similar lines and on this organism could be traced in the literature. The recovered activity of the purified enzyme in the present study has indicated that the enzyme was quite promising for exploitation on a larger scale.

### 4.4.2 GEL FILTRATION ON SEPHADEX G-75

The partially purified enzyme preparation obtained after ammonium sulphate precipitation of the culture filtrate was further purified by sephadex G-75 gel filtration. The elution profile of γ-BHC degrading enzymic protein is shown in Fig.13. Out of the three protein peaks thus obtained, the major one was eluted between the elution volume of 135 to 170 ml. The two minor peaks

**Table 8. Purification scheme of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

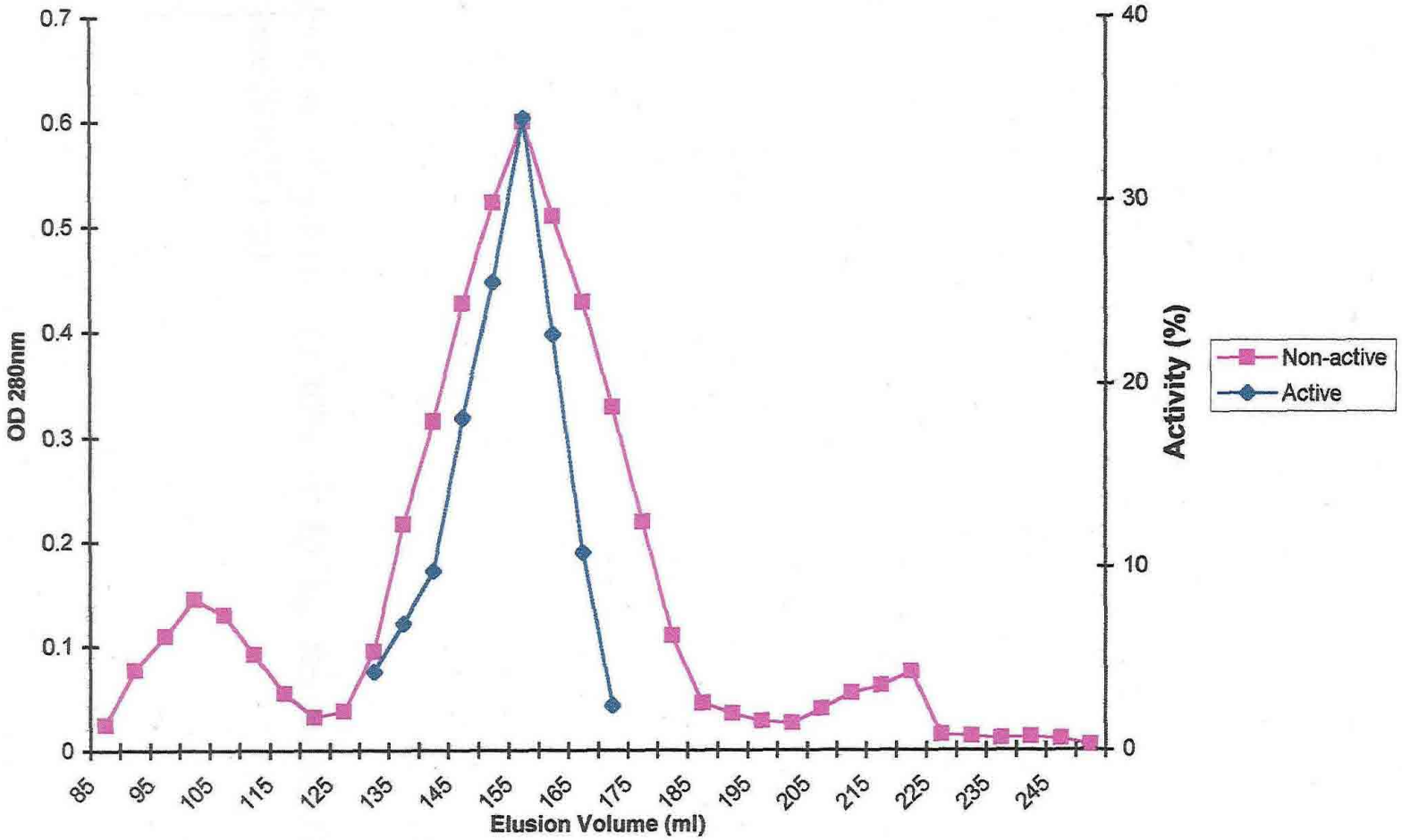
| Purification step | Total protein (mg) | Total enzyme unit | Specific activity | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| Culture filtrate | 180 | 400 | 2.22 | 1 | 100 |
| Ammonium precipitation | 10.40 | 250 | 24.03 | 10.82 | 62.50 |
| Gel filtration (Sephadex G-75) | 1.25 | 50 | 40.00 | 18.01 | 12.50 |

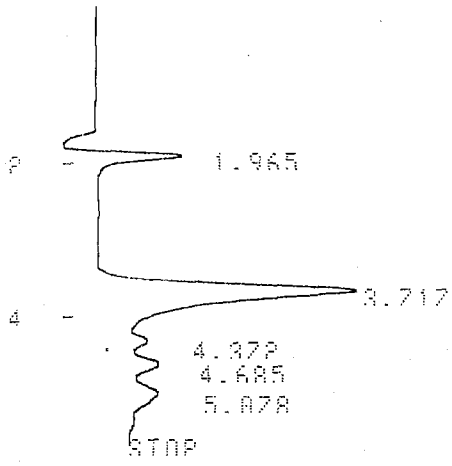



| PEAK | TIME | AREA | MK | TYPE | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 1.923 | 397 | | | 1.7881 | |
| 2 | 3.683 | 4794 | | | 21.5988 | |
| 3 | 4.117 | 1595 | V | | 7.1858 | |
| 4 | 4.487 | 3494 | V | | 15.4368 | |
| 5 | 4.773 | 6113 | V | | 27.5408 | |
| 6 | 5.18 | 2083 | V | | 9.3938 | |
| 7 | 5.445 | 3883 | V | | 17.494 | |
| | TOTAL | 22196 | | | | |

Fig. 12 Effect of ammonium sulphate precipitated enzyme (0.26 mg/ml) on γ-BHC reduction (46.73%) in 1 h at 25°C

**Fig. 13 Gel Filtration chromatogram of the lindane degrading enzyme produced by *Geotrichum candidum* NCDC-228**



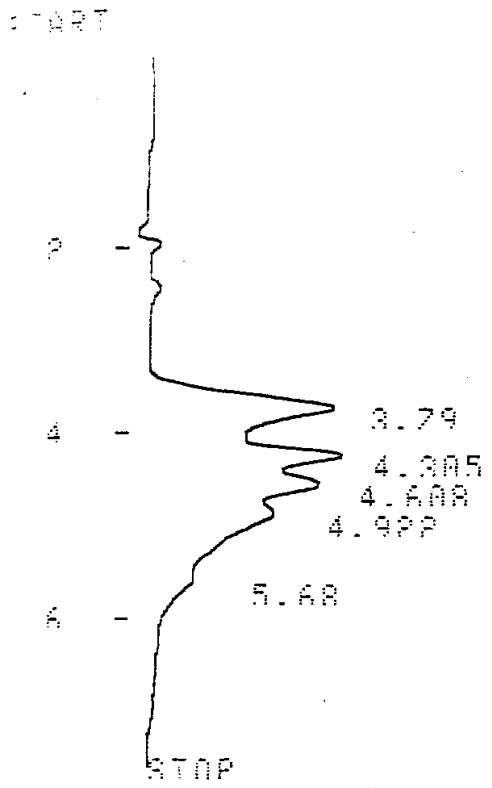



CHROMATOGRAM 1 MEMORIZED

CHROMATOGRAM C-86 FILE 5
SAMPLE NO A METHOD 441
REPORT NO PP74

| PKNO | TIME | AREA | MK | TYPE | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 1.965 | 1624 | | | 12.5051 | |
| 2 | 3.717 | 8478 | | | 65.2837 | |
| 3 | 4.372 | 779 | V | | 6.0003 | |
| 4 | 4.685 | 1087 | V | | 8.3675 | |
| 5 | 5.078 | 1019 | V | | 7.8435 | |
| | TOTAL | 12986 | | | 100 | |

Fig. 14 Reduction of γ-BHC (7.30%, 1 h) by Sephadex G-75 purified enzyme (0.025 mg).

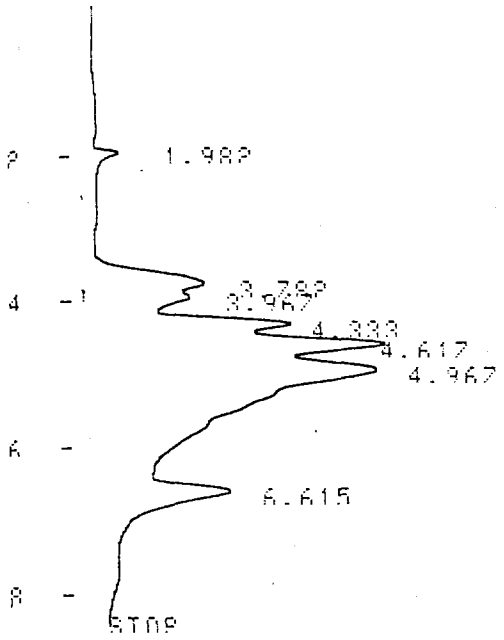



CHROMATOGRAM 1 NORMALIZED

CHROMATOPAC C-R6A FILE 5
SAMPLE NO 0 METHOD 441
REPORT NO P445

| PKNO | TIME | AREA | MK | TYPE | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 3.79 | 6223 | | | 28.904 | |
| 2 | 4.385 | 4918 | V | | 22.8726 | |
| 3 | 4.688 | 4134 | V | | 19.2888 | |
| 4 | 4.992 | 5317 | V | | 24.6992 | |
| 5 | 5.88 | 945 | V | | 4.3886 | |
| | TOTAL | 21529 | | | 100 | |

Fig. 15 Reduction of γ-BHC (31.94%, 4 h) by Sephadex G-75 purified enzyme (0.025 mg protein)




CHROMATOGRAM 1 MEMORIZED

CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 2297

FILE 5
METHOD 441

| PKNO | TIME | AREA | MK | TYNO | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 1.982 | 323 | | | 0.6825 | |
| 2 | 3.782 | 3209 | | | 6.7905 | |
| 3 | 3.967 | 2286 | V | | 4.8486 | |
| 4 | 4.333 | 4471 | V | | 9.4607 | |
| 5 | 4.617 | 8543 | V | | 18.0784 | |
| 6 | 4.967 | 23583 | V | | 49.9045 | |
| 7 | 4.615 | 4922 | V | | 10.4149 | |
| | TOTAL | 47256 | | | 100 | |

Fig. 16 Reduction of γ-BHC (65%, 4 h) by Sephadex G-75 purified enzyme (0.050 mg protein)

lay on either side of the major peak. Among these three peaks, the major peak exhibited the γ-BHC degrading activity. Hence, all the fractions of this particular peak were pooled, concentrated and the protein content and its enzyme activity were measured. Gel filtered fractions had a specific activity 40.00 units/mg protein and this step caused approximately 18.01 fold purification with 12.50 per cent recovery of the activity. Ishida and Dahm (1965) purified the γ-BHC degrading enzyme of housefly on sephadex G-100. Gel filtered product possessed 819 units/mg N of specific activity and a corresponding 61 per cent yield of the activity. Ishida (1968) purified the γ-BHC degrading enzyme of housefly on sephadex G-200 using 0.05 N phosphate buffer (pH 7.4). The sephadex gel filtered fractions represented a single and symmetrical elution curve. The enzyme was eluted between fraction numbers 70 and 90. They did not go for further purification of the enzyme. These workers did not clearly define their enzyme unit. As there is no information available in the literature on the purification aspects of the γ-BHC degrading enzyme from the milk mould, i.e., *G. candidum*, so we were unable to evaluate our results further.

## 4.5 CHARACTERIZATION OF THE ENZYME

The results of the attempts on characterization of the purified enzyme (section 4.4) have been recorded below :

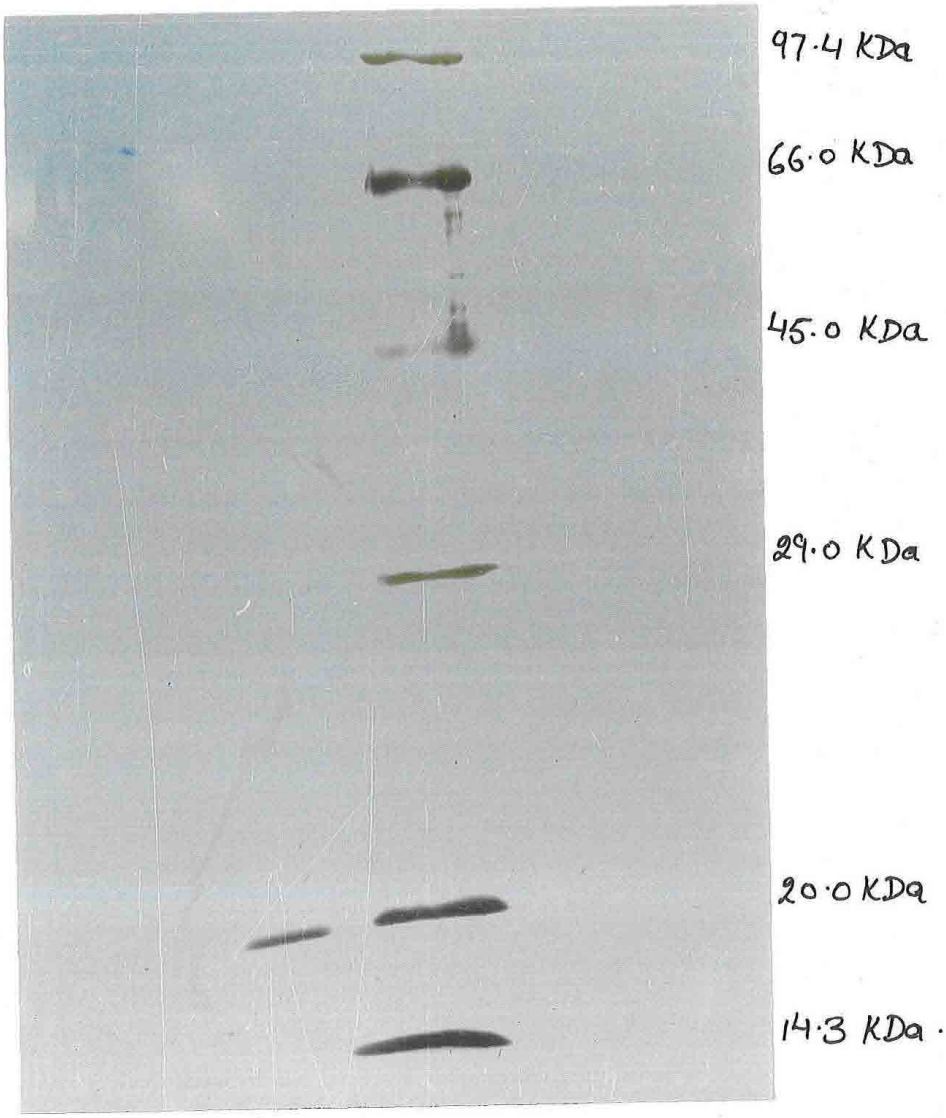
### 4.5.1 DETERMINATION OF MOLECULAR WEIGHT OF γ-BHC DEGRADING ENZYME

The sephadex gel filtered (purified) γ-BHC degrading enzyme was run on sodium doedecyl sulphate polyacrylamide gel electrophoresis (Fig. 17, Photo 1). Based on the results, the molecular weight of the enzyme was estimated to be of the order of 19.5 KD. The dialysis studies indicated that the molecular weight of the enzyme was more than 10 KD as revealed by the presence of γ-BHC degrading enzyme activity in the retentate. In a similar study, Ishida (1968) found that the molecular weight of the enzyme

preparations from housefly and rat liver was 36,000 D. Their findings were in good agreement with Lipke and Kearns (1959) who have described a similar enzyme isolated from housefly and capable of degrading DDT and having a molecular weight of 36,000 D although the substrates for the two enzyme preparations were different. The present enzyme was obtained from a mould which might have been the reason of difference between the molecular weight of Ishida and present enzyme. This aspect requires more and elaborate studies for confirmation.

### 4.5.2 EFFECT OF TEMPERATURE

Temperature plays an important role in the activity of an enzyme. The results of the present study to determine the optimum temperature at which the γ-BHC degrading enzyme in question was active have been presented in Table 9 and [illegible] The enzyme was observed to have an optimum temperature for activity at 25°C where it showed 28.92 per cent γ-BHC degradation corresponding to enzyme activity of 44.30 units/ml. Moving up or down the temperature scale reduced the enzyme activity ($P < 0.01$). It was noted that lowering the reaction temperature had much less deleterious effect than increasing it to 30°C. Some earlier preliminary studies using ammonium sulphate precipitated protein preparation from the culture filtrate of the same organism, i.e., *G. candidum* NCDC 228 also indicated a similar trend and 25°C was found to be the optimum temperature for the enzyme reaction (Ann. Rep. 2000-2001; Mittal, 1999). The present results further showed that the activity curve was not completely smooth or linear. The activity at 20°C was comparatively less with respect to that at 15°C or 25°C. This shoulder formation (Lipke and Kearns, 1959) could not be explained but the sigmoid nature of enzyme activity curves is not very uncommon. However, the overall activity was not that abruptly diminished from the optimum 25°C compared to that it did on raising the temperature to 30°C. Sternburg *et al.* (1954) found that DDT dehydrochlorinase enzyme had maximum activity at 37°C. There was a reduction in the activity at 43°C and it was about half of that at 37°C.




**Photograph 1: SDS-PAGE OF γ-BHC DEGRADING ENZYME**

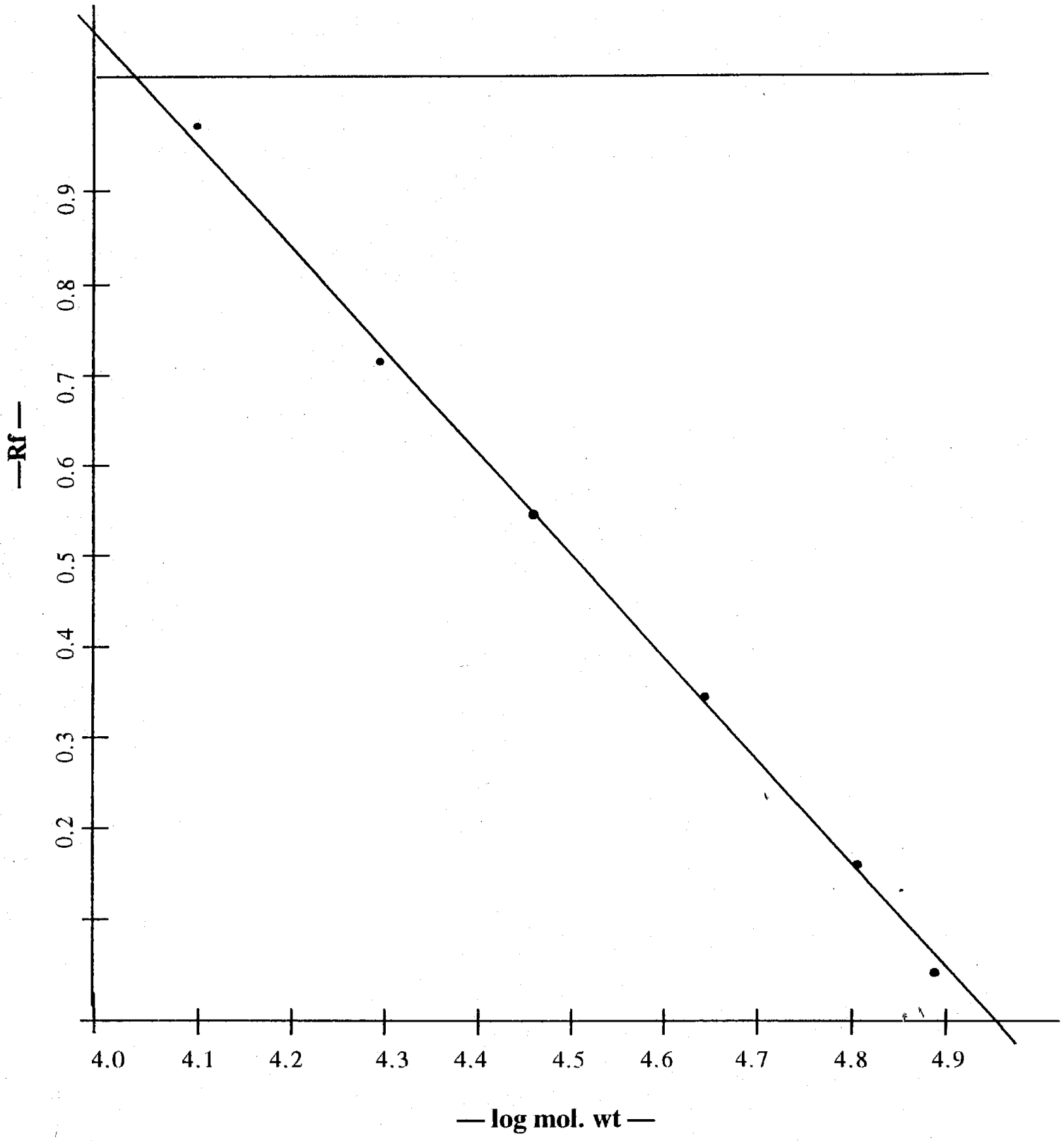



FIG. 17 : Rf versus log molecular weight of protein marker for the determination of molecular weight of enzyme.

**Table 9. Effect of temperature on optimum degradation of γ-BHC by γ-BHC degrading enzyme of *G. candidum* NCDC-228**

| Tube No. | Temperature °C | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|
| | | ppm | % | |
| 1 | 25 | 4.46 | 100.00 | – |
| 2 | 4 | 4.35 | 97.53 | 3.70 |
| 3 | 10 | 3.98 | 89.24 | 16.50 |
| 4 | 15 | 3.73 | 83.63 | 25.10 |
| 5 | 20 | 3.55 | 79.60 | 30.90 |
| 6 | 25 | 3.17 | 71.08 | 44.30 |
| 7 | 30 | 3.76 | 84.35 | 24.00 |

Ishida and Dahm in 1965 found that γ-BHC degrading enzyme from housefly had best activity at 45°C.

### 4.5.3 EFFECT OF pH

The results of the experiments on the influence of pH of the reaction mixture on γ-BHC have been recorded in Table 10 and Fig.19. The enzyme exhibited maximum activity (29.3% reduction, i.e., 49.10 enzyme activity units/ml in γ-BHC content of the reaction mixture) at the pH level of 6.5. This activity decreased to 18.24 per cent at neutral pH and to 27.25 per cent corresponding to 45.70 enzyme activity units/ml at the adjoining lower pH value of 6.0. Evidently, the decline in γ-BHC degradation was not same on these two nearest pH values on either side of 6.5 and was less in the lower pH range. It was further noticed that at still lower pH values (e.g. pH 5.0) the lessening of γ-BHC degradation occurred at slower rates than what it did at the upper side, i.e., up to pH 8.0. Enzyme activity at pH 5.0 was still more than that at pH 7.0. Thus, a pH range of 5 to 6.5 was found to be the best and pH 6.5 was the optimum for the enzyme activity. Its activity decreased enormously at pH 4.0 and 8.0, and was very nominal (3.48%) at pH 3.0 ($P < 0.01$). Sternburg *et al.* (1954) had observed that the optimum pH for DDT dehyhdrochlorinase enzyme activity was 7.4. They also reported a marked loss in enzyme activity at pH 8.5 and 6.5. In an earlier study of a similar nature, Ishida and Dahm (1965) found that the optimum pH for the γ-BHC degrading enzyme of housefly was 7.6.

### 4.5.4 EFFECT OF HEAT TREATMENT

When γ-BHC degrading enzyme under reference was incubated at different levels of temperature for 10 min in Tris-HCl buffer (pH 7.0) in an effort to determine its thermal stability, it exhibited good activity after preincubation at 20° and 25°C (Table 11, Fig. 20). At 30°C and above its activity decreased abruptly ($P < 0.01$) and there was no activity at 50° and 70°C. This indicated the destruction of the enzyme at higher preincubation

**Fig. 18 Effect of temperature on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

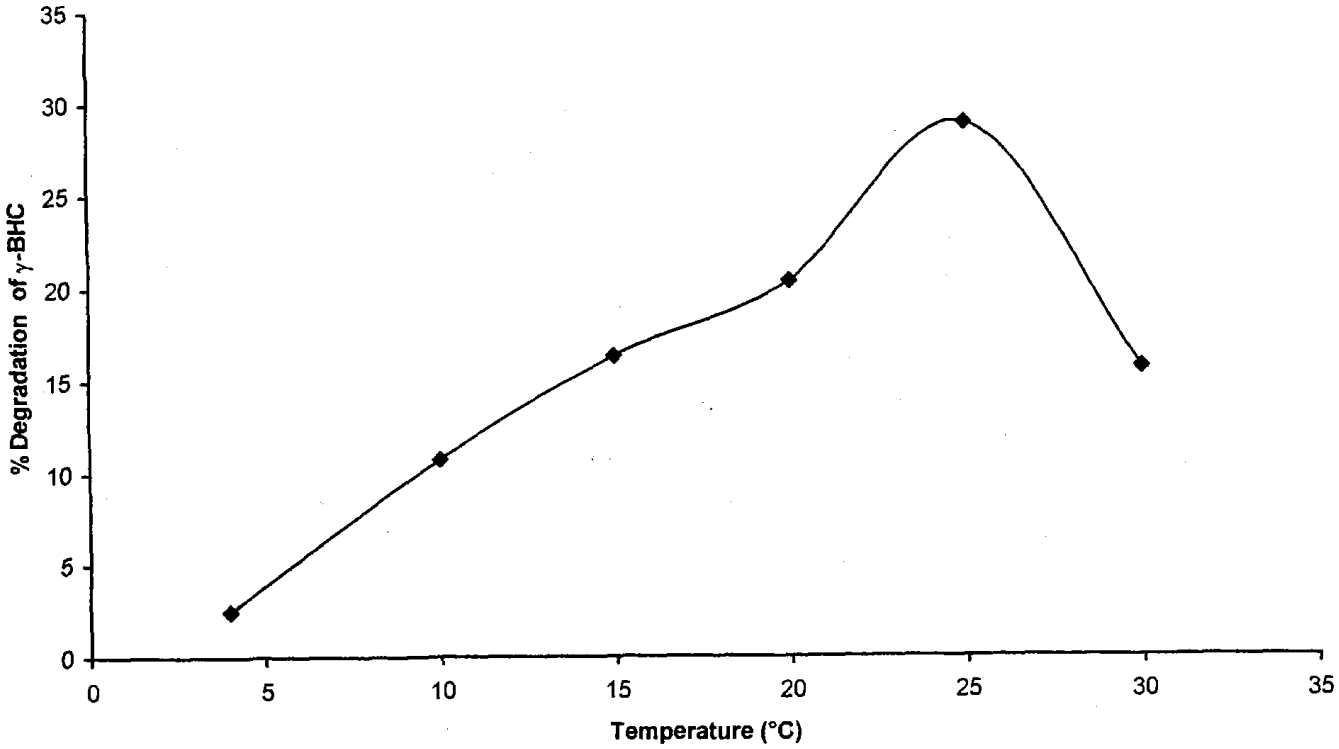


**Table 10. Effect of pH on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

| Tube No. | pH | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|
| | | ppm | % | |
| 1 | 7 | 4.88 | 100.00 | – |
| 2 | 3 | 4.71 | 96.52 | 5.80 |
| 3 | 2 | 4.24 | 86.90 | 22.00 |
| 4 | 5 | 3.79 | 77.68 | 37.40 |
| 5 | 6 | 3.55 | 72.55 | 45.70 |
| 6 | 6.5 | 3.45 | 70.70 | 49.10 |
| 7 | 7 | 3.99 | 80.53 | 29.50 |
| 8 | 8 | 4.31 | 88.32 | 19.60 |

**Table 11. Effect of heat treatment on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

| Tube No. | Temperature °C | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|
| | | ppm | % | |
| 1 | 25 | 4.93 | 100.00 | – |
| 2 | 20 | 3.46 | 70.18 | 50.50 |
| 3 | 25 | 3.47 | 70.44 | 50.20 |
| 4 | 30 | 4.05 | 82.15 | 30.20 |
| 5 | 37 | 4.26 | 86.41 | 23.00 |
| 6 | 50 | 5.21 | 100.00 | – |
| 7 | 70 | 4.97 | 100.00 | – |

Fig. 19 Effect of pH on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228

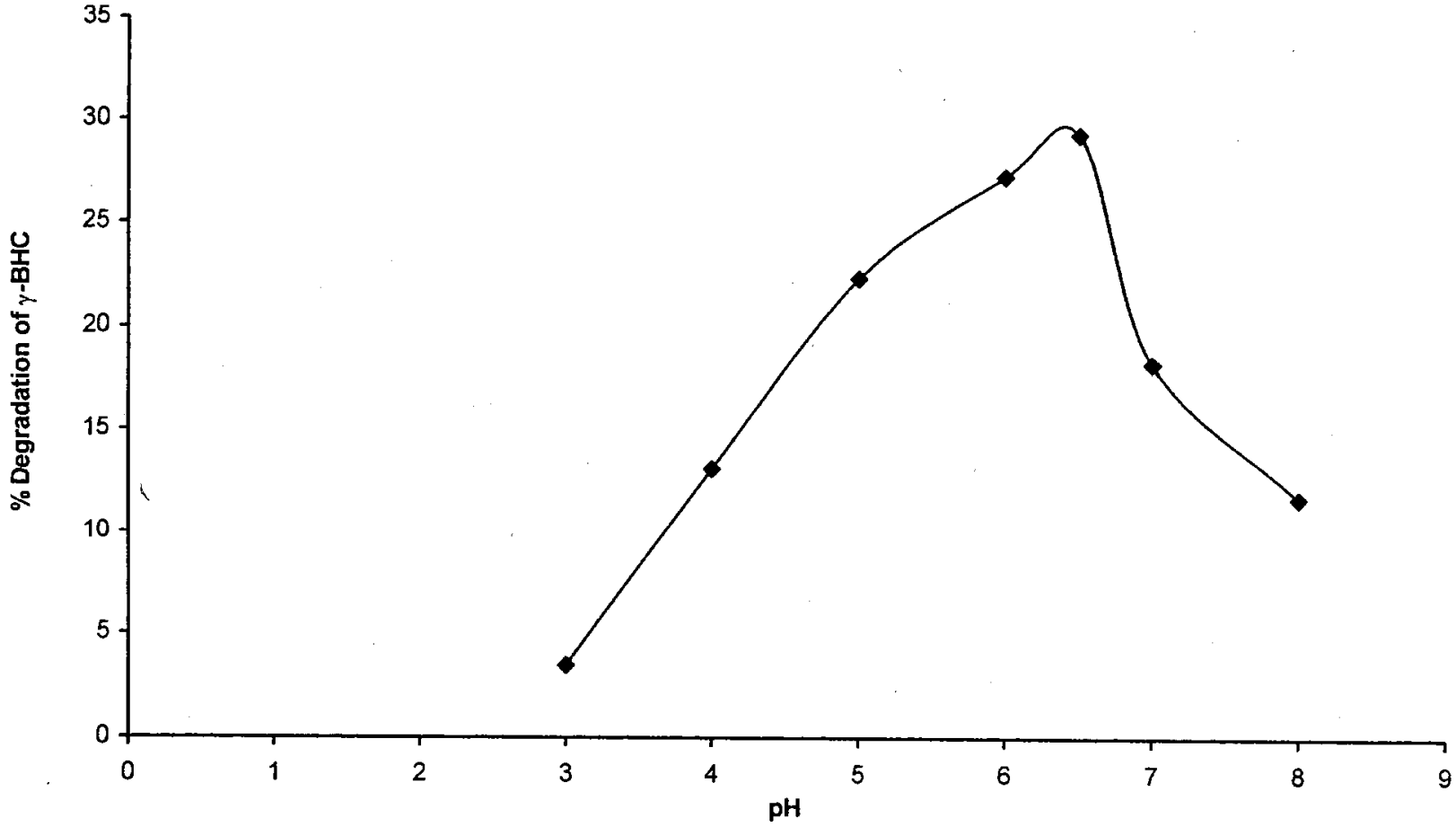
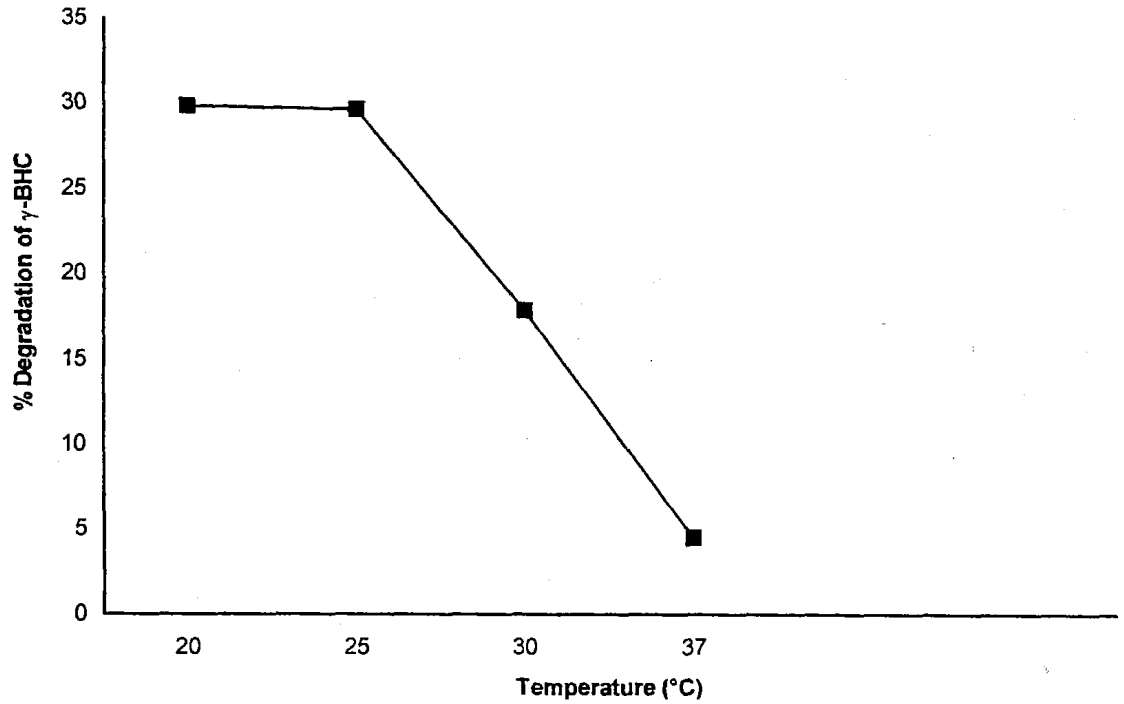


**Fig. 20 Effect of heat stability on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228**



temperature values. After incubation at 25°C, the enzyme caused about 29.61 per cent (enzyme activity units 50.20/ml) reduction in γ-BHC content and it was very close to 29.82 per cent (enzyme activity units 50.50/ml) effected by the enzyme after holding the latter at 20°C. These results were in agreement with those of an earlier work conducted at our institute (Ann. Rep., 2000-2001) wherein it was observed that there was no loss in enzyme activity with preheat treatment up to 25°C for 10 min but at 30°C and above this the loss in enzyme activity progressively increased and a complete loss was observed after preheat treatment at 40°C. In the instant study, some activity was noticed after preheating the enzyme at 37°C. Ishida and Dahm (1965) found that γ-BHC degrading activity in housefly extract was destroyed by heating at 60°C for 5 min. There have been no other reported parallel studies which situation warrants for further investigations.

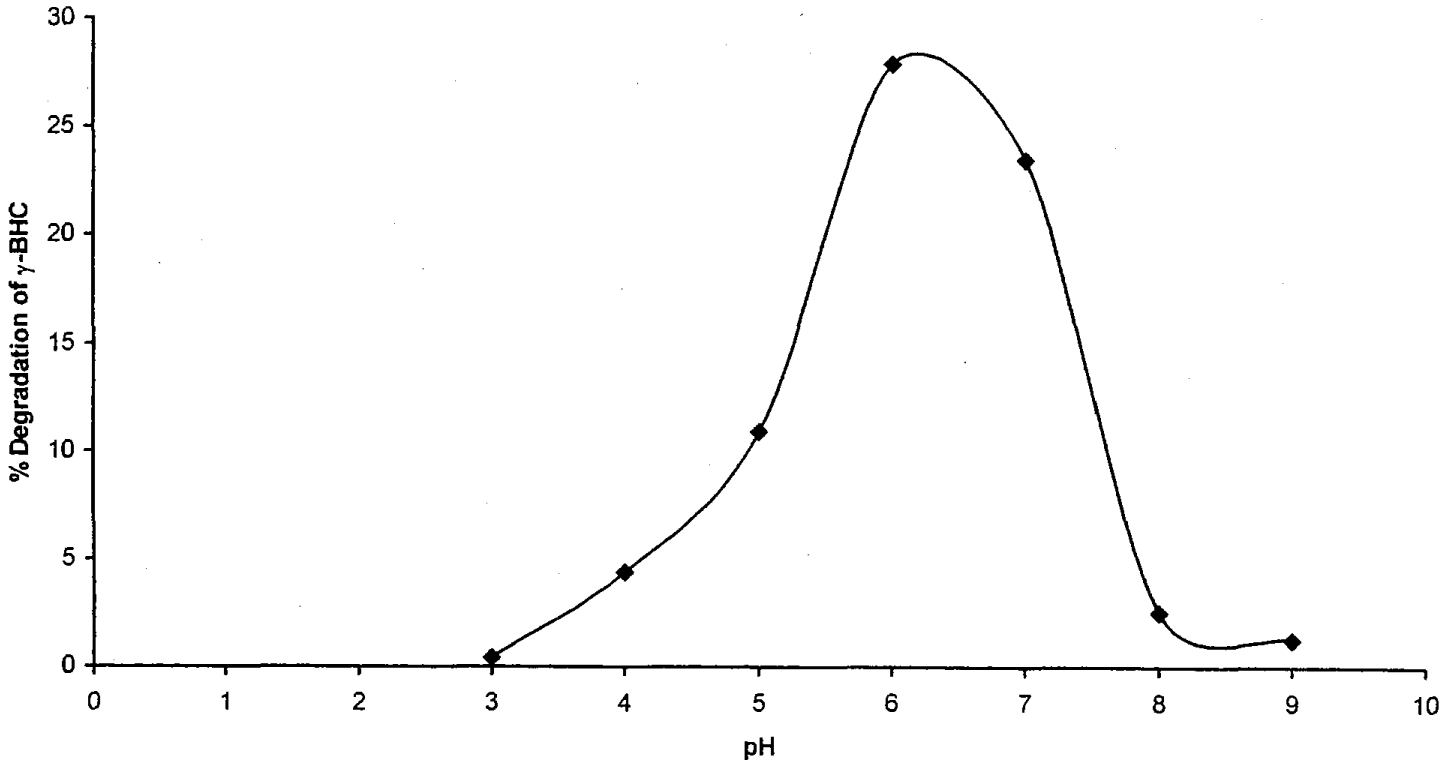
### 4.5.5 pH STABILITY OF ENZYME

The purified enzyme preparation was preliminarily incubated in appropriate buffers at pH levels of 3, 4, 5, 6, 7, 8 and 9 for 60 min at 25°C. The results presented in Table 12 and Fig. 21 revealed that the enzyme was quite stable between pH values of 6 to 7 (enzyme activity units 45.70 and 38.50/ml, respectively). But if there were any deviation on either side of this pH range, there was drastic decrease ($P < 0.01$) in γ-BHC degradation by the enzyme. The highest quantum of degradation obtained (i.e. 27.88%, Fig.12) was after holding the enzyme at pH 6.0, closely followed by that at pH 7.0 (23.48%) in this study. Beyond pH < 5.0 and > 7.0 the insecticide degradation was very close to nil. However, the reaction mixtures were maintained in the initial buffer starting with the test pH and any change in that pH during reaction period of 4 h was not monitored. Earlier studies carried out in the institute had indicated that pH between 6.0 and 7.0 was optimum for maximum stability of the enzyme obtained after $(NH_4)_2SO_4$ precipitation and this fact has lent the best support to the present findings. In an analogous study on the DDT-degrading enzyme isolated from common housefly Lipke and Kearns (1959)

**Table 12. Effect of pH on stability of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

| Tube No. | pH | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|
| | | ppm | % | |
| 1 | 7 | 4.77 | 100 | – |
| 2 | 3 | 4.75 | 99.58 | 0.60 |
| 3 | 4 | 4.56 | 99.50 | 7.20 |
| 4 | 5 | 4.25 | 89.10 | 17.80 |
| 5 | 6 | 3.44 | 72.09 | 45.70 |
| 6 | 7 | 3.65 | 76.47 | 38.50 |
| 7 | 8 | 4.65 | 97.48 | 4.10 |
| 8 | 9 | 4.79 | 98.74 | 2,00 |

Fig. 21 Effect of pH on stability of γ-BHC degrading enzyme of *G. candidum* NCDC-228



observed that the enzyme was most stable at pH 6 to 10 and 10 to 12. However, in the present study, the upper pH limit tested was 9 only. The results obtained could not further be evaluated with similar more studies for lack of such reports.

### 4.5.6 EFFECT OF REACTION TIME ON ACTIVITY OF ENZYME

Table 13 and Fig. 22 represents the results of the enzyme-substrate reaction with respect to time of reaction. The enzyme exhibited practically an ascending straight line relationship between the pesticide degradation and the time of incubation upto 4 h ($P < 0.01$) when the purified enzyme was incubated with the substrate, i.e., γ-BHC, for up to 5 h at 25°C. But incubation beyond 4 h did not improve insecticide degradation which remained around the level that was obtained at 4 h (enzyme activity 45.70 units/ml) incubation. Sternburg *et al.* (1954) found that the rate at which DDT was dehydrochlorinated by a given enzyme varied with source of enzyme and method used in the preparation of enzyme. Such enzyme could sustain linear rates of 60 min, 90 min and as long as 3 h depending on the source of enzyme and beyond which the enzyme activity constantly diminished. A marginal reduction in overall enzyme activity per unit time at 5 h in the present study might not, therefore, be abnormal. Mittal (1999) used a crude enzyme preparation obtained from *G. candidum* culture filtrate and ammonium sulphate precipitation. Although she used higher quantities of the crude enzyme, she got a similar temporal linear trend characteristic of the present investigation.

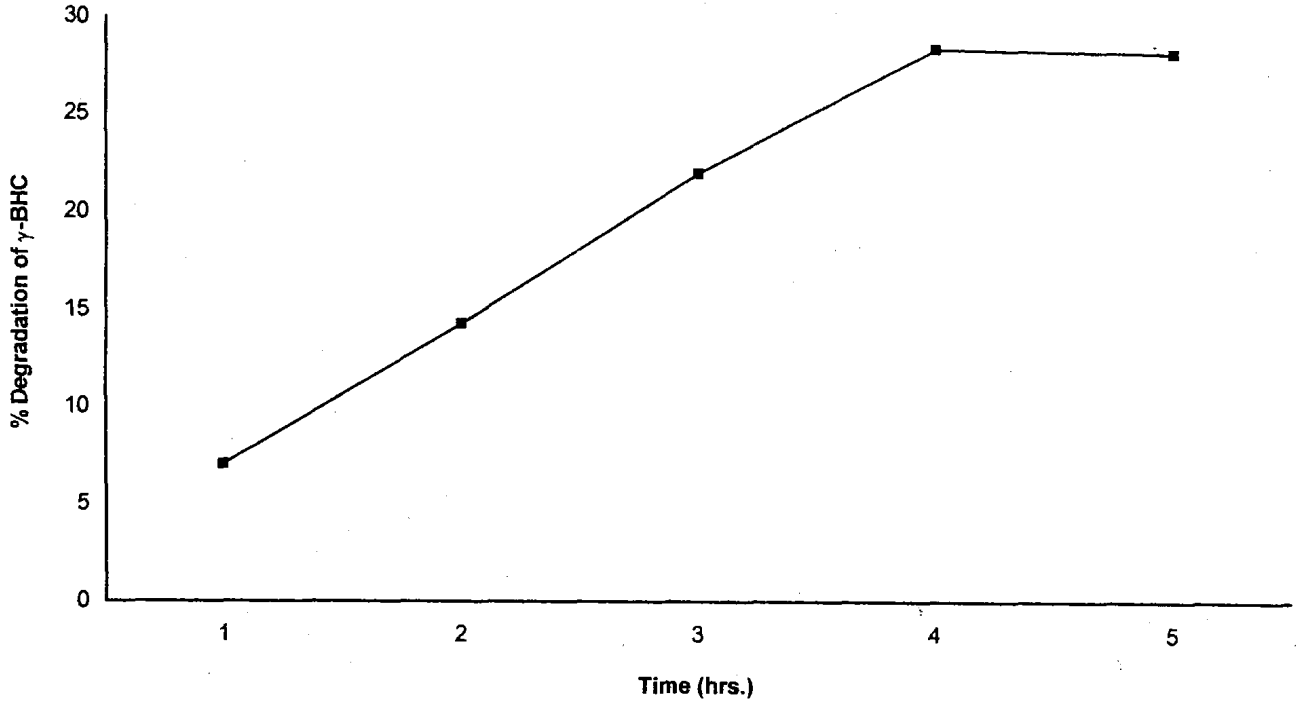
### 4.5.7 EFFECT OF METAL IONS

Among the multitude of environmental factors affecting the substrate utilization properties of an enzyme, metal ions constitute an important niche. The γ-BHC degrading enzyme also showed varying activity in presence of different cations. The results of the investigation have been recorded in Table

**Table 13. Effect of reaction time on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228**

| Tube No. | Reaction time (h) | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|
| | | ppm | % | |
| 1 | 0 | 4.69 | 100 | – |
| 2 | 1 | 4.36 | 92.88 | 11.30 |
| 3 | 2 | 4.02 | 85.62 | 23.00 |
| 4 | 3 | 3.66 | 78.07 | 35.40 |
| 5 | 4 | 3.36 | 71.64 | 45.70 |
| 6 | 5 | 3.33 | 71.11 | 46.70 |

Fig. 22 Effect of time on activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228



14. The enzyme activity to degrade pesticide increased in the presence of $Na^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ ions by 3.40, 7.60 and 4.00 per cent, corresponding to enzyme activity of 55.70, 62.90 and 56.70 units/ml, respectively. On the other hand, $Zn^{2+}$ and $Fe^{3+}$ exhibited an inhibitory effect with the iron cations being stronger (7.8% decrease in enzyme activity compared to 2.2% by $Zn^{2+}$). There has been no such work reported earlier on γ-BHC but Lipke and Kearns (1959), while studying the activity response of a DDT degrading enzyme isolated from common housefly had observed that $Al^{3+}$, divalent cations of Ca, Cu, Fe, Hg, Mg and Mn and some monovalent cations such as Ag and Na, as the salts of nitrate, chloride, aresenate, sulphate, fluoride and also some other co-factors like iodoacetate, N-ethylmaleimide etc. incorporated into the reaction mixture did not adversely or beneficially affect the enzyme activity. Lack of such earlier work has been a constraint in evaluation of the result of the present study. More work is, therefore, required in this direction.

### 4.5.8 EFFECT OF SUBSTRATE CONCENTRATION

The results obtained from the experiments on the effect of γ-BHC concentration on its degradative enzyme activity have been reflected in Table 15. The Lineweaver and Burk plot drawn from the double reciprocal values of reaction rate versus γ-BHC concentration is shown in Fig. 24. The $K_m$ value for γ-BHC degrading enzyme was found to be 29.41 μM and $V_{max}$ 1.45 μmoles at 25°C. As early as in 1959, Lipke and Kearns characterized DDT degrading enzyme from housefly but did not study its $K_m$ and $V_{max}$ aspects. In 1965, a study was conducted by Ishida and Dahm on BHC degrading enzyme from housefly and rat liver. However, these workers also skipped the enzyme kinetics. Getzin and Rosefield (1971) have studied the kinetics of an esterase enzyme isolated from soil and capable of degrading malathion. The highly stable enzyme had a $K_m$ $2.12 \times 10^{-4}$ M and $V_{max}$ 0.28 and 0.58 at 0.75 and 1.5 units. The vast difference in the enzyme kinetic values obtained in the present

**Table 14. Effect of metal ions on activity of γ-BHC degrading enzyme**

| Metal ion ($10^4$ M) | γ-BHC | Per cent inhibition or stimulation | Enzyme activity (units/ml) |
|---|---|---|---|
| Control | 5.00 | – | – |
| $Na^+$ | 3.38 | +3.40 | 55.70 |
| $Ca^{2+}$ | 3.17 | +7.60 | 62.90 |
| $Zn^{2+}$ | 3.66 | –2.20 | 46.00 |
| $Fe^{3+}$ | 3.94 | –7.80 | 36.40 |
| $Mg^{2+}$ | 3.35 | +4.00 | 56.70 |

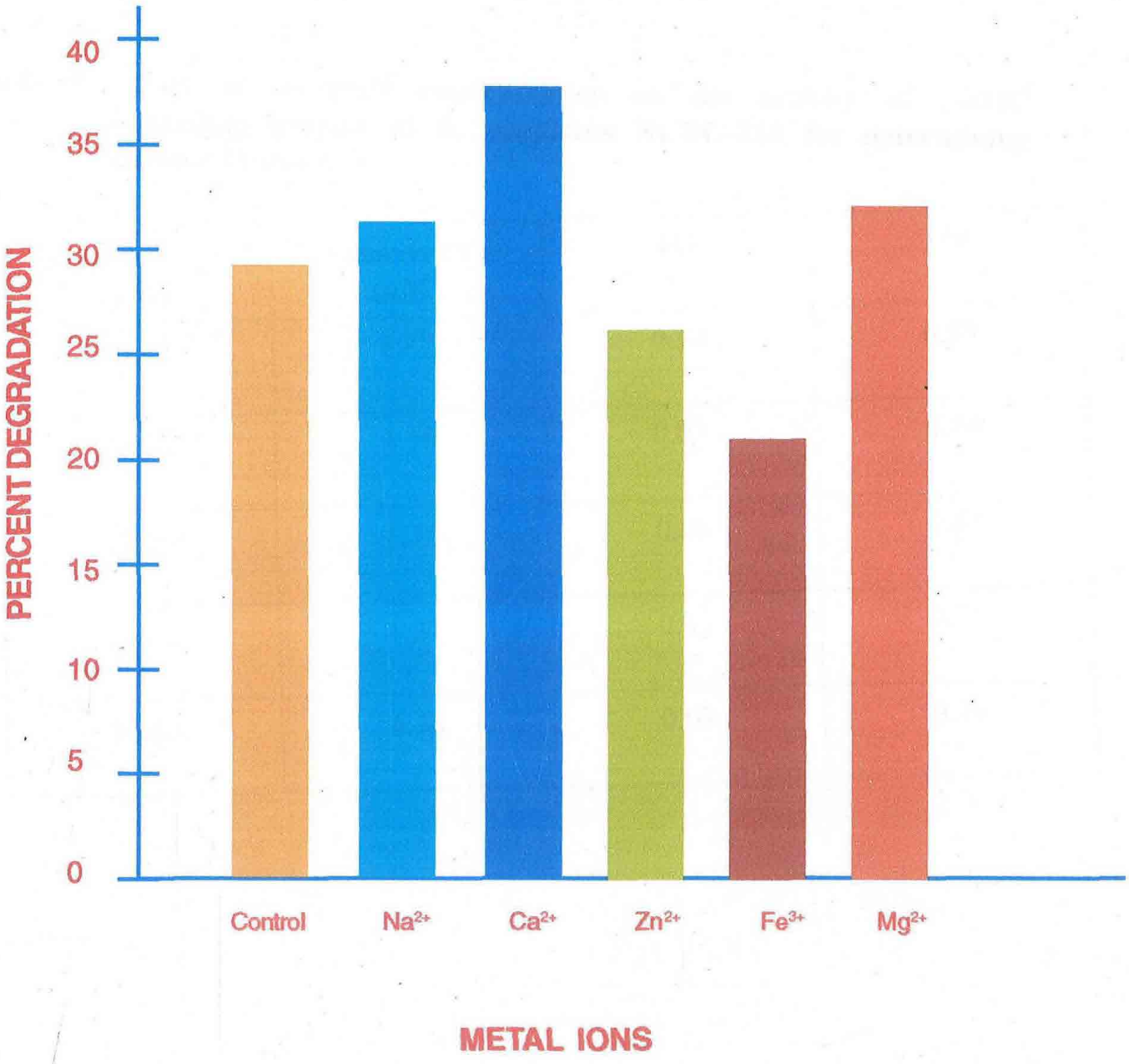



**Fig. 23 : Effect of Metal ions on activity of γ-BHC degrading enzyme.**

**Table 15** **Effect of substrate concentration on the activity of γ-BHC degrading enzyme of *G. candidum* NCDC-228 for determining enzyme kinetics.**

| Substrate (S) (μM) | Velocity (V) (μM) | 1/S | 1/V |
|---|---|---|---|
| 10.32 | 1.12 | 0.10 | 0.89 |
| 13.76 | 1.18 | 0.07 | 0.84 |
| 17.20 | 1.22 | 0.06 | 0.81 |
| 24.08 | 1.27 | 0.04 | 0.78 |
| 34.40 | 1.30 | 0.03 | 0.76 |

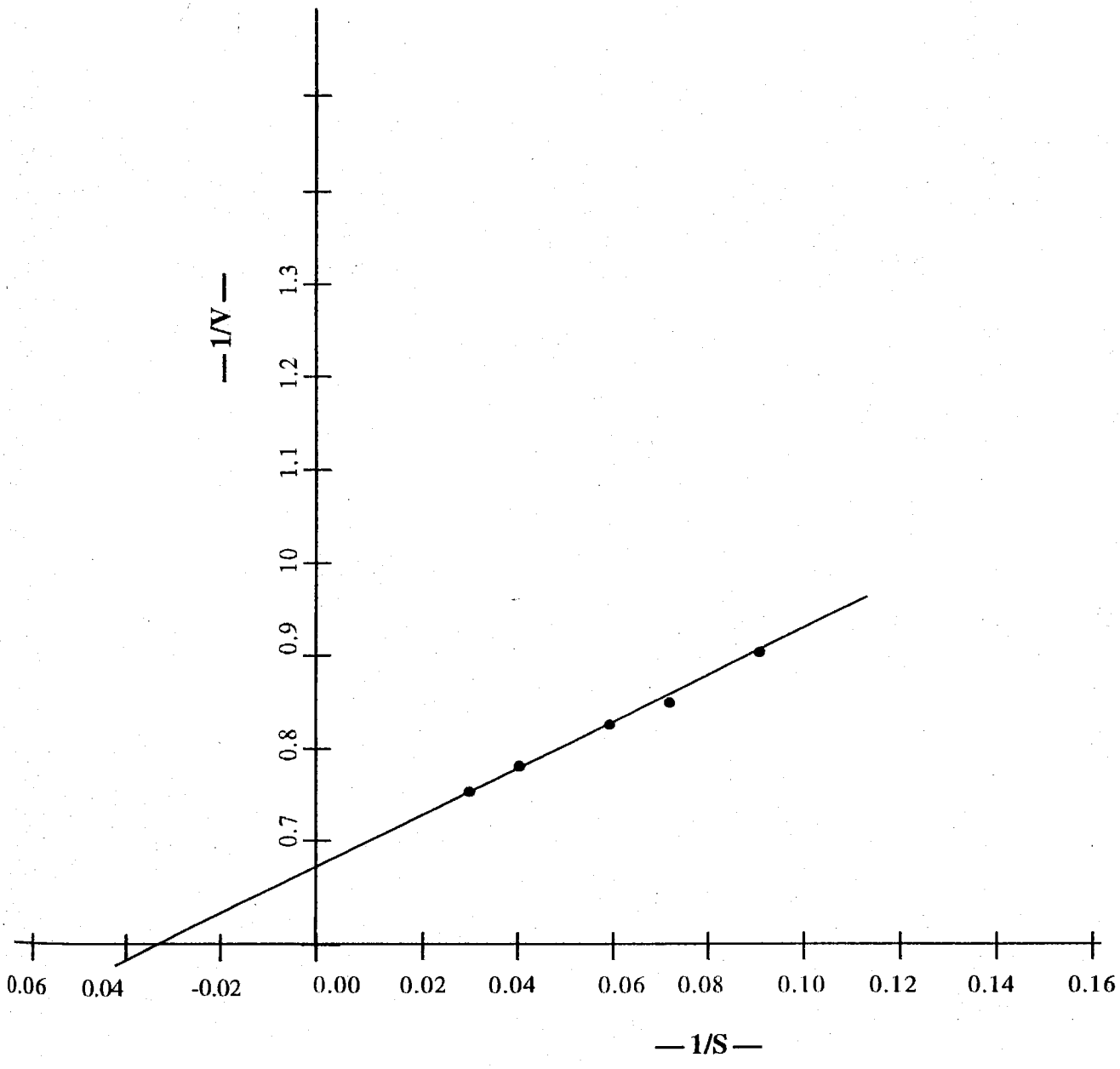


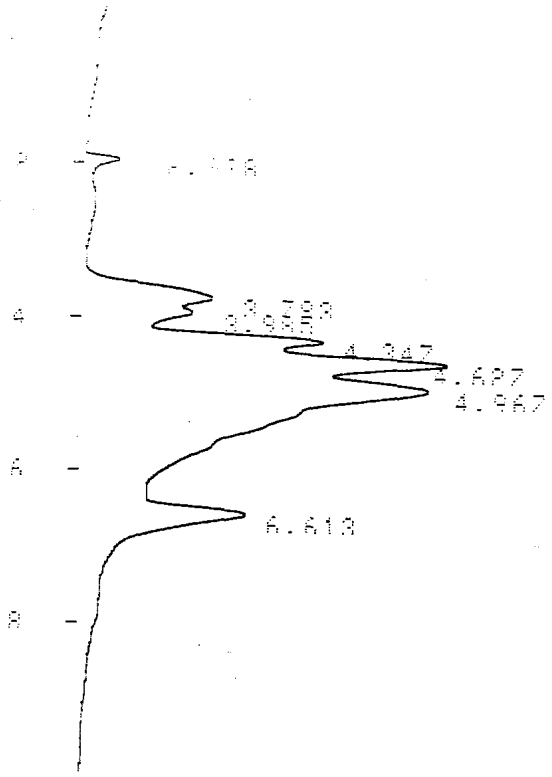

Fig. 24 : Double reciprocal plot of lineweaver and Burk for determination of $K_m$ & $V_{max}$ Values.

**Table 16. Degradtion of γ-BHC level in cow and buffalo milk by γ-BHC degrading enzyme of *G. candidum* NCDC-228**

| Tube No. | Milk | γ-BHC recovered | | Enzyme activity (units/ml) |
|---|---|---|---|---|
| | | ppm | % | |
| 1 | Cow | 4.90 | 100.00 | – |
| 2 | Cow | 3.59 | 73.27 | 45.00 |
| 3 | Buffalo | 4.94 | 100.00 | – |
| 4 | Buffalo | 3.49 | 70.65 | 48.50 |

**Table 17. Quality assurance tests of enzyme treated milk**

| Test sample | Test period (h) | TESTS | | | |
|---|---|---|---|---|---|
| | | COB | % LA | TTC | MBRT time, h |
| PM, PM + E | 0 | – | 0.135 | – | > 5 |
| PM + BHC | 0 | – | 0.135 | + | > 5 |
| PM + C | 0 | – | 0.150 | – | 2½ |
| | 4 | ++ | 0.193 | – | 1½ |
| PM+BHC+E | 0 | – | 0.135 | + | > 5 |
| | 4 | – | 0.136 | ++ | > 5 |
| PM+BHC+C | 0 | – | 0.150 | – | 4 |
| | 4 | + | 0.169 | – | 3 |
| PM+BHC+E+C | 0 | – | 0.150 | – | 3 |
| | 4 | ++ | 0.192 | – | 2 |
| PM+C+E | 0 | – | 0.150 | | 2½ |
| | 4 | ++ | 0.193 | | 1½ |




CHROMATOGRAM 1 MEMORIZED

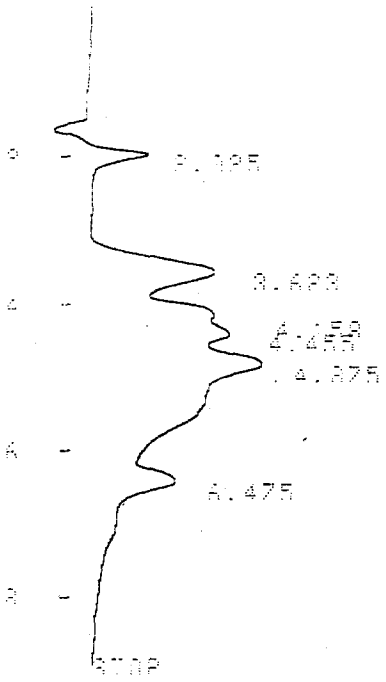
CHROMATOPAC C-R6A
SAMPLE NO. 0
STORAGE NO. PP95

FILE 5
METHOD 441

| PKNO | TIME | AREA | MK | TYPE | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 3.888 | 488 | | | 0.6982 | |
| 2 | 3.783 | 3801 | | | 6.6059 | |
| 3 | 3.985 | 2334 | V | | 4.0654 | |
| 4 | 4.347 | 5693 | V | | 9.8953 | |
| 5 | 4.827 | 10673 | V | | 18.55 | |
| 6 | 4.967 | 27728 | V | | 48.1749 | |
| 7 | 6.613 | 6989 | V | | 12.0084 | |
| | TOTAL | 57537 | | | 100 | |

END

Fig. 25 Application of purified enzyme (0.05 mg protein) in buffalo milk at 25°C *in 4hr*




CHROMATOGRAM 1 MEMORIZED

CHROMATOGR C-RAA FILE 5
SAMPLE NO 3 METHOD 441
REPORT NO PA45

| PKNO | TIME | AREA | MK | TYPE | CONC | NAME |
|---|---|---|---|---|---|---|
| 1 | 3.195 | 3941 | | | 13.7948 | |
| 2 | 3.493 | 4526 | | | 27.8445 | |
| 3 | 4.155 | 2185 | V | | 19.9417 | |
| 4 | 4.455 | 2966 | V | | 13.9505 | |
| 5 | 4.875 | 3574 | V | | 22.8194 | |
| 6 | 4.475 | 1525 | | | 9.4151 | |
| | TOTAL | 16245 | | | 100 | |

END OF REPORT

Fig. 26 Application of purified enzyme (0.05 mg protein) in cow milk at 25°C in 4hr.

contained the insecticide and the enzyme with or without added dahi culture. It was, therefore, implied that γ-BHC residues or the enzyme present in milk, to the extent these were spiked, were undetectable by sensory evaluation and milk could not be graded on that basis. Thus, such milk would ordinarily pass the organoleptic tests of milk quality.

**(ii) COB**

The blank, i.e., pasteurized milk as such as well as that contained 5 ppm of γ-BHC alone or with enzyme did not clot on boiling even after incubation at 35° to 37°C for 4 h. On the other hand, milk inoculated with dahi culture along with γ-BHC clotted on boiling 4 h; but when the enzyme was added, it became strongly COB positive after incubation. The results so obtained indicated that the culture was inhibited, to some extent, by the xenobiotic and the enzyme action removed the inhibition such that the organisms behaved normally. This clearly brought about the utility of the enzyme in effecting depollution of milk.

**(iii) TTC Test**

Test for the presence of inhibitory substances was positive with the milk that contained only the insecticide. In case of such milk as inoculated with *S. salivarius* subsp. *thermophilus* the end point was light red indicating that the organism was not completely inhibited by the pesticide. This result was in consonance with that of Sharma (1994) wherein she had found that *S. salivarius.* subsp. *thermophilus* was not much inhibited by γ-BHC and hence the TTC test was not that dependable in such cases. The quantity (25 μg) of the enzyme added to test milk to degrade γ-BHC in it proved to be quite effective in cleansing of the milk from the pesticide and hence the test organism could grow normally. It, therefore, resulted in a bright cherry red colour comparable to that of the blank milk (Photo 2). The results thus highlight the potential of the enzyme.

**(iv) MBRT test**

Another milk quality assessment test – methylene blue reduction time test was carried out on the milk samples containing the pesticide, the pesticide degrading enzyme or both with or without the added dahi culture. Different combinations reduced the dye in different time periods and it depicted three categories of reduction time. Milk containing either γ-BHC or enzyme or both (i.e., PM, PM + E, PM + BHC, PM + BHC + E) took > 5 h to reduce the dye whereas milk containing BHC and dahi culture (i.e. PM + BHC + C) took 3 to 4 h. It took 3 h or less where the milk contained dahi culture (i.e. PM + C) and the enzyme with or without added γ-BHC (i.e. PM + C + E and PM + BHC + E + C). In the first case, the long reduction time was obviously due to the absence of the culture. In the second case the culture was somewhat inhibited by the insecticide. However, in the third category the reduction time was much less. This implied that the enzyme was quite active and it degraded the insecticide such that the latter's inhibitory effect on the culture was nullified. This observation tallied with the results of the TTC test. Another observation made in these two dye reduction tests was that the enzyme was active at the milk incubation temperature of 37°C. It, therefore, was derived that although the enzyme was not much active at 37°C in pure buffer solution (section 4.5.2) but it retained its activity in milk. More extensive work is required to adjudge the efficacy of the enzyme under such application conditions.

**(v) Lactic acid production**

Lactic acid production by dairy organisms is an important characteristic of these organisms and is an essential step in preparation of fermented dairy products. Presence of γ-BHC has been reported to adversely affect microbial growth and activity as also has been noted in the previous paragraphs. The usefulness of the enzyme produced and purified in the present study was evaluated to free the contaminated milk from the pesticide in terms of per cent lactic acid produced in such milk. Pasteurized milk with γ-BHC or enzyme or


**Photograph 2: TTC OF MILK TREATED WITH ENZYME**

both showed an initial acid content of 0.135 per cent LA after holding the milk for 4 h. The milk inoculated with dahi culture showed an acidity of 0.169 per cent in the presence of γ-BHC after incubation for 4 h. When milk contained γ-BHC, culture and the enzyme, it showed an initial (0 h) acid content of 0.150 per cent LA and final acidity of 0.192 per cent LA after 4 h of incubation which was comparable to 0.193 per cent LA produced by the culture alone or in the presence of the enzyme. The results indicated that the organism could grow well under the test conditions and that it also possessed some inherent resistance to the insecticide. The increased acidity made it clear that the enzyme in question was effective in reducing the inhibitory effect of γ-BHC on dahi culture. These results corroborate well with the above mentioned TTC and MBRT tests. In a similar earlier study, Sharma (1994) had noticed that γ-BHC at concentrations above 1 ppm caused some salutary inhibition of the organism which showed measurable recovery on prolonged incubation. Use of the enzyme effected quick recovery in the present study.

# CHAPTER 5

# *Summary and Conclusions*

# 5. SUMMARY AND CONCLUSION

The deleterious effect of xenobiotic residues encountered in foods and the ever increasing consumer awareness have necessitated the provision of foods having no toxic materials. The dairyman also strives for preventive and curative measures to keep his produce free from such substances. However, as the pesticides have of late become omnipresent, their removal from milk and milk products has become imperative. The milk mould *Geotrichum candidum* has shown its potential in achieving this goal. It produces an enzyme which has been shown to degrade γ-BHC quite efficiently. The present investigation was undertaken to study the various aspects related to production, purification, characterization of this enzyme and its application in dairy industry. This required the optimization of conditions for its production and activity as well as its enzymic chracteristics.

**1. Production .**

*Geotrichum candidum* NCDC-228 was able to withstand the growth restriction by 10 ppm pesticide concentration and gradually started growing normally on prolonged incubation. It could grow well in potato dextrose broth at 25°C and it produced maximum of enzyme in 7 d. Its culture filtrate showed maximum degradation of γ-BHC in 4 h. An initial medium pH 5.0 to 6.0 supported maximum enzyme production, deviation to either side reduced it. Aeration by way of shaking was not beneficial and static culturing under the optimized conditions resulted in a marginally better enzyme yield.

**2. Purification**

Treatment of the culture filtrate with 60 to 70 per cent $(NH_4)_2SO_4$ concentrated the enzymic activity in the resultant precipitate effecting 10.82 fold enzyme purification with a concomitant activity recovery of 62.50 per

cent. Sephadex G-75 chromatography further increased the purification to 18.01 times with a corresponding 12.50 per cent activity recovery.

### 3. Characterization

Characterization was done in buffer. The molecular weight of the purified enzyme was estimated to be beyond 19.5 kD by SDS-PAGE.

Optimum temperature for enzyme activity was found to be 25°C. Lower or higher temperature reduced this activity but high temperature was more deleterious.

Purified enzyme showed good activity between pH 5 to 6.5 and maximum activity at pH 6.5. Higher pH abruptly reduced this activity more rapidly than did the lower pH values.

With respect to time of reaction vis a vis activity the enzyme exhibited ascending straight line relationship up to 4 h beyond which it tended to become horizontal. Its activity decreased abruptly after holding it at 30°C for 10 min and above and it had no activity after pre-treatment beyond 37°C, i.e., at 50°C and 70°C. Holding at 20° and 25°C did not affect its activity.

Storage of purified enzyme at 25°C for 10 min and at different values of pH showed it to be stable between pH 6 to 7 beyond which a rapid decrease occurred in enzyme activity. Maximum stability was at pH 6.0.

$Na^{+}$, $Mg^{2+}$, $Ca^{2+}$ in that order were stimulatory to the enzyme activity but $Zn^{2+}$ and $Fe^{3+}$ were inhibitory; iron cations were more inhibitory than zinc.

The enzyme kinetic studies showed that the purified γ-BHC degrading enzyme had $K_m$ value of 29.41 μM and $V_{max}$ of 1.45 μmoles at 25°C.

### 4. Application : degradation ability in milk

The enzyme when added at a rate of 25 μg/10 ml milk containing 5 ppm γ-BHC caused a 26.73 per cent reduction in the pesticide in cow milk and

29.35 per cent in buffalo milk which was almost equivalent to that caused in buffer (28.92 per cent).

**Quality Assurance Tests**

Sensory evaluation was not affected by the presence of the enzyme in milk. The quality control tests were affected to the extent that on addition of the enzyme the milk polluted with γ-BHC behaved as if it were free from the pesticide.

The present investigation has been a milestone in the human efforts to depollute our environment – the foods being one of the most important causes of pollution. The non-degradability of γ-BHC has resulted in its being banned from use on and in food stuffs with a consequential deprivation of harvesting its usefulness. The present endeavour is one among the various options including its biodegradation using the environmental constituents themselves to depollute our biosphere. The mould is a common saprophytic contaminant of milk and milk fat, its metabolism has no reference of its being toxic to the consumer. The γ-BHC degrading enzyme produced by the mould has proved to be quite efficient in reducing the pesticide residue levels of milk in a short time although pure enzyme may not be so active at that temperature while in buffer. The presence of the enzyme also did not cause any unpleasant or unfavourable effects on milk quality.

Literature on γ-BHC has clearly indicated that its enzymatic degradation products, mainly γ-TCCH and γ-PCCH are highly short lived, hence are further degraded into chlorophenol the easy ring-opening of which results in soluble components which are rapidly excreted. As the enzyme in the present study was produced from *G. candidum* without any induction, the enzyme is constitutive and as per the nature of the producing mould, it also can be safely regarded as harmless. After treatment of milk the residual enzyme can be destroyed by slight heat processing, if so required. To be more economical, use of immobilized enzyme may also be advocated. To provide good and safe

food, incurring of some extra costs, if any, may not be prohibitive. Genetic biotechnology involving transfer of responsible mould genes into compatible organisms including LAB may also be exploited for betterment of life. It can, therefore, be concluded that the study has opened new vistas of application of dairy originated microorganisms and their versatile enzymes for environmental depollution. The vital information so gathered would certainly help to attain the cherished goal earlier.

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