STUDIES ON NOVEL OXALATE OXIDASE PRODUCED BY AN ENDOPHYTIC BACTERIUM *OCHROBACTRUM INTERMEDIUM* CL6

Thesis

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

KUNAL KUMAR

DEPARTMENT OF CHEMICAL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY KARNATAKA

SURATHKAL, MANGALORE – 575 025

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D E C L A R A T I O N

I hereby *declare* that the Research Thesis entitled "**Studies on novel Oxalate Oxidase produced by an endophytic bacterium *Ochrobactrum intermedium* CL6**"which is being submitted to the National Institute of Technology Karnataka, Surathkal in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in the Department of Chemical Engineering, is a *bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

Place: NITK, Surathkal

Date:

Name: KUNAL KUMAR

Register Number: 121160CH12F01

Department of Chemical Engineering

CERTIFICATE

This is to *certify* that the Research Thesis entitled "**Studies on novel Oxalate Oxidase produced by an endophytic bacterium *Ochrobactrum intermedium* CL6**" submitted by **Mr. Kunal Kumar (Register Number: 121160CH12F01)** as the record of the research work carried out by him, is *accepted as the Research Thesis submission* in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**.

Research Guide
Dr. Prasanna B.D
Assistant Professor
Dept. of Chemical Engineering
NITK, Surathkal

Chairman -DRPC

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DEDICATED

TO

MY FAMILY

ABSTRACT

Oxalate Oxidase (EC 1.2.3.4) catalyzes the oxidative cleavage of oxalate to carbon dioxide with the reduction of molecular oxygen to hydrogen peroxide. Oxalate Oxidase (OxO) finds application in clinical assay for oxalate in blood and urine, apart from several potential industrial applications. This research work is about isolation of endophytic bacteria from the tubers of the plants *Colocasia esculenta, Beta vulgaris, Ipomoea batatas* and peel of *Musa paradisiaca* fruit for screening of OxO producing strain. A total of 49 endophytes were isolated and out of which, 4 were OxO producing strains. Based upon the OxO activity produced in nutrient medium, one strain CL6 isolated from tubers of *C. esculenta* was selected and identified as *Ochrobactrum intermedium* by 16 S rDNA sequencing. The effect of media components and process variables on the growth kinetics of the *O. intermedium* CL6 and on the production of the enzyme OxO showed that production is inducible and requires manganese ions in the medium, and very low fill-up volume is beneficial. Characterization of the partially purified OxO revealed many intriguing characteristics. The enzyme is thermostable and remains active for 6 h in the temperature range of 4-80°C. This enzyme is the only known OxO which did not show substrate inhibition up to a substrate concentration of 50 mM. The enzymatic activity was not adversely affected by most of the metal ions and biochemical agents (K^+, Na^+, Zn^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Glucose, Urea, and Lactate). The enzyme appears to be a metalloprotein stimulated by Ca^{2+} and Fe^{2+}. It's Km and kcat for oxalate was found to be 0.45mM and 85 s^{-1} respectively. Chemical modification of OxO revealed that cysteine, carboxylates, histidine and tryptophan residues are part of the active site. A two-fold increase in Oxalate Oxidase activity was observed when histidine residues were modified with 15mM diethylpyrocarbonate. Application studies on the development of a novel enzymatic treatment method to reduce total oxalate content of the extracted starch from Taro flour resulted in 97% reduction and this treatment did not alter any of the desirable physico-chemical properties of the starch.

Keywords: Oxalate Oxidase, Endophytes, *Ochrobactrum intermedium*, Thermostable, Taro, Starch

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ABBREVIATIONS

DEPC - Diethylpyrocarbonate

DMA - Dimethylaniline

DTNB - dithio-bis-(2-nitrobenzoic)

DW - Dry weight

EC - Enzyme Commission

EDAC -1- ethyl-3-(3-dimethylaminopropyl) carbodiimide

EDTA - Ethylenediaminetetraacetic Acid

FW - Fresh weight

HCL - Hydrochloric acid

HPLC – High pressure liquid chromatography

MBTH -3 -Methyl-2-Benzothiazolinone Hydrazone

NBS - N-bromosuccinimide

OD- Optical density

OTR- Oxygen transfer rate

OxO - Oxalate Oxidase

PEG- Polyethylene glycol

PMSF - Phenylmethane sulfonyl fluoride

PPM- Parts per million

RPM - Rotation per minute

SDS–PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

% T - % Transmittance

NOMENCLATURE

K_m - Michaelis Menten constant

k_{cat} - Turn over number

° C - Degree Celsius

M - Molar

mM - Millimolar

h - Hour

gL^{-1} - Gram per liter

mL - Milliliter

U - Unit

kV - Kilo Volt

ET- Enzyme treated

NT - Untreated

μm - Micrometer

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Oxalate Oxidase (oxalate: oxygen oxidoreductase, EC 1.2.3.4) catalyzes the oxidative cleavage of oxalate to carbon dioxide with the reduction of molecular oxygen to hydrogen peroxide. Cupins, functionally diverse proteins that includes seed storage proteins, germins, germin-like proteins and auxin-binding proteins of higher plants, concerned with cell wall, fungal defense, salt tolerance, and floral induction, exhibit Oxalate Oxidase (OxO) activity. Zaleski and Reinhard in 1912 described Oxalate Oxidase for the first time from studies of powdered wheat grains. Since then OxO activity has been detected in several plants viz., Barley (Chiriboga 1966), Beet (Varalakshmi and Richardson 1992), Maize (Vuletic and Sukalovic 2000), Sorghum (Satyapal and Pundir 1993), rice (Dunwell 1998) and in white rot basidiomycete, *Ceriporiopsis subvermispora* (Aguilar et al. 1999) and a bacterium, *Pseudomonas sp*. OX-53 (Koyama 1988) as well.

Among all the physiological importance of Oxalate Oxidase in nature, the most intriguing is its role in coordination of defense responses in plants. The recent demonstration of an Oxalate Oxidase activity for wheat germin suggested specific molecular mechanisms by which the germins might participate in plant defense and development (Dumas et al. 1993; Lane et al. 1993; Lane 1994). Increased Oxalate Oxidase activity has also been found in response to salt stress and fungal infection (Dumas et al. 1995; Hurkman et al. 1991) in plants. This increased activity can result in sustained H_2O_2 production (Zhang et al. 1995), which induces the plants to produce peroxidase enzyme. Peroxidase, in the presence of H_2O_2, catalyses the oxidation of monolignols to free radicals which polymerise to give lignin (Mensen et al. 1998; Kristensen et al. 1999). Lignification of plant cell wall is thus a response to the damage caused by pathogens in plants, which gets initiated by expression of Oxalate Oxidase gene. Thus, high Oxalate Oxidase activity perhaps reveals the close association of the Oxalate Oxidase with development of the cell wall (Sathisraj and Augustin 2012).

Oxalate Oxidase is being extensively used in clinical assays for the estimation of oxalate content in blood and urine (a major component of kidney stones) (Godara and Pundir 2008). Regular assessment of oxalate levels in urine helps to monitor and control hyperoxaluria and Urolithiasis (Dunwell 1998). Removal of oxalic acid from bleaching filtrates using the enzyme Oxalate Oxidase is a potential solution for scaling problem in the pulp and paper industry (Sjode et al. 2008). Oxalate Oxidase has also potential uses in improving disease resistance in plants, reducing oxalate levels in food crops, the bioremediation of oxalate wastes (Dunwell et al. 2000; Escutia et al. 2005). Packaging materials having immobilized OxO and catalase were found effective in scavenging oxygen molecules in packed foods (Winestrandet al. 2013).

Among all, OxO found in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) seedlings are extensively studied and most of the commercially available OxO is purified from barley and wheat plants. However all the reported OxO enzymes showed substrate inhibition and Barley root OxO was the only enzyme, which could show activity in oxalic acid concentration as high as 4mM (Kotsira and Clonis 1997). Interestingly both *Costus pictus* OxO and sorghum leaves OxO showing very high affinity for the substrate (K_m value of 0.065 mM and 0.024 mM respectively) showed substrate inhibition at 0.8 mM and 0.25 mM substrate concentration respectively. Industrial application of this enzyme demands operation in conditions close to 70°C (Cassland et al. 2010) and none of the OxO from barley, wheat and any other plant or microbial sources exhibit the property of thermo stability (Kotsira and Clonis 1997; Vuletic and Sukalovic 2000; Aguilar et al. 1999; Koyama 1988). Therefore finding new source of the enzyme having diverse characteristics could be worthwhile in view of expanding the scope and potential of OxO.

An alternative source of OxO can be of microbial origin and microbial sources are preferred over plants because of technical and economic reasons. Absence of seasonal variation, bulk production, and short generation time in microbes favours their selection over their plant counterpart (Panesar et al. 2010). In addition to this, use of plant species for extracting Oxalate Oxidase enzyme is ecologically unsuitable, as it requires sacrificing the whole plant. In this work, a proposition is made to search an Oxalate Oxidase producing microbial strain from plants abundant in oxalic acid, so that the

enzyme from such microbial sources would have industrially favourable physico-chemical and kinetic properties.

Oxalic acid and its salts are metabolic end product that accumulate in a number of plant tissues (Morrison and Savage 2013). Insignificant amount of oxalic acid is found in most of the plants but few tropical plants have high amount of oxalates in their living tissue as a component of grains, vegetables, tubers, nuts, and fruits (Lane et al. 1993). Highest level of oxalates are found in following families: arum family, for example Xanthosoma (caladium) and Colocasia (taro); amaranth family, for example Amaranthus; ice plant family, for example Tetragonia (NZ spinach); goose foot family, for example Beta (beet or beet root) and Spinacia (spinach); wood sorrel family, for example Oxalis (sorrel, yam); buckwheat family, for example Rhubarb and Sorrel; and the purslane family, for example Purslane (Morrison and Savage 2013). So, some of these plants possessing substantial amount of oxalates in their living tissue might be harbouring endophytes capable of producing oxalic acid degrading OxO.

In general, microbes inhabiting plants have been termed as endophytes and they are more specifically considered as endosymbionts as they do not have any pathogenic effects on plants even after colonizing the interior organs of host plants. The term endophyte denotes to a fungal or a bacterial microorganism (Gunatilaka 2006). The symbiotic association of plant and endophyte is beneficial to both of them. The endophyte produces bioactive metabolites to augment the growth and competitiveness of the plant as well as guards the plant against plant pathogens and grazing herbivores, and in return the host plant gives shelter and nourishes the endophyte (Dreyfuss and Chapela 1994). Endophyte-plant symbiotic associations may have evolved from the time higher plants first appeared on the earth as evident from the fossilized tissues of stems and leaves (Redecker et al. 2000). Endophytes were described for the first time in the Darnel (*Lolium temulentum*) (Freeman 1904) and since then, they have been successfully isolated from different organs of various lower as well as higher plant species, above ground tissues of hornworts, liverworts, lycophytes, mosses, ferns, equisetopsids, and spermatophytes from the tropics to the arctic, and from the wild to domesticated ecosystems (Arnold 2007). All plant species studied till date has been found to harbor at least one endophyte (Kusari et al. 2012).

Endophytes are the chemical producers inside plants (Owen and Hundley 2004). The Endophytes associated with medicinal plants produce different spectrum of secondary metabolites that can be harnessed for the remedy of many ailments (Tejesvi et al. 2007). The unique structures and biological activities possessed by the natural products from endophytic fungi offers a tremendous potential for exploitation in industries as well as in different domains of agriculture (Tan and Zou 2001). These natural products can be classified into several groups including; alkaloids, steroids, terpenoids, glycosides, flavonoids, xanthones, quinones, isocoumarins, lignans, phenyl propanoids, lactones, aliphatic metabolites etc. (Zhang et al. 2006). Fermentative production of bioactive compounds from endophytic microorganisms has numerous advantages, like reliable and reproducible productivity. These bioactive compounds can be exploited commercially by growing them in fermenters for their inexhaustible supply. Production of derivatives and analogues of novel compounds by fermentation requires optimizing various biosynthetic pathways by direct changes in the culture conditions (Strobel et al. 2004).

Endophytes usually produce the enzymes required for the invasion of plant tissues. Several reports propose that most endophytes possess the ability to exploit, at least in vitro, most plant cell components. Most of investigated endophytes produce xylanase and pectinase to utilize complex compounds like xylan and pectin respectively. Production of glucanase (Moy et al. 2002), chitinase (Li et al. 2004) and non-specific peroxidases and laccases (Sieber et al. 1991; Leuchtmann et al. 1992) have also been reported by these endosymbionts of plants.

It is now a well-established fact that all plants harbour at least one endophyte and the potential of these endophytes to produce metabolites of commercial importance has been proved time and again. Hence, it is not unreasonable to hypothesize that oxalic acid rich tropical plants contains endophytes, capable of producing OxO. It is also well established that microbes isolated from ecological niche tend to produce enzymes with novel characteristics. Considering all these factors, this work proposed to isolate bacterial endophytes from oxalic acid rich tropical plants, enhance the production of OxO, characterize the OxO thus produced, and utilize OxO for the production of oxalate free Taro starch.

The research work on OxO was taken up with the following objectives,

- To isolate, select, and identify OxO positive bacteria.
- To study the effect of media components and process variables and optimize OxO production in shake flasks.
- To purify the enzyme and determine its kinetic properties, Thermo – physical properties and biochemical and molecular properties.
- To develop methodology to produce oxalate depleted starch from Taro flour by OxO treatment and study physico-chemical properties of the starch thus produced.

The stated objectives have been addressed in this doctoral work and are being reported in the following chapters:

CHAPTER 1: **Introduction**

This chapter introduces the research work, discusses the background, and significance of the proposed work. The chapter highlights the importance of Oxalate Oxidase in plants and its potential application in industries. The chapter also stresses out the possibility of finding an endophyte which could produce Oxalate Oxidase. The scope and the rationale of the work have been mentioned, and the formulated objectives have been presented.

CHAPTER 2: **Review of literature**

The chapter comprehensively covers a detailed review of the literature pertaining to the research work. Physical, Biochemical, molecular and kinetic characteristics of the enzyme Oxalate Oxidase from different sources have been discussed in detail. Potential application of the enzyme in starch processing industries has been highlighted and the relevant published research works of the field have been summarized.

CHAPTER 3: **Isolation and screening of endophytes for Oxalate Oxidase production and medium and process optimization for enhanced yield by one factor approach**

This chapter gives an account of the isolation of endophytic microorganisms from the tubers of the plants *Colocasia esculenta*, *Beta vulgaris*, *Ipomoea batatas* and peel of *Musa paradisiaca* fruit. It also summarizes the details of screening of the isolated endophytes for their ability to produce Oxalate Oxidase, and of selecting a suitable endophyte for further studies. The effect of media components and process variables on the growth kinetics of the selected microorganisms and on the production of the enzyme Oxalate Oxidase has also been explained in details.

CHAPTER 4: **Purification, Characterization and Chemical modification of Oxalate Oxidase from *Ochrobactrum intermedium* CL6**

This chapter elucidates the strategies employed for purification of Oxalate Oxidase using conventional methods of precipitation and chromatography. This section also deals with the characterization of the partially purified Oxalate Oxidase involving estimation of the molecular weight, studying the influence of various biochemical molecules on functional properties and determination of the kinetic parameters of Oxalate Oxidase. This section in addition to purification and characterization also describes the use of chemical modifications to identify important amino acid residues, which are crucial for the catalytic activity of Oxalate Oxidase produced from the novel endophytic bacterium *Ochrobactrum intermedium* CL6, isolated from *Colocasia esculenta* tubers.

Chapter 5: Production of oxalate depleted starch from Taro flour by enzymatic treatment and evaluating physico-chemical properties of the starch

This section reports development of a novel enzymatic treatment scheme to reduce total oxalate content in the extracted starch from Taro flour and evaluating physico-chemical properties of the starch thus produced.

Chapter 6: Summary and Conclusion

This section offers a brief summary of the presented research. The findings of the research work on studies on Oxalate Oxidase production, purification, characterization and a novel application are concluded with some recommendations for the future work.

CHAPTER 2

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

This section broadly covers a detailed review of the literature relating to the research work. Physical, Biochemical, molecular and kinetic characteristics of the enzyme Oxalate Oxidase from different sources have been discussed in detail. Potential application of the enzyme in starch processing industries has been underlined and the relevant published research works of the field have been summarized.

2.1 OXALATE RICH PLANTS

Plants rich in oxalates are few. Most of the plants synthesize oxalic acid but in small quantity (Bennet-Clark 1933; Olsen 1939). Oxalate may be present as the free oxalic acid, as soluble sodium and potassium salts or as insoluble calcium oxalate crystals (Caliskan 1998). Morrison and Savage (2003) in their brief review on oxalates documented on oxalates level of some oxalate rich plants (Table 2.1). Taro (*Colocasia esculenta*) and sweet potato (*Ipomoea batatas*) were reported to contain 278–574 mg/ 100 g fresh weight (FW) and 470 mg/100 g, respectively. Oxalate levels in yam (*Dioscorea alata*) tubers may not be of dietary concern since three-fourth of the oxalates were present in the water-soluble form and therefore may leach out during cooking. The total oxalate content in yam were in the range 486–781mg /100 g DW. Oca or New Zealand yam (*Oxalis tuberose* Mol.) contains 80–221 mg/ 100 g FW soluble oxalate. Leaves of these root crops have higher levels of oxalates and the skin of these tropical root crops have highest concentrations of oxalate. Coriander leaf (*Coriandrum sativum*) contains oxalates around 1268 mg /100 g; Santhi (*Boernavia diffusa*) and horsegram (*Macrotyloma uniflorum*) contain 3800 mg/ 100 g and 508 mg/ 100 g respectively. Peanut greens, usually consumed in tropical climates, are reported to contain 407 mg /100 g. Nuts such as cashews, peanuts and pecans are relatively high in oxalates. Sesame seeds have been reported to contain high quantities of oxalate, ranging from 350 to 1750 mg/ 100 g FW. Some cultivars of spinach (Universal, Winter Giant) have oxalate content approximately 400–600 mg /100 g, while in others varies

from 700 to 900 mg /100 g, which suggests oxalic acid content varies within same plant species Plants, especially during dry condition accumulates oxalates. Comparison of oxalate content between two cultivars of spinach, Magic (summer) and Lead (autumn), showed that the summer cultivar contained greater amounts of oxalate (740 mg/ 100 g FW) than the autumn cultivar (560 mg/ 100 g FW). Age and ripening stages have been found to affect the accumulation of oxalates in plants. Ripening of the fruits of the goosefoot family increases the proportion of oxalic acid in their leaves. However, in tomatoes, breakdown of oxalic acid takes place during ripening leading to decrease in the total oxalate content.

Table 2.1 Oxalate levels of some tropical and sub - tropical oxalate rich plants

Food stuffs	**Oxalate (mg/100g FW)**
Taro (*Colocasia esculenta*)	278-574
Sweet potato (*Ipomoea batatas*)	470
Yam (*Dioscorea alata*)	486-781
New Zealand yam (*Oxalis tuberose*)	80–221
Red beetroot (*Beta vulgaris*)	121-450
Spinach (*Spinacia oleracea*)	400-900
Amaranth (*Amaranthus polygonoicles*)	1586
Tomato (*Lycopersicum esculentum*)	5-35
Parsley (*Petroselinum sativum*)	140–200
Peanut greens (*Arachis hypogaea*)	407
Coriander leaf (*Coriandrum sativum*)	1268
Horsegram (*Macrotyloma uniflorum*)	508
Santhi (*Boernavia diffusa*)	3800
Sesame seeds (*Sesamum indicum*)	350 - 1750
Coffee (*Coffea arabica*)	50-150
Cashew (*Anacardium occidentale*)	231
Tea (*Thea chinensis*)	300-2000

2.2 ROLE OF OXALATES IN PLANTS

A number of pathways have been proposed for oxalate production in plants. These pathways include the breakdown of isocitrate, oxaloacetate hydrolysis, oxidation of glycolate/glyoxylate, and/or oxidative breakdown of L-ascorbic acid (Hodgkinson 1977). Many researchers find the breakdown of ascorbic acid for oxalate production as the most interesting among all the proposed pathways (Yang and Loewus 1975; Nuss and Loewus 1978; Li and Franceschi 1990; Keates et al. 2000). Calcium oxalate generated by the combination of oxalate with calcium appears in plants in variety of observed crystal shapes and sizes (Nakata and McConn 2000). Although crystal formation has brought interests among scientists for many years, information about crystal development is still incomplete. So far, studies into crystal development and crystal function have depended primarily on cellular (Franceschi et al. 1993; Webb et al. 1995) and biochemical approaches (Arnott and Pautard 1970; Franceschi and Horner 1980; Zindler-Frank 1987; Webb 1999). Cellular and biochemical approaches have provided valuable information about the developmental stages of crystal formation and their ultrastructure. Small number of crystal idioblasts (specialized cells harboring calcium oxalates) present in a given tissue and the technical difficulties connected with isolating intact crystal idioblasts in adequate quantities for experimental study have impeded the current investigation on crystal growth and crystal function (Nakata and McConn 2000). These calcium oxalate crystals often account for a significant portion of the plant's dry weight (McNair 1932). Caliskan (1998) in his review on Metabolism of oxalic acid has stated that both calcium and oxalic acid have a toxic effect when accumulates in excess quantities and insoluble calcium oxalate formation enables plants to remove excess ionically active oxalic acid or calcium. In addition to this, rapid induction of calcium oxalate crystal formation also serves as a storage form for calcium for future needs as evident in Lemma plants (Helper and Wayne 1985).

During calcium deficient conditions in some plants, crystals appear to be dissolved presumably to supply calcium for growth and cell maintenance (Franceschi 1989). Further support for this came from the observation that in some plants calcium is required for the stabilization and activation of certain enzymes; for example release of peroxidase in plant cells depends upon calcium. Peroxidase control cell elongation

since they can rigidify cell walls by their crosslinking activity and their ability to contribute in the formation of lignin and thus calcium has an indirect role in lignification. Thus, for this role they are under the control of cellular calcium levels (Sticher et al. 1981). One of the important roles of calcium in plant cells has been established in the formation of the middle lamella where Ca^{2+} ions form ionic bridges between pectin chains. Many plants are able to hold high concentrations of soluble and free oxalic acid inside their vacuoles suggests that oxalic acid may not be particularly toxic to plant tissues, although it has been indicated that calcium oxalate crystals are a means of detoxifying surplus oxalic acid. However, retention of oxalic acid may have some lethal effect (for example in causing osmotic problems and destabilization of cells), unless it is readily metabolized (Raven and Smith 1976). It has been found that a number of factors play a major role in crystal shape and growth. These factors include macromolecules such as polysaccharides, proteins and lipids as well as macromolecular membrane structures (Arnott and Webb 1983; Horner and Wagner 1980; Webb 1999). More broad analysis is necessary to explain the functional roles of calcium oxalate formation in plants.

2.3 OCCURRENCE OF OXALATE OXIDASE IN DIFFERENT ORGANISMS

Zaleski and Reinhard, 1912 for the first time reported the presence of Oxalate Oxidase in nature. According to Lane et al. 1993, Oxalate Oxidase (OxO, EC 1.2.3.4) catalyzes the oxidative cleavage of oxalate and may regulate the liberation of $Ca^{2+}$and H_2O_2. Both are vital signal molecules in plants (Hu and Guo 2009). Oxalate Oxidase is mostly found in plants, although there are few reports of its presence in microbes also (Table 2.2). Oxalate Oxidases of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) belong to the germin family and have been well characterized. Chiriboga 1963; Sugiura et al. 1979 and Pietta et al. 1982 supported the presence of barley Oxalate Oxidase. The recent description of an Oxalate Oxidase activity for wheat germin (Lane et al. 1993; Dumas et al. 1993;) proposed characteristic molecular mechanisms by which the germins might participate in plant defense and development. Barley OxO has been well characterized and work carried out by Gane et al. 1998; Bernier and Berna 2001; Woo et al. 2000; Requena and Bornemann 1999 confirmed that Oxalate Oxidase is a homohexameric glycoprotein and each subunit, consisting of a β-barrel fold followed

by three α helices in C-terminal, contains one Mn atom which is ligated by three histidine residues, one glutamate residue and two water molecules (Hu and Guo 2009). Kotsira and Clonis (1997) purified the enzyme to homogeneity and found by chemical modification studies that lysine residue, a carboxylate residue and cysteine residues are crucial for its activity. The active site is connected with a channel containing two glutamate residue and one aspartic acid residue to the β-barrel fold (Hu and Guo 2009). Oxalic acid is allowed to enter the channel at optimum pH of 3.8 (Requena and Bornemann 1999; Woo et al. 1998, 2000).

Wheat Oxalate Oxidase exists in three isoforms G, G' and ΨG. The two forms G and G' are present in the root and leaf sheaths of germinated embryos. The third isoform is a pseudogermin and is found in the epidermis and bracts that surround mature grains (Lane et al 1991, 1993). The genetic information for G and G' is encoded by the gene, gf-2.8 (Berna and Bernier 1997). The expressed protein contains a mature peptide with 201 amino acid residues and a putative signal peptide of 23 amino acids (Lane 2000).

The carbohydrate moiety present in G and G' is consisting of xylose and fucose respectively, while the glycan of G' is a paucimannosidic type (Jaikaran et al. 1990; Lerouge et al. 1998). Lane 1994 proposed that wheat Oxalate Oxidase is an oligomeric glycoprotein which shows resistance to proteases and can withstand heat, detergents and extreme pH.

Koyama, 1987 identified the enzyme Oxalate Oxidase in soil bacterium *Pseudomonas sp. OX- 53.* This report of the isolated enzyme from a microbe was probably the first of its kind (Koyoma 1988).

Graz et al. 2008 have reported that in wood rotting fungi, *Abortiporus biennis*, oxalic acid degradation was accomplished by Oxalate Oxidase. When *A. biennis* was cultivated in media containing Cu_2O, ZnO and MnO_2, an increased level of OxO was observed.

All the reported OxO enzymes showed substrate inhibition and barley root OxO was the only enzyme which could show activity in oxalic acid concentration as high as 4mM (Kotsira and Clonis 1997). Interestingly both *Costus pictus* OxO and sorghum leaves

OxO showing very high affinity for the substrate (Km value of 0.065 mM and 0.024 mM respectively) showed substrate inhibition at 0.8 mM and 0.25 mM substrate concentration respectively (Sathisraj and Augustin 2012, Pundir et al. 1984).

Table 2.2 Kinetic properties and inhibitory concentration of substrates for Oxalate Oxidase

Source	**Km (mM)**	**Turn over number (k_{cat})**	**Inhibitory concentration of substrate (mM)**
Hordeum vulgare (Barley)	0.27	22 s^{-1}	4
Sorghum bicolor (Sorghum)	0.024	ND	0.25
Triticum aestivum (Wheat)	0.21		ND
Oryza sativa (Rice)	ND		
Zea mays (Maize)	1.31		
Costus pictus	0.065		0.8
Beta Vulgaris	0.07	ND	ND
Musa acuminate	1.88		
Amaranthus spinosus	2.16		
Bougainvillea glabra	1.6		
Pseudomonas sps. OX-53	9.5		

Ceriporiopsis subvermispora	0.1	88 s^{-1}	2.5
Abortiporus biennis	ND	ND	ND

* ND- Not determined

2.4 APPLICATION OF OXALATE OXIDASE

Oxalate Oxidase has established uses in clinical assays for oxalate in blood and urine (a major component of kidney stones) (Godara and Pundir 2008). Regular assessment of oxalate levels in urine helps to monitor and control hyperoxaluria and Urolithiasis (Dunwell 1998). Oxalate Oxidase has also potential uses in improving disease resistance in plants, reducing oxalate levels in food crops, and bioremediation of oxalate wastes (Dunwell et al. 2000 and Escutia et al. 2005); moreover, structural modelling and docking studies of Oxalate Oxidase (Khobragade 2011) reveals the possible implication in enzyme supplementation therapy for urolithiasis.

Oxalic acid and calcium occur in wood but formation of oxalic acid also takes place during oxidative bleaching of wood polymer in paper and pulp industry. Higher concentration of oxalic acid and calcium leads to the problem of calcium oxalate scaling in pipe work, washing filters, and heat exchangers. Removal of oxalic acid from bleaching filtrates using the enzyme Oxalate Oxidase has a prospect to prevent problems with scaling in the pulp and paper industry (Larsson et al. 2003).

Oxalic acid in plasma and urine can be measured by Oxalate Oxidase. This method of diagnosis is widely used as a medical tool in determining the oxalate concentration in cases of hyperoxaluria. Oxalic acid retention also results in urinary stone formation, which leads to other renal disorders (Hodgkinson 1977). Many of the Oxalate Oxidases identified from moss (Laker et al. 1980), beet stem (Obzansky and Richardson 1983) and barley root (Ichiyama et al. 1985) have been examined with respect to their suitability for measuring urinary oxalic acid. More recently Godara and Pundir, 2007 have found that immobilization of *Amaranthus* leaf Oxalate Oxidase on to affixed glass

beads provided enormous ease in its reuse for determination of oxalate in urinary and serum samples.

2.5 ENDOPHYTES

It is speculated that the relationship of the endophyte to the host plant may have begun to grow from the time of appearance of higher plants on the earth, hundreds of millions years ago (Strobel 2003). Fossilized tissues of stems and leaves gives an evidential argument for plant associated microbes. It is likely to imagine that some of these endophytic microorganisms may have developed genetic systems allowing for the transfer of genetic material between themselves and the higher plants as a result of this long held associations. Endophytes invade specifically to selective plant and exhibits host specificity (Strobel 2003). Such plant specificity indicates that the host and its associated microorganism interact with each other through complex biochemical interactions. A comprehensive investigation of such interactions can provide direction to which endophyte might be carefully chosen in the search for novel medicinal natural products (Strobel 2003; Strobel et al. 2004).

Kusari et al. (2012) and Firakova et al. (2007) have reported in their reviews that nearly all plant species inspected to date were found to harbour endophytic bacteria and/or fungi. Moreover, the invasion of endophytes in bryophytes, mosses and ferns has been also reported by Arnold in 2007. Endophytes are present in almost all organs of a given plant host, and some of them transfer from seeds to next generation plants. (Firakova et al. 2007). For instance asexual Acremonium grass endophytes are spread solely through the seeds of their hosts (Read et al. 2000; Tan and Zou 2001).

Bioactive metabolites of therapeutic use are produced by many endophytes. Many researchers have tapped these microbial resources and exploited them for the production of multifarious known and new biologically active secondary metabolites. These secondary metabolites have antibiotic activity, some examples include Brefeldine A, Phomol (Weber et al. 2004a), Ambuic acid (Li et al 2001); anti-inflammatory activity, one such example is Mevinic acid (Weber et al. 2004b). *Pestalotiopsis microspora* isolated from *Terminalia morobensis* produced isopestacin

and pestacin which have antibiotic and antioxidant activity respectively as reported by Harper et al. (2003) and Strobel et al. (2002). Anti-cancer activity of podophyllotoxin, campothecin produced by endophytes isolated from *Podophyllum hexandrum* and *Nothapodytes foetida* respectively has been reported by Puri et al. (2005). Deoxypodophyllotoxin has been reported by Kusari et al. 2009. Reports of bioactive natural product production by endophytes raises interesting questions regarding the original source of these bioproducts. This bio products were once considered to be synthesized exclusively by plants as plant metabolites. In fact, it is likely that various so-called ‘‘plant metabolites’’ could actually be the biosynthetic products of their endophytes (Kusari et al.2012). Antitumor maytansinoid ansamitocin, originally isolated from higher plants has now been reported to be produced by the Actinomycete *Actinosynnema pretiosum* ssp. auranticum (Yu et al. 2002), proves the remark made by Kusari et al (2012) in their review text.

2.6 PLANT – ENDOPHYTE INTERACTIONS

The fact that endophytes reside within plants and are continuously interacting with their hosts encouraged researchers to consider that plants would have a major influence on the in planta metabolic processes of the endophytes. For example it has been found that biosynthesis of lolitrem in endophytic *Neotyphodium lolii* resident in perennial ryegrass is exceptionally high in planta, but low to undetectable in fungal cultures grown in vitro. This observation offers support to the impression that plant signaling is essential to induce expression (Young et al. 2006). Synthesis of ergoline alkaloids by clavicipitaceous fungi which remains in a symbiotic association with dicotyledonous plants (Convolvulaceae) is another convincing example, and therefore doubt the origin of these compounds in plants (Kucht et al. 2004; Steiner et al. 2006; Leistner and Steiner 2009). *F. solani*, an endophytic fungi from *C. acuminata* produces the anticancer compound camptothecin in planta, however the organism could indigenously only produce the precursors of camptothecin when grown in vitro (Kusari et al. 2009). Absence of strictosidine synthase gene in the genetic constituents of the organism prevented the fungi to produce the compound camptothecin. Supplementation of strictosidine synthase in culture media influenced the production of active camptothecin (Kusari et al. 2011).

Endophytes can also be influenced to produce some important commercial enzymes. Endophyte are good producers of xylanase (Suto et al. 2002). Extracellular cellulase and hemicellulases are also produced but usually restricted to organisms derived from selected hosts or even host tissues (Leuchtmann et al. 1992). Thermostable amylolytic enzymes which have immense potential in industrial processes of starch degradation are being currently investigated among endophytes. The endophytic actinomycete, *Streptosporangium sp* isolated from leaves of maize (*Zea mays* L.) exhibited glucoamylase production which demonstrated thermostable properties (Stamford et al. 2002). The endophyte Neotyphodium from *Poa ampla* was found to produce chitinase enzyme (Li et al. 2004).

2.7 PURIFICATION OF ENZYMES

The introduction of enzymology brings an important revolution in the biotechnology industry, with the global usage of enzymes being nearly U.S. $ 1.5 billion in 2000. The major share of the industrial enzyme market is occupied by hydrolytic enzymes (Begam et al. 2012; Kirk et al. 2002). Purification of these enzymes becomes very important for their application in industries. Characterization of an enzyme's property can be studied accurately in its purest form. Once an enzyme is purified, it is possible to understand its affinity for particular substrates, or dissect its ability to catalyze enzymatic reactions. Such methods have allowed us to understand how biological molecules can act as catalysts in metabolic processes. Proteins with high purity are required in order to determine amino acid sequences and biochemical functions. Moreover, crystals of protein can be grown with pure protein and with such crystals; x-ray data can be obtained that will provide us with the actual functional unit of the protein (Berg et al. 2002).

Physico-chemical properties such as miscibility, mass, ionic charges, and specificity towards ligands of proteins have been exploited to get several thousand proteins purified in active form. Generally, mixtures of protein are subjected to a sequences of purification strategies to produce protein of exceptional purity. At the end of each purification stage, protein concentration and the level of purity is estimated. Sizeable amount of purified proteins are required to fully characterize the molecular structure of

the protein and other physico-chemical properties. Therefore, the most important aspect of any purification strategy is overall yield. Common techniques of purification employed to get proteins of exceptional purity are salting out, dialysis, and chromatography techniques like gel filtration chromatography, ion exchange chromatography, adsorption chromatography, affinity chromatography. High performance liquid chromatography enhances the resolving power of these conventional chromatography techniques. The basic principles of these techniques are mentioned below.

Salting out is a phenomenon of reduction in solubility of proteins at high salt concentration. Salting out is used to fractionate proteins. The most common salt used for this purpose is ammonium sulphate. The salt concentration at which precipitation of proteins takes place differs from one protein to another. For example, Fibrinogen and serum albumin are both proteins of blood but require ammonium sulphate of different concentration to precipitate out. High salt content in concentrated protein fractions obtained after ammonium sulphate precipitation can be removed by Dialysis (Berg et al. 2002).

Dialysis is a method for removal of small molecules from bulk protein molecules through the pores of a semi permeable membrane, such as a cellulose membrane. The membrane acts as a boundary between two solutions of different concentration (Craig 1960).

The concept of size-based separations by chromatography for the first time was speculated by Synge and Tiselius based on their observation that small molecules could be excluded from the small pores of zeolites on the basis of their molecular size. The term ‘‘molecular sieve,’’ was coined by J. W. McBain to describe this property of zeolites, and this technique was later used to describe the size-exclusion or gel filtration chromatography (Hong et al. 2012). Gel filtration chromatography column consists of porous beads made of an insoluble but highly hydrated polymer such as agarose or dextran or polyacrylamide. Sepharose, Sephadex and Bio-gel are the commonly used commercial preparations of these porous beads, which are typically 0.1 mm in diameter. The sample is applied on the top of the drained pre equilibrated column, molecules of

small size can enter into these beads, but large ones fail to do so. Large molecules emerge first as they flow more quickly through the column length (Berg et al. 2002). Small molecules take a longer circuitous path and emerge out last from the column. Intermediate size molecules flow from the column at a midway position and occasionally enter a bead. The first biomolecules to get separated by size-exclusion process were peptides from amino acids. The column used for the purpose was packed with starch (Lindqvist and Storgards 1955).

Proteins are separated by Ion-exchange chromatography on the basis of their net charge. A column of beads containing carboxylate groups at pH 7 will repel proteins with negative charge, whereas a positively charged protein will bind to the column. Elution of positively charged protein bound to such a column can be achieved by elution buffer of high concentration of salt like sodium chloride. In general, two types of ion exchange chromatography system are employed based on the criteria of separation.

1. Cation exchange Chromatography
2. Anion exchange chromatography

Negatively charged proteins (anionic proteins) can be separated by chromatography on positively charged diethyl amino ethyl-cellulose (DEAE-cellulose) columns. On the other hand, negatively charged columns like carboxy methyl-cellulose (CM-cellulose) is frequently used to separate positively charged cationic proteins (Berg et al. 2002).

The liquid chromatography that makes use of biological interactions for the efficient separation of sample components is called as affinity chromatography. Some of the traditional applications of this approach for the direct quantification of solutes include lectin, boronate, protein A or protein G, and immune affinity supports. Antibody-antigen, enzyme-substrate, and enzyme-inhibitor interactions is considered for separation by this technique. With more advancement in the years to come, researchers in this field will become more familiarized with affinity chromatography and more information and applications for this method on clinical samples will appear in clinical chemistry (Hage 1999).

High Performance liquid chromatography has an exceptional capacity of enhancing the resolving power of all the existing conventional chromatography under the influence of high pressure. A combination of factors like finely divided column material and high pressure results in more interaction sites and thus improved resolving power and rapid separation (Berg et al. 2002).

Electrophoresis is a technique by which a complex combination of proteins can be separated under the influence of constant electric field. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to move charged molecules through the sieving action of the gel by means of an electric current. This procedure is frequently used to find out total number of individual protein subunits, confirm the purity of protein samples and determine the molecular weight of the desired protein. The rate of movement of the proteins during SDS-PAGE is determined by the intrinsic charge, molecular weight, and shape of the protein and pore size of the gel matrix (Manns 2005).

The Electrophoretic set up consists of thin, vertical slab of two glass plates and the polyacrylamide gel is carefully casted between the narrow spaces of the glass slab formed by spacers. The samples are loaded on the wells made on the gel and the whole set up is fixed in a tank of buffer. The separation is carried out under the influence of electric current where current flows through the top of the gel to the bottom.

The electrophoretic mobility of protein chains in SDS-PAGE is directly proportional to the logarithm of their molecular mass. Proteins of large size retain at uppermost, near the loading point of the protein mixture, whereas small proteins move quickly through the gel. SDS-PAGE is highly sensitive, fast, and resolve proteins with efficacy. Proteins in the range of micrograms give clear and distinct band when stained with Coomassie blue. Silver stain can detect nano grams of protein in gels. Proteins molecules having molecular weight difference by 2% can usually be distinguished by SDS-PAGE (Berg et al. 2002).

All purification strategies can be examined by studying each fraction obtained from different methods by SDS-PAGE. The first few fractions will display many discrete bands which represents lots of proteins. With each purification step, contaminant

protein will be removed which would be reflected in SDS-PAGE with fewer number of bands in samples from such purification steps when loaded into the gel. Intensity of one of the bands increases and this band will represent protein of interest.

2.7.1 Protein Purification scheme

Success of a protein purification scheme is determined by measuring the specific activity and by performing an SDS-PAGE analysis. The following parameters are calculated at each step:

$$\text{Total Protein} = \text{Protein concentration of each fraction} \times \text{Total volume}$$

$$\text{Total activity} = \text{Enzyme activity of each fraction} \times \text{Total volume}$$

$$\text{Specific activity} = \frac{\text{Total activity}}{\text{Total Protein}}$$

$$\text{Yield} = \frac{\text{Residual enzyme activity after each step}}{\text{Enzyme activity in the initial step}}$$

$$\text{The amount of activity in the initial extract is considered to be } 100\%.$$

$$\text{Purification level} = \frac{\text{Specific activty after each step}}{\text{Specific activty of initial extract}}$$

2.8 CHARACTERIZATION OF ENZYMES

2.8.1 Influence of the pH on enzyme assays

The activity of enzymes depends strictly on the pH in the assay mixture. The activities of most enzymes follow a bell- shaped curve, increasing from zero in the strong acid region up to a maximum value, and decreasing to zero to the strong alkaline region. Two different effects are responsible for this behaviour: (i) the state of protonation of functional groups of amino acids and cofactors involved in the catalytic reaction and (ii) the native, three-dimensional protein structure of the enzyme (Bisswanger 2011).

The pH optima of some enzymes, however, are far away from the usual physiological range. A prominent example is pepsin, the protease of the stomach, with a pH optimum of 2, the optimum of the acid phosphatase is at pH 5.7, that of the alkaline phosphatase at pH 10.5 (Brenda database). Normally the enzyme is fairly stable at its own pH optimum, and so this is recommended not only for testing, but also for storage. This is also of some importance for the performance of enzyme assays, since addition of an aliquot of the enzyme stock solution to the assay mixture will not affect the assay pH. Sometimes, however, the stock solution of the enzyme possesses a different pH, like trypsin, which should be stored at a strong acid pH of 3.0 although it's alkaline pH optimum of 9.5, in order to suppress autolysis (unlike most other enzymes, trypsin tolerates this extreme pH) (Bisswanger 2011).

2.8.2 Dependence of the enzyme activity on the temperature

The temperature dependence of the activity of enzymes resembles in some respect the pH dependence increasing with rising temperature, passing a maximum, followed by a decrease. Therefore this behaviour is frequently described as temperature optimum, although an optimum temperature for the enzyme activity does not necessarily exist at all. Indeed, two counter-acting processes are responsible for this behaviour. The velocity of any chemical reaction increases with temperature, according to an empirical rule two to three times every 10°C. This holds also for enzyme reactions and only boiling of water limits this progression. On the other hand the three-dimensional structure of enzymes is thermo-sensitive and becomes destabilized at high temperature causing denaturation. This process opposes the acceleration of the reaction velocity and is responsible for its decline at high temperature (Bisswanger 2011).

2.8.3 Metal ions in biological catalysis

Metal ions have crucial role in all biological activities (Bertini et al. 2006, Frausto da Silva and Williams 2001). The functional relevance of the metal ions in living systems can be speculated from the fact that majority of enzyme systems require metals for their catalytic activity. The fundamental biological processes of respiration, photosynthesis

and nitrogen fixation require different classes of metal-dependent enzymes (Bertini et al. 2001).

The role played by metals in biochemical reactions catalyzed by enzymes can be mostly classified into two groups. Metal is either involved in non-redox catalysis or it functions as a redox center in the catalysis. In non-redox catalysis, metal functions in the stimulation of reacting species, and in the electrostatic stabilization of intermediates and transition states. Metal induced activation leads to the increase of electrophilicity of a substrate or cofactor; increase the acidity of a substrate or cofactor, which can be involved either in the polarized bond or in a bond adjacent to it. One of the important roles of metal is the generation of both a nucleophile and an electrophile which react with each other. Metal binding brings about the cleavage of a bond involving the ligand atom. The metal ion property of Lewis acidity is exploited here when the substrate or cofactor coordinates the metal; the metal withdraws electrons from the ligand atom, inducing polarization of the bonds involving the ligand atom (Andreini et al. 2008).

Metals are either directly involved in catalytic reaction or perform an auxiliary role in redox catalysis of substrates by enzymes (e.g. copper in superoxide dismutase) (Tainer et al. 1983; Hart et al. 1999) by typically concerned in electron transfer to the active site or from the active site (e.g. the Fe_4S_4 cluster of trimethylamine dehydrogenase (Scrutton 1999). Electrons are therefore either donated to reacting species or accepted from reacting species by the redox-active metal ion and thus takes part in one or more very crucial steps of the enzymatic reaction.

2.8.4 Kinetic Properties

The mechanism of an enzyme catalyzed reaction is understood by studying the rate of the reaction and how it changes in response to changes in experimental conditions. This central approach of determining the rate of enzyme catalyzed reaction in order to understand the mechanism of reaction is called as enzyme kinetics. This central approach is the oldest and the most important approach for understanding the enzyme mechanisms. Most enzymes are believed to have one substrate binding site and have kinetic properties in common. When substrate (S) is brought in the vicinity of an

enzyme, the reaction speedily reaches a steady state in which the rate at which the ES complex forms balances the rate at which it dissociates into product. As S, increases, the steady-state activity of a fixed concentration of enzyme increases in a hyperbolic fashion to reach a characteristic maximum rate, V_{max}, at which essentially the entire enzyme is bound in ES complex. The substrate concentration at which rate of enzyme catalyzed reaction is half the maximum, V_{max} is the Michaelis constant K_m, which is unique for each enzyme acting on a specific substrate (Nelson and Cox 2005).

The Michaelis-Menten equation

$$v_o = \frac{V_{max}[S]}{K_m + [S]}$$

explains the relationship between initial velocity, v_o to S and V_{max} through the Michaelis-Menten constant K_m. Michaelis-Menten kinetics is also called steady-state kinetics. Different enzymes have different K_m and V_{max} values. The limiting rate of an enzyme-catalyzed reaction at saturation is described by the constant k_{cat}, the turnover number. Turnover number is defined as number of substrate molecule converted to product by each molecule of enzyme per second. The ratio k_{cat}/K_m provides a good measure of catalytic efficiency of an enzyme.

2.9 PURIFICATION OF OXALATE OXIDASE

Oxalate Oxidase (OXO) from roots of barley seedlings was purified in 3 steps. The purification process consisted: (i) Heat treatment at 60°C for 10 min, (ii) Dye- affinity chromatography and (iii) affinity chromatography on immobilized lectin concanavalin A. The over-all performance of this three step purification strategy led to 1096-fold purification and recovery of 42%. The specific activity of the purified enzyme was 34 Umg^{-1} at 25°C, and it has five equal subunits of total Mol. wt. 125 kDa (HPLC analysis) showing a single band on SDS-PAGE with a Mol. wt. of 26 kDa after staining the gel with silver nitrate (Kotsira and Clonis 1997).

Oxalate Oxidase (OxO, EC 1.2.3.4.) from wheat (*Triticum aestivum*) seedlings was purified by 4 steps comprising of thermal treatment, ultrafiltration, Sephadex G-100

gel filtration and affinity chromatography with concanavalin A. 66.11-fold fold purification was achieved with a 21.97 % recovery by the 4 sequential steps. The enzyme has five equal subunits of total Mol. wt. of 170 kDa as determined by Sephadex G-150 filtration and showing a single band on SDS-PAGE with a Mol. wt. of 32.6 kDa (Hu and Guo 2009).

Four sequential steps comprising ammonium sulphate precipitation, DEAE Sephacel, Sephadex G-200 and ultrafiltration was adopted to purify Oxalate Oxidase (OxO, EC 1.2.3.4.) from Sorghum seedlings. The enzyme was purified to 109-fold with a total yield of 10%. The native molecular mass of the enzyme was 124 kDa on Sephadex G-200 filtration. The subunit molecular mass of 62 kDa on SDS-PAGE suggested that the enzyme was a dimer of equal molecular weight (Kumar et al. 2011).

The enzyme Oxalate Oxidase of the basidiomycete *Ceriporiopsis subvermispora* was identified in the mycelial extracts of the organism and purified by 3 steps consisting of Q-Sepharose chromatography, precipitation at pH 3.0, and phosphocellulose chromatography. Gel permeation in Sephadex G-200 and SDS–polyacrylamide gel electrophoresis suggested that the enzyme is a 400-kDa hexamer of equal subunits (Aguilar et al. 1999).

Pseudomonas sp. OX- 53 OxO was purified to homogeneity. The native molecular mass of the enzyme was 320 kDa on Sephadex G-200 filtration. The subunit molecular mass of 38 kDa on SDS-PAGE suggested that the enzyme was a homo octamer (Koyoma 1988).

2.10 KINETIC AND BIOCHEMICAL CHARACTERIZATION OF OXALATE OXIDASE

The Michaelis Menten constant K_m of the purified enzyme from barley roots for oxalate was 0.27 mM and the turnover number, k_{cat} was 22 s^{-1} (37°C). The phenomenon of substrate inhibition was witnessed at oxalate concentration of 4 mM or above. Riboflavin and FAD have no impact on its activity and therefore the enzyme contains no non-protein group absorption of light at 370 or 450 nm. Ca^{2+} and Pb^{2+} at 1 mM each had stimulatory effect on the enzyme (Kotsira and Clonis 1997).

The optimum pH for wheat OxO activity was 3.5. Its Km for oxalate was 0.21 mM (Hu and Guo, 2009).

Oxalate Oxidase in *C. pictus* has very high affinity for oxalate with a Km 20 times lesser than the OxO from barley seedling (Sathisraj and Augustin 2012).

Cell wall fraction isolated from maize roots (*Zea mays* L.) also has Oxalate Oxidase activity. The maximum enzyme activity of Oxalate Oxidase from maize was found at pH 3.2 and the enzyme was active at acidic pH. The enzyme was thermostable and unaffected to high salt concentration, anionic detergent (SDS) and proteolytic enzyme (pepsin). The Oxalate Oxidase activity was insensitive to EDTA, KCN and metal ions, but was inhibited by sulfhydryl reagents *N*-ethyl maleimide (NEM), 2-mercaptoethanol (2-ME), and dithiotreitol (DTT) (Vuletic and Sukalovic 2000).

Sorghum seedlings OxO had an optimum pH of 4.5 and optimum temperature of 37 degrees C for maximum activity. Enzyme kinetics study of the OxO from Sorghum seedlings suggested that the first order kinetics of enzyme catalyzed reaction progressed up to 7 min. Km value of the enzyme was 0.22 mM. The enzyme was activated by Cu^{2+} and inhibited by diethyldithiocarbamate, NaCN, EDTA, Na_2SO_4, but sodium chloride at 0.1 mM concentration had no impact on the enzyme. The enzyme activity was enhanced by flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Kumar et al. 2011).

Pseudomonas sp. OX- 53 Oxalate Oxidase contained 1.12 and 0.36 atoms of manganese and zinc per subunit respectively. The enzyme was able to oxidize malic acid and glyoxylic acid at lower reaction rates, besides oxalic acid. However, the enzyme exhibited a high Michaelis constant of 9.5 mM for oxalic acid. The maximum activity of the enzyme was at optimal pH 4.8 and the enzyme exhibited stability from pH 5.5 to 7.0. The enzyme was stimulated by phenylhydrazine, O-phenylenediamine, and flavins and inhibited by B^-, semicarbazide, and hydroxylamine (Koyoma 1988). The isoelectric point of the enzyme was 4.7 as evident from isoelectric focusing studies.

The enzyme Oxalate Oxidase obtained from the extracts of the basidiomycete *Ceriporiopsis subvermispora* mycelium had optimal activity at pH 3.5 and at 45°C.

Isoelectrofocusing revealed a pI of 4.2. The Michaelis-Menten constant of the purified enzyme was 0.1 mM and a turnover number of 88 s^{-1} was found for the enzyme. A low K_m suggests that the enzyme is highly specific for oxalate, although it has exhibited substrate inhibition at 2.5 mM concentrations of oxalic acid (Aguilar et al. 1999).

2.11 CHEMICAL MODIFICATION OF AMINO ACID RESIDUES OF ENZYMES

The design of new biocatalysts is of major significance for biotechnological as well as pharmaceutical applications such as the discovery of biological analytes or the study of complex biomolecular interactions. Covalent modification of these biomolecules can change their native physico-chemical and functional properties (Walsh 2005). For example, alteration at some positions of an enzyme may amend substrate specificities, product distribution, and stereo selectivity or even switch the enzyme's central catalytic action (Diaz-Rodriguez and Davis 2011). There are many reports where chemical modifications of enzymes have blocked the active site essential amino acid, which has led to loss of enzyme activity. Chemical modification have also enhanced the enzyme activity in many cases, below are some of the reviews which will highlight the above statements.

Incorporation of 2 moles of N-ethylmaleimide (NEM), a specific reagent for cysteine modification into TPN-dependent isocitrate dehydrogenase from pig heart leads to inactivation and altered Michaelis constants for the substrate isocitrate and oxalosuccinate (Colman and Chu 1969).

Incubation of Estradiol 17P-dehydrogenase from human placenta with I-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), a specific reagent for modification of carboxyl group in a protein led to the inhibition of enzyme activity (Inano and Tamaoki 1984). Similarly NADPH-cytochrome P-450 reductase (EC 1.6.2.4) purified from rat hepatic microsomal fraction was also inactivated by the carboxyl group specific modifier (Inano and Tamaoki 1985).

2, 4, 6-trinitrobenzenesulfonic acid (TNBS) and phenylglyoxal (PG) were used to modify lysine and arginine residues respectively. This modification inactivated alkaline phosphatases from *Pinctada fucata* (Hong et al. 2005).

Modification of both histidine and tyrosine residues of RrFr-ATPase from the photosynthetic bacterium *Rhodospirillum rubrum* by 5 mM diethyl pyrocarbonate (DEPC) at pH 6.0 and 22 ˚C rapidly and completely inactivated the enzyme. DEPC-modified RrFr-ATPase showed a decrease in absorption at 280 nm and increase in absorption at 242 nm, which suggested modification of both histidine and tyrosine residues (Khananshvili and Gromet-Elhanan. 1983)

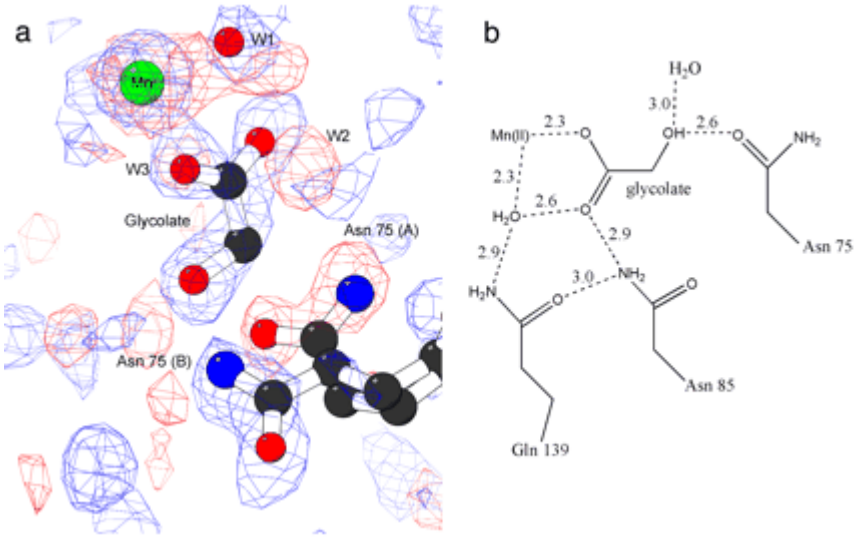
Adenosine deaminase enzyme from bovine spleen and cerebral hemisphere (white and gray matter) was treated with N-bromosuccinimide, a modifier known to oxidize specific tryptophan residues of proteins. Spectroscopy studies confirmed tryptophan modification and enzyme inactivation (Mardanyan et al. 2001).

Porcine pancreatic α-amylase (1, 4- α -n-glucan glucanohydrolase) has both amylase activity (hydrolysis of α -1, 4-D-gluooside bond of starch) and maltosidase activity (hydrolysis of p-nitro phenyl- α -D-maltoside to p-nitrophenol and maltose). By the modification of histidine residues of porcine pancreatic α -amylase with diethylpyrocarbonate (DEP), both amylase and maltosidase activities were decreased in the absence of chloride ion. In the presence of chloride ion, however, maltosidase activity of the modified enzyme was increased to more than 260% of that of the native enzyme (Yamashita et al. 1991).

Modification of histidine residues resulted in enhancement of two fold increase in activity in glutamate dehydrogenase (Wallis and Holbrook 1972). There are few reports of activation of enzyme by sulfhydryl reagents. Activation of rabbit liver fructose 1, 6 diphosphatase by sulfhydryl reagents enhanced the enzyme activity. It was found that for every sulfhydryl group modified, different sulfhydryl reagents stimulated the enzyme to different extents (Little et al. 1968).

2.12 MECHANISM OF OXALATE OXIDASE CATALYSIS

In order to understand the functioning of Oxalate Oxidase action, Opaleye (2006) shed light on the mechanism of Oxalate Oxidase by structural and spectroscopic studies. Glycolate was used as a structural analogue of oxalate to investigate substrate binding in the crystalline enzyme. In this mechanism organization of the substrates (oxalate and dioxygen) and transient reduction of dioxygen is served by the metal ion. The two asparagine and one glutamine residues in the active site of the enzyme are meant to imply important roles in correctly orientating the substrates and reaction intermediates for catalysis. Combined spectroscopic, biochemical, and structural analyses of mutants has confirmed the importance of the asparagine residues in organizing a functional active site complex. Opaleye (2006) provided the basis for understanding a novel mechanism of Oxalate Oxidase catalysis by incorporating the new understandings into carboxylate coordination modes and functions of specific catalytic residues through these combined structural and biochemical studies. According to their work, oxalate is bound to Oxalate Oxidase in its singly ionized monoanionic form as substrate at the optimum pH for the enzymatic reaction (pH 3.5-4), which lies between the two p*Ka* values for oxalic acid dissociation (p*Ka*1-1.25, pK*a*2 - 4.14) (Figure. 2.2). They expected the singly charged oxalate to bind to OXO in the same fashion as glycolate with an additional minor rotation of oxalate to accommodate the additional oxygen atom of the non-coordinated carboxyl group of oxalate. A possible dioxygen binding site is created in the active site, comprising the reduced Mn(II) metal ion and the Gln139 side chain because of such geometry. Monodentate coordination of oxalate displaces one solventmolecule from the Mn(II) centre and allows dioxygen to bind in the inner sphere of the Mn(II) (Figure 2.2 step 1) (W1 in Figure 2.1) to form a Mn(III) superoxide metalloradical complex, stabilized by Gln139., thus stabilizing reduced oxygen species that are predicted to occur as reaction intermediates during turnover through direct coordination to the metal ion.(Figure 2.2, *step 2*). A hydrogen bonding network anchored on the amide side chains of Asn75, Asn85, and Gln139 appears to be essential for correctly orienting dioxygen and oxalate substrates relative to the manganese ion. This nucleophilic superoxide metalloradical generated in this reaction is added to the proximal carboxyl



Courtesy: Journal of Biological chemistry

Figure 2.1 (a) Difference map revealing monodentate binding of glycolate to the active site manganese and movement of Asn75. Positive electron density (+1σ _) is shown in *blue* and *negative* density (-1 σ) in*red*. Asn75 (*A*) labels the conformation present in native crystals, and Asn75 (*B*) labels the conformation seen in the glycolate complex. Additional changes in density are due to the movement of water molecules. There is clear evidence for the binding of glycolate and the expulsion of two waters from the active site (labeled W2 and W3), W1 remains bound to the manganese ion. (*b*) Schematic drawing of the network of interactions between Oxalate Oxidase and glycolate, distances are given in Å units.

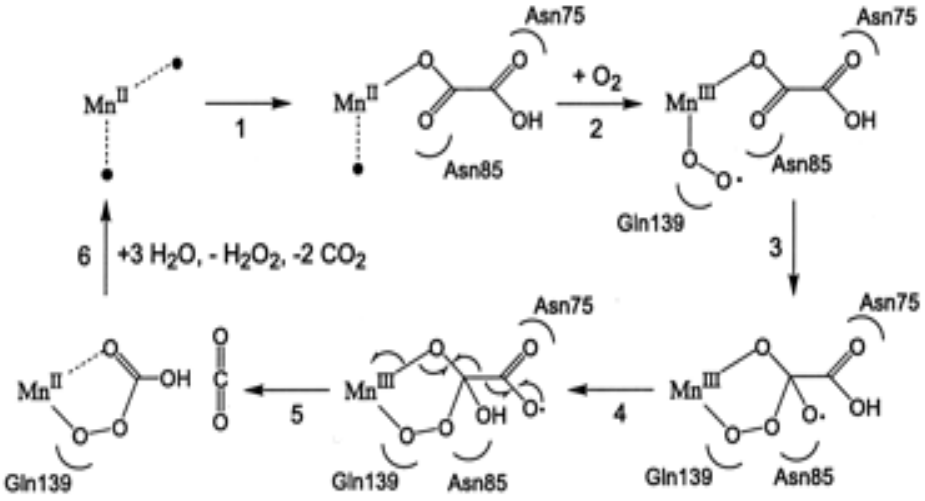


Figure 2.2 Proposed catalytic mechanism for Oxalate Oxidase

group of oxalate (step 3), activating the substrate by hydrogen atom transfer for C–C bond cleavage (step 4). The substrate free radical is thus rearranged with homolytic C–C bond cleavage and reduction of Mn (III) to Mn (II) (step 5). This leads to formation of peroxycarbonate as an initial product of the reaction. Release of CO_2 and percarbonate, or a second molecule of CO_2 and hydrogen peroxide formed by subsequent hydrolysis of percarbonate, completes the turnover cycle.

2.13 STARCH AND ITS INDUSTRIAL APPLICATION

Starch is a major component in many staple foods from cereals to tubers and roots, and its characteristics and interactions with water and lipids in particular, have found significance in the food industry and for human nutrition (Copeland et al. 2009). At present starch is being extracted and processed from various cereal, tuber and root crops worldwide. The annual production of processed starch accounts for 60 million tonnes annually. Of which approximately three- fifth is used in foods (bakery and confectionery products, Dairy products, Drinks and beverages. sugar syrups, Processed meat, snack foods,) and two-fifth in pharmaceuticals (such as antioxidants, colorants, flavours as well as pharmaceutically active proteins) and non-edible purposes, such as in textiles, adhesives, fertilisers, packing material, seed coatings, paper, fabrics, cardboard, bio plastics, diapers, building materials, cement, and oil drilling (Table 2.3) (Burrell 2003, Copeland et al. 2009). Starches from a range of biological origins, such as potato, corn, rice and wheat have gained widespread attention in relation to functional and physico-chemical properties (Madsen and Christensen 1996).

Starches from these cereals, such as various types of rice, wheat, corn, and from tubers or roots, such as potato and cassava (or tapioca) have been commercialized and generates revenue worth of billions of USD (Press release by Market and Market).
The main botanical source of starch is maize, accounting for about 80% of the world market. Maize starch is an important ingredient in the production of foodstuffs, and has been widely used as a thickener, stabilizer, colloidal gelling agent, water retention agent and as an adhesive (Zhu and Wang 2013).

The function and behaviour of starch in various food uses can be interpreted by understanding the fundamental concepts of starch chemistry. Starches are used in the food industry as thickening agents, gel formers, and colloidal emulsifiers. Preferred characteristics can often be enhanced by selection of an appropriate modified starch (Santana and Meireles 2014). Both linear and branched polysaccharides are present in most native starches. The linear polysaccharide is responsible for gel formation and for various syneresis effects, the branched fraction for high colloidal stability and good suspending qualities. The characteristics of modified starches depend on granule structure and on specific size and shape of the component molecules.

Starches, starch derivatives and starch saccharification products are converted as per the requirements of the food industry by hydrolysis. The products obtained from starch hydrolysis are used in a broad range of food products due to both their unique functional and nutritional properties in the food industry (Bertolini 2010).

The physico-chemical properties of starches command their functionality in various applications. Starches with desirable functional properties play important role in improving the quality of different food products and are therefore in great demand in the food industry. These starches with unique desirable properties could replace chemically amended starches that are at present being used in a number of food products (Singh et al. 2003)

The spectrum of desired functional properties of starch can be broadened by the use of new starches from alternative sources which could provide choices for added-value food product development programme. Such alternative sources can be those extracted from roots and tubers (Perez-Pacheco et al. 2014).

With the increasing interest in using native starch for food production in substitute of chemically amended starches in order to meet the industry demand for new technological properties, several non-conventional starch sources, with different properties have been studied (Albano et al. 2014). Currently, during the isolation and separation of small molecule bioactive compounds from different genotypes, these native starches are usually being overlooked and wasted (Yuan et al. 2007). The global starch and starch products market can be influenced with the introduction of novel

starch sources with industrially appealing properties. Such possibility has been drawing the attention of industrialists worldwide (Leonel et al. 2003), though modified native starches are currently being used as an alternative. Thus interest in new value added starch and its products has also encouraged researches towards investigating the physico-chemical and functional properties of starches extruded from different genotypes and botanical sources to meet new processor and consumer market demands.

Table 2.3 Examples of the use of starch

Industries	Applications
Food and drinks	Mayonnaise, Baby food, Bread, Soft drinks, Meat products, Confectionery
Animal feed	Pellets
Agriculture	Seed coating, Fertilizer
Plastic	Biodegradable plastics
Pharmacy	Tablets, Dusting powder
Building	Mineral Fibre, Gypsum board, Concrete
Textile	Warp, Fabrics, Yarns
Paper	Corrugated board, Cardboard Paper
Various	Oil drilling, Water treatment, Glue

Courtesy: Journal of experimental Botany

Physicochemical (e.g., Retrogradation and gelatinization) and functional (e.g., water absorption, swelling, solubility, amylase content and paste clarity) properties of starches determine their potential uses in food systems and other industrial applications (Table 2.4). These properties depend on the molecular structural composition of amylose and amylopectin and their arrangements in starch granules. In food products,

the functional roles of starch could be as an emulsifier, binding agent, thickener, gelling agent or clouding agent (Santana and Meireles 2014).

It is generally beneficial if starches for industrial applications are composed of either amylose or amylopectin. The physicochemical properties of amylopectin and amylase are contrasting, and therefore starches with one or the other are in great demand in industries. Many mutants of cereals and legume species contain starches composed of only amylopectin or very high proportion of amylose (Zeeman et al. 2010)

Industrial processing of native starches at high temperature and excess of water brings physical changes of gelatinization. Starch granules usually swell up during such treatment which primarily also solubilize amylose and amylopectin. Diffusion of amylose out of the swollen granule leads to the formation of continuous gel phase. At the same time aggregation of swollen amylopectin-enriched granules into gel particles, generates a viscous solution forming a two-phase structure, called starch paste. It is desirable for many applications in which processed starches are used as binders or thickeners (Santelia et al. 2011).

2.13.1 Starch from Taro

Colocasia esculenta is a tropical crop generally cultivated for its underground tuber and consumed in tropical and sub-tropical zones of the world. Taro (*Colocasia esculenta)* has been reported to have 70-80% starch with small granules (Aboubakar et al. 2008; Jane et al. 1992). Colocasia tuber starches have never been commercialized for lack of considerable information of their properties as compared to other tuber starches, like potato (Table 2.5). A substantial amount of work has to be done on the specific and unique properties of Colocasia. It is therefore required to characterize physico-chemical and functional properties of Colocasia, along with its proximate composition before considering it as prospective source of starch to use in food and non – edible purposes (Deepika et al. 2013). Deepika et al. (2013) isolated starch from *Colocasia tubers collected from different geographical locations* of Jharkhand, North Eastern State of India. The morphological, physicochemical, structural properties of these starches were studied. The productivity of starches was low and was found in the range of 6.46-13.75%. The starches granules were irregular in shape with a diameter of 5-10 μm. The

physico-chemical properties like amylose content, swelling and solubility power, water hydration capacity showed significant variations among different cultivars of Colocasia. Studies by Deepika et al. (2013) revealed that these starches have possible applications for pharmaceutical industries. *In vitro studies* revealed the delayed release of all tablets made from these starches when compared with maize starch and therefore could be used as drug excipient in continuous release dosage form with minimum modification (Deepika et al. 2013) and thus holds huge potential to be used in pharmaceutical industries.

Hawaiian and Cameroonian varieties of Taro flour and starches has been extensively studied for their physical, chemical and functional properties (Jane et al. 1992; Sugimoto et al. 1986; Mbofung et al. 2006; Njintang et al. 2007a). The microscopic and thermal properties of some Hawaiian taro flours and starches have also been reported by Jane et al. (1992). In addition, the functional properties of raw and precooked taro flours have been reported by Tagodoe and Nip (1994). The effect of pre-cooking time and drying temperature on the physico-chemical characteristics and in vitro carbohydrate digestibility of Cameroonian taro flour has been inspected by Njintang (2003) and Mbofung et al. (2006). More recently, the relationship between the biochemical characteristics of taro flour and creep recovery, colour and sensory characteristics of the paste made from it has been investigated by Njintang et al. (2007a, 2007b). In the same vein, the influence of taro flour reconstitution on the texture profile of achu was evaluated by Njintang et al. (2007c). The functional and alveographic properties of wheat flour and dough was also studied in details on addition of taro flour. Regardless of growing interest in taro, studies on the properties of its flour and starch continued to remain limited. Aboubakar et al. (2013) prepared Taro flour from six varieties of taro corms from Ngaoundere, West central Africa and coded as CE, CN, KW1, KW2, RIN and RIE. The Starch yields percentages prepared from the flours were in concordance with previous reports of Jane et al. (1992) and varied between 66.5% for KW2 to 86.6% for RIE. The proteins content of the sample flour varied from 2.9% for KW1 to 4.9% for CN. The small granular sizes, common characteristics of Taro starches were also found in these varieties. Differential scanning calorimetry (DSC) method was used to estimate the total amylose contents and it varied from 14.7 ± 1.64% to 30.85 ± 0.63%.

Table 2.4 Important physico-chemical properties of few commercial starches

Starch Type	Amylose content (%)	Amylopectin content (%)	Rate of granular swelling	Size (µm) and shape	Total lipid (%)	Solubility (%)	WBC
Potato	20–21[1]	79–80[1]	Fast[1]	15-110, Oval and spherical[3]	0.19[4]	82[4]	83[4]
Wheat	25–30[1]	70-75[1]	Slow[1]		-	15[2]	-
Tapioca	16–17[1]	83–84[1]	Fast[1]	5.4, round[4]	0.1[4]	26[4]	
Corn	25–28[1]	72–75[1]	Slow[1]	11.4-11.7, oval to polyhedral[3]	0.67-0.69[3]	6-8[3]	101-110[3]
Rice	17–30[1]	70–83[1]	Slow[1]	5.2-5.7, Polyhedral[3]	0.25-0.33[3]	2-4[3]	107-115[3]
Yam	21-29[4]	-	-	6-100, oval[4]	0.05[4]	7.8 – 16.8[4]	103[5]

[1] Gregorova and Bohacenko, 2005
[2] Charles et al. 2007
[3] Ali et al. 2014
[4] Hoover 2000
[5] Huang et al. 2016

The granular diameter of each of the starch samples and between the samples were irregular and showed wide variations. The lipid content and ash recovery were also found as a part of proximate estimation, and were 0.2–0.6% and 2% respectively. The onset gelatinisation temperatures of the Taro flours varied from 55.2 to 65.49 °C, whereas those of the starches are between 48.08 ± 2.46 for KW2 and 64.37 ± 2.35 °C for KW1. A water absorption capacity of Taro flour and Taro starch was found to be very high. It was evaluated to be 240% to 470% for Taro flour and 60% to 250% for Taro starch. Taro starches had lower solubility index than their flour counterparts. Among the varieties, CE and RIN starches had the highest solubility whereas KW1 starch had the lowest (Aboubakar et al. 2013).

Table 2.5 Physico-chemical properties of Taro starch from Africa and India

Geographical Location	**Amylose content (%)**	**Water Binding capacity (%)**	**Swelling Power (g/g)**	**Solubility (%)**	**Granular Size (µm)**	**References**
Ngaoundere, West central Africa		250*		27-37**	1-5	Aboubakar et al. 2008
Jharkhand, India	13.5-27.6	328-506*	0.89-1.4*	42-48**	5-10	Deepika et al. 2013
Ghana, West Africa					0.23-0.25	Sefa-Dedeh and Sackey, 2002
Punjab, India	20.4	70.14*	12.47*	7.42*		Pramodrao and Riar, 2014

Experiment were carried out at 60°C (*) and 90-100°C (**)

2.13.2 Anti-nutritional factors present in food crops

One of the concerns that revolve round the beneficial effect of the nutrients in food crop is presence of anti-nutrients which masks the nutritional quality of food crop. When plants containing these compounds are consumed by humans, adverse physiological

and biochemical effect such as pancreas enlargement and growth inhibition results (Alonso et al. 2000), and oxalate is one such anti-nutrient (Almanza and Begum 1996). Oxalate are strong cheaters (Anju et al. 2015) which interact with minerals like calcium, magnesium, zinc, copper, and therefore these minerals cannot be absorbed during the course of digestion and assimilation in the intestine. Such complexes are excreted, thus reducing the bioavailability of minerals to the body (Kelsey 1987). Oxalises, a condition of calcium oxalate deposits in body tissues can be fatal to humans and other animals on consumptions of foods rich in oxalates. A central role in calcium oxalate kidney stone disease in humans is consumption of more moderate amounts of oxalate. Such moderate amount of consumptions lead to absorption in intestines and excretion in urine (Holmes and Kennedy 2000). So removal of such anti-nutrients from our food crop will enhance the quality and possibility of consumer acceptance of processed food products from such food crops.

2.13.3 Strategies to reduce oxalate content in Taro flour

Published literature suggests that several methods are available for reducing oxalate content in food materials. Boiling, blanching with hot water, steeping with sodium bicarbonate and subsequent water leaching are some of the popular methods to reduce oxalate content in food materials (Table 2.6).

Oxalic acid and its salts are metabolic end product that accumulates in number of plant tissues. High levels of soluble and insoluble oxalates were markedly found in some leafy plants and some root crops. Studies on oxalate content in food crops has been extensively done by Savage et al. (2000) for different root and leafy vegetables of New Zealand. Among all the vegetables grown in New Zealand, roots and brassicas appear to contain relatively low levels of oxalates whereas leafy vegetables such as NZ spinach and silver beet seems to approach and exceed levels found in rhubarb stalks. Rhubarb stalks contains highest level of oxalates among all the vegetables. Savage et al. (2000) quantified oxalate content in cooked vegetables commonly consumed in New Zealand and found that the oxalate content of the cooked food is drastically reduced by leaching losses into the cooking water (Savage et al. 2000)

Mukpo" (*Mauna flagellates*) seed flour is one of the soup thickeners used in most rural Igbo-speaking communities of Southern Nigeria. Preparation of this soup thickener is usually associated with long cooking time which is required to soften the cotyledon before grinding as well as reduce the anti-nutritional components like oxalic acids of the seed. Oneugbu et al. 2013 therefore aimed at determining the effect of boiling time on reduction of oxalic acid in addition to some functional properties and other anti-nutritional properties of theukpo seed flour. The oxalate reduced from value of 0.838% in the raw sample to low values of 0.140% after 60 min boiling. This is as a result of thermal breakdown of these compounds and subsequent leaching of soluble components into the boiling medium (Oneugbu et al. 2013).

Colocasia esculenta (Taro) tubers from different cultivars of the world also contains substantial amount of oxalates in their tissues, which have been reported by researchers worldwide. The chemical composition of cocoyam (*Colocasia* and *Xanthosoma sps*) corms and cormels and the effect of processing and particle size on the physicochemical and organoleptic properties of the flours for use as soup thickener were examined by James et al. 2013 for few African varieties. Fresh cocoyam corms and cormels were peeled, sliced, washed, and divided into four parts that were variously blanched, sulphited and sulphited/blanched. The proximate composition, ascorbic acid, anthocyanin and oxalic acid contents, pH, bulk density, water and oil absorption capacities and the sensory properties of colour and texture of the flour was analyzed before and after various treatment. Ascorbic acid, anthocyanin and oxalic acid contents were respectively reduced from averages of 30.35, 31.58 and 173.88 mg/ 100 g (dry weight) in the corm/cormel to ranges of 8.95-16.28, 9.58-15.90 and 141.69-160.68 mg/ 100 g in the flours. Bulk density was enhanced by blanching and particle size. The water and oil absorption capacities were increased by blanching. Sulphiting, blanching and decreasing particle size improved the colour preference. Acceptability of soups from flours were not affected by treatments and particle sizes (James et al. 2013).

Studies on the reduction of calcium oxalate content from taro corms of Semarang, Indonesia through soaking and boiling of taro corm chips in baking soda solution has been carried out. Soaking and boiling of taro corm chips in baking soda solution was still unable to reduce the calcium oxalate content to safe level. From the technical and

economics point of views, soaking taro corm chips in 10 % w/w baking soda solution at ratio 1:4 (w/v) for 10 hours at ambient temperature followed by boiling at 98 °C for 60 minutes was relatively good condition to reduce calcium oxalate content to obtain taro flour with similar functional properties of American wheat flour (Kumoro et al. 2014).

Steeping at 30°C, boiling at 90°C and roasting at 165°C of flours from three cultivars of *Colocasia sps*. (coca-india, ede-ofe and inimbu) and one *Xanthosoma* sp. (ede-uhie) of Imo State, Nigeria were compared with those of untreated flours for oxalate reduction and physic-chemical properties. Results showed that 40 min of boiling brought highest rate of change of oxalate reduction in inimbu. The three different treatments had decreased the gelatinization temperature, but water and oil absorption capacities were subsequently improved. Boiling and roasting affected reduction in cold-paste viscosity and on the other hand steeping led to an inconsistent trend (Iwuoha and Kalu 1995).

Another study of chemical composition as well as the effect of processing on the cormels of *Colocasia esculenta* and two *Xanthosoma sagittifolium* species from Ghana, West Africa was carried out by Sefa-Dedeh and Sackey (2002). Standard analytical methods were employed to assess the oxalate content of various cormels before and after processing of the tubers. The apical portion of all the species had high protein while the distal region had high levels of ash, fibre and minerals. Thus the results showed that the chemical composition were considerably different among the different sections of the cocoyam cormels. Potassium was the most abundant mineral (763–1451 mg/100 g). The concentration of magnesium (46.7–85.0 mg/100 g), phosphorus (41.6–63.1 mg/100 g) and zinc (17–51.1 mg/100 g) was also substantial. Oxalate compositions of the fresh samples were in the range of 328–460 mg/100 g for the *Colocasia esculenta*, 254–381 mg/100 g for the *X. sagittifolium* (red-flesh), and 302–323 mg/100 g for the *X. sagittifolium* (white-flesh). Oven-drying and solar-drying samples could not bring any significant differences in oxalate content of the samples. However, oxalate levels were reduced to approximately 50% by drum drying (Sefa-Dedeh and Sackey, 2002).

Several attempts have been made to reduce oxalate content in Taro. Although it has been reported that traditional methods of drying reduce oxalate content, it does not eliminate completely (Sefa-Dedeh and Agyir-Sackey 2004). Soaking and blanching of Taro tuber flour could reduce oxalate content by 18% (James et al. 2013). Substantial reduction in oxalate content (around 80%) has been reported by cooking for 40 minutes at 90°C (Iwuoha and Kalu 1995).

The present doctoral work also aims at producing oxalate free starch from taro flour, which would add value to the processed starch. However, if the objective is to extract oxalate free starch, the methods employed by previous researchers is unacceptable as cooking and other stringent treatment conditions may compromise with the quality of the extracted starch (Liu et al. 2013). Native starches cannot withstand the harsh processing conditions of high temperature, which lead to abundant granule disruption and detrimental product properties. When heated in the presence of water, starch undergoes an irreversible order disorder transition termed gelatinization and leads to decomposition of glucose rings (Liu et al. 2013), formation of lumping and affects the physical properties like expansion ratio, water absorption, water solubility (Dunford 2012), and also affect the mouth feel property of the starch (BeMiller and whistler 2009).

Therefore, alternative method to reduce oxalate content of Taro starch, which would not compromise with the quality, will certainly encourage food industry to explore the possibility of utilizing this cheap and abundant source. This research was therefore aimed at developing an enzymatic treatment to reduce oxalate content in the extracted starch and evaluating physico-chemical properties of the starch thus produced. Absence of reliable information on the characteristics of these starches has contributed to restricted utilization of these starches in industry. Information on characteristics of the starches from these crops therefore would unravel the prospects offered by these root crops and help their utilization.

Table 2.6 Total oxalate content of few food stuffs and % reduction of oxalate content after treatment

Food stuffs	**Total oxalate content (mg/100 g WM)**	**% Reduction of total oxalate content after treatment**	**Method employed for reduction**	**References**
Silverbeet leaves	525.5	44	Cooked by boiling in water	Savage et al. 2000
Spinach	329.6	53		
Rhubarb stalk	986.7	23.3		
Beetroot tubers	45.6	Increment in oxalate content after treatment		
Broccoli	16.1	37		
Colocasia corms and cormels	173.88	7.5	Sun Dried	James et al. 2013
		9.8	Blanching for 5 min in boiling water	
		9.9	Soaking in 250 ppm sodium metabisulphite solution for 4h	
		18.5	Sodium meta bisulphite treatment and blanching (combination of 3rd and 4th treatment)	

Food stuffs	**Total oxalate content (mg/100 g WM)**	**% Reduction of total oxalate content after treatment**	**Method employed for reduction**	**References**
Taro Corms	770	61	Soaking in 10% (w/w) baking soda solution for 2 hours followed by boiling at 90 °C for 60 minutes	Kumoro et al. 2014
Cocoyam flour	691	82.1	Boiling at 90°C for 40 minutes	Iwuoha and Kalu (1995)
		46.5	Roasted at 165°C for 40-45 minutes	

CHAPTER 3

ISOLATION AND SCREENING OF ENDOPHYTES FOR OXALATE OXIDASE PRODUCTION AND MEDIA AND PROCESS OPTIMIZATION FOR ENHANCED YIELD BY ONE FACTOR APPROACH

CHAPTER 3

ISOLATION AND SCREENING OF ENDOPHYTES FOR OXALATE OXIDASE PRODUCTION AND MEDIA AND PROCESS OPTIMIZATION FOR ENHANCED YIELD BY ONE FACTOR APPROACH

Judging from the fact that more than one endophyte often inhabits a single plant, new and interesting endophytic microorganisms are likely to be found from the nearly 300,000 plant species that inhabit the diverse environments and ecosystems of the earth (Kusari et al. 2012). The endophytic microorganisms were thus explored from tubers of plants which possess abundant oxalic acid. The isolated endophytic population exhibited different morphology, which were screened for Oxalate Oxidase production and identified by molecular characterization technique.

The use of plant species for extracting Oxalate Oxidase enzyme is ecologically unsuitable as it requires sacrificing the whole plant. Over these many years, no other renewable sources have been found which account for the commercial scale Oxalate Oxidase production. In order to meet the high demand for the enzyme in future for its potential in different industries, a novel process for the production of Oxalate Oxidase by a cheaper fermentation method has been studied, which would pave way for initiating commercial production of the enzyme in large scale. Kinetics of Oxalate Oxidase production as a function of culture growth conditions was also investigated and is being reported in this section.

3.1 MATERIALS AND METHODS

3.1.1 Selection of plants

Plants selected for the study were Taro, Banana, Sweet Potato and Beet root. The reason behind selecting these plants was presence of appreciable amount of oxalic acid in the stem, leaves and tubers of these plants.

3.1.2 Isolation of endophytes

Bacterial endophytes were isolated from the four different plant sources. Tubers of Taro, Sweet Potato, and Beet root and peel of Banana fruit was taken for the isolation of Oxalate Oxidase producing endophytes.

The respective parts of the sample plants were cut into small pieces of approximate 1.5 cm length. The explants were washed with distilled water and surface sterilized by dipping the explants in 70% (v/v) ethanol for 5 minutes, followed by 5% (w/v) sodium hypochlorite solution for 5 minutes, and 70% (v/v) ethanol for 30 s. The surface sterilized samples were washed in sterile distilled water three times to remove surface sterilization agents (Araujo 2002).The explants were then cut into small pieces of 0.5 cm length with a flame sterilized scalpel and transferred to sterilized tryptic soy agar medium supplemented with antibiotic cyclohexamide of 10 ppm concentration for the isolation of bacteria. To check the efficacy of surface sterilization, the water from the final rinse was inoculated in tryptic soy agar plates. All the Petri plates were then incubated at 30° C for 7 – 20 days.

3.1.3 Screening of Oxalate Oxidase positive endophytes

Isolates were screened for Oxalate Oxidase activity by streaking the isolates on solidified basal mineral medium (Appendix I). Basal mineral media was autoclaved at a pressure of 15psi for 15 minutes in an autoclave. The media was then poured in 90mm Petri plates. Solidified plates were then streaked with the isolates and incubated at 30º C for 3- 4 days. Control plate was kept to avoid any false result.

3.1.4 Maintenance and preservation methods

The positive isolates after the screening stages are maintained in tryptic soy slants and the organisms are being preserved for long storage in 20% glycerol stock supplemented with 1% host plant extract (v/v). The host plant extract was prepared by crushing 1g of small pieces of tubers of *Colocasia esculenta* in a mortar and pestle with 10mL of distilled water. The homogenate was centrifuged for 10 min at 8,000 RPM in a

refrigerated centrifuge and the recovered supernatant was filter sterilized using 0.2 µm filter membrane.

Table 3.1 Lists of plants utilized for the present work

S.No	Plant	Common name	Image of the plant	Image of the plant parts used for the study.
1.	*Colocasia esculenta*	Taro		
2.	*Beta vulgaris*	Beet root		
3.	*Ipomoea batatas*	Sweet Potato		
4.	*Musa paradisiaca*	Banana		

3.1.5 Physical and molecular characterization of the positive strain

The colony morphology of the organism was studied on TSA agar plates and cell wall composition was determined by Gram's Method of differential staining.The phylogeny and taxonomy of the organism was determined by 16S rRNA gene sequencing.Samples were sent to IMTECH Chandigarh for molecular identification by 16 S rRNA gene Sequencing

3.1.6 Inoculum medium

Inoculum for all the shake flask studies was prepared in tryptic soy broth supplemented with 2.5 gL^{-1} sodium oxalate having an initial medium pH of 6.5. The medium was inoculated with a loop full of culture from slant, incubated at 30˚ C in an incubator shaker at 150 rpm. The culture was harvested after 18 h of growth, added as inoculum at the rate of 2 % (v/v).

3.1.7 Media design for production of Oxalate Oxidase

All the shake flask trials were conducted in 250 ml Erlenmeyer flasks, incubated at 30˚ C in an incubator shaker at 150 rpm, unless otherwise stated. The unoptimized medium had the following (gL^{-1}): KH_2PO_4 – 3, Na_2HPO_4 – 6, NaCl – 5, NH_4Cl – 2, $MgSO_4.7H_2O$ – 0.1, $MnSO_4$ – 0.05, Disodium oxalate- 5 and Biotin- 0.0015, with the initial pH of 6.5. Samples were taken at definite time intervals to analyse cell growth and Oxalate Oxidase production. Growth was monitored by measuring the optical density at 600 nm and the cell free supernatant obtained after centrifugation (Remi coolong centrifuge, C-24 BL) at 8,000 RPM for 15 min in a refrigerated centrifuge, was analyzed for Oxalate Oxidase activity. The obtained optical density values were converted to dry cell weight (gL^{-1}) using a calibration curve (Prabha et al. 2014).

3.1.8 Enzyme Assay procedure

3.1.8.1 Principle

Oxalate Oxidase will be assayed by 3-Methyl-2-Benzothiazolinone Hydrazone (MBTH) method (Laker et al. 1980). The Hydrogen peroxide produced by the oxidation

of oxalate by Oxalate Oxidase enzyme combines with MBTH and DMA (N, N-Dimethylaniline) in the presence of peroxidase to form purple colour indamine dye. The concentration of indamine dye is directly proportional to the concentration of hydrogen peroxide which in turn proportional to the activity of Oxalate Oxidase enzyme. The colour produced due to indamine dye formation will be read at 600 nm.

$$\text{Oxalate} + O2 \xrightarrow{\text{Oxalate Oxidase}} H_2O_2 + 2CO_2$$

$$H_2O_2 + \text{MBTH} + \text{DMA} \xrightarrow{\text{Peroxidase}} \text{Indamine dye dye} + 2H_2O$$

3.1.8.2 Reagents

A**.** 50 mM Succinate buffer: It is prepared 100 ml in deionized water using Succinic Acid, and adjusted to pH 3.8 at 30°C with 1 M NaOH.

B. 200 mM Oxalic Acid Solution, pH 3.8 at 30°C: It is prepared 10 ml in deionized water using Oxalic Acid, Free Acid, and adjusted to pH 3.8 at 30°C with 1 M NaOH.)

C. 100 mM Ethylenediaminetetraacetic Acid Solution (EDTA): It is prepared 10 ml in deionized water using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate).

D. Colouring reagent: 50 mM Succinate buffer, with 0.79 mM N,NDimethylaniline and 0.11 mM 3-Methyl-2- Benzothiazolinone Hydrazone, pH 3.8 at 30°C (MBTH): It is prepared 100 ml in deionized water using Succinic Acid, 3-Methyl-2 Benzothiazolinone Hydrazone, Hydrochloride and N,N-Dimethylaniline, and adjusted to pH 3.8 at 30°C with 1 M NaOH).

E. Peroxidase Enzyme Solution (POD): It is prepared immediately before use containing 1 mg/ml of Peroxidase, in cold deionized water.

3.1.8.3 Procedure

The assay of Oxalate Oxidase was carried out in tubes wrapped with aluminium foil. To each tube 1.7 ml succinate buffer (Reagent A, 0.05 M, pH 3.8.0), 0.04 ml oxalic acid solution (Reagent B, 0.2 M) and 1 ml fermentation broth were added. The reaction mixture was incubated at 55ºC for 30 min. After incubating it at 55ºC for 30 min, 0.1

ml of 100 mM EDTA (Reagent C) was added. The tubes were vortexed and 1.0 ml colour reagent (Reagent D) and 3U of 0.02 ml of peroxidase enzyme (Reagent E) was added to each tube and kept at room temperature for 15 min in dark to develop the colour. The reaction was stopped by the addition of 0.5 ml of 10% (w/v) TCA (Trichloro acetic acid) solution. Absorbance was read at 600 nm against the zero setting blank of 0.05 M succinate buffer pH 3.8. A control was maintained which contains all the reagents except the substrate solution. One unit of Oxalate Oxidase was defined as the amount of enzyme required to produce 1 μmol of H_2O_2 per min at 55ºC. Standard curve was prepared using commercially available hydrogen peroxide solution dissolved in succinate buffer 0.05 M, pH 3.8.0. The amount of hydrogen peroxide produced from the sample due to Oxalate Oxidase activity on oxalic acid was determined by extrapolating the standard curve prepared using hydrogen peroxide in the range of 0.001–0.030 mmol.

3.1.9 Effect of nutritional parameters on Oxalate Oxidase production

3.1.9.1Effect of different carbon sources

The effect of different carbon sources on the production of Oxalate Oxidase and biomass were investigated in shake flasks containing above explained nutrient medium supplemented with 20 gL^{-1} carbon sources (sucrose, glucose, glycerol). Once the best carbon source was determined, the effect of supplemented carbon source concentration was determined by varying the selected carbon source (sucrose) in the range of 20-60 gL^{-1}.

The condition maintained were incubation at 30ºC, RPM 150, initial medium pH of 6.5 and one-half fill up volume of shake flask.

3.1.9.2 Effect of different nitrogen sources

The effect of nitrogen sources was determined using the nutrient medium explained above supplemented with 20 gL^{-1} sucrose and 2 gL^{-1} of various organic and inorganic nitrogen sources (Yeast extract, peptone, casein hydrolysate, Ammonium chloride, sodium nitrate). Once the best nitrogen source was selected, the effect of concentration

of selected nitrogen source (NH_4Cl) on enzyme production was determined by varying its concentration in the range of 2-8 gL^{-1}.

3.1.9.3 Effect of Mn^{2+} ions

Several reports suggest presence of Mn^{2+} ions is important for functional Oxalate Oxidase enzyme production in plants (Kotsira and Clonis 1997) and in fungus *Ceriporiopsis subvermispora* (Aguilar et al. 2009). So the effect of Mn^{2+} was studied for its role in the production of functional Oxalate Oxidase by incorporating $MnSO_4$ ($0.025 – 0.1gL^{-1}$) in the media.

3.1.10 One factor study of Non-nutritional parameters

3.1.10.1 Temperature

Effect of temperature on the growth of the organism and production of enzyme was studied. The temperature range was kept between 20ºC to 35ºC.

3.1.10.2 pH

Effect of pH on the growth of the organism and production of enzyme was also studied. The pH range was kept 5 to 8. Medium pH was adjusted using either dilute HCl or dilute NaOH before autoclaving the medium.

3.1.10.3 Fill up volume

Effect of fill up volume on the overall growth and enzyme production was studied. Fill up volume of one-half, one-fourth, one-fifth, one-tenth were maintained in different flasks and the biomass and enzyme production was estimated for each of the flasks.

3.1.10.4 Agitation speed

Effect of agitation speed for 150,175,200 RPM on the growth and enzyme production was determined.

3.1.10.5 Inoculum age

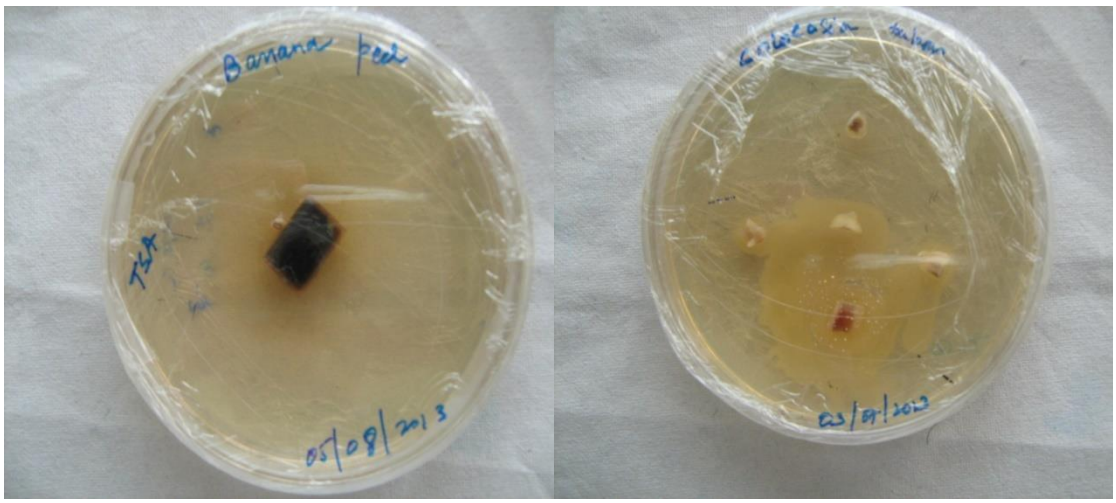
Effect of Inoculum from inoculum age of 12, 18 and 24 h was studied for enhanced production of enzyme.

3.2 RESULTS AND DISCUSSION

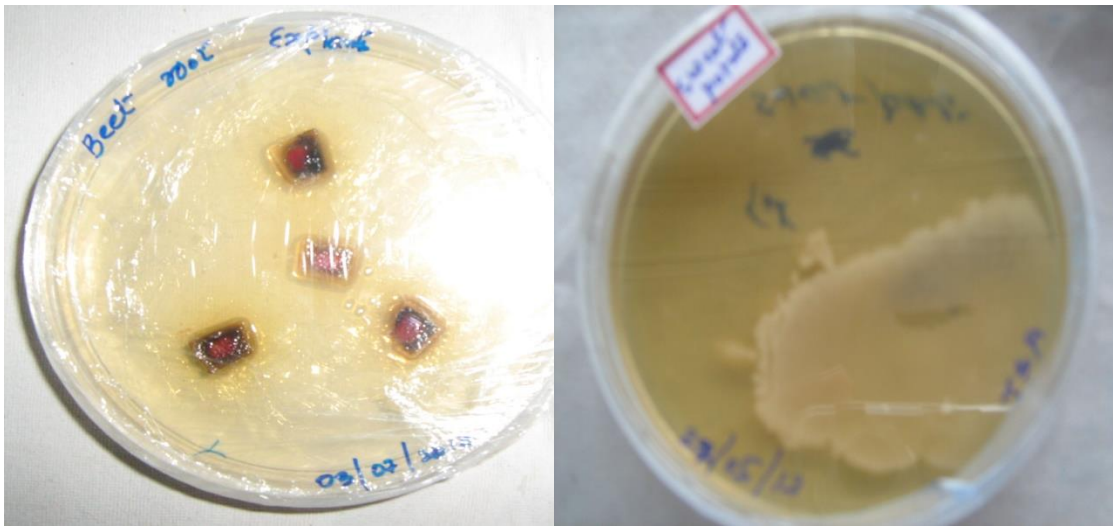
3.2.1 Isolation of endophytes

A total of 49 endophytic bacteria were isolated from tubers of *Colocasia esculenta*, *Beta vulgaris, Ipomoea batatas* and peel of *Musa paradisiaca* after implanting the surface sterilized tubers and peel of approximately 0.5 cm × 0.5 cm size in growth medium as explained in section 3.1.2. Some of the strains were pigmented- yellow, red and pink colonies. Some of the bacteria formed very minute colonies and were slow growing (Table 3.2). The isolates were subjected to screening minimal mineral medium (Appendix I**)** agar plates containing 0.5 % calcium oxalate as the only source of carbon. Colonies which were able to solubilize calcium oxalate in screening medium were selected for further studies. Solubilisation of calcium oxalate was evidenced by the formation of clear zones around the colonies due to dissolution of calcium oxalate.

Seven bacterial cultures were selected for shake flask studies based on their ability to form clear zones by dissolution of calcium oxalate during primary screeningon minimal mineral medium agar plates supplemented with 5 gL^{-1} calcium oxalate. These were one from *Musa paradisiaca* (Banana), two from *Beta vulgaris* (Beet root) and four from *Colocasia esculenta* (Taro). These seven bacterial isolates were grown in the production media supplemented with 0.5 % sodium oxalate and the cell free supernatant was assayed for Oxalate Oxidase activity. Out of these seven, four isolates were Oxalate Oxidase positive strains. OxO positive strains were one from *Musa paradisiaca* (Banana), two from *Beta vulgaris* (Beet root) and one from *Colocasia esculenta* (Taro) (Table 3.3). One isolate, CL6 exhibited large clear zone on screening plate (Figure 3.2) and good growth and noticeable Oxalate Oxidase production on sodium oxalate supplemented production medium in initial shake flask studies. The activity of the enzyme produced by CL-6 was better than all other strains and therefore the strain CL-6 was chosen for rest of the study.



A B



C D

Figure 3.1 Bacterial endophytes growing out on TSA plates from explants of Banana peel (A), Colocasia (B), Beet root (C) and Sweet Potato (D)

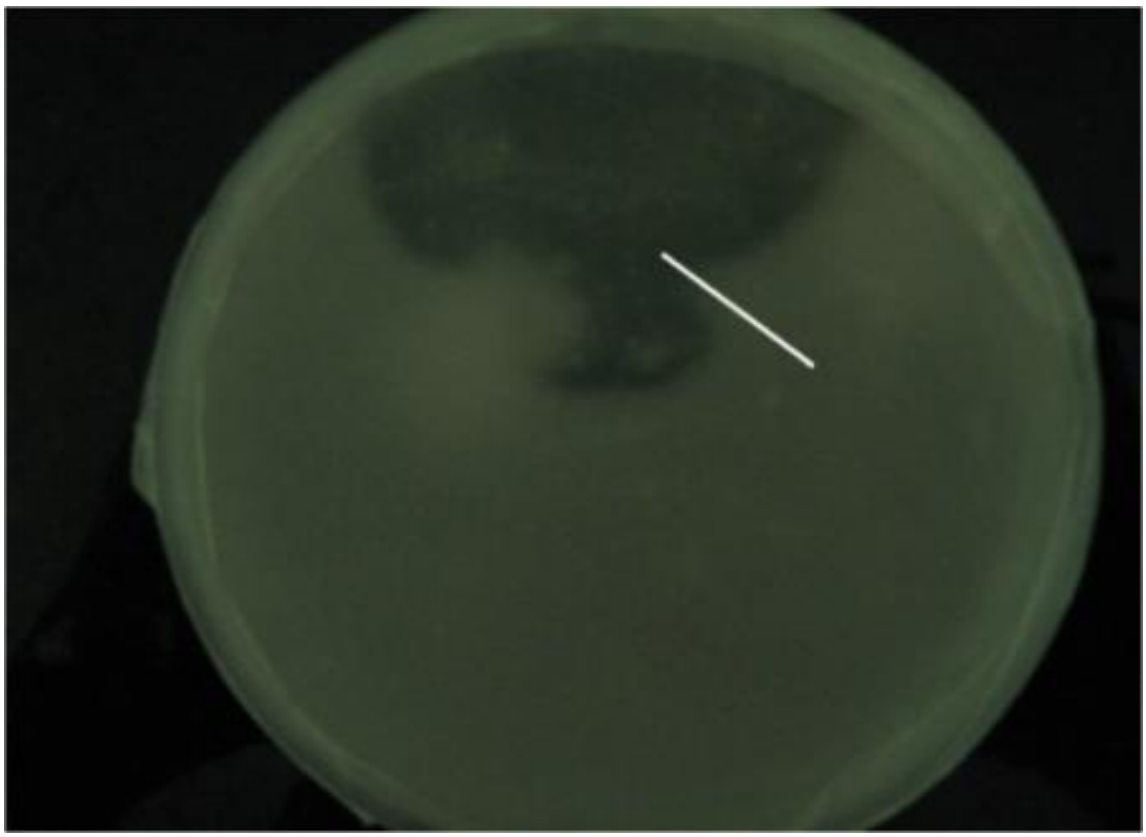


Figure 3.2 Screening plate of *Ochrobactrum intermedium* CL6 showing clear zone due to dissolution of calcium oxalate

Table 3.2 Endophytic isolates from the explants of studied plant sources and their morphology

Isolate source	Microorganism type	Total no of isolates	Bacterial Isolates Characters
Colocasia esculenta	Bacteria	17	Creamish, yellowish coloured colonies, slightly raised, colonies, Few were bordered with pink pigment, Few were minute colonies and slow growing
Beta vulgaris	Bacteria	05	
Musa paradisiacal	Bacteria	10	
Ipomoea batatas	Bacteria	17	

Table 3.3 Preliminary Oxalate Oxidase activity observed in few bacterial endophytes

S.No	Isolates Name	Source	Activity(U/ml)
1	BN 1	Peel of *Musa paradisiaca*	0.05
2	BR 2	Tuber of *Beta vulgaris*	0.06
3	BR 4	Tuber of *Beta vulgaris*	0.02
4	CL6	Tuber of *Colocasia esculenta*	0.1

3.2.2 Physical and molecular characterization

The colony morphology of CL-6 showed creamish colonies, which were oval in shape and slightly raised. Differential staining showed that the organism is Gram negative and small rod shaped (Figure 3.3).

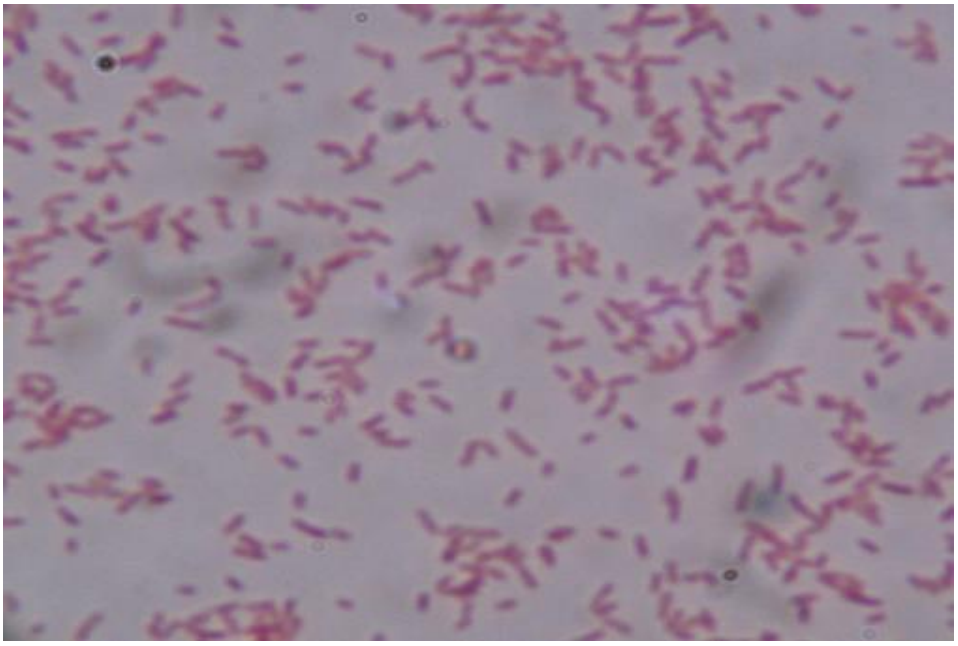


Figure 3.3 Differential staining of CL-6

The 16S rRNA gene sequencing and phylogenetic analysis result showed the test organism belongs to rhizobiales order of alphaproteobacteria. The 16S rRNA sequencing result showed that the query sequence obtained from amplification and sequencing of the hypervariable region of 16S rRNA has 99.86 % similarity with the 16S rRNA nucleotide sequence of *Ochrobactrum intermedium* and the number of mismatch of nucleotide between the total 16S rRNA nucleotide of the test organism and *Ochrobactrum intermedium* is 2 (Table 3.4). The number of mismatch between the total 16S rRNA nucleotide of the test organism and all other closely related organisms of rhizobiales order is higher, which is evident from the table below. Hence the test organism has been identified as *Ochrobactrum intermedium.*

TACCTTGTTACGACTTCACCCCAGTCGCTGACCCTACCGTGGTCGCCTGCC
TCCTTGCGGTTAGCACAGCGCCTTCGGGTAAAACCAACTCCCATGGTGTG
ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT
CCGCGATTACTAGCGATTCCAACTTCATGCACTCGAGTTGCAGAGTGCAA
TCCGAACTGAGATGGCTTTTGGAGATTAGCTCACACTCGCGTGCTCGCTGC
CCACTGTCACCACCATTGTAGCACGTGTGTAGCCCAGCCCGTAAGGGCCA
TGAGGACTTGACGTCATCCCCCACCTTCCTCTCGGCTTATCACCGGCAGTC
CCCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGGGCGAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCAT
GCAGCACCTGTCTCCGATCCAGCCGAACTGAAGGATAGTGTCTCCACTAA
CCGCGATCGGGATGTCAAGGGCTGGTAAGGTTCTGCGCGTTGCTTCGAAT
TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTT
TTAATCTTGCGACCGTACTCCCCAGGCGGAATGTTTAATGCGTTAGCTGCG
CCACCGAAGAGTAAACTCCCCAACGGCTAACATTCATCGTTTACGGCGTG
GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGC
GTCAGTAATGGTCCAGTGAGCCGCCTTCGCCACTGGTGTTCCTCCGAATAT
CTACGAATTTCACCTCTACACTCGGAATTCCACTCACCTCTACCATACTCA
AGACTAACAGTATCAAAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACC
CCTGACTTATTAGCCCGCCTACGTGCGCTTTACGCCCAGTAAATCCGAACA
ACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGG
GCTTCTTCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAA
CCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCC
ATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCT
CAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTATGGATCGTCGCCT
TGGTAGGCCTTTACCCCACCAACTAGCTAATCCAACGCGGGCCGATCATTT
GCCGATAAATCTTTCCCCTTTCGGGCTCATACGGTATTAGCACAAGTTTCC
CTGAGTTATTCCGTAGCAAATGGTACGTTCCCACGCGTTACTCACCCGTCT
GCCGCTCCCCTTGCAGGGCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAG
CGTTCGTTCTGAGCCAGGATCAAACTCT

Figure 3.4: 16S rRNA gene sequencing of *Ochrobactrum intermedium*

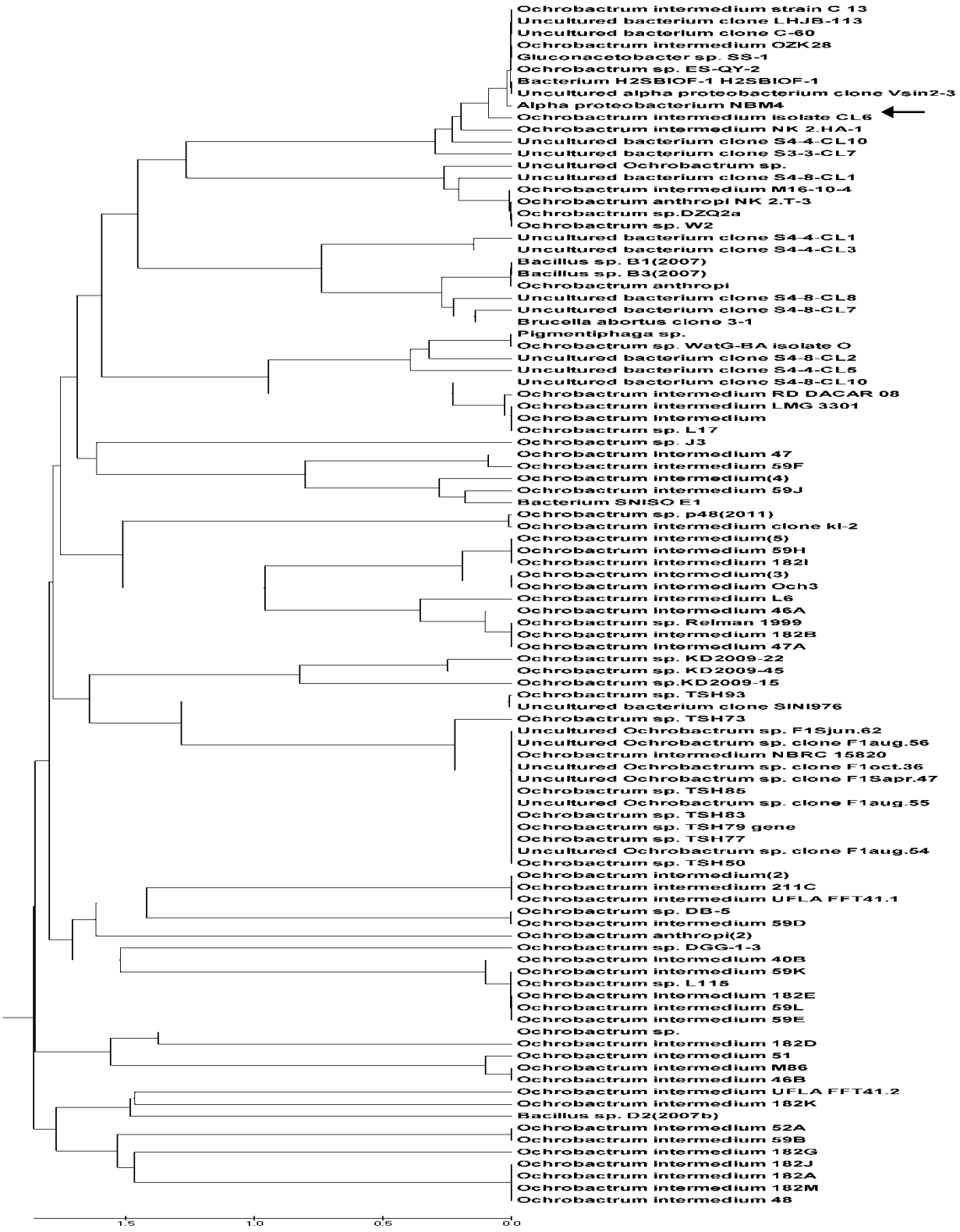


Figure 3.5: Phylogenetic position of *Ochrobactrum intermedium*

Table 3.4 Pairwise similarity index of test organism and other organisms of rhizobales

Rank	Name	Pairwise Similarity (%)	Diff/Total nt	Completeness (%)
1	*Ochrobactrum intermedium*	99.86	2/1443	100
2	*Ochrobactrum ciceri*	99.38	9/1442	100
3	*Brucella inopinata*	99.03	14/1443	100
4	*Brucella abortus*	98.68	19/1443	100
5	*Brucella canis*	98.68	19/1443	100
6	*Brucella suis*	98.68	19/1443	100
7	*Brucella microti*	98.68	19/1443	100
8	*Brucella ovis*	98.68	19/1443	100
9	*Brucella melitensis*	98.68	19/1443	100
10	*Brucella neotomae*	98.61	20/1443	100
11	*Brucella ceti*	98.60	20/1430	100
12	*Brucella pinnipedialis*	98.60	20/1430	100
13	*Ochrobactrum tritici*	98.21	25/1397	98.04

Rank	***Name***	**Pairwise Similarity (%)**	**Diff/Total nt**	**Completeness (%)**
14	*Ochrobactrum daejeonense*	98.07	26/1345	95.73
15	*Ochrobactrum cytisi*	97.99	29/1441	100

Differential staining of the test microorganism revealed that it is Gram negative and small rod shaped. The work carried out by Wackerow-Kouzova, 2006 and Velasco *et al.* 1998 suggests that *Ochrobactrum intermedium* is Gram negative and small rod shaped. It is clear from table 3.5 that the test organism is also closely related to *Ochrobactrum ciceri*. Ochrobactrum species have been found to be associated with rhizosphere and root nodules of many plants. There are also reports of Ochrobactrum being inhabitated within the internal tissues of some plants. Imran et al. 2010 reported the isolation of *Ochrobactrum ciceri*from nodules of chickpea (*Cicer arietinum*). An endophyte, *Ochrobactrum oryzae,* a type strain, designated as MTCC 4195T was isolated from surface-sterilized seeds and plant tissue from deep-water rice (*Oryza sativa*) cultivated in Suraha Tal Lake in northern India by Tripathi et al. 2006. *Ochrobactrum tritici*, a type species has been isolated from wheat rhizoplane by Lebuhn et al. *2000. Ochrobactrum intermedium* has also been reported by Faisal and Hasnain 2006 for promoting the growth of mung bean, *Vigna radiata.* The endophytic colonization of *Ochrobactrum anthropi* Mn1 strain was investigated microscopically using green fluorescent protein in *Jerusalem artichoke* tissues through the root, and was found to possess plant growth-promoting (PGP) effects of symbiotic nitrogen fixation, root morphological optimization and enhanced nutrient uptake (Meng et al. 2014). Another strain of *Ochrobactrum anthropi* was isolated from (rice) Oryza sativa as an endophyte by Mano & Morisaki (2008). Nitrogenase activity was observed in endophytes from wild sugarcane plants, which had close genetic makeup with that of *Ochrobactrum intermedium (*Muangthong et al. 2015)

The taxonomy of Ochrobactrum reveals that it belongs to the order rhizobiales, which also comprises of bacteria of rhizosphere. The test organism *Ochrobactrum*

intermedium has been isolated from the tubers of Colocasia as endophyte, so it can be stated that the test organism *Ochrobactrum intermedium* might have invaded the colocasia plant tissue over a period of time from its rhizosphere to the internal tissues of colocasia tuber. Literature suggests that microbial flora of rhizosphere can be endosymbionts or endophytes of plants. It has been speculated that endophytic root bacterial communities comprise a subset of colonists originating from the surrounding rhizosphere soil (Cocking 2003 & Hallmann 1997), and the resulting community composition is affected by the surrounding soil and environmental properties. Nitrogen-fixing bacteria are able to enter into roots from the rhizosphere, particularly at the base of emerging lateral roots (Cocking 2003). Endophytic bacteria originate from the epiphytic bacterial communities of the rhizosphere (Hallmann 1997). The work of Hui et al. 2014 reveals that the bacterial community structure and diversity of the rhizosphere and endophytic bacteria in *Stellera chamaejasme* compartments were dominated by the members of the *Proteobacteria* (43.2%), *Firmicutes* (36.5%) and *Actinobacteria* (14.1%) in both samples (Jin et al. 2014).

During the colonization of plant roots by soil bacteria, microorganisms from the bulk soil undergo selective enrichment in the plant rhizosphere in response to different root exudate components. Because different plant species release different types and quantities of exudates, plants exert species-specific effects on the soil microbial community that result in broad spectrum of microflora (Lynch 1990). Different plant species select different bacterial communities in the proximity of their roots and that these plant-specific enrichments can be increased by repeated cultivation of the plant species in the same field (Smalla 2001).

The oxalate-degrading bacteria form a close association with plant roots that are rich in oxalic acid, where the oxalate exudates form a source of carbon for a variety of microorganisms in the rhizosphere (Sahin 2005). The yam (*Dioscorea alata*, *Typhonium trilobatum* and *Amorphophallus*) tubers are one such example. A new type of oxalate-utilizing Paracoccus sp. was isolated from Amorphophallus rhizoplane (Kolandaswamy et al. 2007). Oxalic acid is often accumulated as a metabolic end product in plant tissues or is released by root systems as a free organic acid or mineral salts such as calcium, iron, or magnesium oxalate (Cailleau et al. 2005).

Rhizobacteria like Pseudomonas and Rhizobium follow Entner-Doudoroff pathway for glucose catabolism and prefer organic acids over glucose by repressing enzymes of glucose utilization (Collier et al. 1996; Mandal and Chakrabartty 1993).

It can be argued that endophytic microbial community of *C. esculenta* might have also been influenced by high amount of oxalate in its living tissue. It appears that *O. intermedium* CL6 isolated from *Colocasia* tuber have evolved the enzyme system, Oxalate Oxidase in order to utilize oxalate available in host plant tissues.

In order to understand the revealing role of the *Ochrobactrum intermedium* as an endophyte in *Colocasia esculenta* and a competent source of Oxalate Oxidase, a strong relationship between role of an endophyte and Oxalate Oxidase with evidential argument has to be highlighted. *Ochrobactrum intermedium* and other species of the genus Ochrobactrum from previous works has been established as an endophyte (Faisal and Hasnain 2006). Endophytes enhance the growth and competitiveness of the host plant and protect it from herbivores and plant pathogens apart from producing diversity of natural bioactive products (Gunatilaka et al., 2006). Hydrogen peroxide which gets generated by the catalytic action of Oxalate Oxidase (OxO, EC 1.2.3.4) on oxalate in presence of O_2 in all tropical and subtropical plants has several defensive roles . These defensive roles for H_2O_2 in plants have been proposed and proved time and again. (Lamb and Dixon 1997). For example, H_2O_2 in plant tissues may reach levels that are directly toxic to microbes (Peng and Kuc 1992). H_2O_2 may contribute to the structural reinforcement of plant cell walls (Bolwell et al. 1995) and trigger lipid peroxide and salicylic acid (SA) synthesis (Leon et al. 1995). Moreover, H_2O_2 appears to have roles in signal transduction cascades that coordinate various defense responses, such as induction of HR (hypersentivity reaction) and synthesis of pathogenesis-related (PR) proteins and phytoalexins (plant physiology). Thus *Ochrobactrum intermedium* CL6 in this report might have the capability of rendering support for fight against pathogens in *Colocasia esculenta* plants in the capacity of an endophyte by triggering its Oxalate Oxidase gene to produce H_2O_2 in response to attack from an invading pathogenic microorganism. This finding reinforces the fact that endophytes do have a significant role in plant defense.

3.2.3 Effect of nutritional parameters

3.2.3.1 Effect of different carbon sources

Initially the maximum biomass of the strain *Ochrobactrum intermedium* CL6 obtained with sodium oxalate as an only source of carbon was 0.3 gL^{-1} after 120 h of incubation but a comparative higher growth was obtained with sucrose as well as with glucose as additional carbon sources within 48 h of incubation (Figure 3.6). Sucrose as a carbon source exhibited maximum enzyme activity for Oxalate Oxidase at 48 h of cultivation time. However, glucose as a carbon source was not very effective for enzyme production as it took 60 h to exhibit the maximum enzyme activity for Oxalate Oxidase. There was no change in biomass content and enzyme activity with glycerol as a carbon source. Sucrose was therefore selected for further study as it could reduce the fermentation time from 120 h to 48 h. The Oxalate Oxidase production with different concentrations of sucrose was also tested. Sucrose at 20 gL^{-1} exhibited the maximum Oxalate Oxidase activity 0.128 U mL^{-1} (Figure 3.7). Presence of organic acid like oxalic acid in the fermentation broth may lead to catabolite repression of glucose and therefore glucose, being a readily available source of carbon did not contribute much to biomass of the organism (Singh et al. 2008; Bruckner and Titgemeyer 2002). Similar phenomenon have been reported in rhizobacteria like Pseudomonas and Rhizobium, both follow Entner-Doudoroff pathway for glucose catabolism and prefer organic acids over glucose by repressing enzymes of glucose utilization (Collier et al. 1996; Mandal and Chakrabartty 1993). Succinate is one of the major organic acids present in root exudates of plants in rhizosphere and repression of glucose utilization by succinate is termed as succinate-mediated catabolite repression (SMCR) (Bringhurst and Gage 2002).

The result showed that test organism was utilizing sucrose. In most bacteria, the uptake of sucrose is mediated via the phosphoenolpyruvate-dependent carbohydrate: phosphotransferase system (PTS), yielding sucrose 6-phosphate, which is cleaved by an intracellular hydrolase into glucose 6-phosphate and fructose (Reid and Abratt 2005) and glucose 6-phosphate and fructose can be directly assimilated by contemporary pathways inside the cell. *Ochrobactrum anthropi*, a close relative of Ochrobactrum

intermedium also utilize sucrose by phosphotransferase system yielding sucrose 6-phosphate (Courtesy KEGG pathway).

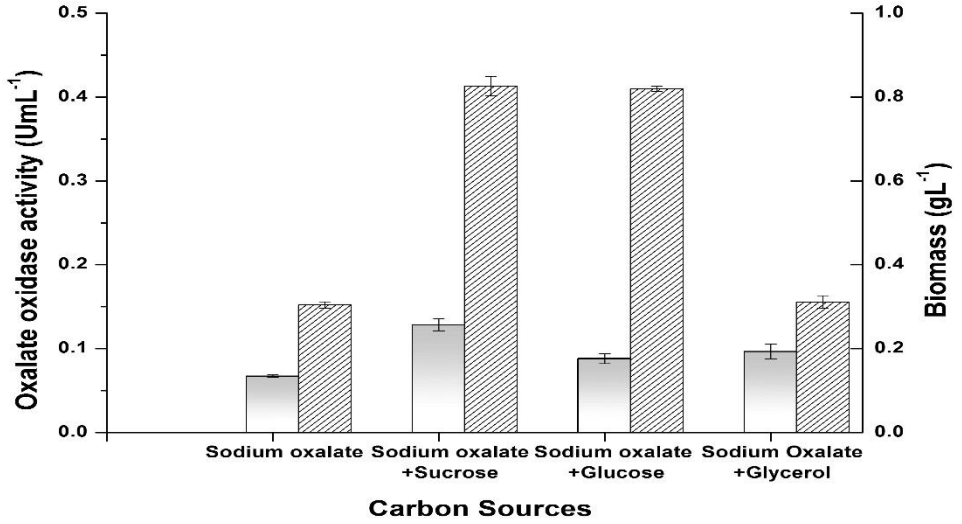


Figure 3.6 Effect of various sugars in medium on biomass growth (▨) and enzyme activity (□). Medium contains 20 gL^{-1}carbon source, 5 gL^{-1} Sodium oxalate and 2 gL^{-1} NH_4Cl along with basal salts. The peak biomass concentration and enzyme activity in the figure is after 120 h, 48 h, 60 h and 120 h of cultivation time for sodium oxalate (sole source of carbon), sodium oxalate + sucrose, sodium oxalate + glucose, and sodium oxalate + glycerol respectively.

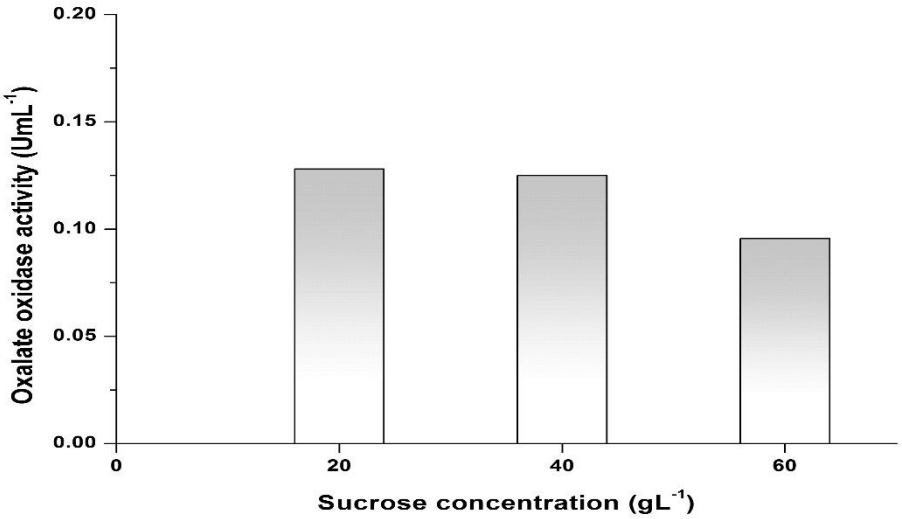


Figure 3.7 Effect of sucrose concentration on the enzyme activity was studied by changing its concentration in the medium having 5 gL^{-1} Sodium oxalate and 2 gL^{-1} NH_4Cl along with basal salts

3.2.3.2 Effect of different nitrogen sources

NH_4Cl was found to be the best for the maximum production of Oxalate Oxidase. NH_4Cl at 2 gL^{-1} exhibited an Oxalate Oxidase activity of 0.128 UmL^{-1}. Enhancement of enzyme activity was low in case of Yeast extract and Peptone. The only organic nitrogen source which showed some enzyme production was casein hydrolysate (Figure 3.8). NH_4Cl was selected for further study. Varied concentration of NH_4Cl didn't contribute in further improving the Oxalate Oxidase activity. The enzyme activity remained same for NH_4Cl concentration of 2-6 gL^{-1}. However, as the nitrogen source concentration increased, peak enzyme activity got shifted from 48 to 84 h. At NH_4Cl concentration of 8 gL^{-1}, reduction in peak enzyme activity was observed at 84 h (Figure 3.9).

The reason behind good enzyme activity with NH_4Cl as nitrogen source could be traced from the available literature on the active site composition of Oxalate Oxidase. Many literature has documented the presence of glutamate and glutamine at the active site of the functional enzyme. (Bernier and Berna 2001; Gane et al. 1998; Requena and Bornemann 1999; Woo et al. 2000).Reduced nitrogen in the form of NH_4^+ is assimilated

into amino acids and then into other nitrogen-containing biomolecules. Two amino acids, glutamate and glutamine**,** provide the critical entry point. The most important pathway for the assimilation of NH_4^+into glutamate requires two reactions. First, glutamine synthetase catalyzes the reaction of glutamate and NH_4^+ to yield glutamine. In bacteria and plants, glutamate is produced from glutamine in a reaction catalyzed by glutamate synthase (Patriarca et al. 2002). The result also showed that the organism couldn't utilize organic nitrogen to produce the enzyme in high concentration. The reason could be because of the fact that organic nitrogen contains a rich source of different amino acids and these pools of amino acids allosterically inhibit glutamine synthetase, which indirectly aid in the production of glutamate. Thus decrease in the glutamate concentration from the pool of the amino acid may prevent the organism to incorporate glutamate in the active site of the enzyme Oxalate Oxidase. Alanine, glycine, histidine and at least six end products of glutamine metabolism are allosteric inhibitors of the enzyme glutamine synthetase (Nelson and Cox. 2005).

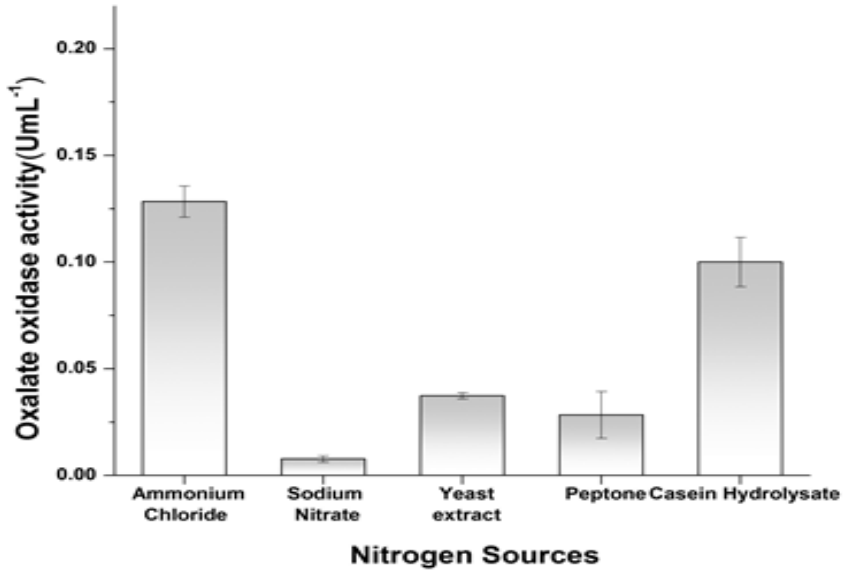


Figure 3.8 Effect of various nitrogen sources in medium on enzyme production. The medium contains 20 gL^{-1} of sucrose as carbon source, 2 gL^{-1} nitrogen source and 5 gL^{-1} sodium oxalate as inducer along with basal salts. The peak enzyme activity for all nitrogen sources was obtained at 48 h of cultivation time.

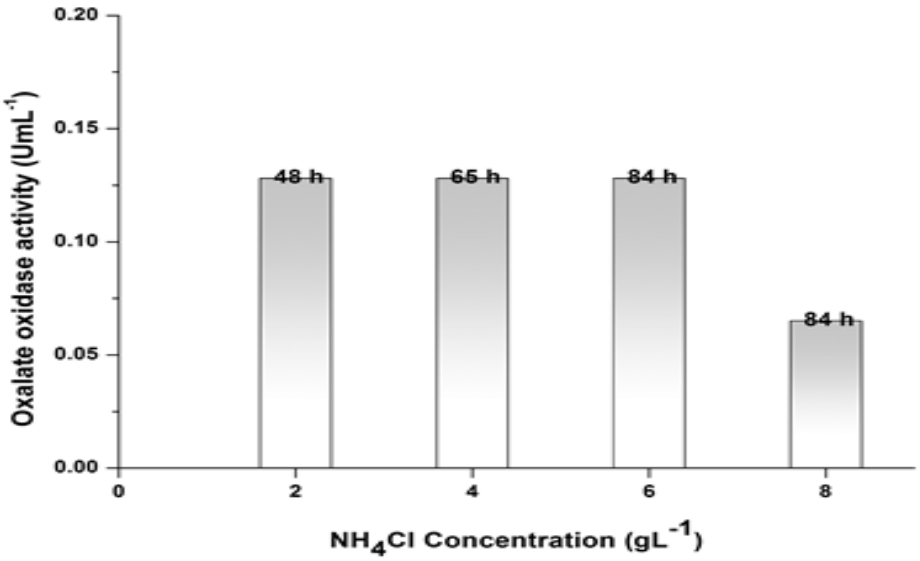


Figure 3.9 Effect of NH_4Cl Concentrations on Oxalate Oxidase activity was studied by changing its concentration in the medium having 20 gL^{-1} of sucrose and 5 gL^{-1} sodium oxalate along with basal salts

3.2.3.3 Effect of Mn^{2+}

Mn^{2+} ions were found to be extremely important for active form of enzyme production. There was no activity of Oxalate Oxidase, when source of Mn^{2+} ions in the form of $MnSO_4$ was not supplemented in the production media. The concentration of $MnSO_4$ was varied from 0.025gL^{-1} to 0.1gL^{-1}. Optimum concentration of $MnSO_4$ was found to be 0.05 gL^{-1}. Higher concentration of the $MnSO_4$ supplementation in the media was leading to lower activity of the enzyme (Figure 3.10). Manganese sulphate concentration of 0.05 gL^{-1} was selected for further study. There are reports where presence of manganese at the active site of the enzyme Oxalate Oxidase has been documented. *Ceriporiopsis subvermispora* Oxalate Oxidase was the first bicupin enzyme identified that exhibit manganese dependent oxidation of oxalate (Moomaw et al. 2013).Barley Oxalate Oxidase, a homohexameric glycoprotein, contains one Mn atom which is ligated by three histidine residues, one glutamate residue and two water molecules (Requena and Bornemann 1988, Woo et al. 2000, Gane et al. 1998).Koyama has reported the presence of 1.12 atoms of manganese and 0.36 atoms of zinc per

subunit in Oxalate Oxidase produced from the soil bacterium *Pseudomonas* sp. OX- 53 (Koyoma 1988).

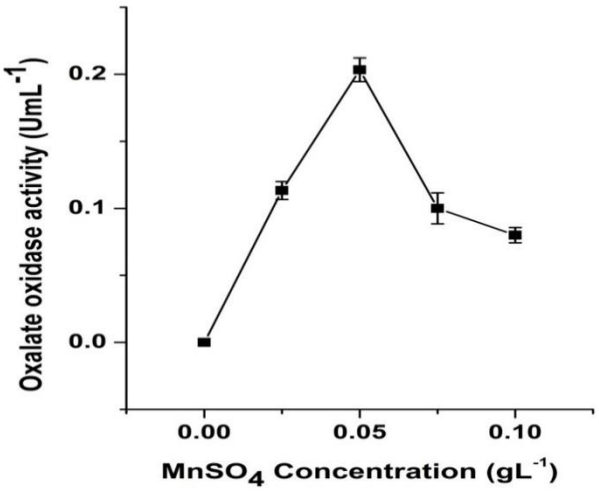


Figure 3.10 Effect of Mn^{2+} ions on the activity of the Oxalate Oxidase in the medium containing20 gL^{-1} of sucrose as carbon source, 2 $gL^{-1}$of NH_4Cl as nitrogen source and 5 gL^{-1} sodium oxalate as inducer along with other basal salts. The peak enzyme activity was obtained at 48 h of cultivation time.

3.2.4 One factor study of physical parameters

3.2.4.1 Effect of fill up volume

Fill up volume of one-half, one-fourth, one-fifth, one-tenth of shake flask was tried in four different experiments. The observations revealed that fill up volume of one-fifth and one- tenth gave higher biomass in short duration (Figure 3.11). There was a progressive increase in enzyme activity as the fill up volume is reduced from one- half to one-tenth. An Oxalate Oxidase activity of 0.5 UmL^{-1} was obtained at one-tenth of fill up volume. From this we can infer that oxygen requirement of *Ochrobactrum intermedium* CL6 is high and meeting the requirement is crucial. Higher oxygen requirement could be attributed to the fact that oxygen is a co-substrate utilized during oxalate catabolism (Opaleye et al. 2006). Moreover it is the proved fact that in shake flasks, the oxygen transfer rate (OTR) is reversely proportional to the volume of culture (Maier and Buchs 2001).Higher OTR is obtained in flasks with less culture volume or by increasing the agitation rate, however one should take in account that very low

amounts can only be used for short-term fermentations otherwise the medium will evaporate (Machado et al. 2013).

Regarding bacterial cell growth, lower volume of medium in the flasks resulted in higher cell density as determined by the optical density at 600 nm. For all the cases, the OD increased with increasing volume ratios with values of 1.1, 1.4, 1.8 and 2.2 for volume ratios of 1:2, 1:3, 1:4 and 1:5, respectively (Machado et al. 2013)

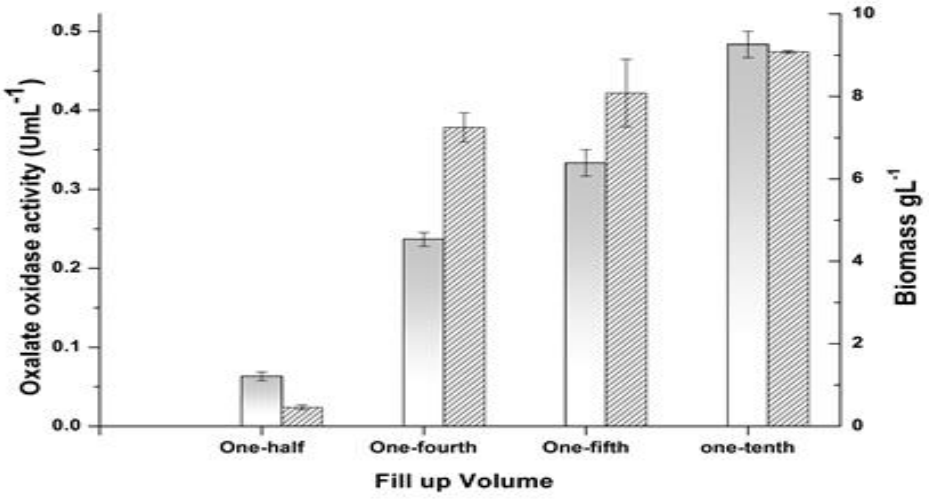


Figure 3.11 Effect of fill up volume of the culture flask on the growth ( ) of the microorganism and activity of Oxalate Oxidase ( )

3.2.4.2 Effect of temperature on biomass and enzyme production

Temperature of incubation during growth had a significant influence on the enzyme production. Observation suggests that the organism was growing very well at temperature of 35°C during the first few hours after inoculation. But after 24 h, the growth profile of the organism at 35°C and 30°C was almost same. Temperature of 25°C was suboptimal for enzyme production. The peak enzyme activity observed at 30°C and 35°C was almost similar (Figure 3.12)

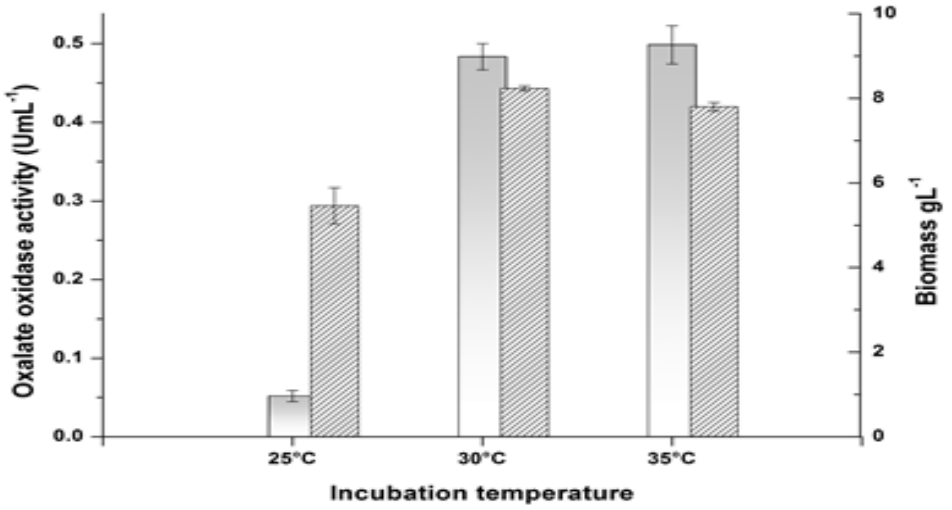


Figure 3.12 Effect of temperature on the growth of the organism (▨) and activity of the enzyme Oxalate Oxidase (□)

3.2.4.3 Effect of pH on the growth of the organism and activity of the enzyme

It was observed that the growth pattern of the organism was almost similar in initial medium pH range of 6.5, 7.5 and 8. However, the activity of the enzyme was relatively higher in pH 6.5 (Figure 3.13). The result showed that the yield of the enzyme Oxalate Oxidase by strain *Ochrobactrum intermedium* CL6 was relatively less at initial medium pH 7.5. The enzyme activity profile revealed that maximum activity of 0.2 UmL^{-1} was obtained for initial medium pH 8, which is very similar to the observations of Koyoma (1988), while studying production of Oxalate Oxidase in *Pseudomonas* sp. OX-53.The optimum pH of 6.5 for maximum production of enzyme is an advantage as it is nowhere close to the optimum pH of enzyme activity which is 3.8. This optimum pH of enzyme activity prevents the rapid formation and thereby accumulation of the hydrogen peroxide in the fermentation medium during the incubation period, which otherwise would have affected the growth of the microorganism and production of Oxalate Oxidase as well. Hydrogen peroxide is known to have bactericidal effect. It is thus beneficial to have a system which will give an edge over suppressing the inhibitory factors and improves the prospect of production of Oxalate Oxidase.

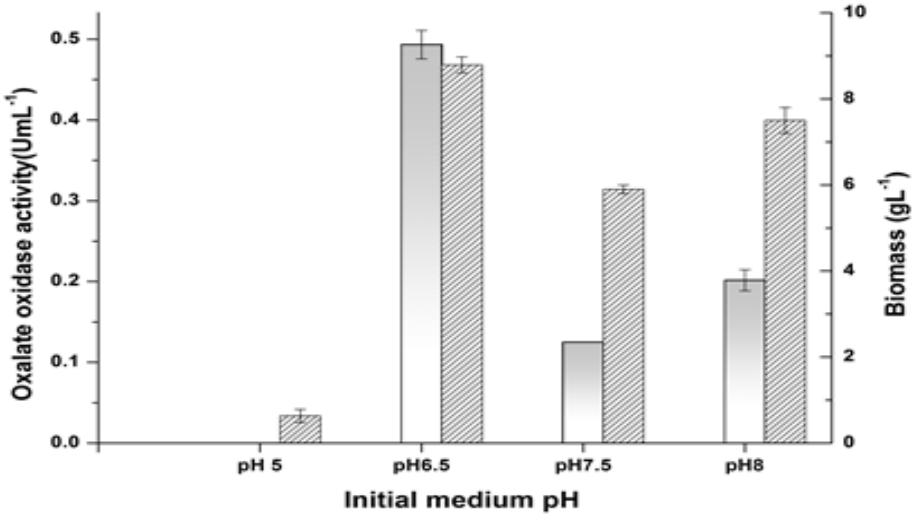


Figure 3.13 Effect of pH on the growth of the organism (▨) and activity of the enzyme Oxalate Oxidase (□)

3.2.4.4 Effect of Agitation speed and Inoculum age

There was an increase in biomass content as the agitation speed was increased from of 150 to 200 RPM. However there was no significant increase in Oxalate Oxidase production with increase in agitation speed (Figure 3.14). Higher agitation rates did not bring about the increase in production, probably because at a high agitation rate, the structure of enzyme would be altered (Roychoudhury et al. 1988).

It has been found in many fermentation process that agitation mode gives significant increase in biomass and product formation as compared to stationary condition. Although increase in agitation may provide better mixing and mass transfer effects, an excess might result in high shear stress and in turn lead to many adverse effects. Lipase production from *Pseudomonas* sp. BWS-5 showed a progressive increase with the increase in agitation rate up to 150 RPM and thereafter, a decrease in enzyme activity was recorded (Sooch and Kauldhar 2013). Pabai et al. (1996) also witnessed that 150 RPM agitation rate as the best for lipase production by *Pseudomonas fragi*, *P. putida* and *P. fluorescens*. Both oxygen availability and availability of other nutrients in the medium is affected by agitation rate. Low enzyme activity at higher agitation rates may be credited to the effect of shear stress on microbial cells as well as on the

enzyme structure (Calderbank and Moo-Young 1959). The effects of agitation rate on the production of protease and bacterial growth were investigated. The highest protease production and bacteria growth were obtained when agitated at 200 RPM. At this speed, the aeration of the culture medium was increased, and this further led to a sufficient supply of dissolved oxygen in the media. The production of protease was found to decrease when shaken at 250 RPM, and even the static condition almost inhibited its production (Abhusham et al. 2009).

Yield of Oxalate Oxidase was found to be dependent on inoculum age. Inoculum of age 24 h exhibited poor production in comparison to 18 h old inoculum (Figure 3.15). Initial growth studies of the organism *Ochrobactrum intermedium* CL6 in inoculum medium exhibited onset of stationary phase before 24 h, and the cells were in exponential phase during 16-22 h (Appendix II). Studies demonstrated the importance of inoculum age on bio product production by bacterial cultures. Improper starter culture age leading to reduced product formation has been observed in many fermentation process (Prabha et al. 2014). Cell growth and product formation is limited in few cases where late inocula are used. This is because of the inability of late inocula containing a high proportion of spores to return to the vegetative cell cycle. On the other hand overall fermentation time is prolonged due to insufficient activated cells of lag phase of seed culture. Therefore bacterial inocula must be transferred in the log phase of growth, during which cells are still metabolically active (Gurung et al. 2013). Similar observations were made by Wang and Shih (1999), while studying in the production of keratinase yield by *B. licheniformis*. Fermentation cycle duration for Clavulanic acid production by Streptomyces clavuligerus was reduced from 139 h to 96 h when younger and more active inocula was used (Neves et al. 2000).

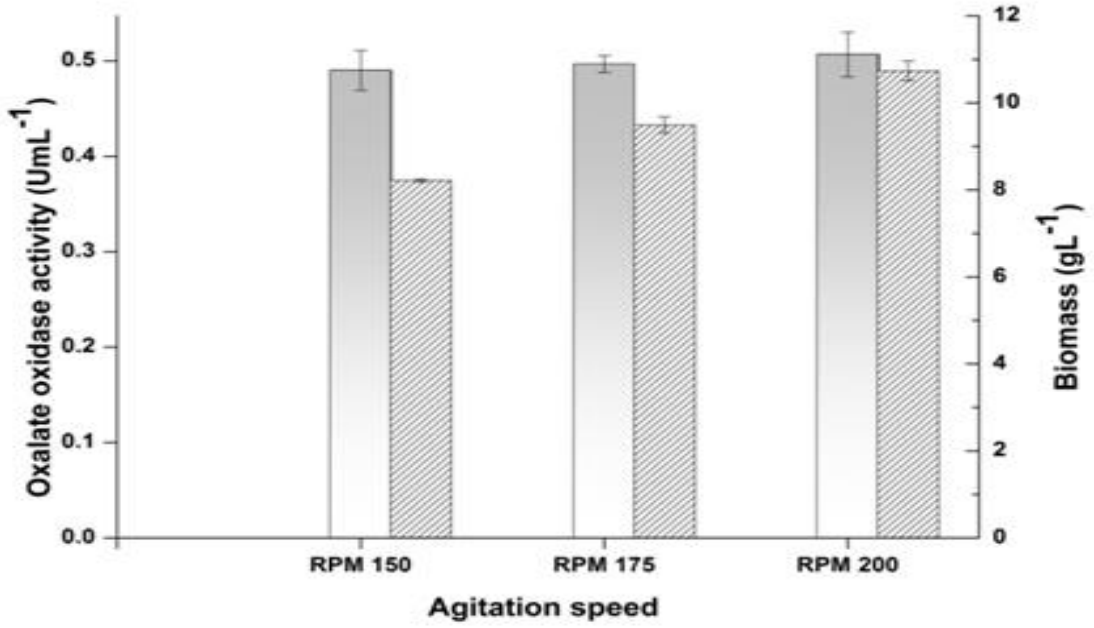


Figure 3.14 Effect of agitation speed on the growth of the organism (▨) and activity of Oxalate Oxidase (▭)

Figure 3.15 Effect of inoculum age on activity of the enzyme Oxalate Oxidase

3.2.5 Time course study of Oxalate Oxidase production

Enzyme activity with the present optimized condition of 20 gL^{-1} sucrose as additional carbon source, 5 gL^{-1} sodium oxalate as inducer, 2 gL^{-1} NH_4Cl as nitrogen source, 0.05 gL^{-1} $MnSO_4$, fill up volume of one-tenth, pH of 6.5, fermented with inoculum age of 18 h, 30ºC at 150 RPM showed maximum activity of 0.5 UmL^{-1} after 65 h of fermentation (Figure 3.16). The 25 mL culture supernatant yielded 27.5 mg of protein with total Oxalate Oxidase activity of 12.5 U and specific activity of 0.454 Umg^{-1}.

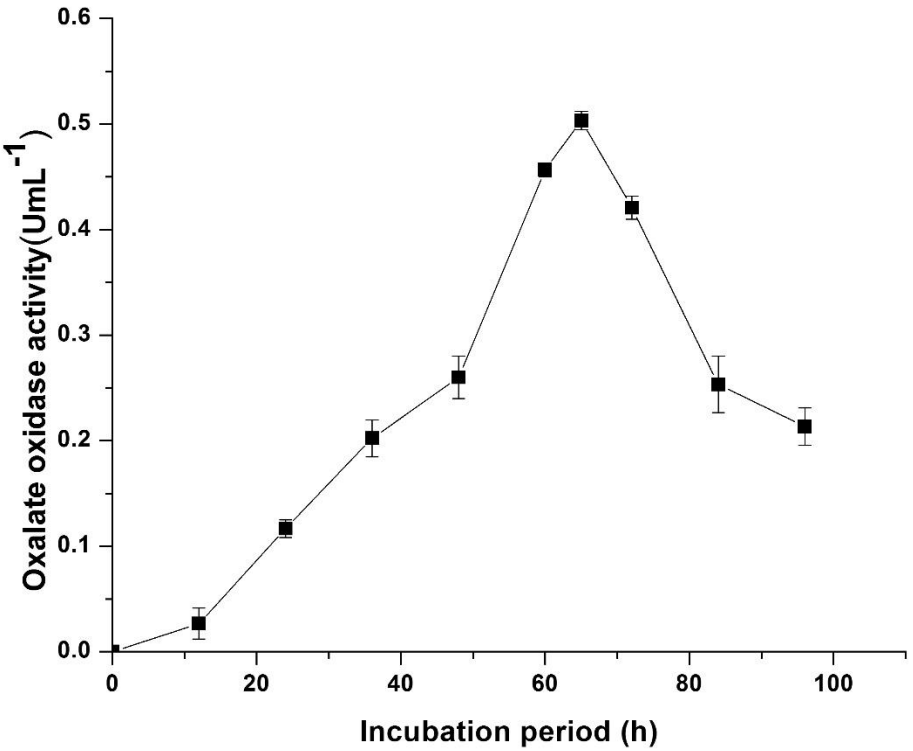


Figure 3.16 Time course study of Oxalate Oxidase production in optimized medium and conditions. Medium having 20 gL^{-1} of sucrose, 5 gL^{-1} sodium oxalate and 2 gL^{-1} NH_4Cl, 0.05 gL^{-1} $MnSO_4$ along with basal salts was used. Experiments were conducted at 30°C, fill up volume of one-tenth, agitation rate of 150 RPM and initial medium pH of 6.5 with inoculum age of 18 h

Comparison of specific activity of Oxalate Oxidase from *O. intermedium* CL6 and other sources suggests that specific activity of Oxalate Oxidase obtained in the present study was 0.454 Umg^{-1} (Table 3.5), which is much higher than that of other microbial sources (Koyoma 1988) as well as plant sources (Kotsira and Clonis 1997, Hu and Guo 2009, Sathishraj and Augustin, 2012).The total enzyme activity from wheat seedling and barley roots was also lower than the total enzyme activity obtained from the culture supernatant of *O. intermedium* CL6. Higher specific activity in the cell free broth also indicates that the enzyme purification could be relatively easy.

Table 3.5 Comparison of Oxalate Oxidase activity from *Ochrobactrum intermedium* CL6 and other sources

Culture supernatant /crude extract	**Specific activity (Umg^{-1})**
Ochrobactrum intermedium CL6	0.454
Pseudomonas sps. OX-53	0.29
Barley roots	0.031
Wheat seedlings	0.107
Costus pictus leaves	0.0374

3.3 SUMMARY

A growing body of evidence suggests that the plant-associated microorganisms, especially endophytic and rhizosphere bacteria and fungi, represent a huge and largely untapped resource of natural products with chemical structures that have been optimized by evolution for biological and ecological relevance (Gunatilaka 2006, Kusari et al. 2012). Since endophytes were first described in the Darnel (*Lolium temulentum*) (Freeman 1904), they have been isolated from various organs of different plant species, above ground tissues of liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns, and spermatophytes from the tropics to the arctic, and from the

wild to agricultural ecosystems (Arnold 2007) and to date, all plant species studied have been found to harbour at least one endophyte (Kusari et al. 2012).Endophytes usually produce the enzymes necessary for the colonization of plant tissues. It was demonstrated that most endophytes are able to utilize most plant cell components at least *in vitro*. As *Colocasia esculenta* tubers were found to contain large amount of oxalate crystals; it was hypothesized that at least few endophytes inhabiting tuber will have the ability to produce oxalate degrading enzyme, Oxalate Oxidase. Successful isolation of four bacterial strains supports our hypothesis. Presence of substantial amount of oxalic acid in the tubers of *Colocasia* might have put selective pressure on these endophytes to develop a means to use these energy rich molecules. This first report of isolation of *O. intermedium*, a novel Oxalate Oxidase producing bacterium from *Colocasia esculenta* also reiterate the fact that plants harbour endophytes that have the potential to produce many novel metabolites of human importance.

Four Oxalate Oxidase producing bacteria were isolated as result of our effort. The bacterium which showed highest Oxalate Oxidase activity was identified as *Ochrobactrum intermedium*. The nutritional and non-nutritional factors were optimized in shake flasks. Sucrose at 20 gL^{-1}, NH_4Cl at 2 gL^{-1}, $MnSO_4$ at 0.05 $gL^{-1}$and sodium oxalate as inducer at 5gL^{-1}, fill up volume in shake flask at one – tenth, incubation temperature of 30°C and initial medium pH of 6.5 and inoculum age of 18 h was found to be optimum. Oxalate Oxidase activity of 0.5UmL^{-1} and specific activity of 0.454 $Umg^{-1}$was recorded in fermentation broth after 65h of fermentation under optimal conditions.

CHAPTER 4

PURIFICATION, CHARACTERIZATION AND CHEMICAL MODIFICATION OF OXALATE OXIDASE FROM *OCHROBACTRUM INTERMEDIUM* CL6

CHAPTER 4

PURIFICATION, CHARACTERIZATION AND CHEMICAL MODIFICATION OF OXALATE OXIDASE FROM *OCHROBACTRUM INTERMEDIUM* CL6

Purification of enzymes becomes very important for their application in industries. Characterization of an enzyme's property can be studied accurately in its purest form. Once an enzyme is purified, it is possible to understand its affinity for natural substrates, or scrutinize its ability to catalyze enzymatic reactions. Such methods have allowed us to understand how molecules of biological origin can act as catalysts in metabolic processes. Starting from pure proteins, we can determine amino acid arrangements and evolutionary relationships between proteins in diverse organisms and we can inspect a protein's biochemical function.

In this chapter, the candidate endophytic bacterium selected by the screening process in chapter 3 was cultured in optimized medium in order to obtain target putative novel Oxalate Oxidase. Herein, the purification, characterization, possible amino acid composition at the active site of the enzyme Oxalate Oxidase from *Ochrobactrum intermedium* CL 6 and influence of various biochemical molecules on functional properties of the enzyme are described

4.1 MATERIALS AND METHODS

4.1.1 Purification of Oxalate Oxidase

The enzyme Oxalate Oxidase (OxO) was produced by the *Ochrobactrum intermedium* CL6, an endophytic bacterium isolated from *Colocasia esculenta*. The organism was cultured in the optimized medium containing the following components (gL^{-1}): sucrose - 20; NH_4Cl - 2; sodium oxalate - 5;KH_2PO_4 - 3;Na_2HPO_4 - 6; NaCl - 5;NH_4Cl - 2;$MgSO_4.7H_2O$ - 0.1; $MnSO_4$ - 0.05; biotin- 0.0015, with an initial pH of 6.5 and 2% (v/v) inoculum volume. Sterilized medium (25 mL) in 250 mL Erlenmeyer flasks was

incubated at 30 °C, 150 rpm in an incubator shaker for 65 h. Cell free broth was collected by centrifugation at 8000 RPM for 10 min for enzyme recovery.

Extra cellular Oxalate Oxidase in the culture supernatant was concentrated by precipitation techniques. Common precipitation techniques of ammonium sulphate precipitation, precipitation by non-ionic polymers like polyethylene glycol and organic solvent precipitation were tried out to concentrate our protein of interests. Enzyme precipitation was followed by gel filtration chromatography to elute Oxalate Oxidase of relatively high purity. Enzyme purity and molecular weight were determined using 10% polyacrylamide SDS–PAGE, as described previously by Laemmli (1970). Samples for SDS PAGE were run under denaturing and reducing conditions. Proteins gels were stained with Silver nitrate method (Chevallet et al. 2006). The enzyme activity in all steps of purification were estimated according to the protocol mentioned in section in 3.1.8.3. The total protein content was calculated by Lowry's assay (Appendix III) with bovine serum albumin as standard.

4.1.1.1 Precipitation of Oxalate Oxidase

4.1.1.1.1 Ammonium sulphate precipitation

Oxalate Oxidase in the crude culture was precipitated down by ammonium sulphate method using different concentrations (0-100%). 100 mL of crude fermentation broth was taken and centrifuged at 8000 RPM to obtain cell free supernatant. The supernatant was then transferred to 500 mL beaker with a magnetic bead and kept over a magnetic stirrer. The stirring was maintained at a slow speed. Amount of ammonium sulphate for different salt cuts (0-25%, 25-50%, 50-80%, 80-100%) was calculated, weighed and ground to powder using a mortar and pestle. The required amount of ammonium sulphate was gradually added to the enzyme sample while maintaining cold condition and gentle agitation. After each salt cut, the supernatant was centrifuged at 10,000 RPM to get pellet from each concentration. The sediment was re-suspended in buffer solution (pH 3.8). The protein contents and enzyme activity of both the supernatant and precipitate for all the fractions obtained after each salt cut were measured to decide the next set of experiments.

4.1.1.1.2 Precipitation by non – ionic polymer

Precipitations were carried out by mixing 10mL of enzyme supernatant and 10 mL of PEG 6000 solutions to make a final concentration of 25% PEG in the solution. The mixture was kept in an ice bath for 30 min and then centrifuged at 10,000 rpm. The supernatant was separated and the sediment was dissolved in 2 mL of 50 mM succinate buffer, pH 3.8. Enzyme activity and protein concentration were measured in both dissolved pellets and supernatants.

4.1.1.1.3 Organic solvent precipitation

Organic solvent precipitation of enzyme was accomplished by absolute acetone and ethanol in strictly cold condition (0-4°C).

4.1.1.1.3.1 Acetone precipitation

10mL of crude enzyme supernatant was poured in a beaker, immersed in an ice bath and stirred until the temperature reaches 4°C. 6 mL of precooled acetone was added to the enzyme supernatant drop wise with constant stirring and at such a rate that the temperature doesn't rise above 4°C. After the addition of acetone, stirring for 10 minutes was continued with constant control of temperature. The precipitated protein was removed by centrifugation at 4°C for 10 minutes at 10,000 RPM using precooled centrifuge tubes. The volume of the combined supernatant was measured and poured back into the beaker in the ice bath. Further 0.25mL of acetone/mL of protein solution was added with all the precautions described above. After further saturation of solution with acetone, the precipitated protein was recovered by centrifugation and the supernatant was poured off and the centrifuge tubes were inverted over filter paper to drain and completely blot any drops of solution adhering to the walls of the tubes. The pellet was suspended in 50 mM succinate buffer of pH 3.8. The enzyme activity and protein content for the different pellets and final supernatant were assayed.

4.1.1.1.3.2 Ethanol precipitation

100 mL of crude enzyme supernatant was poured in a beaker, immersed in an ice bath and stirred until the temperature reaches 4°C. 60 mL of precooled ethanol was added to the enzyme supernatant drop wise with constant stirring and at such a rate that the temperature doesn't rise above 4°C. After the addition of ethanol, the solution was allowed to stand overnight at 4°C. Precipitated protein was collected after centrifugation at 10,000 RPM at 4 °C for 15 min with pre cooled centrifuge tubes. The supernatant was poured off and the centrifuge tubes were inverted over filter paper to drain and completely blot any drops of solution adhering to the walls of the tubes. The pellet was suspended in 50 mM succinate buffer of pH 3.8. The enzyme activity and protein content for both the pellets and final supernatant were assayed.

4.1.1.2 Reverse dialysis and dialysis

Cellulose dialysis tubing was pre-treated with sodium bicarbonate and EDTA before use. The ends of the tubes were clipped with adjustable clips after loading 5 mL of sample. The dialysis bag with the sample was laid onto bed of PEG (polyethylene glycol) and covered with PEG. Continuously, tubing was checked until desired reduction in volume is achieved. Standard dialysis against 0.1 M phosphate buffer was carried out to remove PEG.

4.1.1.3 Gel filtration chromatography

4.1.1.3.1 Preparing the gel

Sephadex G 100 from Sigma Aldrich was procured for the gel filtration chromatography. 1 g of the sephadex G100 was weighed and was allowed to swell in excess of 50 mM succinate buffer (pH3.8). The slurry was kept in a water bath at 90°C for 5 h to completely swell up and occasionally given gentle stirring. During swelling excessive stirring was avoided as it would have broken the beads. Succinate buffer used for the swelling of sephadex beads was degassed in an ultra sonicator.

4.1.1.3.2 Filling the column

The suspension of gel was adjusted to make fairly thick slurry. Fine particles were removed by decantation. The column of 1 cm diameter was mounted on a stable laboratory stand. The column was tilted and poured with the well-mixed gel suspension down the inside wall of the column in a single operation. Immediately the column was readjusted to vertical position. The flow was started soon after filling the column to obtain even sedimentation.

4.1.1.3.3 Equilibrating the bed

Two or three column volumes of eluent was passed through the column in order to stabilize the bed and the column was equilibrated with the same eluent buffer.

4.1.1.3.4 Sample application and fraction collection

The column bed height after the equilibration process was found to be 20 cm. The total volume of the column bed was 15.7 mL. The volume of the precipitated enzyme sample loaded was 2.5% of the column bed volume and was loaded on a drained column bed surface. The sample was allowed to drain into the bed and enough precautions were taken to prevent the bed from drying. Immediately the sample on the bed surface and on the column wall was washed into the bed with a small amount of eluent. The column was refilled with eluent and a constant head on top of the gel was maintained. The bound proteins were eluted by the same 50 mM sodium succinate buffer (pH 3.8) at a flow rate of 0.5 mL min^{-1}. Each fraction of 3 mL volume was collected and those with high activity were pooled.

4.1.1.4 Protein purity and molecular mass estimation

Enzyme purity and molecular weight were determined using 10% polyacrylamide SDS-PAGE, as described previously by Laemmli (1970). Samples for SDS-PAGE were run under denaturing and reducing conditions, and non-reducing conditions. Proteins gels were stained with Silver nitrate method (Chevallet et al. 2006).

4.1.2 Characterization of Oxalate Oxidase

4.1.2.1 Effects of pH and temperature on enzymatic activity

The effect of pH on enzymatic activity was assayed at 55°C in a 50 mM sodium succinate buffer (pH 3.0–6.0). To investigate the optimum temperature for enzymatic activity, enzymatic activity was measured across a temperature range of 25–100°C in the same sodium succinate buffer (pH 3.8). With respect to pH stability, the purified enzyme was diluted in following buffers at 0.1 M concentration: glycine- HCl buffer (pH 2.2), citrate buffer (pH 4.0), acetate buffer (pH 6.0), phosphate buffer (pH 8.0), and sodium carbonate – Bicarbonate buffer (pH10.0). The diluted enzyme solutions were incubated at room temperature and aliquots were taken at every 2 h for 6 h, and then the activity was measured under standard conditions as described above. The thermal stability of the purified enzyme was determined by incubating enzyme samples across a 4°C–100°C temperature range at pH 3.8 and aliquots were taken every 2h for 6h. After incubation, enzyme solutions were cooled immediately, and activity was assayed under standard conditions as mentioned in section 3.1.8.3. Data shown in the results are mean ± standard error of three replicates.

4.1.2.2 Effects of different metal ions and biochemical reagents on enzymatic activity

Oxalate Oxidase is being used in estimating oxalate content in serum and urine. Serum and urine contains many metal ions and other biochemical. It is expected that those biochemical and metal ions which exist in appreciable quantity will affect the enzyme activity. Hence experiments were designed to assess the effect of prominent metal ions and biochemical of serum and urine on OxO activity. Therefore the metal ions, biochemical and their concentration range were chosen based on their concentration in serum and urine for the experiments. The effects of metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) at 1mM concentration on enzymatic activity were investigated. Furthermore, the concentration of K^{+}, Na^{+} at 50 mM and 150mM respectively were considered for examining their influence on OxO activity. In addition, the effects of biochemical reagents EDTA, glucose, xylose and urea at 1, 2.5, 5 and 10 mM

concentration on enzymatic activity was also determined. The additives were added to the reaction mixture in a sodium succinate buffer (pH 3.8) and enzymatic activity was determined according to the standard methods in section 3.1.8.3. Here, the activity assayed in the absence of metal ions or reagents was defined as 100%.

4.1.2.3 Effect of different carboxylic acids on enzyme activity

Carboxylic acids (Glutaric acid, Malonic acid, Succinic acid, Malic acid, Pyruvic acid, Citric acid and lactic acids) were added in reaction mixture at 2.5-20 mM concentration in presence of oxalic acid to analyze their potential role in activating or inhibiting the enzyme by competing with oxalic acid for active site or binding to one or other sites on the enzyme. Effect of succinic acid was checked only in 0.1M citrate buffer (pH 3.8) and for all other organic acids, sodium succinate buffer was used. Experiments were also carried out with these Carboxylic acids as only source of substrates in absence of oxalic acid to check the oxidizing power of the enzyme Oxalate Oxidase for these organic acids. The additives were added to the reaction mixture in a sodium succinate buffer (pH 3.8) and enzymatic activity was determined according to the standard methods described in section 3.1.8.3. Here, the activity assayed in the absence of other carboxylic acids was defined as 100%.

4.1.2.4 Effect of substrate analogue

The catalytic prowess of the enzyme Oxalate Oxidase in presence of glycolic acid, a substrate analogue for oxalic acid was checked for four different concentrations 0.1, 0.5, 1and 3 mM in 50 mM sodium succinate buffer (pH 3.8). In another experiment glycolic acid was used as the only source of substrate and enzymatic activity was determined according to the standard methods described in section 3.1.8.3.

4.1.2.5 Effect of Riboflavin

0.1 – 1µmoles/L of different concentrations of riboflavin were used to determine their effect on the catalysis of oxalic acid. The additive was added to the reaction mixture in a sodium succinate buffer (pH 3.8) and enzymatic activity was determined according

to the standard methods in section 3.1.8.3. Here, the activity assayed in the absence of riboflavin was defined as 100%.

4.1.2.6 Effect of bleaching agents

The effect of bleaching agents like chlorate, sulphite on Oxalate Oxidase was also studied. Previous studies have ascertained the application of Oxalate Oxidase in paper and pulp industry. Processing of raw materials in this industry requires the use of bleaching agents and combined effect of pulping and bleaching result in the formation of low molar mass lignin-oxidation compounds from lignins. It is expected that those bleaching agents and Low molar mass lignin-oxidation compounds which exist in the process water will affect the enzyme activity. Hence experiments were designed to assess the effect of Chlorate, sulfite and lignin model vanillin on OxO activity. Sodium salts of chlorate (ClO_3^-) and sulfite (SO_3^{2-}) were used for the study with concentrations chosen emanate from earlier results (Cassland et al. 2009). The effect of chlorate was studied at 0.1, 1 and 5 mM, while the effect of sulfite was studied at 0.01, 0.1, and 1 mM. The lignin model vanillin was studied at 0.1, 1.0, and 5.0 mM.

4.1.2.7 Determination of kinetic parameters

Various oxalate concentrations (0.09–10.0 mmol/L) were used to determine the initial reaction rates. Enzyme reactions were conducted in optimum conditions (pH 3.8 and 55°C), Michaelis–Menten constant (Km) and rate of reaction (V_{max}) were determined according to Lineweaver–Burk plot (Nelson and Cox 2005). Turnover number (k_{cat}) of the enzyme was also calculated from V_{max} values. Turnover number (k_{cat}) was calculated by the equation, $k_{cat} = V_{max}/ [E_0]$, where $[E_0]$ is the molar concentration of enzyme in the reaction mixture.

4.1.2.8 Substrate inhibition of Oxalate Oxidase

The effect of high substrate concentration on the catalytic ability of the enzyme was studied with substrate concentration in the range of 0.09-50 mM.

4.1.3 Chemical modification on Oxalate Oxidase

Purified OxO was taken for chemical modifications. Cysteine, carboxylates, histidine, serine and tryptophan residues were subjected to modifications in five different sets of experiment and their effect on enzyme activity was studied. Modification reactions were carried out in a reaction volume of 1 mL and incubated at 37°C. Strength of important reagents used in each reaction and reaction time were varied to find the optimum reaction conditions. The modified enzymes were then assayed for activity at optimum temperature of 80°C as per the method described in section 3.1.8.3 and compared with the activity of the native enzyme. Data shown in the results are mean ± standard error of three replicates.

4.1.3.1 Modification of cysteine residues

Cysteine residues of OxO were modified using 5, 5-dithio-bis-(2-nitrobenzoic) acid (DTNB) as described by Hu and Guo (2009). A stock of 10 mM DTNB solution was prepared fresh in 100 mM phosphate buffer (pH 8.0) for use. The modification reaction mixture had comprised of 20 mM phosphate buffer of pH 8, with 0.017 U OxO and 0.2–0.8 mM DTNB. Modified OxO activity was then estimated at two different temperatures (55° and 80°C).

4.1.3.2 Modification of carboxylates

The importance of carboxylate groups in the activity of OXO was investigated by modifying them using 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Kotsira and Clonis 1998). Freshly prepared stock of 500 mM 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in ethanol was used for the reaction. The modification reaction was carried out in 10 mM potassium acid phthalate/NaOH buffer of pH 4.1 containing 0.017 U OxO and 10–100 mM EDAC in 1 ml reaction volume. Modified OxO activity was then estimated at 80°C.

4.1.3.3 Modification of histidine residues

Histidine residues were modified using 150 mM diethylpyrocarbonate (DEPC) stock solution prepared in ethanol. The reaction was carried out by incubating 0.017 U OxO

with 1–15 mM DEPC in 20 mM phosphate buffer of pH 8 in a reaction volume of 1mL (Hu & Guo 1999). Modified OxO activity was then estimated at two different temperatures (55°C and 80°C).

4.1.3.4 Modification of serine residues

For modification of serine residues, a stock of 150 mM phenylmethane sulfonyl fluoride (PMSF) prepared in ethanol was used. The reaction was conducted at 37°C by incubation of 0.017 U OxO with 1–30 mM PMSF in 20 mM phosphate buffer of pH 8 in a reaction volume of 1 mL (Hu and Guo 1999). Modified OxO activity was then estimated at 80°C.

4.1.3.5 Modification of tryptophan residues

For modification of tryptophan residues, a stock of 1mM N-bromosuccinimide (NBS) was prepared in 100 mM phosphate buffer (pH 8.0). The reaction was carried out at 37°C by incubation of 0.017 U OxO with 0.01–0.05 mM NBS in 20 mM phosphate buffer of pH 8 in a reaction volume of 1 mL (Wen et al. 1999; Hu and Guo 1999). OxO activity was then estimated at 80°C.

4.1.3.6 Estimation of kinetic parameters after modification

The kinetic parameters K_m and V_{max} were determined by Lineweaver-Burk double reciprocal plot and the turnover number (k_{cat}) were calculated for native OxO and histidine-modified OxO. Oxalic acid in the range of 0.09–10.0 mM was used as the substrate for native enzyme whereas 0.025-10.0 mM oxalic acid was used for histidine-modified enzyme under optimal assay conditions (pH 3.8 and 80° C). The concentration of enzyme was maintained at 0.073µM for the entire assay. The catalytic efficiency of native and histidine modified enzyme was calculated by computing the ratio of k_{cat} to K_m.

4.2 RESULTS AND DISCUSSION

4.2.1 Purification of Oxalate Oxidase

The enzyme was purified by sequential steps of precipitation and gel filtration chromatography.

4.2.1.1 Precipitation

Precipitation techniques like ammonium sulphate precipitation, precipitation by non-ionic polymer and organic solvent precipitation was employed as part of this strategy.

4.2.1.1.1 Ammonium sulphate precipitation

The enzyme Oxalate Oxidase could not be precipitated by ammonium sulphate method even at 100% saturation of the supernatant with ammonium sulphate. Only 4.5 % of the total units of enzyme could be precipitated out. The result was completely unexpected as it is one of the most common methods of precipitation and is widely used in enzyme purification strategies. Though it was found that there was an increase in enzyme specific activity of the supernatant as 25 % of contaminant protein was precipitated out at 100 % ammonium sulphate saturation (Table 4.1). This report of solubility of Oxalate Oxidase from *Ochrobactrum intermedium* CL 6 in 100% saturated ammonium sulphate solution is first of its kind among the different proteins from prokaryotes, though such proteins are present in humans and in other vertebrates. These solubilized proteins in humans and other vertebrates are grouped as S-100 proteins, and are so called because of their solubility in 100% saturated solution of $(NH_4)_2SO_4$ (Schaub and Heizmann 2008) These proteins are acidic and have different metal ion-binding properties. It was further decided that since the specific activity of the supernatant has increased two fold, reverse dialysis of the supernatant would be taken up to concentrate the supernatant.

Table 4.1 Summary of Ammonium sulphate precipitation

Stages	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)
Before Precipitation	47.5	101.4	0.468
After Precipitation			
Supernatant	44.69	75.6	0.5911
Pellet	2.81	25.8	0.1089

4.2.1.1.2 Reverse dialysis

Reverse dialysis of the supernatant was carried out to further concentrate the supernatant. After the process of reverse dialysis, the initial volume of 5 mL of sample was reduced to 1 mL, but there was unexpectedly 80% loss of enzyme activity. In many instances, significant loss of enzyme activity occurs during the process of dialysis if the enzyme concentration is low. Some researchers claim that non-specific adsorption of the targeted analyte to the dialysis membrane is the primary cause for observation of decreased recovery for some proteins (Ller 2012).

4.2.1.1.3 Precipitation by non- ionic polymer

Precipitation by PEG 6000 also couldn't yield better result as bulk of the enzyme Oxalate Oxidase remained in the supernatant.

4.2.1.1.4 Organic solvent precipitation

Acetone precipitation led to the complete denaturation of the enzyme and no activity was seen in the pellet as well as in the supernatant, on the other hand the ethanol precipitation effectively separated the enzyme from most cellular proteins, and increased the specific activity 14.60 fold, resulting in a yield of 89 %. The notable part of this purification was precipitation of the most of the desired protein by 30% ethanol saturation of the fermentation broth was attained; leaving behind 94% of the

contaminant proteins in the supernatant and a purification fold of 14.60 was achieved after the precipitation step (Table 4.2). The result indicated that the enzyme Oxalate Oxidase was very efficiently being precipitated out by ethanol. Acetone on the other hand was ineffective and caused complete denaturation. It was expected that the system may behave in a similar fashion as both the organic solvent has different properties and are placed far by in order of polarity. In other words it can also be stated that acetone has strong destabilizing effect, and on other hand ethanol has stabilized the enzyme. Some organic solvents exhibit a strong stabilizing effect, while others show either no effect or a strong destabilizing effect on an enzyme (Toshio et al. 1977 and Ogino and Ishikawa 2001). Toshio (1977) reported in his work that the addition of ethanol (2.5%, v/v) totally inhibited the denaturation of HbS, while the addition of toluene (0.25%, v/v) increased the rate of denaturation 4-fold. Acetone denaturation of the enzyme could be because of binding of ketone group to the active site of the enzyme. Organic molecules bind to specific sites in enzymes such as an active site, and then the enzymes lose their activity (Ogino and Ishikawa 2001).

4.2.1.2 Gel filtration Chromatography

After gel permeation chromatography step, the enzyme was purified 58.74 fold, with a protein yield of 83%. The specific activity increased from 0.456 U/mg in the crude supernatant to 26.78 U/mg in the chromatography step (Table 4.2). The total activity and protein content of the pooled fraction was 1.125 U and 0.042 mg for 400 µL of crude precipitated sample loaded onto the column. This accounts to 93.6% and 25 % of the total units of enzyme and protein loaded onto the column respectively. After gel filtration on sephadex G-100 column, two peaks of proteins, one large peak followed by a small peak, were eluted from the column (Figure 4.1). The enzyme activity was detected in the first peak, indicating lots of proteins were removed by the gel filtration (A total of 75% of the total protein loaded was removed). The final yield and purification fold of these two steps purification is better than many other purification strategies where there was considerable loss of product with each purification step (Guo and Hu 2009 and Aguilar et al. 1999). On the other hand, Kotsira and Clonis (1997) and Koyoma (1988) were also able to get higher yield in two step of purification.

Table 4.2 Summary of the purification steps

Purification step	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	Yield %
Crude supernatant	47.5	104	0.456	1	100
Ethanol Precipitation	42	6.3	6.66	14.60	89
Gel-filtration Chromatography	39.375	1.47	26.78	58.74	83

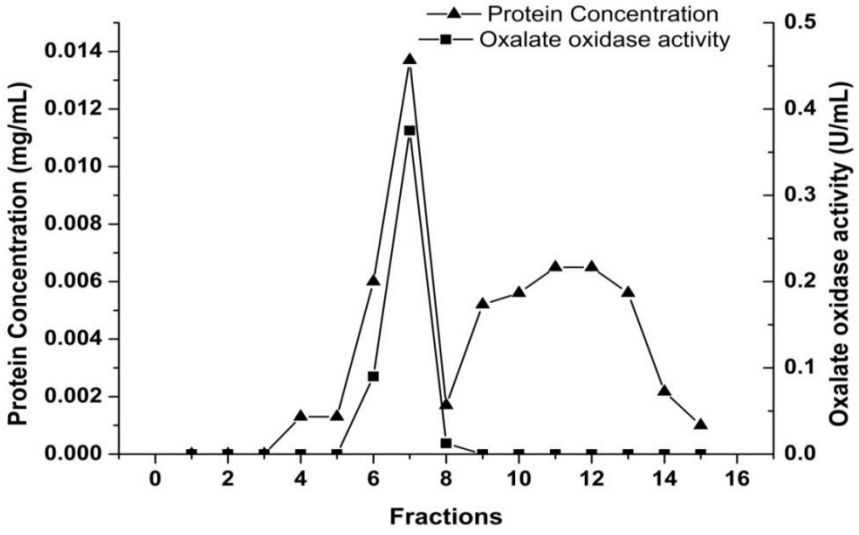
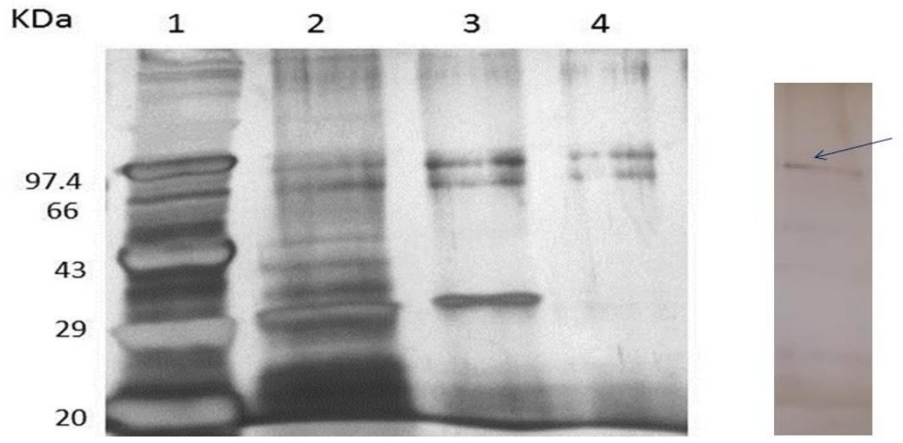


Figure 4.1 Chromatography of *Ochrobactrum intermedium* OxO on sephadex G-100. Four hundred micro litre of enzyme solution was applied on a column (1cmx20cm). The column was equilibrated and eluted with 50 mM succinic acid/NaOH buffer (pH 3.8)

4.2.1.3 Protein purity and molecular mass estimation

Pure enzyme showed two bands on SDS-PAGE. On the basis of molecular weight marker ran parallel with proteins during each purification steps in SDS-PAGE, target protein was found to have two sub units under reducing and denaturing conditions, and molecular weight of the subunits were found to be 102 and 89 kDa (Figure 4.2) . The figure also shows the level of purity at each stage of purification. Many discrete bands of different intensity were observed in the crude supernatant. The gel image of the resolved protein of first stage of purification exhibited fewer bands than the crude supernatant. The lane containing purified protein had two bands which are the subunits of the purified enzyme (Figure 4.2). Literature suggests that mostly Oxalate Oxidase is a pentamer with subunits of equal molecular weight (Kotsira and Clonis 1997 & Hu and Guo 2009) which is contrary to our result. *C. subvermispora* OxO is a homohexamer of molecular weight of 400 kDa (Aguilar et al. 1999). Pseudomonas ox-53 OxO has molecular weight of 320 kDa.



(A) (B)

Figure 4.2 SDS-PAGE of the purified Oxalate Oxidase from *Ochrobactrum intermedium* CL6. (A) SDS-PAGE under reducing and denaturing conditions. Molecular weight marker (lane 1), crude enzyme in culture supernatant (lane 2), crude enzyme in precipitate (lane 3), active fractions after sephadex G-100 gel filtration chromatography (lane 4). (B) SDS-PAGE under non-reducing conditions showing a single band of purified OXO from *Ochrobactrum intermedium* CL6.

On the other hand when purified enzyme sample was run under non-reducing condition (without βME), SDS-PAGE exhibited single band of undissociated enzyme subunits (Figure 4.2 (B))

4.2.2 Characterization of Oxalate Oxidase

4.2.2.1 Effect of pH and temperature on enzyme activity and stability

Optimum pH for enzyme activity was found to be 3.8. There was a sudden drop in enzyme activity at pH above 5 (Figure 4.3). This enzyme was stable in the pH range of 2.0 to 8.0 and no loss of activity was found for 2 h (activity at pH 3.8 for succinate buffer was considered to be 100%). However, considerable loss of activity was observed in next 4 h at various pH. Beyond pH 8.0, there was a sharp decline in activity and the enzyme almost lost 80% of its residual activity (Figure 4.4).

The OxO had maximum activity at pH 3.8 which is similar to the optimum pH 3.8 or 4.0 of barley root OxO (Kotsira and Clonis 1997 & Requena and Bornemann 1999) and 3.5 for wheat OxO (Hu and Guo 2009). The *pseudomonas sp*. OxO has pH optimum at 4.8 (Koyoma 1988). Oxalate Oxidase binds the singly ionized oxalate monoanion as substrate at the optimum pH for the enzymatic reaction (pH 3.8), which lies between the two p*Ka* values for oxalic acid dissociation (p*Ka*, 1 - 1.25, pK*a*,2 - 4.14). Several researchers have reported that binding of the singly charged oxalate to Oxalate Oxidase favours catalysis (Moomaw et al. 2013 and Moussatche et al. 2011). Moreover the carboxylate residues present at the active site need to be unprotonated for enhanced catalysis. The pH of 3.8 is close to the pKa value of side chain COOH group of asparatic and glutamic acids. The pH dependence of this reaction suggests that the dominant contribution to catalysis comes from the monoprotonated form of oxalate binding to a form of the enzyme in which an active site carboxylic residue must be unprotonated (Moussatche et al. 2011).

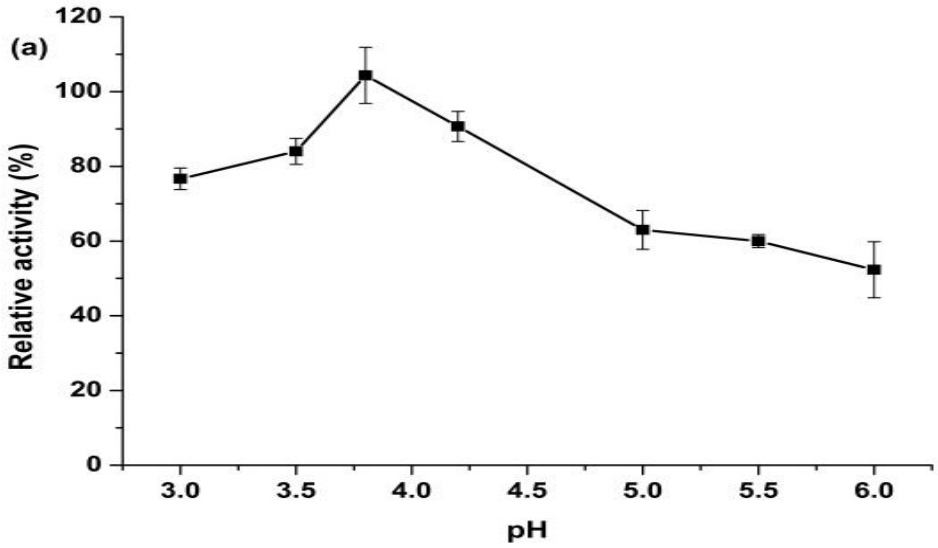


Figure 4.3 Effects of pH on the activity of the purified enzyme OxO

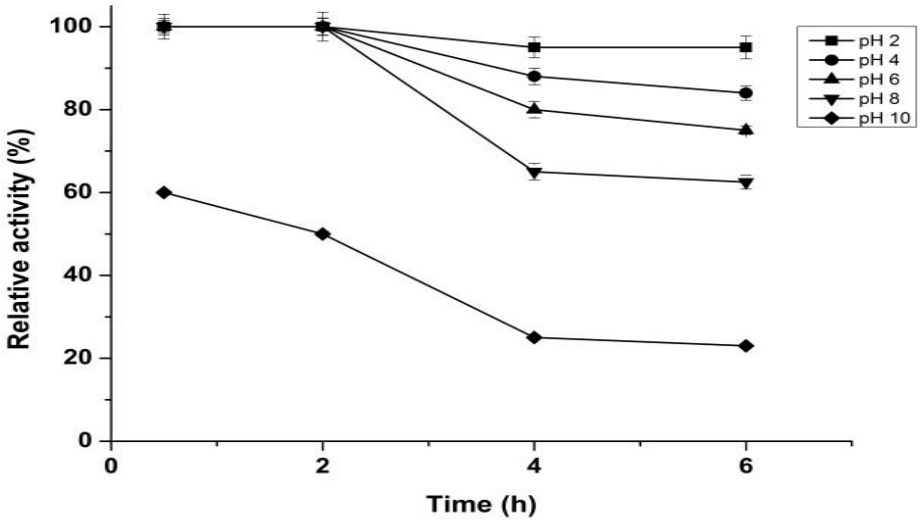


Figure 4.4 Effects of pH on the stability of the purified enzyme OxO

Optimum activity of the purified enzyme was found at 80°C (Figure 4.5) and it remained active over a temperature range of 25–80°C, retaining almost 100% of its residual activity (activity at optimum temperature was considered to be 100%) (Figure 4.6). Thermal stability profile of Oxalate Oxidase in form of relative activity has been presented in Figure 4.6. The enzyme showed no loss of activity after incubating the enzyme at 4 - 80°C for 6 h and retained more than 80% activity at 85 °C for 4 h incubation. At 100°C a sharp fall in residual activity was observed with increase in pre-incubation time. Maize Oxalate Oxidase was also found to retain 100 % activity at 80°C (Vuletic and Sukalovic 2000).The thermal stability of this enzyme will broaden its industrial application as using ordinary thermo labile enzyme as a catalyst in chemical reactions has been very cost ineffective, due to its low stability. *Pseudomonas sp*. OX 53 Oxalate Oxidase has low stability at a temperature above 65°C (Koyoma 1988) whereas barley roots and *Costus pictus* OxO has low thermal stability above 50°C when incubated for more than 30 minutes (Kotsira and Clonis 1997 & Satishraj and Augustin 2011). Perhaps this is the first report of any OxO showing complete thermal stability in a range of 4 - 80°C for 6 h. A combination of different factors leads to thermostability of enzymes. Thermophilic proteins can exhibit higher core hydrophobicity (Schumann et al. 1993), greater numbers of ionic interactions (Vetriani et al. 1998), increased packing density (Russell et al. 1997), additional networks of hydrogen bonds (Jaenicke and Bohm 1998), decrease in solvent-exposed hydrophobic surface, and anchoring of "loose ends" (i.e., the N and C termini and loops). Metals have long been known to stabilize and activate enzymes at high temperatures. Xylose isomerases have two metal binding site and possess high thermal stability (Marg and Clark1990 &Whitlow 1991). *Thermoactinomyces vulgaris* subtilisin-type serine-protease thermitase contains three Ca^{2+} binding sites; one of them is not present in its mesophilic homologues (Frommel et al. 1990). The ferredoxin from *Sulfolobus* sp. strain 7 contains an extra 40-residue N-terminal extension that is linked to the protein core by a Zn binding site (Fujii et al. 1997). These observations suggest that major stabilizing forces are associated with the presence of metal in the holoenzyme

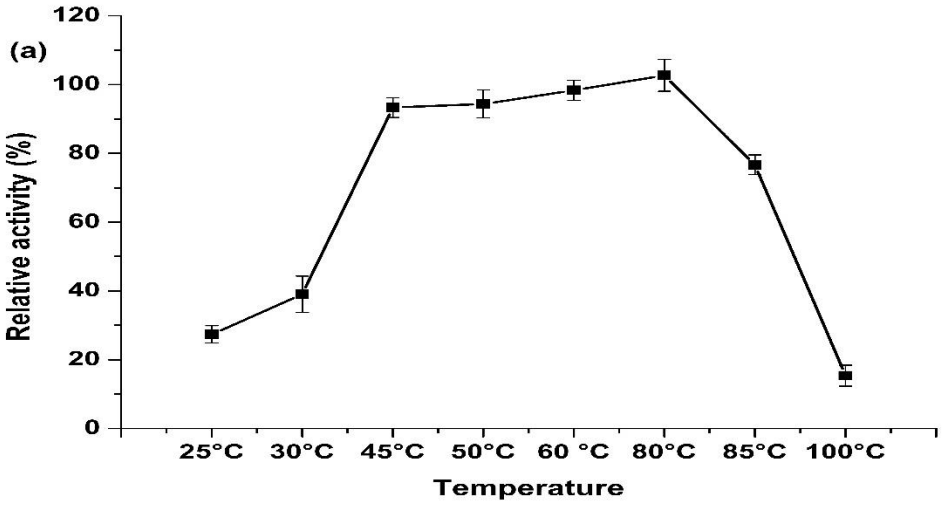


Figure 4.5 Effects of temperature on the activity of the purified enzyme OxO

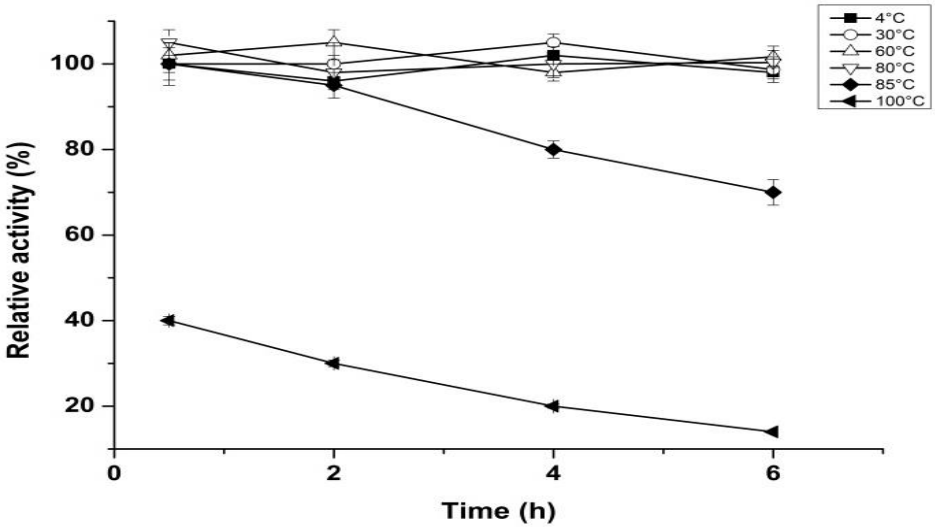


Figure 4.6 Effects of temperature on the stability of the purified enzyme OxO

4.2.2.2 Kinetic determinations

Michaelis–Menten constant (K_m) and rate of reaction (Vmax) were determined according to Lineweaver–Burk plot (Nelson and Cox 2005). Apparent Km and Vmax values, determined for Oxalate Oxidase by using oxalic acid as substrate were 0.45 mM (Figure 4.7) and 28 U mg^{-1} proteins respectively.

Barley root OxO had Km at 0.27 and 0.42 mM, respectively, in two different investigations (Chiriboga1966 and Kotsira and Clonis 1997). In contrast, OxO from barley seedlings had Km at 1.3 mM (Requena and Bornemann 1999). Pseudomonas OxO had K_m of 9.5 mM (Koyoma 1988). A high K_m indicates low affinity of the enzyme towards the substrate. The K_m of *C. subvermispora* was 0.1 mM (Aguilar et al. 1999). The k_{cat} of *Ochrobactrum intermedium* CL6 OxO was found to be 85 s^{-1}, which is similar to the k_{cat} of *C. subvermispora* OxO (88 s^{-1}) (Aguilar et al. 1999) and four times higher than the barley OxO (22 s^{-1}) (Kotsira and Clonis 1997). The low k_{cat} indicates that Oxalate Oxidase is not a fast catalyst.

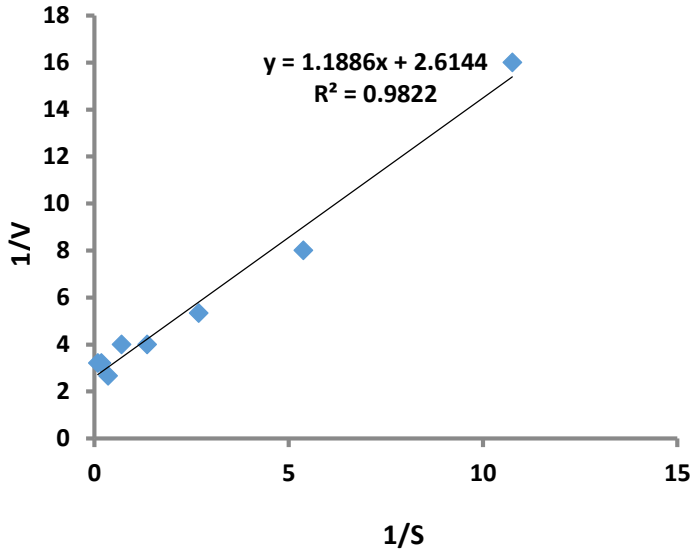


Figure 4.7 Lineweaver – Burk Plot (1/S vs 1/V)

4.2.2.3 Effect of metal ions, inhibitors and other significant chemicals

Metal ions present in blood, urine, grains and vegetables samples may have profound effect on the activity of Oxalate Oxidase if such samples are being used for monitoring the oxalate content. The effect of different metal ions was thus studied to investigate their possible influence of on Oxalate Oxidase activity. Effect of different metal ions studied on enzyme activity is shown in Table 4.3. Cu^{2+} has considerable inhibitory effect on the enzyme Oxalate Oxidase. Contrary to this, the Oxalate Oxidase from sorghum leaves and roots (Pundir and Nath 1984 & Pundir and Kuchhal 1989) required Cu^{2+} for maximal activity whereas *Pseudomonas sp.* OX 53 OxO (Koyoma 1988) also got inhibited with 0.1 mM $CuSO_4$ concentration. 80% of residual activity was found with Mg^{2+}, whereas 60% increase in activity was observed when Ca^{2+} and Fe^{2+} at 1 mM concentration was added to the reaction mixture in two different experiments indicating stimulatory effect of Ca^{2+} and Fe^{2+} (Table 4.3). It was also found that the enzyme is stable at very high concentration of sodium and potassium ions (Table 4.4).

EDTA has complete inhibitory effect at a minimum concentration of 2.5 mM in the enzyme substrate reaction mixture (Table 4.3) indicating that OxO of *O. intermedium* CL6 is a metalloprotein. Sorghum leaves and roots OxO were also inhibited by EDTA (Pundir and Nath 1984 & Pundir and Kuchhal 1989). Glucose at 10mM and xylose at 5mM concentration has 30% inhibitory effect. Urea at 10 mM concentration has 40% inhibitory effect.

Thus it is evident from the published reports that OxO in general is a metalloprotein inhibited by metal chelating molecules such as EDTA. There are quite a few reports available indicating stimulatory role played by few metal ions such as Ca^{2+}, Cu^{2+} , Fe^{2+} and Mn^{2+} in case of Barley root OxO, Sorghum leaves OxO and *Pseudomonas sp.* OxO respectively. In general the effect of various metal ions on activity is highly variable and differs for OxO purified from different sources.

Table 4.3 Effect of different metal ions and other agents on the activity of the Oxalate Oxidase purified from *Ochrobactrum intermedium* CL6

Metal ions and other agents	Relative activity (%)
Ca^{2+}	160
Mg^{2+}	83.3
Mn^{2+}	95
Fe^{2+}	160
Fe^{3+}	80
Zn^{+}	90
Na^{+}	100
K^{+}	100
Cu^{2+}	44
Glucose	70
Xylose	70
Urea	60
EDTA	Complete inhibition
Riboflavin	200

Table 4.4 Effect of various concentration of sodium and potassium ions on the activity of the Oxalate Oxidase purified from *Ochrobactrum intermedium* CL6

Concentration (mM)	Relative activity (%)	
	Na^+	K^+
1	100	100
2	100	100
5	100	100
10	100	100
25	100	75
50	85	75
100	85	-
150	85	-

4.2.2.4 Effect of different tricarboxylic and dicarboxylic acid on enzyme activity

Succinic acid and its structural analogue malonic acid have profound effect on increasing the activity of the enzyme to 200% of the residual activity. 75% residual activity was seen when citric acid at 10 mM was added as an additive to the enzyme substrate reaction mixture whereas in presence of pyruvic acid at 20mM, the enzyme activity decreased to 28% of the residual activity. Other organic acids have no effect on Oxalate Oxidase (Table 4.5). The enzyme could not oxidize any of the organic acids, when these organic acids were replaced with oxalic acid in the reaction mixture as a source of substrate indicating the enzyme does not have broad substrate specificity and is highly specific to oxalate only. Activation of the *Ochrobactrum intermedium* CL6 OxO by succinate and its structural analogue indicates that both bind to a specific common site on the enzyme. Koyoma (1988), on the other hand reported that besides oxalic acid, the enzyme Oxalate Oxidase from *Pseudomonas sps*. OX 53 oxidized glyoxylic acid and malic acid at lower reaction rates.

Table 4.5 Effect of different carboxylic acids on the activity of Oxalate Oxidase purified from *Ochrobactrum intermedium* CL6

Carboxylic acids	**Relative activity (%)**	
	10mM	20mM
Malonic acid	150	200
Glutaric acid	100	100
Succinic acid	177	200
Malic acid	100	100
Pyruvic acid	71.7	28
Citric acid	75	70
Lactic acid	100	100

4.2.2.5 Substrate inhibition of Oxalate Oxidase

The effect of high substrate concentration on the catalytic ability of the enzyme was studied with substrate concentration in the range of 0.09-50 mM (Figure 4.8). High oxalate concentration up to 50 mM in the enzyme substrate reaction mixture was not detrimental for *Ochrobactrum intermedium* CL6 Oxalate Oxidase. The result is in contrast to many plant Oxalate Oxidase where substrate inhibition of Oxalate Oxidase is reported. Barley root Oxalate Oxidase showed substrate inhibition when oxalic acid concentration reached to 4 mM (Chiriboga1966 and Kotsira and Clonis 1997). In contrast, Oxalate Oxidase from barley seedling did not show substrate inhibition even at 200 mM substrate concentration (Requena and Bornemann 1999) but the enzyme from barley seedling has very high K_m of 1.3 mM. Interestingly both *Costus pictus* OxO and sorghum leaves OxO showing very high affinity for the substrate (K_m value of 0.065 mM and 0.024 mM respectively) showed substrate inhibition at 0.8 mM and 0.25 mM substrate concentration respectively (Sathisraj and Augustin 2012; Pundir and Nath 1984).

Oxalate Oxidase from *C. subvermispora,* was inhibited at concentrations of substrate above 2.5 mM. In comparison to the kinetic characteristics reported so far, *Ochrobactrum intermedium* CL6 enzyme is the only OxO showing such a high affinity

for Oxalic acid (K_m value of 0.45 mM) without being inhibited by the substrate, oxalic acid up to 50mM concentration.

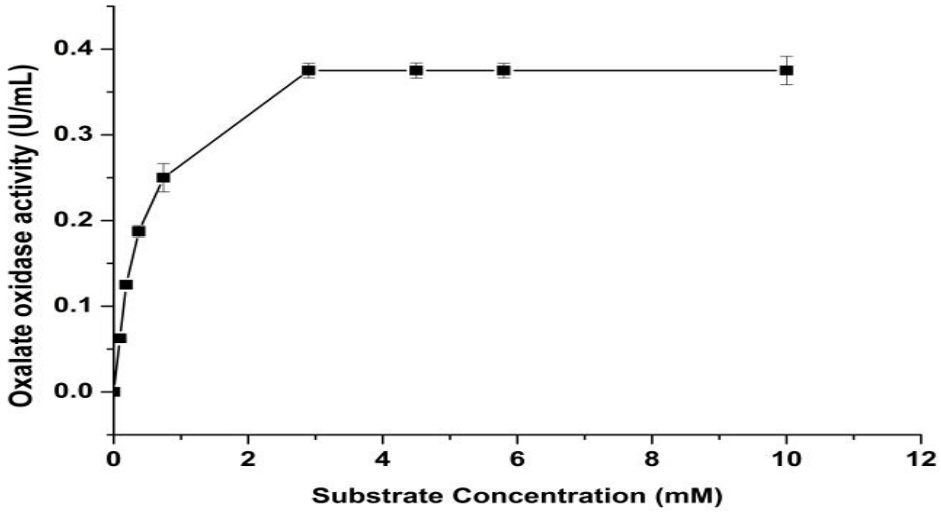


Figure 4.8 Effect of substrate concentration on the activity of OxO

4.2.9 Effect of glycolic acid

Enzyme activity was studied for different concentrations of glycolic acid as shown in the figure 4.9. Results showed that glycolic acid has no inhibitory role on Oxalate Oxidase for any of the concentrations used in the experiments. An experiment was also carried out to check the oxidizing ability of the enzyme Oxalate Oxidase for glycolic acid, where glycolic acid was used as an only source of substrate. The enzyme Oxalate Oxidase showed negligible activity for the substrate analogue glycolic acid, which resembles oxalic acid structurally. The oxidizing ability of the enzyme Oxalate Oxidase for glycolic acid was thus neglected in the present study and the enzyme is specific to oxalic acid. However previous reports showed marginal enzyme activity in case of *Pseudomonas OX-53* Oxalate Oxidase (Koyoma 1988). Glycolate did not work as a substrate and did not inhibit turnover when included in the assay at equimolar (3mM) concentrations of oxalate, implying that glycolate is bound less tightly than the substrate. Opaleye et al. 2005 have demonstrated in crystallography studies, glycolate as a structural analogue of oxalate to investigate substrate binding in the crystalline

enzyme. The monodentate binding of glycolate to the active site manganese ion of Oxalate Oxidase is consistent with mechanism involving C–C bond dissociation driven by superoxide anion attack on a monodentate coordinated substrate.

Most substrate analogues have been reported to be potent inhibitors of enzyme activity (Berg et al. 2002). For eg., Tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a substrate analog for chymotrypsin and inhibits the enzyme by binding at the active site and reacts irreversibly with a histidine residue at that site. The compound 3 bromoacetol mimics the normal substrate, dihydroxyacetone phosphate for the enzyme triose phosphate isomerase (TIM) and binds at the active site; Then it covalently modifies the enzyme such that the enzyme is irreversibly inhibited (Berg et al, 2002).

However few enzymes are not susceptible to inhibition by their substrate analogues like the one in the present study. Succinate, fumarate, maleate and β- and γ-hydroxybutyrate which are the structural analogues of L-malate, had no effect on the enzyme activity of NADP-malic enzyme purified from *Zea mays* L. leaves (Spampinato et al. 1994). Likewise, mushroom tyrosinase cannot catalyze the oxidation of 3,4-dihydroxybenzonitrile (a), 3,4-dihydroxybenzaldehyde (b), 3,4-dihydroxybenzoic acid (c) and 2,3- dihydroxybenzoic acid (d). On the contrary, compounds a, b and c can inhibit the activity of tyrosinase for the oxidation of DOPA, while compound d had no effects on enzyme activity (Xie et al (2007).

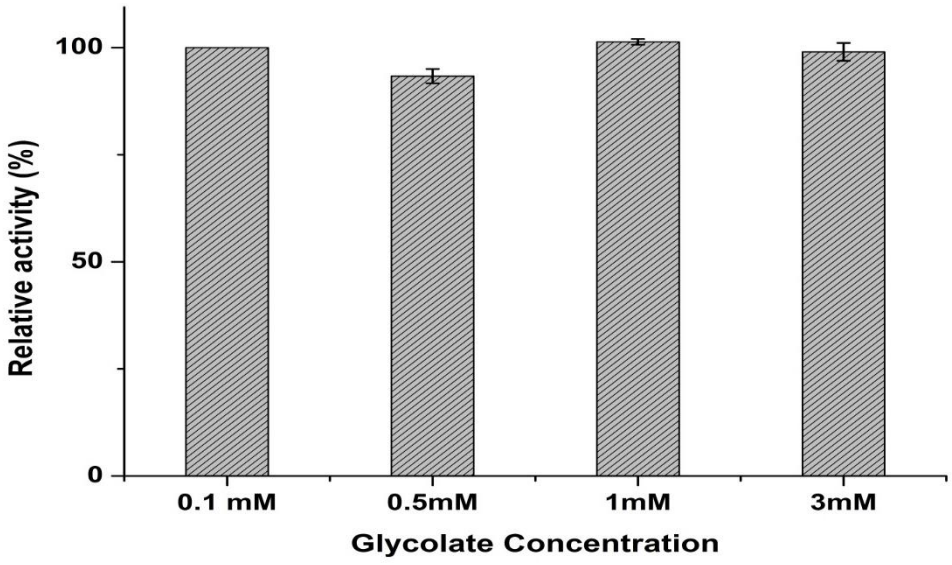


Figure 4.9 Effect of substrate analogue, Glycolate on Oxalate Oxidase

4.2.2.7 Effect of Riboflavin

Riboflavin has considerable enhancing effect on the enzyme Oxalate Oxidase activity. There was increase in two fold activity at 0.1 µmolar riboflavin concentration (Table 4.3). Studies carried out at higher concentration of riboflavin had no positive effect. Enhancement of the activity by riboflavin suggests that the enzyme could be a flavoprotein. Few classical examples of enhancement of enzyme activity by riboflavin are methylenetetrahydrofolate reductase, glutathione reductase.5-methyltetrahydrofolate which serves as a methyl group donor in the conversion of homocysteine to methionine is produced from 5, 10-methylenetetrahydrofolate by the flavoenzyme methylenetetrahydrofolate reductase (MTHFR). In rats, experimental riboflavin deficiency led to low MTHFR activity and reduced levels of 5-methyltetrahydrofolate. The mutant MTHFR in humans has lower affinity for its flavin cofactor than the wild-type enzyme, and exhibited reduced enzyme activity. Reddi (1986) has demonstrated that riboflavin-deficient diabetic rats were found to have reduced glutathione reductase activity. The activity of the enzyme augmented dramatically in the presence of flavin-adenine dinucleotide (FAD) to 72%. Moreover all enzyme activities were normalized after riboflavin treatments which suggested that riboflavin enhanced the synthesis of erythrocyte and hepatic FAD and riboflavin deficiency decreased erythrocyte and hepatic flavoprotein enzyme activities.

These results advocate that the activity of flavo enzymes is enhanced by riboflavin. Flavoproteins are known to have maxima absorbance peaks at 370 and 450 nm (Rider et al. 2009), but the enzyme Oxalate Oxidase in the current work showed no noticeable absorbance peaks at 370 and 450 nm. Therefore the enzyme in our study cannot be considered as a flavoenzyme though it has tremendous enhancement in activity by riboflavin. This result is in agreement with Pseudomonas Oxalate Oxidase (Koyoma 1988), however riboflavin was found to have no effect on barley Oxalate Oxidase (Kotsira and Clonis 1997).

4.2.2.8 Effect of bleaching agents and lignin model compound

Chlorate inhibited Oxalate Oxidase from Ochrobactrum species of the present study at a concentration of 0.1 mM to around 45 % of control. No strong inhibition was observed

on further increasing the concentration of sodium chlorate to 5 mM in the reaction mixture (Figure 4.10). In contrast, filtrates from Swedish pulp mills containing 1.5 mM chlorate almost completely inhibited Oxalate Oxidase (97% inhibition) from barley (Cassland et al. 2010). In another study, chlorates present in paper and pulp filtrates severely decreased the Oxalate Oxidase activity at 1.5 mM (Larsson et al. 2003). Winestrand et al. (2009) reported 92% decline in activity of barley Oxalate Oxidase in a reaction mixture containing 1 mM chlorate. All these previous studies suggest that chlorate is a strong inhibitor for barley Oxalate Oxidase and probably cannot be used for breaking down oxalate in those filtrates, which have been extracted after bleaching with chlorate in the paper and pulp industries.

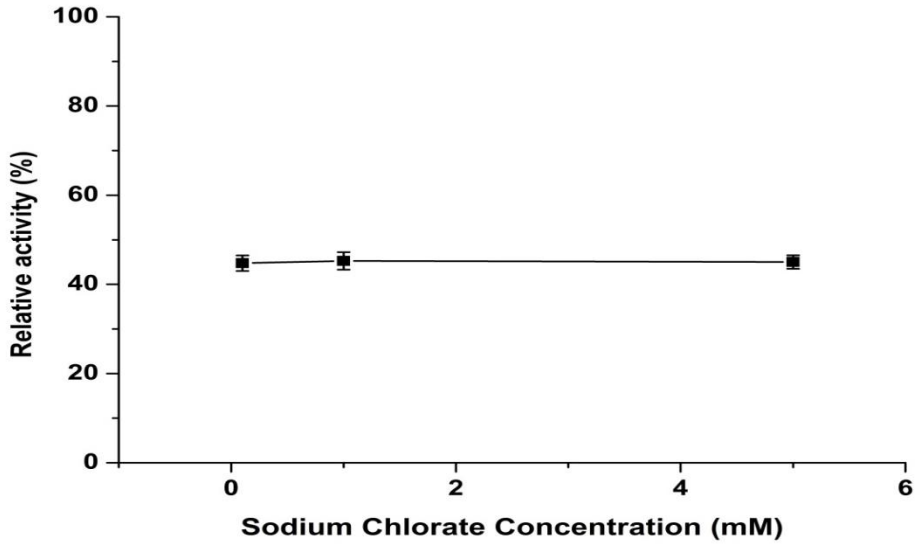


Figure 4.10 Effect of sodium chlorate on Oxalate Oxidase

The activity of Oxalate Oxidase decreased to 50 % and 34% of the original when the concentration of sulfite was 0.01 and 1 mM, respectively in the reaction mixture (Figure 4.11). Earlier, it has been shown that 0.1 mM sulfite extinguishes the activity of Oxalate Oxidase (Larsson et al. 2003). Sulfite at a concentration of 1 mM completely inhibited Oxalate Oxidase from barley (Winestrand et al. 2009). It is clear that sulfite strongly inhibits Oxalate Oxidase. Fortunately, sulfite is in general not a significant component

in filtrates, except when mechanical pulp is reductively bleached with dithionite (Cassland et al. 2010).

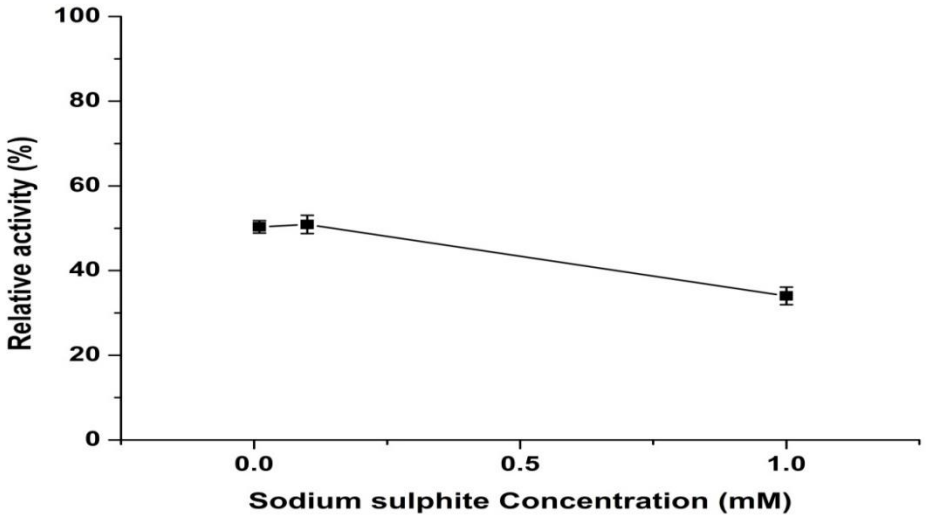


Figure 4.11 Effect of sodium sulphite on Oxalate Oxidase

Lignin model compound vanillin had strong inhibitory effect on Oxalate Oxidase and the residual activity was observed to be around 19% for 0.1 mM vanillin in the reaction mixture. On further increase in vanillin concentration to 5mM, the residual activity came down to 13 % of the original (Figure 4.12). Cassland et al. (2010) selected four frequently occurring lignin model compounds vanillic acid, vanillin, acetovanillone, and syringol, and tested them as inhibitors for Barley Oxalate Oxidase. No inhibition was observed at 5 mM for any of the lignin models with Oxalate Oxidase. Oxalate Oxidase from *Ochrobactrum intermedium* showed considerable loss of activity for lignin model compound, which may restrict its application in paper and pulp industry. But the concentration of lower molar mass lignin- oxidation compounds are strongly diminished as bleaching continues, and the amount of monomeric phenols are low in the in Kraft pulp bleaching filtrates (Liukko et al. 1999; Fuhrmann al. 2000; Dahlman et al. 1995). Extraction of acidified filtrates by Methyl tertiary butyl ether, followed by silylation and analysis by Gas Chromatography (Cassland et al. 2010), revealed only low concentrations. Thus the laboratory results may be dissatisfactory for lignin models but in situ use of the *Ochrobactrum intermedium* Oxalate Oxidase may not possess any

undesirable threat as long as concentration of lignin oxidised compounds are low in the final processed filtrates after completion of bleaching.

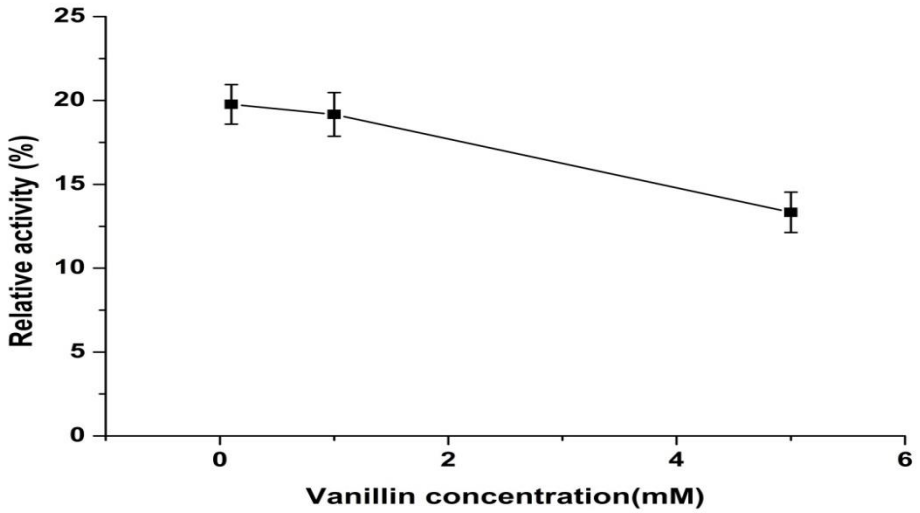


Figure 4.12 Effect of Lignin model compound Vanillin on Oxalate Oxidase

Application of enzyme-based control of oxalic acid in mills would be eased by operation of enzymes that are active at temperatures close to 70˚C in order to avoid the need for cooling of filtrates and the risk for further precipitation of calcium oxalate (Cassland et al. 2010). Barley, *Costus pictus* or wheat Oxalate Oxidase (Kotsira and Clonis 1997; Sathishraj and Augustin 2012; Hu and Guo 2009) may not find application in paper pulp industry, as all this Oxalate Oxidase from different sources are not thermostable to find use in industry, which demands high temperature of 70˚C for processing of raw materials. High thermostability of Oxalate Oxidase from *Ochrobactrum intermedium* at a temperature of 80°C for 6 h could be a desirable property to be exploited in these industries and improves its prospect of utilization in paper and pulp industry in spite of inhibition by lignin model compounds. Moreover, this enzyme from *Ochrobactrum intermedium* unlike other Oxalate Oxidase does not get completely inhibited by 5mM chlorate concentration and retains near to 50% of the original activity, which is otherwise not observed in barley Oxalate Oxidase, where complete inhibition was observed at 1.5 mM concentration of Chlorate.

Some bleaching filtrates from kraft pulp mills have a pH that is more suitable for the Oxalate Oxidase. Filtrates with pH as high as 11 will probably need dilution or neutralization before the enzymatic treatment (Cassland et al. 2010).

4.2.3 Chemical modifications of amino acid residues of enzyme

4.2.3.1 Modification of cysteine residues

Modification of cysteine residues of *Ochrobactrum intermedium* CL6 OxO resulted in the slight increase in activity. Modification carried out by using different concentrations of DTNB (0.2-0.8 mM) for different time intervals (0-40 min) showed that 0.4 mM of DTNB with incubation for 20 min was optimum, which resulted in an increase in activity by 10% when assayed at 55°C (Figure 4.13). However, modified enzyme did not show any enhancement of activity when assayed at 80°C (as compared to native enzyme), indicating modified enzyme is not thermostable unlike its native form. The increase in activity and loss of thermostable property could be attributed to considerable changes in conformation of the enzyme. This could be possible due to the reaction between thiol groups of proteins and DTNB leads to the formation of mixed disulphide (Riddles et al. 1983). The mixed disulphide formed are themselves highly reactive compound (Garman 1997), and might have been used to link to other thiols to form a new disulphide. Some reversible thiol-disulphide interchange reactions among proteins precede via mixed disulphide intermediates and can lead to migration of disulphide bonds to other locations in the same or separate protein which further forms disulphide linkages with other free thiols in a protein (Klomsiri et al. 2011). Formation of new disulphide bonds might bring functional change through a conformational change or steric blockage (Nagy 2013) and might have enhanced the activity of the enzyme Oxalate Oxidase towards the substrate oxalate, and this change in conformation might have contributed to its loss of thermostability. A similar phenomenon was reported in the case of Fructose 1, 6-Diphosphatase, where modification of the enzyme by DTNB led to the increase in enzyme activity (Little et al. 1969).

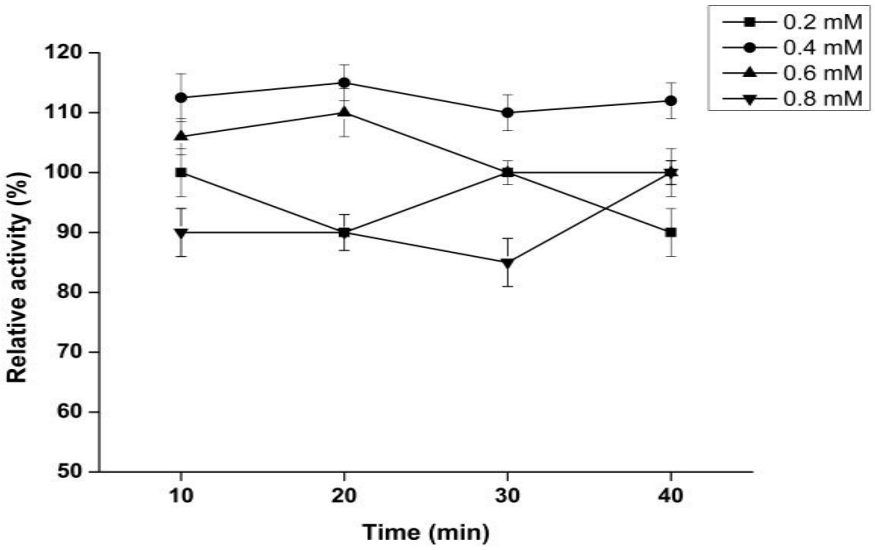


Figure 4.13 Effect of 5, 5-dithio-bis-(2-nitrobenzoic) acid (DTNB) concentration and reaction time on the activity of Oxalate Oxidase(OxO) of *Ochrobactrum intermedium* CL6

4.2.3.2 Modification of carboxylate residues

The modification of carboxylate residues of OxO using 10–100 mM1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) resulted in complete loss of activity (Figure 4.14). The result indicated that with an increase in the concentration of EDAC, there is a progressive decrease in activity of Oxalate Oxidase. The enzyme lost its complete activity at 100mM of EDAC with 30 minutes of reaction time. Complete loss of activity was also observed with 30mM EDAC with 90 min reaction time. The result points to the fact that carboxylate residues are essential for OxO activity, which is in agreement with wheat and barley, root OxO (Kotsira and Clonis 1998; Hu & Guo 1999). In the initial reaction, carbodiimide reacts with the carboxyl group of a protein to form an intermediate compound, O-acylisourea complex, subsequent reaction of the intermediate with amine forms an amide linkage (Wong and Jameson 2011). This reaction causes inactivation of the modified proteins and decreases the catalytic efficiency.

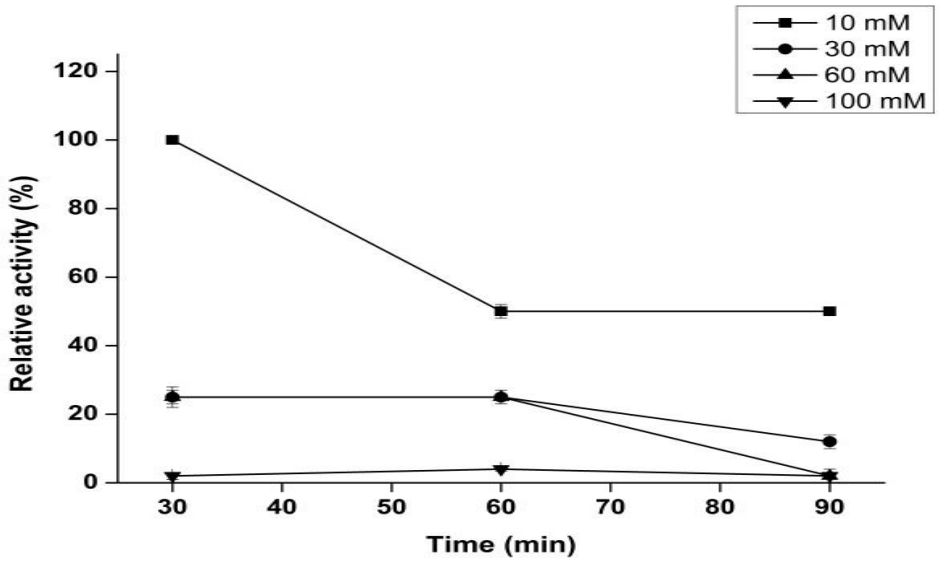


Figure 4.14 Effect of 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) concentration and reaction time on the activity of enzyme

4.2.3.3 Modification of histidine residues

There was an enhancement of catalytic activity of OxO when diethylpyrocarbonate (DEPC) at 1-15mM concentration was used to modify the enzyme's histidine residues. A two-fold increase in OxO activity was observed when the enzyme was reacted with 15mM concentration of DEPC for 60 min (Figure 4.15). Interestingly the modified enzyme showed same activity at 55°C and 80 °C, the trend seen in its native form. Oxalate Oxidase purified from wheat seedlings and barley root, on chemical modification of their histidine residues were also found uninhibited (Kotsira and Clonis 1998; Hu and Guo 1999) but unlike *Ochrobactrum intermedium*, such modification didn't lead to any improvement of enzyme's catalytic prowess. Modification of histidine by DEPC results in carbethoxylated derivatives (Mendoza and Vachet 2009), which may lead to loss of acid – base behaviour of histidine, and the ability of imidazole ring to coordinate with metal ions (Jr 1990; Zhao and Hutchens 1994; Eren et al. 2006). The OxO from *Ochrobactrum intermedium* CL6 might be having Mn^{2+} incorporated in the active site of the enzyme like most other Oxalate Oxidases (Opaleye et al. 2006). Modification of histidine prevents the coordination of Mn^{2+} with its imidazole ring and thus Mn^{2+} becomes partially free to interact more effectively with

the substrate oxalic acid. It has been reported that Mn^{2+} takes part in catalytic interaction with the substrate during the catalysis process by Oxalate Oxidase (Opaleye et al. 2006). Modification of histidine resulted in the two-fold increase in activity in glutamate dehydrogenase (Wallis & Holbrook 1973) and 2.6 fold increase in pancreatic α-amylase (Yamashita et al. 1991) to that of native form.

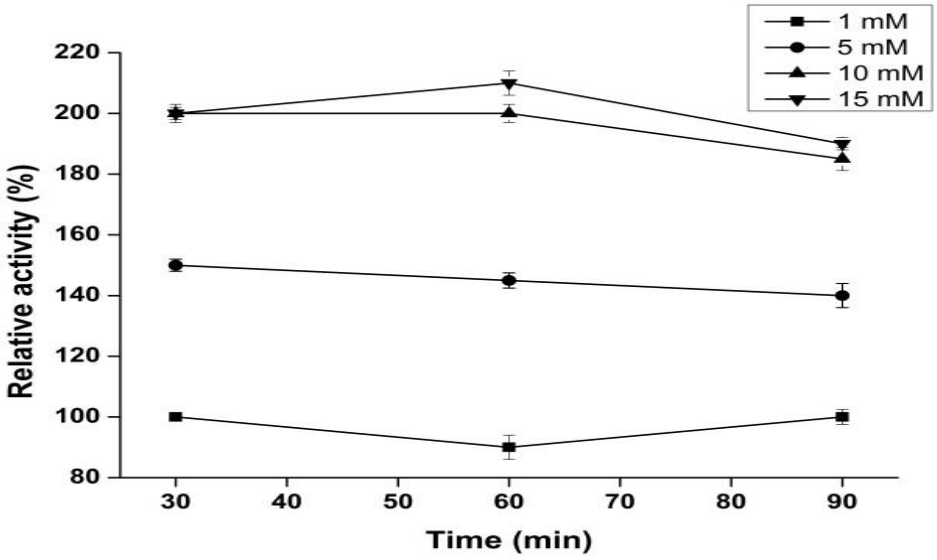


Figure 4.15 Effect of diethylpyrocarbonate (DEPC) concentration and reaction time on the activity of enzyme

4.2.3.4 Modification of serine residues

Modification of Serine residues by reacting OxO with Phenyl methanesulfonyl fluoride (PMSF) did not result in any loss of activity. At the same time, no enhancement and reduction in enzyme activity were noticed even after changing PMSF concentration and reaction time (Figure 4.16). This shows that Serine residue is not a part of the active site of OxO.

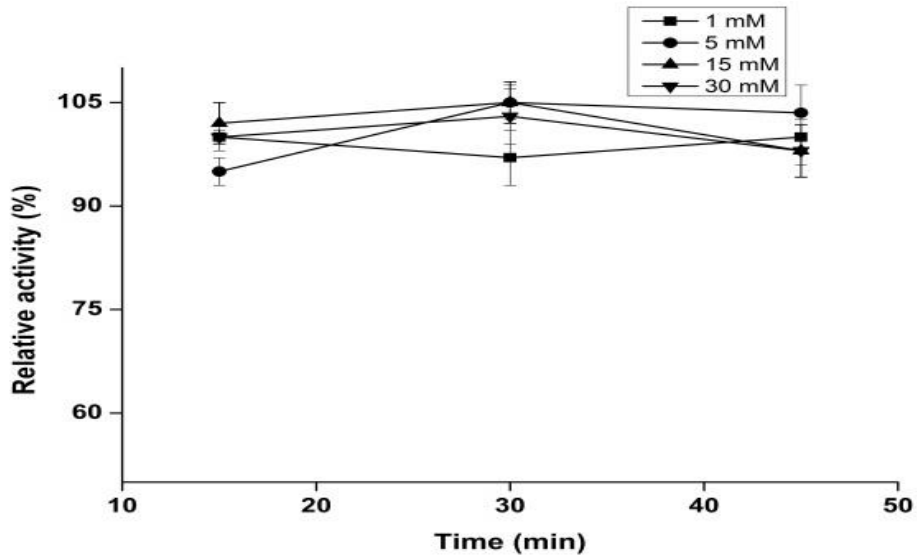


Figure 4.16 Effect of sulfonyl fluoride (PMSF) concentration and reaction time on the activity of enzyme

4.2.3.5 Modification of tryptophan residues

Tryptophan residues of *Ochrobactrum intermedium* CL6 OxO was modified by reacting with N-bromosuccinimide (NBS) of varying strength (0.01–0.05 mM). About 65 % of native activity was inhibited within 15 min of reaction time with 0.05 mM NBS. The enzyme lost its activity completely when it was reacted with 0.025 mM NBS for 45 min (Figure 4.17). The result shows that tryptophan residues are essential as they could be present at the active site of the enzyme. There are no reports on inhibition of OxO by modification of tryptophan residues of OxO.

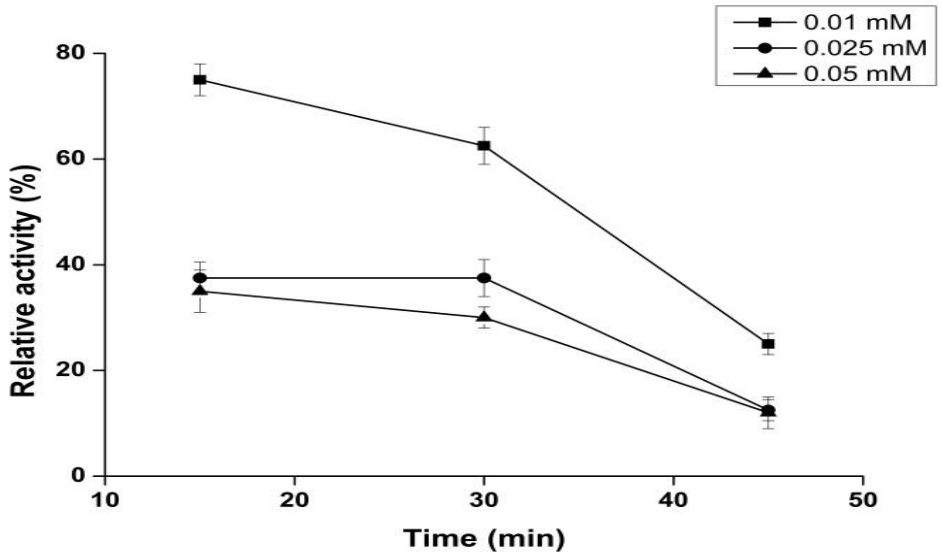
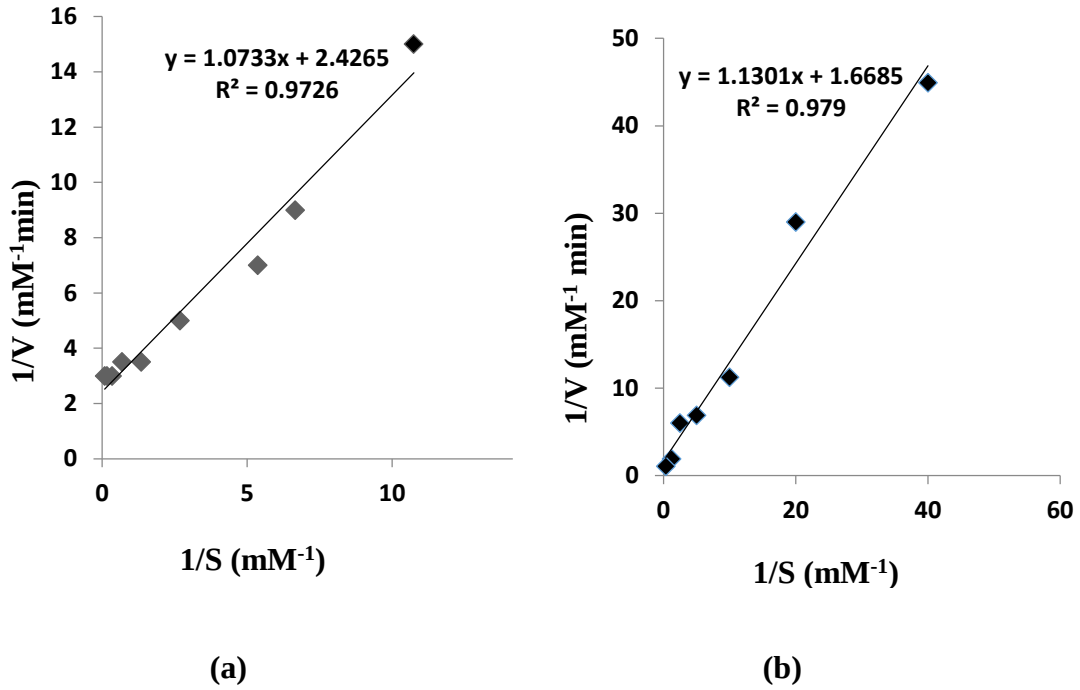


Figure 4.17 Effect of N-bromosuccinimide (NBS) concentration and reaction time on the activity of enzyme.

4.2.3.6 Kinetic studies of native OxO and histidine modified enzyme

The K_m value obtained from the Lineweaver-Burk plot for native and histidine modified enzyme was found to be 0.45 mM and 0.68 mM respectively and V_{max} was 413 $\mu Mmin^{-1}$ and 625 $\mu Mmin^{-1}$ respectively (Figure 4.18). There is a slight decrease in the affinity of the histidine modified enzyme towards its substrate oxalic acid, as evident from the increase in K_m value. The turnover numbers (k_{cat}) of native and histidine-modified enzyme were 91 s^{-1} and 140 s^{-1} respectively. The catalytic efficiency of histidine-modified enzyme was found to be $20.59x10^4$ $M^{-1}S^{-1}$, against $20.22x10^4$ $M^{-1}S^{-1}$ of native enzyme. Thus, the chemical modifications of histidine residues lead to the increase in turnover number (k_{cat}) which was however counteracted by increase in K_m value. As a result, a marginal increase (1.82%) in catalytic efficiency was found due to chemical modification. Nevertheless, the catalytic efficiency recorded here is 2.5 times that of OxO from Barley roots (Kotsira and Clonis 1997) indicating its better catalytic prowess. Moreover, the *Ochrobactrum intermedium* CL6 OxO, which is studied here, had exhibited no substrate inhibition up to 50 mM concentration making it an interesting enzyme for commercial applications.



(a) **(b)**

Figure 4.18 Kinetic studies of native and histidine modified Oxalate Oxidase (OxO) of *Ochrobactrum intermedium* CL6. Lineweaver – Burk Plot for (a) native enzyme. (b) Histidine-modified enzyme

4. 3 SUMMARY

In this study, OxO produced by a bacterial endophyte, *Ochrobactrum intermedium* CL6 was purified from fermentation broth by a two-step process, involving ethanol precipitation followed by gel filtration chromatography. The purified enzyme showed remarkable pH stability (pH 2 - 8) and thermostability (4 - 80°C) with a pH and temperature optima of 3.8 and 80°C respectively. The enzymatic activity was not adversely affected by most of the metal ions and biochemical agents (K^+, Na^+, Zn^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Glucose, Urea, Lactate), with Cu^{2+} being the exception. Interestingly, Ca^{2+} and Fe^{2+} showed stimulatory effect on the enzyme. Complete inhibition observed in the presence of EDTA proves that enzyme is a metalloprotein like already reported OxO of barley and wheat. Bleaching agents like chlorate and sulphite had inhibitory effect on the enzyme. Enzyme exhibited high affinity for substrate and absence of substrate inhibition up to 50 mM substrate concentration. High thermostability, good

kinetic parameters and absence of substrate inhibition shown by this enzyme make it interesting from commercial application point of view. Chemical modification of amino acid residues of enzyme revealed that cysteine; carboxylates, histidine and tryptophan residues are part of the active site and modification of cysteine and histidine residues had resulted in the enhancement of enzyme activity. Kinetic studies revealed that chemical modification of histidine residues resulted in the enhancement of turnover number (k_{cat}) by 1.53 times compared to native enzyme. The catalytic efficiency was 2.5 times higher than the OxO from Barley roots showing the potential of this enzyme for clinical and industrial applications. Hence, it is worthwhile improving catalytic efficiency of OxO produced from *Ochrobactrum intermedium* CL6 by site directed mutagenesis.

The enzyme was almost soluble in 100% ammonium sulphate saturated solution, and this characteristic of the enzyme could be attributed to its thermo stable properties. It was found that some of the thermostable enzyme do not possess any surface hydrophobic groups unlike other thermolabile proteins as all their hydrophobic amino acids are tightly packed into the core of the protein and gives necessary rigidity to withstand high temperature. It is possible that Oxalate Oxidase from *Ochrobactrum intermedium* CL6, which also has thermostable attributes may not possess surface hydrophobic patches, and it is because of this that the enzyme remained soluble at 100% ammonium sulphate solution and could not be precipitated by ammonium sulphate. Precipitation by ammonium sulphate actually takes place because of hydrophobic interaction between hydrophobic patches. The enzyme maintains its activity at high Na^+ and K^+ concentration which brightens the prospect of application of this enzyme in diagnosis of hyperoxaluria and treatment of urolithiasis. The enzyme has narrow substrate specificity and oxidizes oxalic acid specifically. The oxidizing ability of the enzyme Oxalate Oxidase for even the substrate analogue, glycolic acid was thus neglected in the present study and the enzyme is specific to oxalic acid.

CHAPTER 5

PRODUCTION OF OXALATE DEPLETED STARCH FROM TARO FLOUR BY ENZYMATIC TREATMENT AND EVALUATING PHYSICO-CHEMICAL PROPERTIES OF THE STARCH

CHAPTER 5

PRODUCTION OF OXALATE DEPLETED STARCH FROM TARO FLOUR BY ENZYMATIC TREATMENT AND EVALUATING PHYSICO-CHEMICAL PROPERTIES OF THE STARCH

Interest in new value added starch products has encouraged researches towards investigating the Physico-chemical and functional properties of starches produced from different genotypes and botanical sources to meet food industry demands. Modification of native starches are an alternative which have been used for some time, however, the possibility of introducing novel starch raw material sources with industrially interesting characteristics, has been drawing the attention of industrialists as it could influence the global market (Leonel et al. 2003). Finding new starch sources, developing process technology to extract starch, knowledge on physico-chemical and functional properties of the starches from these crops therefore would benefit food industry immensely.

Taro *(Colocasia esculenta)* is one such starch sources finds less usage in food industry due to reasons, including lack of considerable information on its physico-chemical and functional properties and presence of oxalate crystals, which is an antinutritional factor. Taro is a tropical tuber crop mostly produced for its underground corms and consumed in tropical and sub-tropical zones of the world. The total taro production in the world is about 9.22 million tons from an area of 1.57 million hectares (Ammar *et al*. 2009). Taro has been reported to have 70-80% starch with small granules (Jane et al. 1992). Substantial amount of work remains to be done to understand Physico-chemical and functional properties of Taro before considering it as potential source of starch to use in food and non - food industries (Deepika et al. 2013). Moreover, the presence of oxalates in Taro, limit its widespread usage. The oxalate present in Taro imparts acrid taste or cause irritation when foods prepared from them are eaten. Ingestion of foods containing oxalates has also been reported to cause caustic effects, irritation to the intestinal tract and absorptive poisoning. Oxalates are also known to interfere with the bio-availability of calcium (Sefa-Dedeh and Agyir-Sackey 2004). Oxalates interact with minerals like calcium, magnesium, zinc, copper, and therefore these minerals cannot be absorbed during the course of digestion and assimilation in the intestine. Such

complexes are excreted, thus reducing the bioavailability of minerals to the body (Kelsay 1987). The consumption of large amounts of plant oxalate and its absorption can be fatal to humans and other animals because of oxalosis, a condition of calcium oxalate deposits in body tissues. The consumption of more moderate amounts of oxalate appears to play a central role in calcium oxalate kidney stone disease because of its absorption and excretion in urine (Holmes and Kennedy 2000). Therefore, removal of oxalate from Taro starch is crucial to make it acceptable for food industry applications.

Several attempts have been made to reduce oxalate content in Taro. Although it has been reported that traditional methods of drying reduce oxalate content, it does not eliminate completely (Sefa-Dedeh and Agyir-Sackey 2004). Soaking and blanching of Taro tuber flour could reduce oxalate content by 18% (James et al. 2013). Substantial reduction in oxalate content (around 80%) has been reported by cooking for 40 minutes at 90°C (Iwuoha and Kalu 1995). However, if the objective is to extract oxalate free starch, the methods is unacceptable as cooking may compromise with the quality of the extracted starch (Liu et al. 2013). Therefore, alternative method to reduce oxalate content of Taro starch, which would not compromise with the quality, will certainly encourage food industry to explore the possibility of utilizing this cheap and abundant source. This doctoral work was therefore also aimed at finding a solution by developing an enzymatic treatment to reduce oxalate content in the extracted starch and evaluating physico-chemical properties of the starch thus produced. This section reports the treatment of the Taro tuber flour after processing in laboratory with purified Oxalate Oxidase from *Ochrobactrum intermedium* CL6, the test organism of the present study. Thereafter physico-chemical properties of the starch extracted after treatment was evaluated.

5.1 MATERIALS AND METHODS

5.1.1 Collection and Processing of Taro tubers

Colocasia esculenta (Taro) tubers were purchased from the local market of Mangalore, India. The tubers were thoroughly washed with tap water, peeled, and sliced into pieces. The slices were dried in a hot air oven at 60° C for overnight. The dried slices were

ground to powder in a mortar and pestle and passed through 500 µm screen to get fine flour.

5.1.2 Treatment of Taro tubers flour

Known weight of Taro tuber flour was taken (0.125 g), mixed with OxO enzyme solution (6 mL), incubated at 55° C in an incubator shaker. The OxO solution was prepared by dissolving known quantity of lyophilized enzyme in 6 mL of 50 mM sodium succinate buffer of pH 3.8. To study the effect of incubation period on the reduction in oxalic acid, Taro flour was mixed with 2 Units of OxO activity and incubated for varying period of incubation (15-120 min). Similarly, the effect of enzyme loading on the reduction in oxalic acid content was assessed by mixing Taro flour with increasing activity of enzyme (1-10 Units) and incubated for 120 min. After optimizing the enzyme load, trials were conducted to ascertain the effect of peroxidase enzyme (Sigma-Aldrich) on oxalate reduction kinetics. Two different concentrations of peroxidase enzyme (5 and 10 units) were used for the study.

5.1.3 Determination of oxalic acid content

The oxalic acid is found to exist in both soluble and insoluble forms in the plant materials. The soluble oxalic content and total oxalic acid content were determined as described below and the insoluble oxalic acid content was calculated by subtracting soluble oxalic acid content from total oxalic acid content.

To calculate soluble oxalic acid content, 0.125 g of sample of Taro tuber flour was weighed and dispersed in 6 mL of distilled water. The slurry was incubated on a water bath at 80°C for 15 min. The extract was allowed to cool and then volume of mixture was made to 12.5 mL with distilled water and left undisturbed for 30 minutes prior to oxalate estimation (Savage et al 2000). For determining total oxalic acid content, 0.125 g of sample of Taro tuber flour was weighed and suspended in 6 mL of 2M HCL. The slurry was incubated on a water bath at 80°C for 15 min. The extract was allowed to cool and then volume of mixture was made to 12.5 mL with 2M HCL and left undisturbed for 30 minutes prior to oxalate estimation.

The extracts were centrifuged at 3000 RPM for 10 minutes and 10mL of the supernatant was filtered through a 0.45 µm cellulose acetate membrane. 20 µL filtrate was analyzed using a HPLC system (Shimadzu, Japan), and UV/ VIS detector set at 254 nm. Data capture and processing were carried out using the Lab Solution software. The chromatographic separation was carried out using an RP C18 (4.6 mm I.D×250mm) analytical column attached to a guard column, using an isocratic elution at 0.5 mL/min with 0.3% acetic acid as a mobile phase. The analytical column was held at 30°C and the column was equilibrated at a flow rate of 0.5mL/min prior to use and in between sample sets. The oxalic acid peak was identified by comparison of the retention time to a range of oxalic acid standards.

5.1.4 Production and Partial purification of the enzyme Oxalate Oxidase

The enzyme Oxalate Oxidase (OxO) was produced by the *Ochrobactrum intermedium* CL6, the endophytic bacterium isolated from *Colocasia esculenta* and partially purified by two step method as previously described in chapter 4

5.1.5 Extraction of starch

Starch was extracted from Taro flour by the method of Aboubaker et al. (2008) with modifications. After incubation with OxO, the suspension was centrifuged at 5000 RPM for 10 min. The supernatant was discarded and sediment was washed twice in distilled water and dried at 60 °C for 12 h. The dried sediments were collected and steeped in (5mL) distilled water at 35 °C overnight. The slurry obtained was homogenized for 15 minutes using a commercial blender. The suspension obtained was filtered using a filter medium having pore size of 150 µm. The filter cake was discarded and filtrate was allowed to sediment for 24 h. The sediment was then collected and washed twice with water, dried for 48h at 60 °C and stored in air tight container. Starch was also extracted from Taro tuber flour in a similar fashion without the addition of enzyme as mentioned above.

5.1.6 Studies on physico-chemical properties of starch

5.1.6.1 Water binding capacity (WBC)

WBC of starches was determined, using the method described by Huang et al. (2016) with some modifications. A suspension of 0.2 g starch (dry weight) in 5 mL distilled water was agitated at regular intervals for 1 h at four different temperatures (30°C, 65°C, 80°C and 100°C). After centrifugation at 5000 RPM for 10 min, the supernatant was removed carefully and drained for 10 min. then the wet starch in the sediment was weighed.

5.1.6.2 Determination of paste clarity

Paste clarity of the starch suspension was determined by measuring light transmittance of the starch paste using the method of Correia et al. (2012). Starch aqueous suspension (2% w/v) was heated in a boiling water bath with stirring for 1 h. After cooled to room temperature, the light transmittance of the starch paste was determined at 640 nm with a UV-Vis spectrophotometer.

5.1.6.3 Swelling power and solubility

The swelling power and solubility were measured by stirring an aqueous suspension of starch (2% w/v) in a water bath kept at 65 °C, 80°C, 100 °C for 1 h, respectively. The suspension were cooled to room temperature and centrifuged at 5000 RPM for 10 min. The liquid supernatant was decanted out carefully and evaporated overnight at 100 °C. The swollen starch residue was weighed. Swelling power and solubility were calculated using the method described in Gomand et al. (2010).

Swelling power and solubility were calculated as follows:

$$\text{Swelling power (g/g)} = (SW \times 100)/Starch_{dwb} \times (100\% - \%SOL) \quad (1)$$

$$\text{Solubility (SOL \%)} = \text{Weight of dried supernatant} \times 100/Starch_{dwb} \quad (2)$$

Where, SW is weight of wet sediment, and $starch_{dwb}$ is the dry water basis starch weight.

5.1.6.4 Determination of amylose content

The blue value and amylose content of isolated starches was determined following the colorimetric method described by Morrison and Laignelet (1983). 70 mg of starch sample was placed in a test tube and 10 mL of urea (6 M)-DMSO solution (1:9) was added with continuous stirring. The samples were heated for 10 min in boiling water and kept in an oven at 100 °C for 1 h and then cooled at room temperature. Then 0.5 mL of the solution was taken into volumetric flask containing 25 ml distilled water +1 ml of I_2/KI (100 mg I_2 and 1000 mg KI in 50 ml distilled water) and final volume was made up to 50 mL using distilled water and mixed thoroughly.

The absorbance of the samples was measured at 635 nm in a UV spectrophotometer, against a blank (prepared by allowing chemicals and distilled water to stabilize for 15 min).

Blue value = (Absorbance/2 × g solution × mg starch) ×100

% Amylose = Blue value× 28.414

5.1.6.5 Microscopical examination of starch granules

Starch granule morphology was studied by using Scanning electron microscope. For Scanning electron microscopy, samples were scattered on the double sided adhesive tape mounted on metal stub and it was then coated with gold to make the sample conductive and images were examined at an accelerating potential of 10 kV.

5.1.6.6 X-ray diffraction (XRD)

X- ray diffraction of starches were recorded by Rigaku miniflex 600 X- ray powder diffraction (Rigaku, Tokyo, Japan) with Nickel filtered Cu Kα radiation (λ = 1.54056 Å) at a voltage of 40 kV and current 15 mA. The scattered radiation was detected in the angular range of 3-40° (2θ), with a scanning speed of 2° (2θ)/min and step size of 0.02° (2θ). The degree of crystallinity was calculated approximately by the software origin 8.5 based on the method reported by Nara and Komiya (1983).

For each of the treatments and measured parameters, observations were made for three replicates and thus the resultant values were the mean (±SE) of 3 replicates. In each

characterization study, a commercially available laboratory starch was taken as a control for comparison of results.

5.2 RESULTS AND DISCUSSION

Oxalate Oxidase (OxO) catalyzed the oxidative cleavage of oxalate of Taro flour to carbon dioxide with the reduction of molecular oxygen to hydrogen peroxide (Kotsira and Clonis 1997). Hydrogen peroxide, which is relatively unstable molecule subsequently, decomposed into water and molecular oxygen leaving behind no undesirable residues. In the current study, initially the Taro flour was produced from Taro tubers and then taro flour was treated with OxO. The incubation time and OxO load was optimized by one-factor-at-a-time method to achieve maximum oxalate reduction in Taro flour. Then starch was extracted from enzyme treated Taro flour. Various physico-chemical properties of starch thus produced was studied.

5.2.1 Effect of incubation period, Oxalate Oxidase load and peroxidase enzyme

The effect of incubation period on the oxalate content of Taro flour is presented in Table 5.1. The result shows that with the increase in incubation time, the oxalate content decreased progressively. After 2 h of incubation with 2 Units of OxO, a reduction of 23 % oxalate content was noticed. However, about 20% oxalate reduction was achieved within 45 minutes and rest in remaining 75 minutes. The results indicate that there is a scope to enhance oxalate reduction by increasing enzyme load. Moreover, the slowing down of oxalate conversion during the later stage of incubation suggests that OxO is being inhibited by one or the other component of either Taro flour or the products of enzymatic conversion. However, the addition of peroxidase enzyme to the slurry of Taro flour and OxO enzyme could not alter oxalate reduction kinetics, ruling out the possibility of feedback inhibition. Further, addition of Taro tuber water extract (freshly prepared from wet tuber) to the OxO solution quickly diminished the enzyme activity indicating the presence of inhibitors in the Taro flour. The reduction in oxalate conversion rate during the last phase of incubation suggests the presence of either small quantity of inhibitors or slower inhibition kinetics. To enhance the oxalate conversion further, increased OxO load was tried in the next set of experiments. With the increase in OxO load from 1-10 Units of activity, progressive reduction in oxalate content was

observed (Table 5.1). About 65% oxalate conversion was observed with the enzyme load of 6 Units, and further increase in enzyme load to 10 units, increased the oxalate conversion by another 4%.

After treating the Taro flour with 6 Units of OxO for 120 minutes, the starch was extracted and the oxalate content was estimated (Table 5.2). Total oxalate content was reduced by 97% in the extracted starch. Both soluble and insoluble oxalates got reduced due to enzymatic processing.

Published literature suggests that several methods are available for reducing oxalate content in food materials. Boiling, blanching with hot water, steeping with sodium bicarbonate and subsequent water leaching are some of the popular methods to reduce oxalate content in food materials. Cooking reduces the oxalate content of common foodstuffs like Silverbeet, Spinach, Rhubarb, Beetroot, Broccoli and Mucuna seeds by leaching losses into the cooking water in the range of 15 % to 83% for different cooking time (Savage et al. 2000 and Oneugbu et al. 2013). In case of Taro, soaking and blanching was not found to be suitable method to reduce total oxalate content above 18% (James et al. 2013). Stringent treatment conditions of Taro flour with sodium bicarbonate could reduce the oxalate content to 61% (Kumoro et al. 2014). However, a substantial reduction of around 80% was achieved by Iwuoha and Kalu (1995), by cooking Taro slices for 40 minutes at 90°C. Reduction of 80% oxalate content of Taro, by cooking looks attractive, but the methods is unacceptable as cooking will affect quality of the extracted starch if the objective is to extract oxalate free starch from Taro flour. Native starches cannot withstand the stringent processing conditions of high temperature, which lead to abundant granule disruption and undesirable product properties. When heated in the presence of water, starch undergoes an irreversible order disorder transition termed gelatinization and leads to decomposition of glucose rings (Liu et al. 2013), formation of lumping and affects the physical properties like expansion ratio, water absorption, water solubility (Dunford 2012), and also affect the mouth feel property of the starch (BeMiller and whistler 2009). Under this scenario, oxidative cleavage of oxalate in Taro flour by OxO is worthwhile, which would prevent detrimental physical modifications of starch.

Table 5.1 Effect of incubation time and Oxalate Oxidase load on the oxalate reduction in Taro flour

	% Reduction of oxalic acid	Total oxalate (mg/100g DW)
Incubation time		
0 min	0	790±5.77
15 min	6.76±1.13	736.4±5.99
45 min	20±1.15	631.8±4.503
75 min	21.06±1.55	623.2±8.22
120 min	23.73±0.635	602.44±2.14
Enzyme load (U)		
0	0	790±5.77
1	6.86±1.07	736.4±5.99
2	21.37±1.62	620.66±13.48
4	50.83±1.92	388.48±16.34
6	64.83±0.440	277.83±2.68
8	68.66±0.66	247.6±6.86
10	69.33±1.33	242.4±12.03

Table 5.2 Oxalate content of Taro flour, Starch extracted from Oxalate Oxidase treated Taro flour

Sample	Total oxalate (mg/100g DW)	Soluble oxalate (mg/100g DW)	Insoluble oxalate (mg/100g DW)
Taro flour	790±5.77	639±5.8	150±11.56
Extracted starch after enzymatic treatment	24.2±0.2	22.42±0.02	1.78±0.21

5.2.2 Studies on physico-chemical properties of starch

5.2.2.1 Water binding capacity (WBC) and paste clarity

Water binding capacity (WBC) of NT was highest at every temperature (30-100 °C) measured, compared to ET and control. Interestingly the WBC profile of ET starch was also showed a similar trend (Figure 5.1). However, the WBC of ET was less than NT indicating, small changes occurred in the starch during enzymatic treatment. The WBC of control was same as ET till 65 °C, diminished linearly with the increase in temperature beyond 65 °C, unlike ET and NT.

Interestingly the WBC determined here for Taro starch (ET & NT) is almost half the value reported by Deepika et al. (2013). While working on *Colocasia* species of Jharkhand, India. The loose association of amylose and amylopectin molecules in the native starch granules has been studied to be responsible for high WBC (Soni et al. 1987).

Water binding capacity is extremely important for thickening properties of starch, and thereby influences the quality of food products. High water binding capacity of starch molecule is a desirable characteristic as it stabilizes food products against effects such as syneresis and thus offers resistance to undesirable starch retrogradation (Baker et al.1994).

The Paste clarity (% Transmittance) was 9.38±0.19 % and 10.44±0.79% for ET and NT respectively (Figure 5.4) indicating marginal increase in opaqueness due to enzymatic treatment. Compared to control, taro starch in general showed very low transmittance indicating higher opaqueness. Paste clarity obtained here for Taro starch (ET & NT) found to be in agreement with the findings of Deepika et al. (2013).

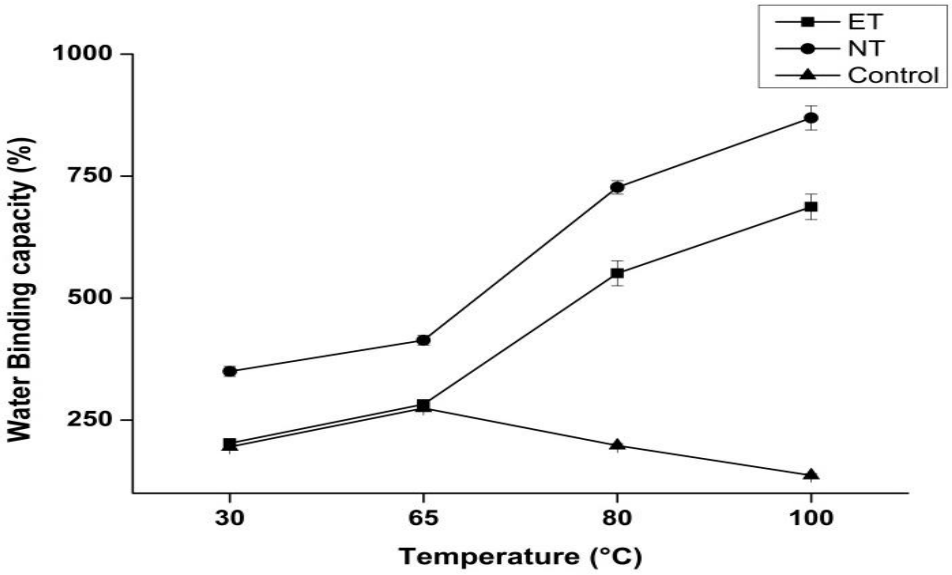


Figure 5.1 Effect of temperature on Water binding capacity of untreated (NT), treated (ET) and control starch

5.2.2.2 Swelling power and solubility

Swelling power of NT increased almost linearly with the increase in temperature from 65 °C to 100 °C (Figure 5.2). In case of ET, Swelling power did not change from 65°C to 80 °C, increased linearly though, from 80 °C onwards. Control showed low swelling power of 8.17±0.09 at 100 °C as against 20.66±0.84 and 15.32±0.37 for NT and ET respectively. The swelling power of NT and ET increased with temperature, signifying their hydrating ability under different temperature and excess water, which was otherwise not seen with the control. Solubility of NT was quite low at a temperature of 65°Cand was comparable with ET only at 100°C (Figure 5.3). Control however showed a linear increase in solubility with temperature reaching 83 % at 100 °C, which was about 4 times higher than ET and NT.

In general, swelling power and solubility of starches and fibre residues increases gradually with the increase in temperature in a particular range. Perhaps, this temperature range signifies the onset of gelatinization. Indeed, gelatinization promotes hydrogen bond cleavage as well as irreversible progressive relaxation in the bonding forces within granules leading to water absorption (Carcea and Acquistucci 1997).

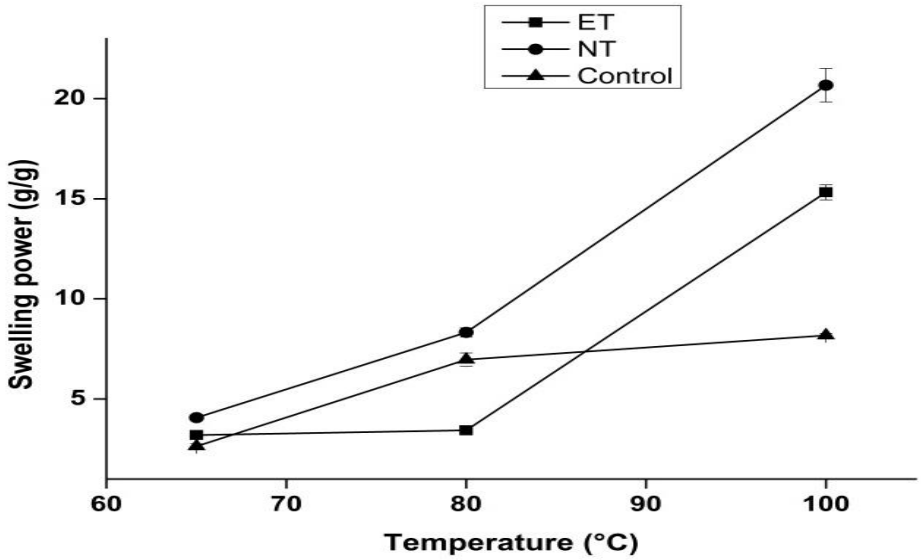


Figure 5.2 Effect of temperature on swelling power of untreated (NT), treated (ET) and control starch

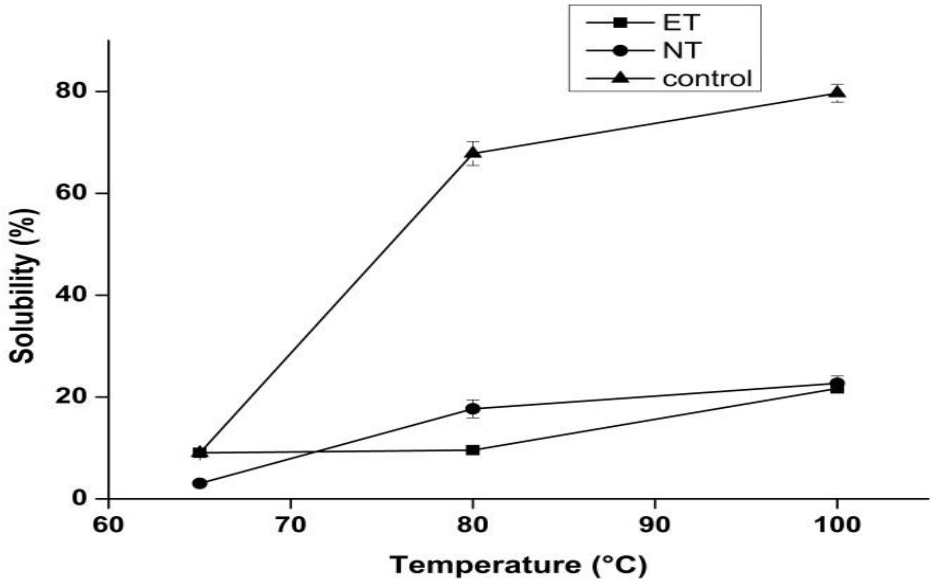


Figure 5.3 Effect of temperature on solubility of untreated (NT), treated (ET) and control starch

5.2.2.3 Amylose content

The amylose content of the three varieties of starches calculated from blue value is represented in Figure 5.4. Both treated and untreated starch has similar but low amylose content of around 7% which is three times less than that of the control (Figure 5.4). The low amylose content of the starch from Taro tubers corroborates the results of water binding capacity and swelling power of the starch varieties. The high water binding capacity of the starch in the present study can be attributed to low amylose content (Arendt and Bello 2011). Comparison of the swelling power and amylose content of Taro tuber starch and control suggests that swelling power is negatively correlated to amylose content. Amylose acts as both a diluent and an inhibitor of swelling (Tester and Morrison 1990). It has been suggested that amylose plays a role in restricting initial swelling, but swelling proceeds rapidly after leaching of amylose molecules. The extent of leaching of amylose mainly depends on the lipid content of the starch and the ability of the starch to form amylose–lipid complexes. The amylose-lipid complexes are insoluble in water and require higher temperatures to dissociate (deWilligen (1976a, 1976b), Raphaelides and Karkalas 1988; Singh et al. 2003). Therefore in the present study increase in water binding capacity and swelling power with increasing temperature could be phenomena of low amylose content and progressive dissociation of insoluble amylose-lipid complex if any with rise in temperature. Such low amylose starch has excellent solution stability, including freeze-thaw stability, and prevents retrogradation. The starch is useful in a wide variety of food, pharmaceutical, and industrial applications, either with or without chemical modification (Jeffcoat et al. 2003). Low amylose starch increases the shelf life of products in bread making industry, improve the texture of noodles and common Chinese sphageti. In addition to this low amylose starch finds application in textile, paper and adhesive industry (Tuberosa et al. 2013).

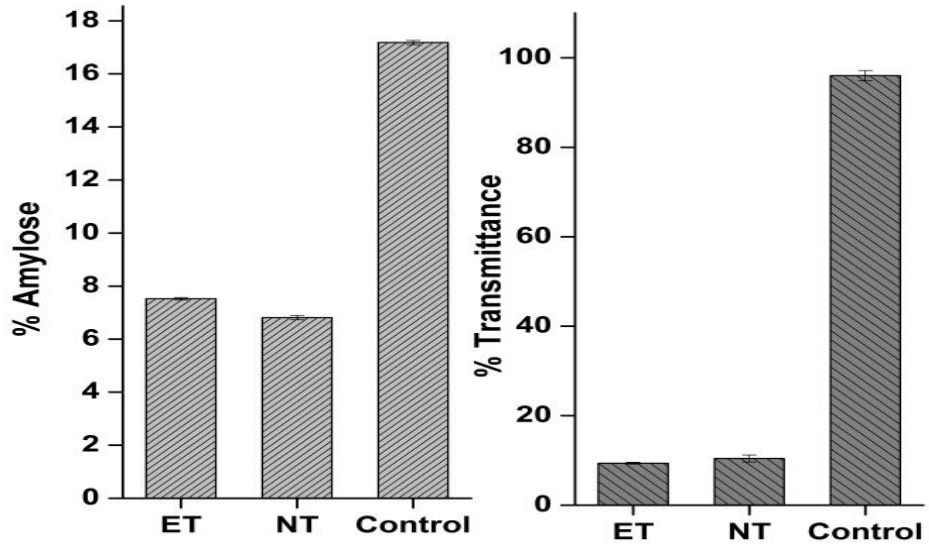


Figure 5.4 Physicochemical properties: Amylose % and paste clarity of untreated (NT), treated (ET) and control starch

5.2.2.4 Crystalline properties

The starch crystalline information is obtained by the x-ray diffraction patterns in Figure 5.5, in the diffraction spectra of the treated as well as untreated starch; there were three strong diffraction peaks (2θ) at 15°, 17.8°, 23.2°, and an additional sharp peak at 26° for untreated sample. Figure 5.5 show a typical A-type pattern of starch granular arrangement, with strong reflections at 2θ, 15° and 23° and an unresolved doublet 17°, 18° 2θ. A-type crystals tend to be more resistant to enzyme digestion than the B-crystal form (Copeland et al. 2009). Starch that is not degraded rapidly by human digestive enzymes in the upper gut has been associated with health benefits due to a slower release of glucose into the blood stream resulting in reduced postprandial glycemic and insulin responses (Copeland et al. 2009). The corresponding crystallinity levels calculated from the ratio of diffraction peak area and total diffraction area starches were about 9.63%, 10% and 15% for treated, untreated and control, respectively.

Additional sharp peaks in NT (indicated by arrows in Figure 5.5) are characteristics peaks of calcium oxalate monohydrate crystals (Okada et al. 2008). This strongly suggests that insoluble crystalline calcium oxalate remains as contaminant in the processed starch and necessary treatment is required to obtain oxalate free starch.

Absence of characteristics peaks of calcium oxalate in case of ET gives a qualitative insight of the whole strategy of insoluble crystalline calcium oxalate reduction in the present study.

The reduction of insoluble crystalline calcium oxalates in ET due to enzymatic treatment of Taro flour followed by extraction is quite unusual. The enzymes are expected to attack only soluble substrates. However, it is rarely found that an enzyme can bound to its crystalline substrate. There are few reports of crystalline cellulose being hydrolyzed by cellulase enzyme (Gao et al. 2013). We suspect a similar mechanism could be responsible for the reduction in insoluble crystalline calcium oxalates to a small extent. The major reason for the reduction in insoluble crystalline calcium oxalates could be due to the starch extraction procedure.

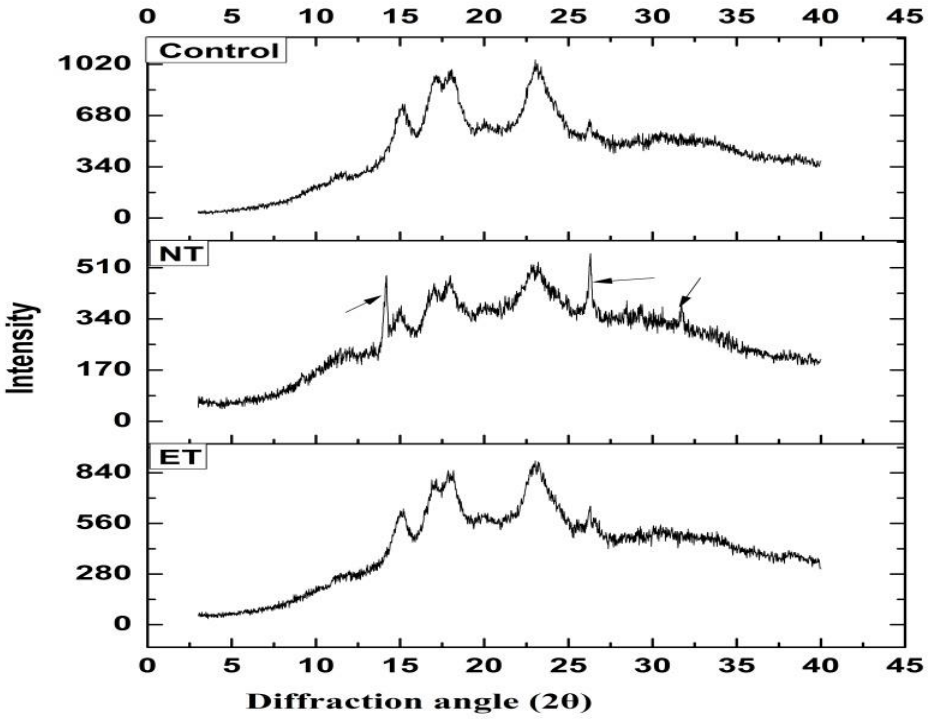


Figure 5.5 XRD patterns of Taro tuber starch. Untreated native starch (NT, Treated starch (ET) and control (Commercial starch). Peaks with arrow in case of NT represent characteristics peaks of the calcium oxalate.

The calcium oxalate crystals might be bound to the fibres of Taro (Savage et al. 2000) which remained entangled with the discarded filtrate. The XRD spectra proves that insoluble crystalline calcium oxalate content was significantly low in ET also corroborates the HPLC analysis (Table 5.2).

5.2.2.5 Scanning electron micrograph

The morphological characteristics of treated, untreated and control starches using scanning electron microscopy are represented in Figure 5.6, 5.7 and 5.8 respectively. The SEM of both treated and untreated starch starch showed the smooth surface and irregular shapes of granules without any damage or fissures. The starch granules of control were observed to be round, elliptical and possessed rough surface. The mean diameter of both the treated and untreated starch granules were approximately 1-2 µm, whereas that of control ranging from 5-15 µm. The variation in starch granule morphology may be due to the biological origin and physiology of the plant and the biochemistry of the amyloplast. This may be also due to the variations in the amylose and amylopectin content and its structure, which in turn play an important role in the control of the starch granule size and shape (Kaur et al. 2007). The granule size is variable and ranges from 1 – 120 µm among starches from different cultivars (BeMiller and Whistler 2009). Variation in the size of the granules influence light transmittance, water holding capacity and swelling power of the starch molecules (Badenhuizen 1969). The granule size from other sources of Taro tubers as reported in literature also varied from 1-10 µm in diameter (Deepika et al. 2013 and Aboubaker et al. 2008). The Small granule size of starch from Taro tubers in the present investigation possess large surface area and therefore exhibit higher water holding capacity (Arendt and Zannini 2013)

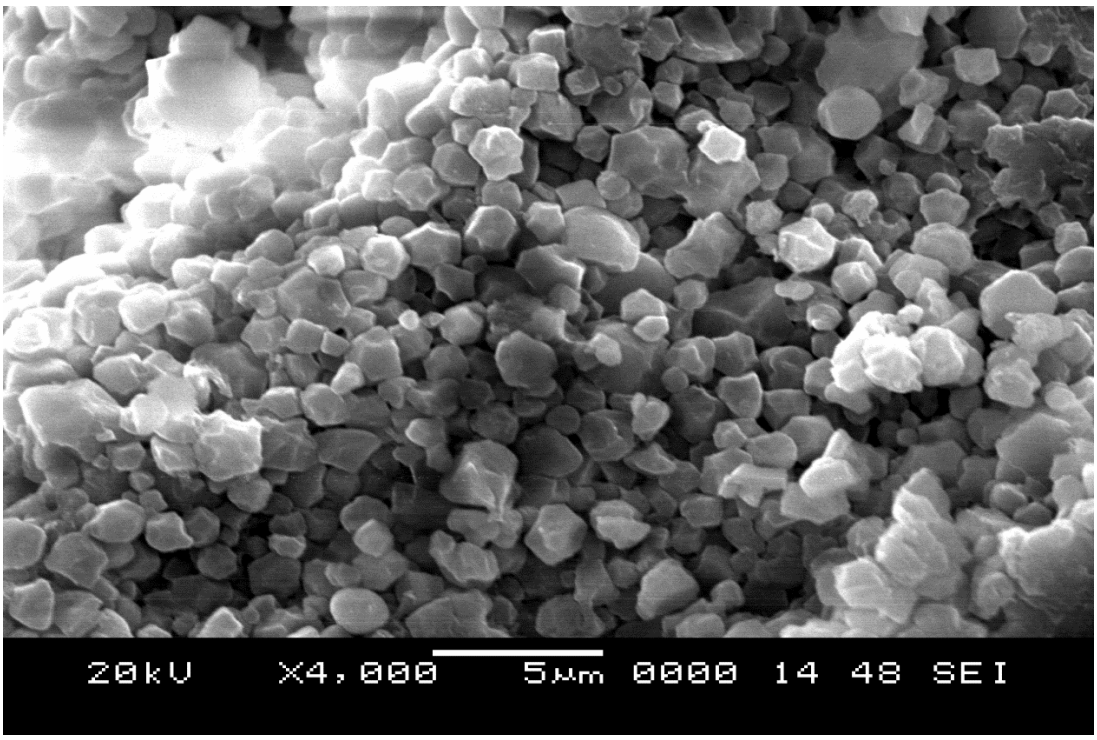


Figure 5.6 SEM micrographs of enzyme treated starch, ET (4000× magnification)

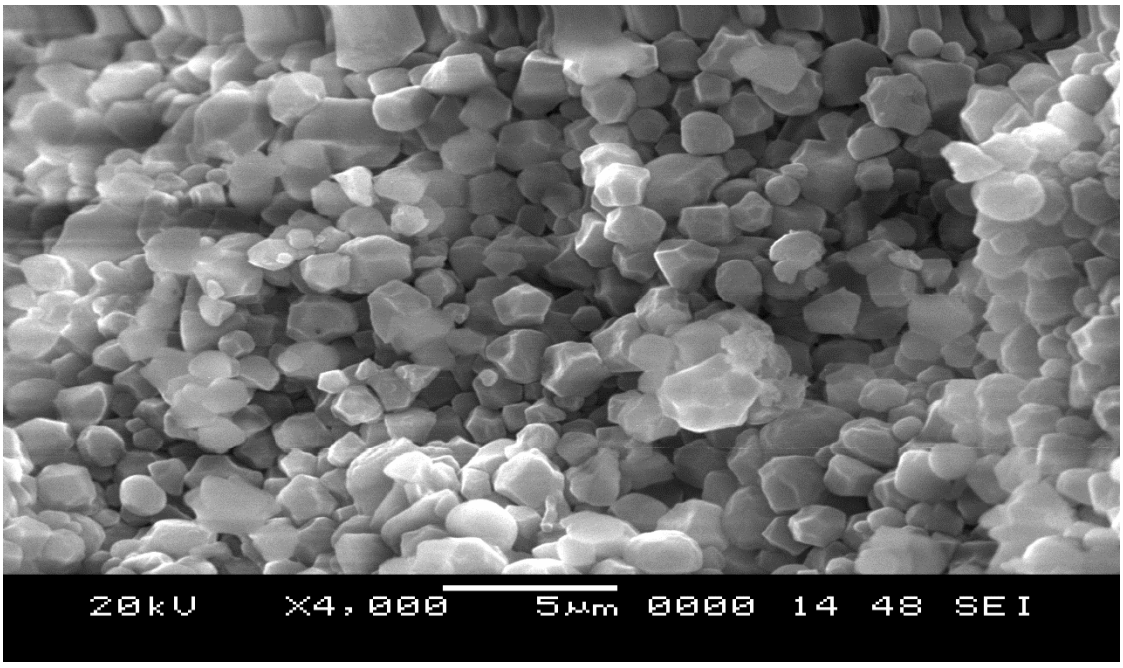


Figure 5.7 SEM micrographs of untreated starch, NT (4000× magnification)

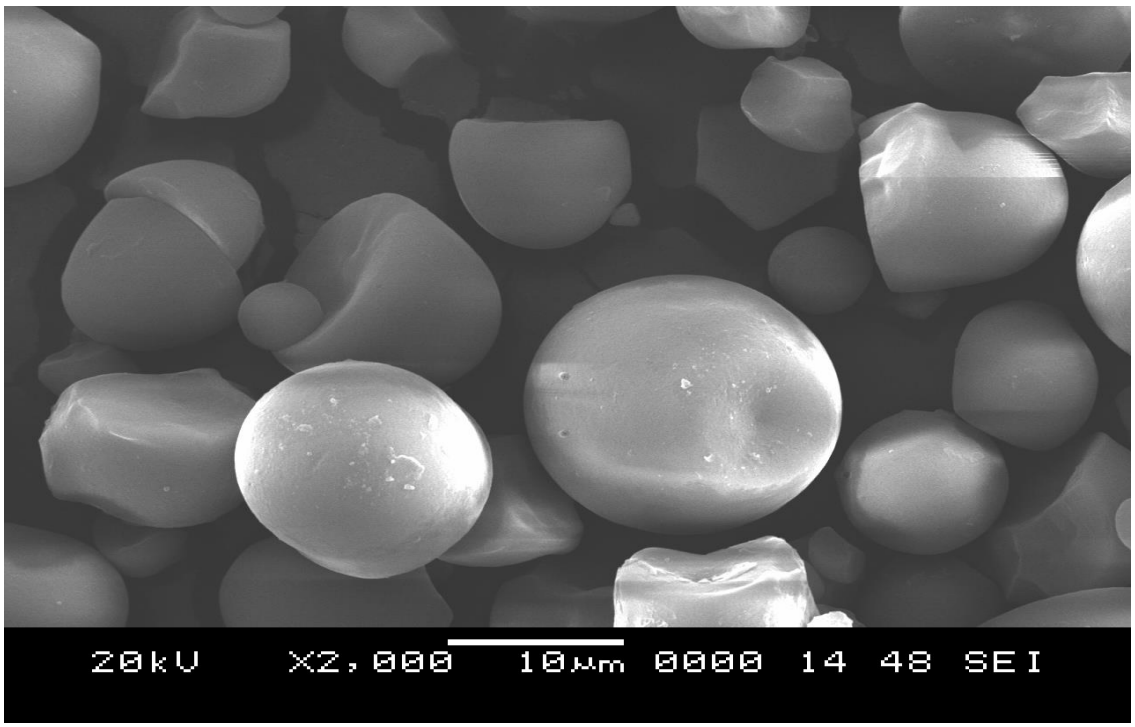


Figure 5.8 SEM micrographs of Control (2000× magnification)

Table 5.3 Comparison of physicochemical properties of starches from different genotypes

Starch Source	Water* binding capacity (%)	Amylose content (%)	Swelling ** power (g/g)	Solubility* * (%)	Paste clarity (% T)	Granule pattern	size (µm)
Taro starch Present Study	202.33±5.04	7.52±0.049	3.19±0.153	9.02±0.268	9.38±0.198	A	1-2
Taro Starch	328- 506[a]	13.5-27.66[a]	0.89-1.40[a]	7.42[b]	10.6[b]	-	5-10[a]
Potato Starch	83.05[b]	20.1–31.0[c]	19.39[b]	6.3[b]	96[c]	B[c]	1-110[c]
Sweet Potato	80.52[b]	-	15.48[b]	6.47[b]	8.0[b]	B[c]	-

[a] Deepika et al. 2013 [b]Pramodrao and Riar, 2013 [c] Singh et al. 2003

[*] Water binding capacity was estimated at 30°C. [**] Swelling power and solubility % were estimated at 65°C for the present study and at 60 °C for all other reported study.

5.3 SUMMARY

From the Taro tuber (*Colocasia esculenta*), flour was produced, was then treated with OxO enzyme and subsequently the starch was extracted. Oxalate content reduced from 790±5.77 mg to 24.2±0.2 mg in 100 g starch (dry weight basis), due to this novel strategy. Total oxalate level obtained here (24.2±0.2 mg in 100 g starch) is less than 71 mg/ 100 g, which is the permissible oxalate levels in food (Sefa-Dedeh and Agyir-Sackey 2004; Kumoro et al.2014). Several physico-chemical properties such as water binding capacity, paste clarity, swelling power, solubility, amylose content, granule pattern and size of starch produced out of enzyme treatment (ET) and without enzyme treatment (NT) were studied. The data suggests that all the studied parameters of ET closely resembled NT, indicating minor changes. All the studied parameters were compared with the physico-chemical properties of potato and sweet-potato starch (Table 5.3). The starch produced out of enzyme treatment showed higher water binding capacity, very low transmittance indicating higher opaqueness, high swelling power, very low solubility, and low amylose content at a temperature range of 80 – 100 ° C, as compared to potato and sweet-potato starch. Moreover the starch possesses A-crystalline form, unlike B-crystalline form found in potato and sweet-potato starch.

Some of the desirable properties of Taro starch are high water binding capacity, low amylose content, and high swelling power. These properties are desirable from food industry perspective as well, because these properties impart higher shelf life, higher solution stability, including freeze-thaw stability, and prevent retrogradation to the food. A-type crystal structure of Taro starch is another most desirable unique property known to be associated with health benefits due to a slower release of glucose into the blood stream resulting in reduced postprandial glycaemic and insulin responses. Thus, oxalate depleted Taro starch produced by Oxalate Oxidase enzyme treatment could be another source of starch for many food-processing industries.

CHAPTER 6

SUMMARY AND CONCLUSIONS

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The extensive demand of vast and diverse assortment of new substances for various human applications has led to continuous screening for novel chemical structures. Natural products produced by microorganisms have aided as potential resources to accomplish this important demand, because they are highly effective, possess low toxicity, and have a negligible environmental impact. Nevertheless, the coherent selection of high-quality screening sources is essential for further efficient screening of bioactive compounds for the benefit of human race. On this logical context, present work was taken up to identify a defining source of Oxalate Oxidase, which would meet up the future demand of the enzyme world.

This doctoral work on "Studies of Oxalate Oxidase from endophytes" has been described in the thesis in five chapters. Following is the summary of the study.

6.1 SUMMARY

Four oxalate degrading endophytic bacteria were isolated from oxalate rich tubers of *Colocasia esculenta*, *Beta vulgaris* and *Ipomoea batatas*, and peel of *Musa paradisiaca*. Based upon the Oxalate Oxidase (EC 1.2.3.4) production in screening medium, one bacterium was selected and identified as *Ochrobactrum intermedium* by 16 S rRNA gene sequencing.

Studies on effect of nutritional and non-nutritional parameters showed that Oxalate Oxidase production is inducible and requires manganese ions in the medium, and very low fill-up volume is beneficial. The medium comprising of sucrose, ammonium chloride, sodium oxalate along with basal salts gave 0.5 UmL^{-1} Oxalate Oxidase activity and 0.454 Umg^{-1}specific activity after 65 h of fermentation in shake flasks.

Oxalate Oxidase produced by this endophytic bacterium, *Ochrobactrum intermedium* CL6 was purified by ethanol precipitation followed by gel filtration chromatography.

This approach resulted in 58.74-fold purification with 83% recovery. Specific activity of the final purified enzyme was 26.78 U/mg protein. Characterization studies revealed some fascinating characteristics of enzyme. The enzyme displayed an optimum pH and temperature of 3.8 and 80℃ respectively and high stability at 4℃ to 80℃ for 6 h. The enzymatic activity was not influenced by metal ions and chemical agents (K^+, Na^+, Zn^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Glucose, Urea, Lactate) commonly found in serum and urine, with Cu^{2+} being the exception. The enzyme was stimulated by Ca^{2+}, Fe^{2+} and riboflavin. Complete inhibition observed in the presence of EDTA proves that enzyme is a metalloprotein like already reported OxO of barley and wheat. The enzyme was partially inhibited to nearly 50% by chlorate and sulphite ions. Model lignin compound also inhibited the enzyme and reduced the activity to 19% of the original. Substrate analogue glycolic acid did not cause any inhibition. It's K_m and k_{cat} for oxalate was found to be 0.45mM and 85 s^{-1} respectively. This enzyme is the only known Oxalate Oxidase which did not show substrate inhibition up to a substrate concentration of 50 mM. Chemical modifications of Oxalate Oxidase using amino-acid specific reagents revealed that cysteine, carboxylates, histidine and tryptophan residues are part of the active site. Moreover, modification of cysteine and histidine residues had resulted in the enhancement of enzyme activity. A two-fold increase in Oxalate Oxidase activity was observed when histidine residues were modified with 15 mM diethylpyrocarbonate for 60 min. Histidine-modified enzyme exhibited k_{cat} value of 140 s^{-1} against 91 s^{-1} of native enzyme. However, catalytic efficiency did not increase substantially (1.82%) due to the counteracting increased K_m value.

The novel Oxalate Oxidase enzyme produced by the endophyte, *Ochrobactrum intermedium* CL6 was used to treat Taro tuber flour. Novel process comprising treatment of Taro tuber flour with Oxalate Oxidase enzyme, and subsequent extraction of starch resulted in 97% reduction in total oxalate content. Further, several physico-chemical properties such as water binding capacity, paste clarity, swelling power, solubility, amylose content, granule size of starch produced out of enzyme treatment were studied and compared with properties of Taro starch produced without Oxalate Oxidase treatment. Comparison study revealed that enzyme treatment did not bring appreciable changes in the studied parameters. The Taro starch produced out of enzyme

treatment showed higher water binding capacity (673.83±38.55%), very low paste clarity (9.38±0.19 %), high swelling power (15.32±0.37 g/g), low solubility (21.66±0.66 %), and low amylose content (7.52±0.049 %) at 100 °C in comparison to potato and sweet-potato starches. X-ray diffraction data revealed that taro starch possesses A-crystalline form, unlike B-crystalline form found in potato and sweet potato starch. To the best of our knowledge, this is the first work reporting the use of Oxalate Oxidase to produce oxalate free Taro starch.

6.2 SIGNIFICANT FINDINGS

- Oxalate rich tropical plants harbour many endophytic bacteria capable producing appreciable amount of Oxalate Oxidase. An endophytic strain *Ochrobactrum intermedium* CL6 isolated from the tuber of *Colocasia esculenta* was found to be a novel source of Oxalate Oxidase enzyme.
- Oxalate Oxidase from *Ochrobactrum intermedium* CL6 possesses many intriguing characteristics. The enzyme is thermostable showed negligible loss of activity after 6 h of incubation at 80°C. Enzyme exhibited absence of substrate inhibition up to 50 mM substrate concentration. The enzymatic activity was not adversely affected by most of the metal ions and biochemical agents (K^+, Na^+, Zn^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Glucose, Urea, and Lactate).
- There was two-fold increase in Oxalate Oxidase activity on modification of histidine residues on Oxalate Oxidase enzyme with 15 mM diethylpyrocarbonate.
- The treatment of Taro tuber (*Colocasia esculenta*) flour with Oxalate Oxidase enzyme and subsequent extraction of starch led to 97% reduction of Oxalate content (790±5.77 mg to 24.2±0.2 mg in 100 g starch). The physico-chemical properties of oxalate-depleted starch were found to be highly desirable from food industry perspective. The Oxalate depleted starch is expected to impart higher shelf life, higher solution stability, including freeze-thaw stability, and prevent retrogradation to the food.

6.3 CONCLUSIONS

- Oxalate rich tropical plants harbour endophytes capable of producing Oxalate Oxidase enzyme.
- Oxalate Oxidase produced by the endophytic bacterium *Ochrobactrum intermedium* CL6 is a novel enzyme possessing several intriguing characteristics. This vindicated the theory that enzymes produced by microbes isolated from ecological niche possess novel characteristics.
- Chemical modifications of amino acid residues of enzyme could serve as a tool to explore the importance of various amino acid residues in catalysis. Chemical amendment of the residues showed enhancement of Turnover number.
- Usage of Oxalate Oxidase to deplete oxalate content in Taro starch was found to be beneficial. No adverse change in the desirable properties of Taro starch was evidenced.

6.4 SCOPE FOR FUTURE WORK

- The significant variables influencing the production of Oxalate Oxidase obtained by one-factor approach can be used to optimize the media composition by design of experiments
- The amino acid sequence of the enzyme can be elucidated by proteomic studies.
- Use of enzyme as a therapeutic agent for treating conditions of hyperoxaluria and Urolithiasis can also be studied.

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APPENDICES

APPENDIX I

SCREENING MEDIUM COMPOSITION

Composition	**(gL^{-1})**
KH_2PO_4	3
Na_2HPO_4	6
NaCl	5
NH_4Cl	2
$MgSO_4.7H_2O$	0.1
$MnSO_4$	0.05
Calcium Oxalate	5
Biotin	0.0015

APPENDIX II

BACTERIAL BIOMASS ESTIMATION

Bacterial growth in shake flask was monitored by measuring the optical density at 600 nm. The obtained optical density values were converted to dry cell weight (gL^{-1}) using a calibration curve. For calibration curve, bacterial cultures from log phase of their growth were diluted to different dilutions with sterile growth medium and the absorbance was measured. The same diluted known quantity was centrifuged at 8,000 rpm for 10 minutes to get pellets and the pellets were kept in hot air oven for 12 h for dry weight measurement.

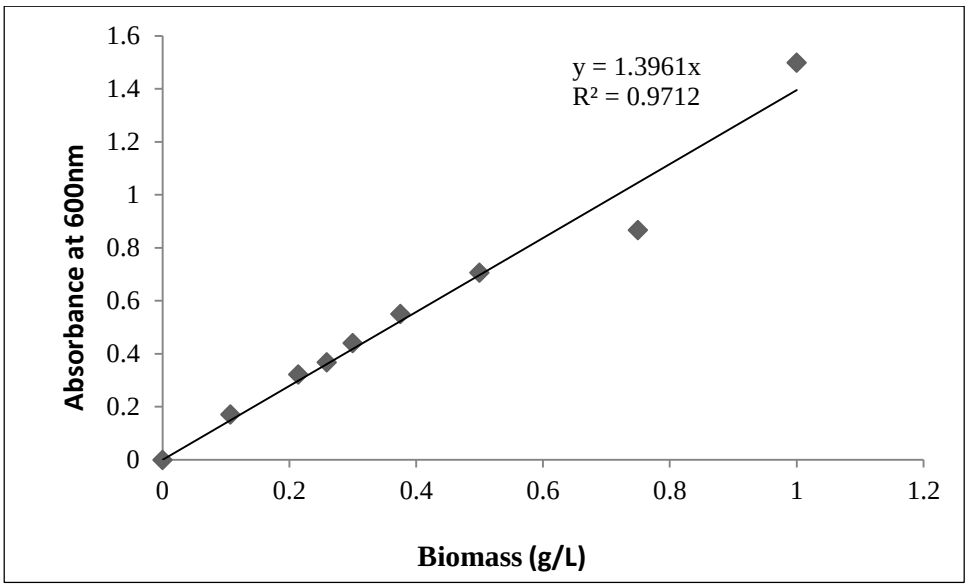

Figure A II (A) Calibration plot for bacterial biomass estimation

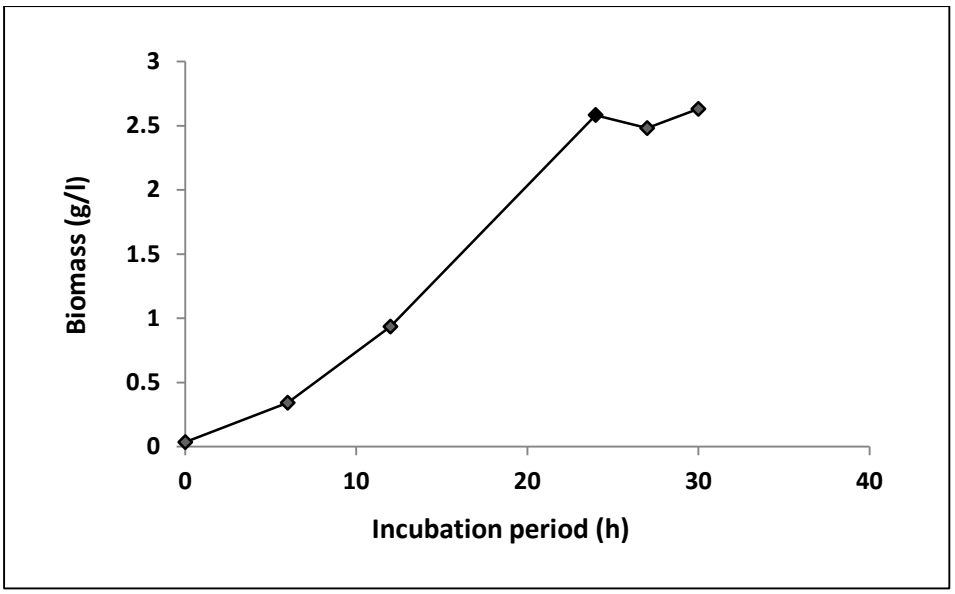

Figure A II (B) Growth curve of *Ochrobactrum intermedium* CL6 in growth medium

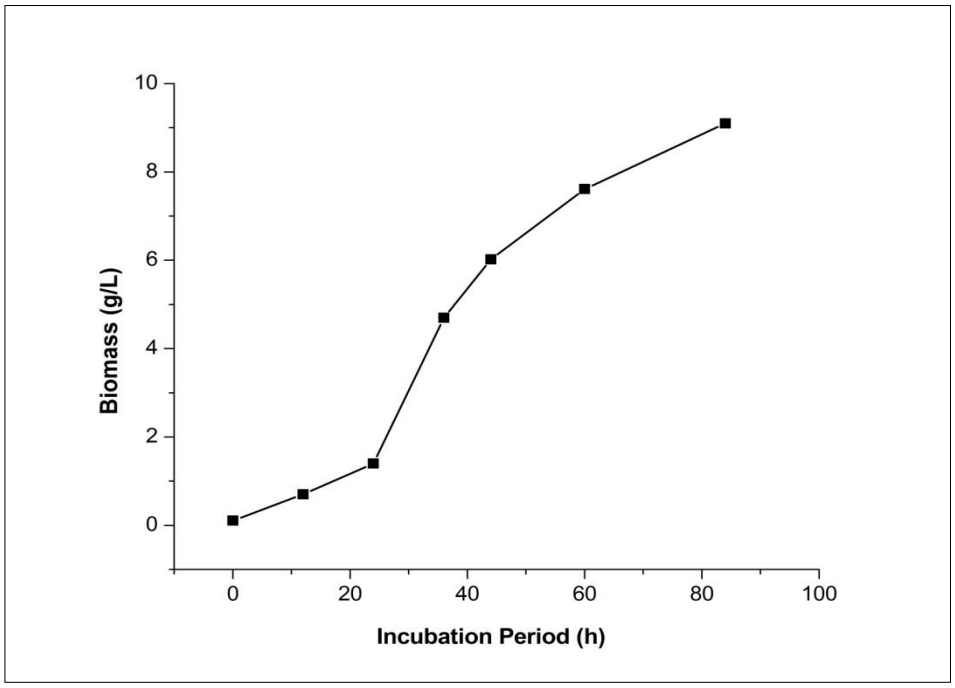

Figure A II(c) Growth curve of *Ochrobactrum intermedium* CL6 in production medium

APPENDIX III

STANDARD CURVES FOR QUANTITATIVE ESTIMATION

Standard curve of hydrogen peroxide

Standard curve was prepared using commercially available hydrogen peroxide solution dissolved in succinate buffer 0.05 M, pH 3.8.0. Addition of 1 mL of colouring reagent comprising 0.1 mM MBTH (3-Methyl-2-Benzothiazolinone Hydrazone) and 0.72 mM DMA (N, N-Dimethylaniline) along with 3 U of 20 µL of peroxidase (Sigma Aldrich) solution in the test tubes containing hydrogen peroxide solution results in formation of purple colour indamine dye. The colour produced due to indamine dye formation was read at 600 nm. The amount of hydrogen peroxide produced from the different samples due to oxalate oxidase activity on oxalic acid was determined by extrapolating the standard curve prepared using hydrogen peroxide in the range of 0.001–0.030 mmol.

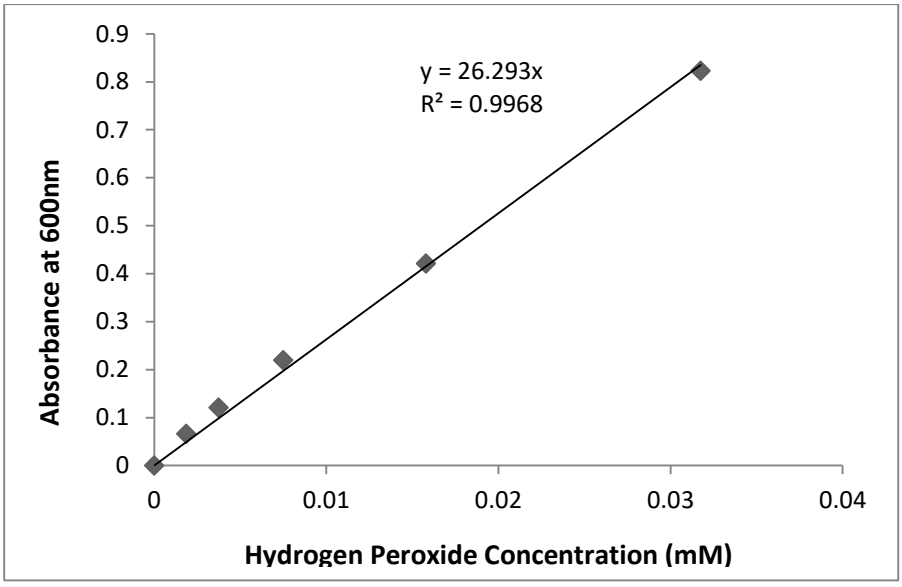

Figure A III (A) Standard graph of hydrogen peroxide

Standard protein curve for total protein estimation of unknown samples

Bovine serum albumin (BSA) was used to prepare the standard stock solution (500 µg/mL). A series of BSA solutions in the concentration range of 0-500 µg/mL was prepared by mixing BSA stock solution with 0.05 M sodium succinate buffer (pH3.8) in different test tubes. This standard curve for protein estimation of unknown samples was made by using Folin- Lowry's method.

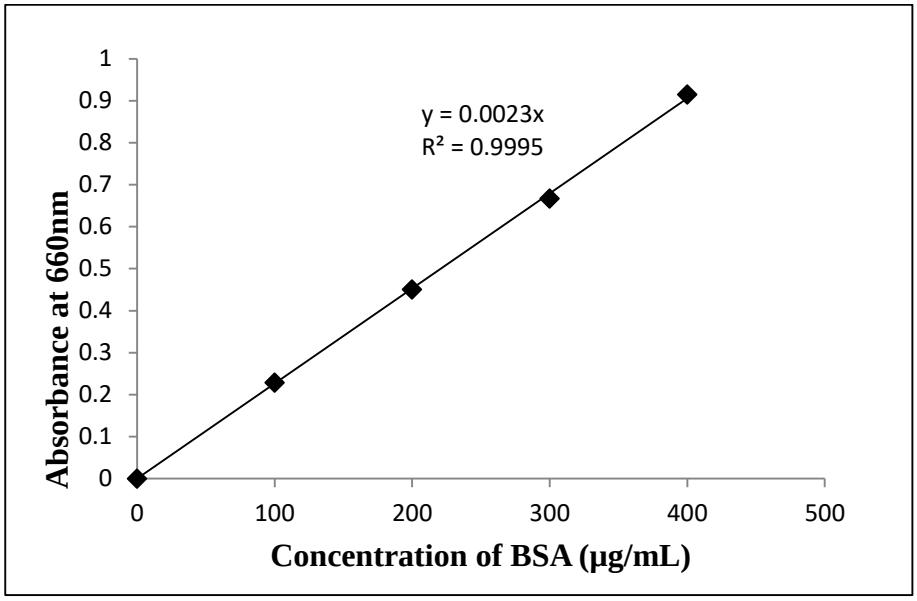

Figure A III (B) Standard protein curve by Folin- Lowry's method.

Standard oxalic acid curve

The range of oxalic acid standards (0.0125 – 0.05 mg/mL) was prepared in distilled water and filtered through a 0.45 µm cellulose acetate membrane. 20 µL filtrate was analysed using a HPLC system (Shimadzu, Japan), and UV/ VIS detector set at 254 nm. Data capture and processing were carried out using the Lab Solution software. The chromatographic separation was carried out using an RP C18 (4.6 mm I.D×250mm) analytical column attached to a guard column, using an isocratic elution at 0.5 mL/min with 0.3% acetic acid as a mobile phase. The analytical column was held at 30°C and the column was equilibrated at a flow rate of 0.5mL/min prior to use and in between standard sample sets. The oxalic acid peak and the retention time were

compared for the entire range of oxalic acid standards. The standard oxalic acid curve was plotted with area under the peak and concentration of oxalic acid.

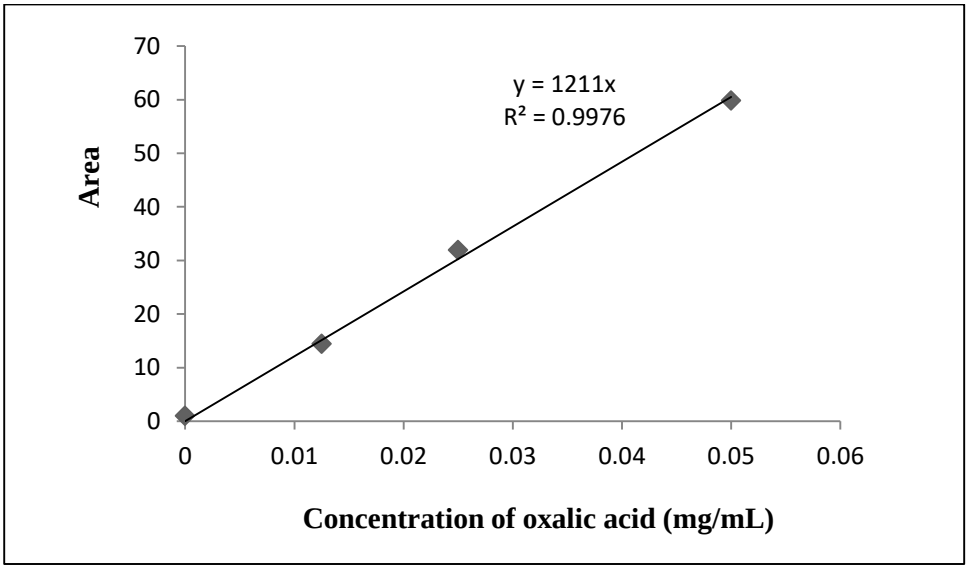

Figure A III (C) Standard oxalic acid curve by HPLC method

Standard curve for molecular weight determination by SDS page

Standard proteins run in SDS PAGE were of medium range (97.4 – 20KDa). On the basis of molecular weight marker ran parallel with proteins during each purification steps in SDSPAGE, target protein molecular weight was calculated with the help of standard curve generated from log molecular weight of standard proteins and their corresponding relative mobility. The protein markers and their corresponding molecular weight are tabulated below.

Table A III (A) Standard proteins and their molecular weight

Protein marker	**Molecular weight (kDa)**
Phosphorylase b	97.4
Bovine Serum Albumin	66
Ovalbumin	43
Carbonic Anhydrase	29
Soyabean Trypsin Inhibitor	20

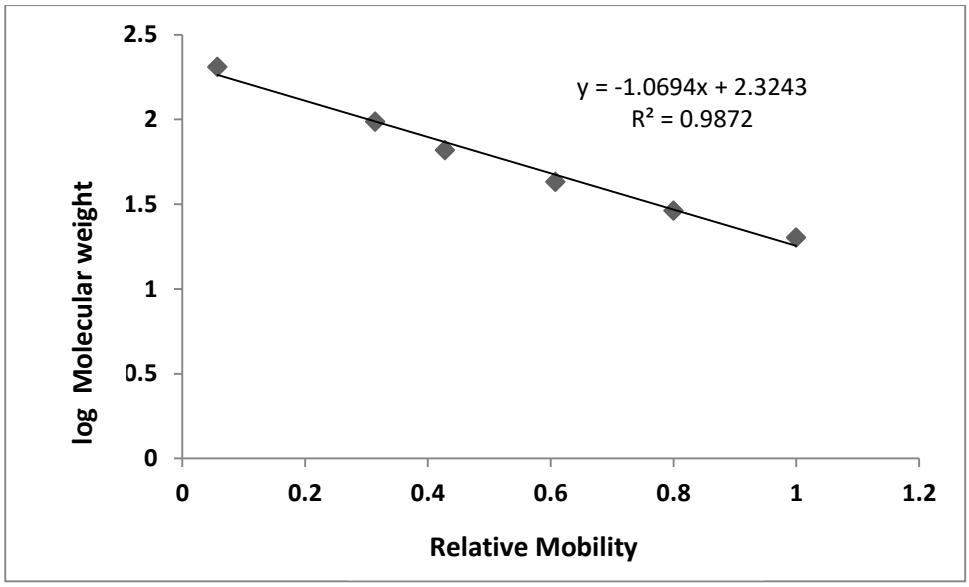

Figure A III (D) Standard curve for molecular weight determination by SDS PAGE

APPENDIX IV

DETERMINATION OF PROTEIN CONTENT BY USING FOLIN-LOWRY METHOD

Reagents

A. 2% Na_2CO_3 in 0.1N NaOH
B. 0.5% $CuSO_4$ in 1% Na-K Tartarate solution
C. Solutions A and B are mixed in the ratio 50:1 just before use.
D. Commercially available Folin-Ciocalteau reagent diluted with distilled water in the ratio 1:1 just before use.

To 1 ml of sample, 5.5 ml of solution C was added and incubated at room temperature for 10 minutes followed by the addition of 0.5 ml of solution D and it was incubated in the dark for 30 minutes.

SDS-PAGE OF PROTEINS FOR THE DETERMINATION OF THE MOLECULAR WEIGHT

SDS-PAGE is a routine method used for qualitative characterization of proteins in biological preparations and for the estimation of protein molecular weight.
A strong anionic detergent sodium dodecyl sulphate (SDS) is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured protein binds to SDS and become negatively charged while exhibiting a consistent charge to mass ratio. The SDS – polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

Assembling the gel moulding cassette for the SDS-PAGE

The glass plates along with the comb and spacers were cleaned thoroughly and rinsed with deionised water followed by ethanol and was dried under dust free conditions. The glass plates were aligned in the holders while achieving a leak proof condition.

Preparation of the gel

The resolving gel was prepared according to the recipe mentioned in section 2 below and the contents were mixed thoroughly before dispensing it into the gap assembled glass cassette with care as to not form any air bubbles. Enough space was left for casting the stacking gel and water was poured over the edge of the gel to prevent dehydration of the gel. The gel was left undisturbed in a vertical position for polymerization to occur.

The stacking gel was prepared according to the recipe mentioned in section 3 below and mixed thoroughly and carefully poured till the edge of the casting gel without the formation of any air bubble. The comb was inserted carefully into the stacking gel. The gel was left undisturbed in a vertical position for polymerization to occur.

Mounting the gel in the electrophoresis apparatus and electrophoretic separation

After the polymerization was complete, the comb was carefully removed from the polymerized gel and the wells were washed with the SDS-PAGE running buffer (section 4) to remove any unpolymerized acrylamide. The gel assembly was removed from the clamp and placed onto the electrophoresis unit. The electrophoresis buffer was added to the unit till the edge of the gel cassette was completely immersed in it. The samples were loaded into the wells ensuring that the samples did not overflow from the respective wells. The electrical connections were made according to the manufacturer's specification.

The electrophoresis was performed at a constant voltage of 50 V till the dye front reached the end of the gel. The process was stopped once the dye front reached the edge of the gel and the assembly was removed from the unit. The glass cassette containing the gel was separated and the gels were stained.

Preparation of the samples (under denaturing and non-reducing conditions)

Before loading the sample on the SDS-PAGE, 50-100 μL of the sample buffer with an equal volume of each sample was mixed and denatured by heating at 100 ˚C for 3 min.

Silver nitrate staining

The gel after electrophoresis of samples was stained by silver nitrate method as described in next section.

Composition of gels and buffers used for SDS-PAGE

1. **Acrylamide-bis –acrylamide solution**
    - 30% acrylamide
    - 0.8% bisacrylamide

2. **Resolving gel (10%), 20 mL**
    - 6.6 mL Acrylamide-bis –acrylamide solution
    - 5 mL 1.5 M Tris- HCl, pH 8.8
    - 0.2 mL 10% SDS
    - 0.1 mL 10% APS
    - 10 μL TEMED
    - Rest deionised water

3. **Stacking gel (5%), 10 mL**
    - 1.66 mL Acrylamide-bis –acrylamide solution
    - 2.5 mL 0.5 M Tris- HCl, pH 6.8
    - 0.1 mL 10% SDS
    - 0.05 mL 10% APS
    - 4 μL TEMED
    - Rest deionised water

4. **Running buffer (0.025M Tris pH 8.3, 0.192 M glycine, and 0.1 % SDS)**
    - 3g tris
    - 14.4 g glycine
    - 10mL 10% SDS

The reagents were mixed in 500 mL distilled water and made up the final volume to 1 liter after adjusting the final pH to 8.3 using conc. HCl.

5. **Sample buffer, 2X (20 mL)**
    - 5 mL stacking gel buffer
    - 8 mL 10% SDS
    - 4mL Glycerol
    - 0.8 mL 1% Aqueous bromophenol blue
    - Rest distilled water

SILVER NITRATE STAINING METHOD

1. The gel was fixed in 10% methanol, 10% acetic acid for 30 minutes.
2. The gel was washed 4 times in water for 5 minutes per wash.
3. The gel was incubated in 0.02% sodium thiosulfate solution for one minute.
4. The gel was again washed 3 times quickly with water.
5. 0.2% silver nitrate solution was used to impregnate the washed gel from step 4 for 20 minutes.
6. The gels was then immersed in a tray containing water for 1 minute and quickly transferred into developer solution (10 g potassium carbonate, 20 ml sodium thiosulfate solution from step 3, and 250 µl 40% formaldehyde in 500 ml).
7. When adequate degree of staining was achieved, the gel was transferred to the Tris stop solution (4% w/v Tris and 2% Acetic acid) for atleast 30 minutes.
8. Finally the gel was washed again in water and stored in water for several days.

APPENDIX V

ENZYME ACTIVITY AND RELATIVE ENZYME ACTIVITY OF OXALATE OXIDASE (OXO)

Table A V (A) Effect of various sugars in medium on biomass growth and Oxalate Oxidase activity

Carbon sources	Enzyme activity (U/mL)	Biomass (g/L)
Sodium oxalate	0.06733	0.304
Sodium oxalate +Sucrose	0.12833	0.826
Sodium oxalate +Glucose	0.08833	0.819
Sodium oxalate + Glycerol	0.09667	0.31133

Table A V (B) Effect of sucrose concentration on the Oxalate Oxidase activity

Sucrose Concentration (mM)	Enzyme activity (U/mL)
20	0.128
40	0.125
60	0.0955

Table A V (C) Effect of various nitrogen sources in medium on Oxalate Oxidase production

Nitrogen sources	Enzyme activity (U/mL)
Ammonium Chloride	0.12833
Sodium Nitrate	0.00767
Yeast extract	0.03733
Peptone	0.02833
Casein Hydrolysate	0.1

Table A V (D) Effect of NH_4Cl Concentrations on Oxalate Oxidase activity

Ammonium Chloride Concentration (mM)	Enzyme activity (U/mL)	Time of Peak Enzyme activity (h)
2	0.128	48
4	0.128	65
6	0.128	84
8	0.065	84

Table A V (E) Effect of Mn^{2+} ions on the activity of the Oxalate Oxidase

Manganese concentration (g/L)	Enzyme activity (U/mL)
0.025	0.10333
0.05	0.15033
0.075	0.095
0.1	0.08

Table A V (F) Effect of fill up volume of the culture flask on the growth of the microorganism and activity of Oxalate Oxidase

Fill up volume	Enzyme activity (U/mL)	Biomass (g/L)
One-half	0.06333	0.4595
One-fourth	0.23667	7.25
One-fifth	0.33333	8.08
one-tenth	0.48333	9.08

Table A V (G) Effect of pH on the growth of the organism and activity of the enzyme Oxalate Oxidase

pH	Enzyme activity (U/mL)	Biomass (g/L)
5	0	0.63333
6.5	0.493	8.78667
7.5	0.125	5.90067
8	0.20	7.5

Table A V (H) Effect of temperature on the growth of the organism and activity of the enzyme Oxalate Oxidase

Temperature	Enzyme activity (U/mL)	Biomass (g/L)
25°C	0.05183	5.45833
30°C	0.48333	8.23133
35°C	0.49833	7.79867

Table A V (I) Effect of agitation speed on the growth of the organism and activity of Oxalate Oxidase

RPM	Enzyme activity (U/mL)	Biomass (g/L)
150	0.05183	8.21467
175	0.48333	9.49067
200	0.49833	10.734

Table A V (J) Time course study of Oxalate Oxidase production in optimized medium and conditions

Incubation Period	Oxalate Oxidase activity (U/mL)
0	0
12	0.027
24	0.117
36	0.202
48	0.26
60	0.456
65	0.503
72	0.420
84	0.253
96	0.213

Table A V (K) Effects of temperature on the stability of the purified Oxalate Oxidase

Temperature	Relative activity (%)			
	Time (h)			
	0.5	**2**	**4**	**6**
4°C	100.20	96.47	102.23	98
30°C	100.33	100.45	105.43	98.66
60°C	102.32	105.21	98.27	101.66
80°C	105.54	98.33	10.09	100.33
85°C	100.33	95.17	80.05	70
100°C	40.45	30.43	20.26	14

Table AV (L) Effect of temperature on the activity of the purified Oxalate Oxidase

Temperature	Enzyme activity (U/mL)	Relative activity (%)
25°C	0.108	27.33
30°C	0.156	39
45°C	0.372	93.33
50°C	0.376	94.33
60 °C	0.392	98.33
80°C	0.41	102.66
85°C	0.384	96.66
100°C	0.06	15.33

Table A V (M) Effects of pH on the stability of the purified Oxalate Oxidase

pH	Relative activity (%)			
	Time (h)			
	0.5	**2**	**4**	**6**
2.2	100.05	100.17	95.5	95
4	100.32	100.23	88.4	84
6	100.45	100.29	80.37	75
8	100.23	100.21	65	62.5
10	60	50.86	25	23

Table A V (N) Effects of pH on the activity of the purified Oxalate Oxidase

pH	Enzyme activity (U/mL)	Relative activity (%)
3	0.308	77
3.4	0.336	84
3.6	0.36	90
3.8	0.4	100
4.0	0.36	90
4.2	0.34	85
5	0.252	63
5.5	0.24	60
6	0.208	52.33

Table A V (O) Data for Lineweaver – Burk Plot (1/S vs 1/V)

Substrate concentration,S (mM)	Enzyme activity,V (U/mL)	1/[S]	1/[V]
0.093	0.0625	10.75	16
0.15	0.1	6.66	10
0.186	0.125	5.37	8
0.373	0.1875	2.68	5.33
0.74	0.2	1.35	5
1.45	0.25	0.689	4
2	0.285	0.5	3.5
2.9	0.375	0.344	2.66
5.8	0.375	0.172	2.66
11.6	0.375	0.086	2.66

Table A V (P) Effect of substrate analogue, glycolate on Oxalate Oxidase

Glycolate concentration (mM)	Enzyme activity (U/mL)	Relative activity (%)
0.1	0.375	100
0.5	0.349	93.33
1	0.38	101.33
3	0.371	99

Table A V (Q) Effect of sodium sulphite on Oxalate Oxidase

Sulphite concentration (mM)	Enzyme activity (U/mL)	Relative activity (%)
0.01	0.1875	50.33333
0.1	0.190	50.9
1	0.126	34

Table AV (R) Effect of sodium chlorate on Oxalate Oxidase

Chlorate concentration (mM)	Enzyme activity (U/mL)	Relative activity (%)
0.1	0.167	44.77
1	0.169	45.26
5	0.168	45.03

Table A V (S) Effect of Lignin model compound Vanillin on Oxalate Oxidase

Vanillin concentration (mM)	Enzyme activity (U/mL)	Relative activity (%)
0.1	0.073	19.76
1	0.0718	19.16
5	0.049	13.33

Table A V (T) Effect of 5, 5-dithio-bis-(2-nitrobenzoic) acid (DTNB) concentration and reaction time on the activity of Oxalate oxidase(OxO) of *Ochrobactrum intermedium* CL6

Concentration of DTNB		Pre incubation Time (min)			
		10	**20**	**30**	**40**
0.2 mM	**Enzyme activity (U/mL)**	0.4	0.36	0.4	0.36
	Relative activity (%)	100	90	100	90
0.4 mM	**Enzyme activity (U/mL)**	0.45	0.46	0.44	0.448
	Relative activity (%)	112.5	115	110	112
0.6 mM	**Enzyme activity (U/mL)**	0.424	0.44	0.4	0.4
	Relative activity (%)	106	110	100	100
0.8 mM	**Enzyme activity (U/mL)**	0.36	0.36	0.34	0.4
	Relative activity (%)	90	90	85	100

Table A V (U) Effect of diethylpyrocarbonate (DEPC) concentration and reaction time on the activity of Oxalate Oxidase

Concentration of DEtPC		Pre incubation Time (min)		
		30	60	90
1 mM	Enzyme activity (U/mL)	0.4	0.36	0.4
	Relative activity (%)	100	90	100
5 mM	Enzyme activity (U/mL)	0.6	0.58	0.56
	Relative activity (%)	150	145	140
10 mM	Enzyme activity (U/mL)	0.8	0.8	0.74
	Relative activity (%)	200	200	185
15 mM	Enzyme activity (U/mL)	0.8	0.84	0.76
	Relative activity (%)	200	210	190

Table A V (V) Effect of 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) concentration and reaction time on the activity of Oxalate Oxidase

Concentration of EDAC		Pre incubation Time (min)		
		30	60	90
10 mM	Enzyme activity (U/mL)	0.4	0.2	0.2
	Relative activity (%)	100	50	50
30 mM	Enzyme activity (U/mL)	0.1	0.1	0.048
	Relative activity (%)	25	25	12
60 mM	Enzyme activity (U/mL)	0.1	0.1	0.008
	Relative activity (%)	25	25	2
100 mM	Enzyme activity (U/mL)	0.008	0.016	0.008
	Relative activity (%)	2	4	2

Table A V (W) Effect of N-bromosuccinimide (NBS) concentration and reaction time on the activity of Oxalate Oxidase

Pre incubation Time (min)	Concentration of NBS					
	0.01 mM 0.02		0.025 mM		0.05 mM	
	Enzyme activity (U/mL)	Relative activity (%)	Enzyme activity (U/mL)	Relative activity (%)	Enzyme activity (U/mL)	Relative activity (%)
15	0.3	75	0.15	37.5	0.14	35
30	0.25	62.5	0.15	37.5	0.12	30
90	0.1	25	0.05	12.5	0.048	12

Table A V (X) Effect of sulfonyl fluoride (PMSF) concentration and reaction time on the activity of Oxalate Oxidase

Concentration of PMSF		Pre incubation Time (min)		
		30	**60**	**90**
1 mM	**Enzyme activity (U/mL)**	0.4	0.388	0.4
	Relative activity (%)	100	97	100
5 mM	**Enzyme activity (U/mL)**	0.38	0.42	0.412
	Relative activity (%)	95	105	103.5
15 mM	**Enzyme activity (U/mL)**	0.408	0.42	0.392
	Relative activity (%)	102	105	98
30 mM	**Enzyme activity (U/mL)**	0.4	0.412	0.392
	Relative activity (%)	100	103	98

Table A V (Y) Physicochemical properties of treated (ET) and untreated (NT) Taro starch

Water binding capacity (%)			
Samples	65°C	80°C	100°C
ET	282.33±2.33	551±15.57	673.83±26.07
NT	413.33±10.03	727±13.86	869.33±24.77
Swelling power (g/g)			
ET	3.19±0.153	3.43±0.233	15.32±0.376
NT	4.06±0.144	8.32±0.230	20.66±0.837
Solubility (%)			
ET	9.02±0.268	9.56±0.348	21.66±0.66
NT	3.03±0.257	17.66±1.76	22.66±1.45
	% Amylose content	**Paste clarity**	**Granule size (µm)**
ET	7.52±0.049	9.38±0.198	1-2
NT	6.81±0.083	10.44±0.79	1-2

APPENDIX VI

HPLC CHROMATOGRAM

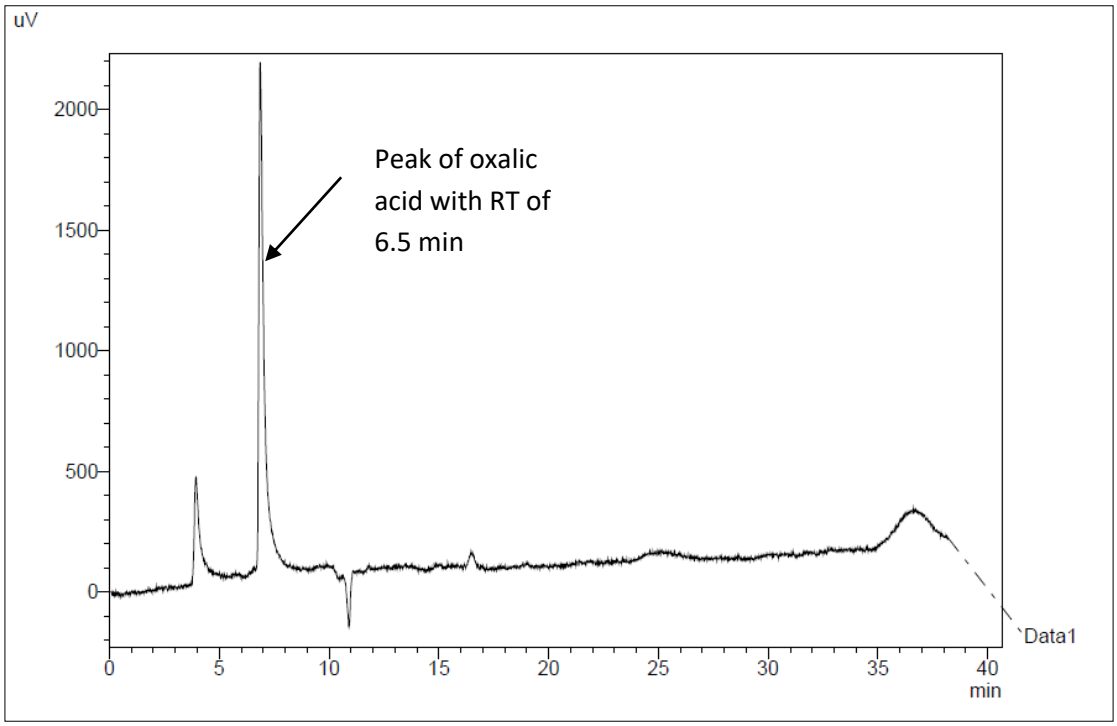

Figure A VI (A) Chromatogram of standard oxalic acid

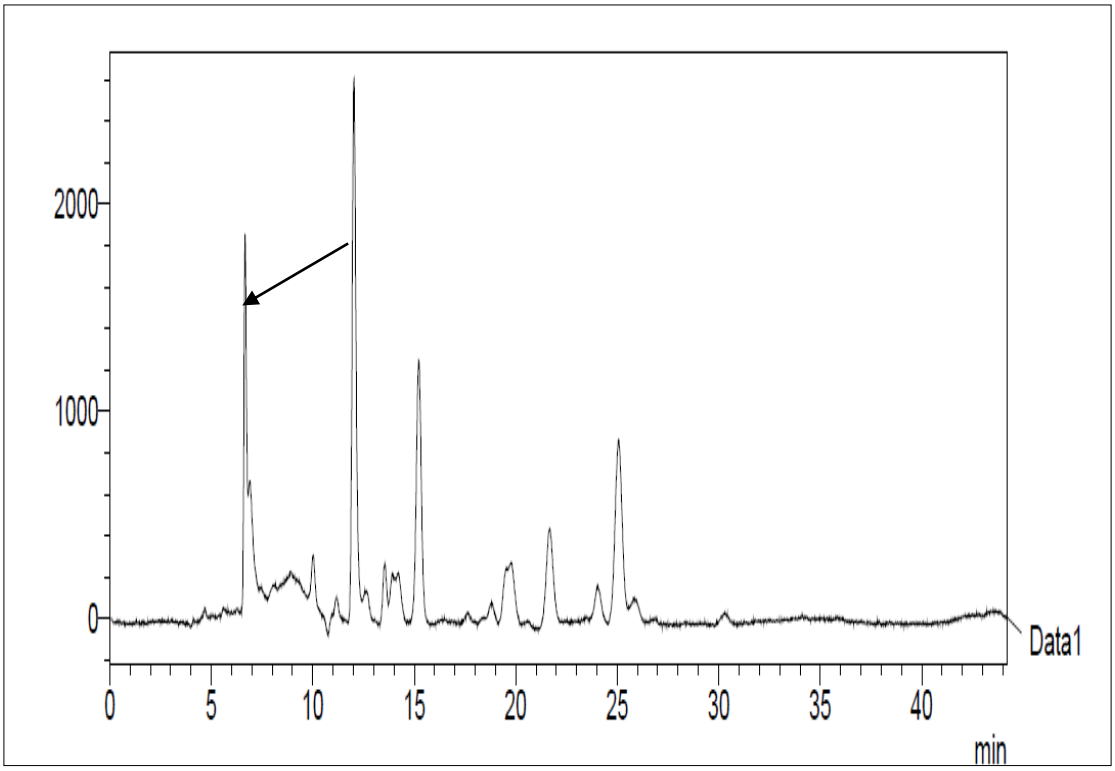

Figure A VI (B) Chromatographic separation of samples made out from Taro flour. Arrow indicates the peak of oxalic acid eluted out from the column.

LIST OF PUBLICATIONS BASED ON THIS RESEARCH WORK

1. Kumar, K. and Belur, P. D. (2016). “A new extracellular thermostable oxalate oxidase produced from endophytic*Ochrobactrumintermedium* CL6: Purification and biochemical characterization.” *Prep. Biochem and Biotechnol.*, 46 (7), 734-739. (Taylor and Francis Journal)
2. Kumar, K. And Belur, P. D. (2016). “Production of oxalate oxidase from endophytic *Ochrobactrum intermedium* CL6.”*Indian J. Biotechnol.*, (Accepted)
3. Kumar, K. And Belur, P. D. (2017). “A new insight of *Ochrobactrum intermedium* CL6 Oxalate oxidase catalytic prowess through Chemical modification.” *Asian J. Biochem.*, DOI: 10.3923/ajb.2017. (Early print view).

MANUSCRIPT UNDER REVIEW

1. Kumar, K. And Belur, P. D. (2016). “A novel enzymatic process to produce oxalate depleted starch from Taro and study of its physico-chemical properties.”*J. Food Sci. and Technol.*(A Springer Journal)

CONFERENCE PROCEEDINGS

1. Kumar, K. and Belur, P.D. (2015). “A novel oxalate oxidase from an endophytic bacterium exhibits unique solubility property in saturated ammonium sulphate solution.” *International Conference on New Horizons in Biotechnology (NHBT2015)*, NIIST, Trivandrum, India (Nov 22-25).

2. Kumar, K. And Belur, P. D. (2014). “Studies on Oxalate oxidase from endophytes.” National Conference on Empowering Mankind with Microbial Technologies (EMMT 2014), TNAU, Coimbatore, India (Nov 12-14).

BIO-DATA

Kunal Kumar

Email: kumarkunal123@gmail.com
Mobile No: 9740643293

DOB: 4th Jan, 1982

Permanent address: B/3 New Dalma View Colony
Road no : 7 extension, Sonari
Jamshedpur Pin: 831011
Jharkhand

Educational Qualification:

- **Ph.D (8.6/10), 2012-2017**
  National Institute of Technology Karnataka Surathkal,
  Srinivasnagar Post, Mangalore, Karnataka

- **M.Tech in Biotechnology (8.63/10), 2006-2008**
  VIT University, Vellore, Tamil Nadu

- **M.Sc in Zoology (63.25%), 2003-2005**
  Jamshedpur Co-operative College, Jamshedpur, 572106,
  Ranchi University, Ranchi, Jharkhand

- **B.Sc in Zoology (69%), 2000-2003**
  Jamshedpur Co-operative College, Jamshedpur, 572106,
- Ranchi University, Ranchi, Jharkhand

Research papers published and accepted:

- Kumar, K. and Prasanna, D. B. (2016). "A new extracellular thermostable oxalate oxidase produced from endophytic *Ochrobactrum intermedium* CL6: Purification and biochemical characterization". *Preparative Biochemistry and Biotechnology*, 46 (7), 734-739
- Kumar, K. and Prasanna, D. B. (2015). "Production of oxalate oxidase from endophytic *Ochrobactrum intermedium* CL6". *Indian Journal of Biotechnology*(Accepted)

- Kumar, K. and Prasanna, D. B. (2016). "A new insight of *Ochrobactrum intermedium* CL6 Oxalate oxidase catalytic prowess through Chemical modification". *Asian Journal of Biochemistry* (Early print view)

- Siva, R., Kumar, K. and Rajasekaran, C. (2013). "Genetic diversity study of important Indian rice genotypes using biochemical and molecular markers."*African Journal of Biotechnology* 12 (10), 1004-1009.

Books Chapter

- Siva, R., Kumar, K. (2009). "Application of Computer softwares in Molecular Systematics." In: Plant Genetic Diversity, A molecular approach.

Research papers Communicated:

- Kumar, K. and Prasanna, D. B. (2016). "Enzymatic removal of oxalate content in Taro starch and study of its physico-chemical characteristics*"Journal of Food Science and Technology.*

Conference Proceedings:

- **Presented a poster entitled.** "A novel oxalate oxidase from an endophytic bacterium exhibits unique solubility property in saturated ammonium sulphate solution." **International Conference on New Horizons in Biotechnology (NHBT 2015) held on 22-25, Nov 2015 at Trivandrum, Kerala.**

- **Presented a poster entitled.** "Studies on Oxalate oxidase from endophytes." **National Conference on Empowering Mankind with Microbial Technologies (EMMT 2014) held on 12-14, Nov 2014 at TNAU, Coimbatore, Tamil Nadu.**

Experience

- 4 years of teaching experience in the post of Asst. Professor in the Department of Biotechnology. V.M.K.V Engineering College, Salem, Tamil Nadu.

- Organized a two day National Symposium on Application of Biotechnology in Medical Field- current scenario and Future prospects **fundedby DBT, DRDO, CSIR- New Delhi and TNSCST- Chennai** on September 2011.

Area of Interests

Bioprocess technology, Downstream Processing of bioproducts, Fermentation Technology, Enzyme Technology

Laboratory skills

- Sound knowledge in molecular biology techniques , DNA isolation methods; Polymerase chain reaction; Protein profiling study by SDS PAGE; Isozyme Analysis; DNA Fingerprinting analysis by RAPD techniques.
- Practical knowledge on Enzyme extraction and purification, **HPLC, FPLC, Labscale bioreactor, Fermentation Technology.**
- Knowledge of immunotechnology methods like ELISA, Western blotting, Immunohistochemistry.
- Ability to analyse the level of Genetic diversity with the help of softwares like NTSYS.
- Dissection of vertebrate and invertebrate animals & permanent slide preparations.
- **Experience in handling Bio-Chemistry lab, Downstream processing lab and Chemical Engineering lab**.

Training, Seminar, Symposium, Conferences attended and Paper Presented

- Attended two weeks Faculty Development Program on Entrepreneurship at R.V. College of Engineering, Bangalore from 9th Jan 2012 to 21st Jan 2012.
- Participated in a workshop organized by Indian Association of Biomedical Scientist at Punjab University, Chandigarh on 10th- 12th Nov 2011.
- Presented a paper on "Antimicribial activity of *Rosa indica*" in the National Symposium on Application of Biotechnology in Medical Field held at the VMKV Engg. College Salem Tamil Nadu on 28th and 29th Sept 2011.
- Presented a paper on "Isozyme forms of Indian rice varieties" in the National Seminar on Application of Biotechnology in Human Welfare held at the VMKV Engg. College Salem Tamil Nadu on 14th Oct 2009.

- Presented a paper on "Effect of food additives on the activity of the enzyme Amylase" in the National Seminar on Challenges in Biotechnology held at the VMKV Engg. College Salem Tamil Nadu on 28th March 2009.

- Participated in National workshop on Three Dimensional structure determination of Drug like molecules using X- Ray Crystallography, Sponsored by DBT, Govt of India, January 2009.

- Participated in a National seminar on Protein engineering at VIT university Feb 2007 .

- Participated in Lecture cum workshop on 'Environmental Hazards' at Jamshedpur womens college on 14th January 2004.

Declaration:

I hereby declare that all the information mentioned above is true to the best of my knowledge.

Kunal Kumar