Characterization of a novel Thermostable Caffeine dehydrogenase from *Pichia manshurica* strain CD1 isolated from Kombucha tea

Thesis submitted to

Jadavpur University

By

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JU Index No.: 21/18/Life Sc. /25

In the partial fulfilment of the requirements for the degree of

Doctor of Philosophy (Ph.D.) in Science

Department of Life Science &Biotechnology

Jadavpur University, Kolkata-700032, India

2023

যাদবপুর বিশ্ববিদ্যালয়
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Certificate from the supervisor

This is to certify that the thesis entitled **"Characterization of a novel Thermostable Caffeine dehydrogenase from *Pichia manshurica* strain CD1 isolated from Kombucha tea"** submitted by Rubia Parvin who got her name registered on 05.02.2018 with registration number [Index No.: 21/18/Life SC/25] for the award of **Ph.D. (Science)** degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Prof. Ratan Gachhui** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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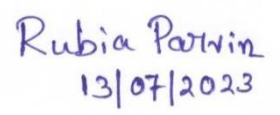
Declaration certificate by the candidate

I declare that the thesis entitled **"Characterization of a novel Thermostable Caffeine dehydrogenase from *Pichia manshurica* strain CD1 isolated from Kombucha tea"** Submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of **Prof. Ratan Gachhui** has not formed the basis for the award of any degree, diploma, fellowship, titles in this or any other University or other institution of Higher learning. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.



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Dedicated to ….

My family

Acknowledgements

I am very glad to have this opportunity to express my thanks to all of the people who have been part of my Ph. D. I thank Almighty *Allah* for giving me the opportunity to pursue research, for all the blessings and also for the courage given to me to withstand all difficulties and hardships.

I wish to thank my supervisor Dr. Ratan Gachhui, Professor, Department of Life Science and Biotechnology for his unending support, valuable guidance, and outstanding motivation during my Ph. D. years and for giving me the opportunity to work on interesting research topics. He always gave me considerable freedom in choosing the direction of my research and provided a lot of useful hints to improve my work and has always been encouraging when difficulties arose.

Besides my supervisor, I would like to thank my Research Advisory Committee Member Dr. Joydeep Mukherjee, School of Environmental Studies, Jadavpur University for his timely guidance, suggestions and encouragement throughout my work.

My special thanks to the faculty members of our department Prof. Parimal Karmakar, Prof. Biswadip Das, Dr. Paltu Kr. Dhal, Dr. Arunima Sengupta, Dr. Arghya Adhikary, and Dr. Sougata Roy Chowdhury for their whole hearted and unfailing assistance throughout my research work.

I am always grateful to all nonteaching staff, librarian of this department for their time and support at the various phases of my Ph. D.

I like to extend my sincere thanks to all my seniors, Writachit Chakraborty, Semantee Bhattacharya, Soumyadev Sarkar, and Avishek Mukherjee. I express my sincere thanks to all my lab mates, Jhilam Majumdar, Rinky Bhadra, Sayanta Sarkar, Bidhan Roy, and Sontu

Haldar. I have admired Jhilam di and Rinky di for their never-ending support and enthusiasm during the most difficult times. It has been a pleasure to work and sharing lab with you. Your cheerful accompany have provided a friendly environment inside and outside the laboratory, and made this journey smooth.

I warmly thank to my friends, Debbithi Bera and Arunima Mondal for their mental support and encouragement whenever I was distraught with the failures in my research work.

I would also like to thank the other research fellows of our department: Pranamita kunda, Debjit Dey, Tilak Nayak, Bhaswar Nandi, Sunirmal Paira, Shreya Das, Indraneel Sengupta and Gargi Das for their friendship.

Finally, I would like to thank my family members, who witnessed every step of the way and provided me support and confidence in all aspects to let me accomplish my Ph. D. I owe my deepest gratitude to my parents, Mr. Rabiul Haque (Papa) and Mrs. Monjura Begam (Maa) for their love, prayers, support and encouragement that kept me going through the past years. I would also like to acknowledge my sisters Priya and Mim for their love and support. I know only "thanks" can't express my feelings.

I would like to especially thank to my husband, Dr. Sahel Md. Delabul Hossain for his love, support and encouragement. He is always with me in all times, and his unconditional faith and blind trust in me have made my journey easier. In this whole life, I am so lucky having him beside me.

My parents, husband, and siblings form the backbone of my happiness and I dedicate my thesis to them.

Rubia Parvin
13/07/2023

(Rubia Parvin)

List of abbreviations

°C	:	Degree Celsius
%	:	Percent
A	:	Absorbance
ANOVA	:	Analysis of variance
APS	:	Ammonium per sulphate
ATCC	:	American Type Culture Collection
BLAST	:	Basic Local Alignment Search Tool
BP	:	Base Pair
BSA	:	Bovine Serum Albumin
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy nucleotide triphosphates
DTT	:	Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic acid
Fig	:	Figure
H	:	Hour
HPLC	:	High Performance Liquid Chromatography
IPTG	:	Isopropyl β-D thiogalactoside
ITS	:	Internal Transcribed Spacer
kDa	:	Kilo Dalton
mL	:	Mililitre
mM	:	Milimolar
µL	:	Microlitre
NBT	:	Nitro blue tetrazolium chloride
OD	:	Optical Density

PAGE	:	Polyacrylamide Gel Electrophoresis
PCR	:	Polymerase chain reaction
rpm	:	Rotation perminute
SDS	:	Sodium dodecyl sulphate
SEM	:	Scanning electron microscopy
sp.	:	Species
TEMED	:	Tetramethylethylenediamine
UV	:	ultra violet
X-Gal	:	5-bromo 4-chloro 3-indolyl β-D thiogalactoside
YPD	:	Yeast extracts peptone dextrose

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Abstract

Caffeine, an alkaloid purine (1,3,7-trimethyl xanthine), is one of the important ingredients of many popular beverages, especially tea and coffee. Excessive consumption of caffeine through beverages can be dangerous. It is a well-known central nervous system stimulant. Moreover, caffeine-contaminated waste has a negative impact on the environment. Awareness of these side effects has increased the demand for decaffeination of both foods and waste products.

Kombucha tea is a beverage produced by fermenting sugared black tea with a consortium of yeasts and acetic acid bacteria for about 7-14 days. In the present study, we observed a reduction in caffeine content during the course of the fermentation of kombucha tea which is mainly attributed to the activity of the microorganisms with the help of enzymes. Caffeine degrading strain CD1 was isolated from the Kombucha biofilm and was later purified and identified as *Pichia mansurica* strain CD1. It was able to grow in caffeine broth when there was no source of nitrogen and carbon and degrade the caffeine by producing some novel enzymes through the enzymatic pathway.

A novel thermostable caffeine dehydrogenase enzyme was isolated and purified from *Pichia manshurica* strain CD1. With further characterization it was found that the caffeine dehydrogenase was a monomer of approximately 85 kDa and had optimal activity at pH 7.5. The enzyme also exhibits high activity and stability at a wide range of temperatures (30 °C to 100 °C). A Comprehensive analysis was carried out to monitor the thermodynamic parameters (E_a, ΔG, ΔH, and ΔS) of the enzyme caffeine dehydrogenase. The conformational changes of caffeine dehydrogenase in both its native and thermally treated states were

investigated by circular dichroism and intrinsic tryptophan fluorescence spectroscopy. Applications of thermostable enzymes are also becoming more popular because they are less harmful to the environment than chemical methods. The outcomes of this study should be highly beneficial to biotechnological processes like waste treatment, biocatalysts for decaffeination and the development of biosensors.

CHAPTER I

Introduction & Review of Literature

1.1. Caffeine

The purine alkaloid caffeine (1,3,7-trimethylxanthine) is a naturally occurring chemical that is found in many different plant species, including seeds, tea, coffee, cola, cocoa (Ashihara and Crozier 2001). The main modes of entry of caffeine in human systems are through beverages like tea, coffee and caffeinated soft drinks and numerous food products like chocolates and desserts. It is also widely used in pharmaceuticals as an adjuvant to the analgesic actions of aspirin and as a cardiac, neurological and respiratory stimulant as well as a diuretic(Güneş and Demirer 2023). Caffeine is an active psychostimulant which increases alertness and sustains concentration by overcoming fatigue(Lorist and Tops 2003). It boosts our alertness and energy level due to its central nervous system stimulant activity(Nehlig et al. 1992). Although it has many health benefits but consuming too much of it can be dangerous. The global consumption of caffeine ranges from 80 mg to 400 mg per person per day(Reyes and Cornelis 2018). Furthermore, excessive caffeine consumption can lead to anxiety, mutation, cardiovascular disease, liver disease and infertility(Quinlan et al. 2000; Lovallo et al. 2005). Caffeine contaminated waste has an adverse effect on the environment that causes soil infertility, inhibits seed germination, kills microbes and insects and also affects the aquatic life(Gummadi et al. 2011; Fernandes et al. 2017). A majority of coffee waste products like pulp and husk are discarded into the environment, contributing to the pollution of water bodies(Buerge et al. 2003; Chen et al. 2008). For this adverse effects of caffeine, that may leads to the development of a decaffeination process

1.2. Sources and consumption of caffeine

Caffeine, a plant alkaloid, was discovered in the leaves and fruits of many plant species, where it acts as a normal pesticide, killing and paralyzing certain insects that feed on them.

Caffeine and other purine alkaloids are naturally produced by at least 13 different plant orders(Nathanson 1984; Ashihara and Crozier 2001).

Camellia sinensis (tea), *Coffea arabica* (coffee), *Theobroma cacao* (cacao, or cocoa), *Cola nitida* (Cola), *Paullinia cupana* (guarana), and *Ilex paraguariensis* (yerba mate) are among the nearly 100 species in the kingdom(Ashihara and Crozier 2001; Ashihara et al. 2008). Most studies on caffeine-producing plants have focused on species from the genera Coffee and Camellia. Caffeine and other related compounds such as theobromine and theophylline are primarily found in tea and coffee plants. Caffeine sources include guarana and yerba mate plants, which are used in the preparation of caffeinated beverages (cola drinks) and teas in some cases. Guaranine and mateine are two other possible names for caffeine that were derived from guaranine and mateine plants, respectively. Caffeine is also present in some non-alcoholic beverages. Caffeine is also found in foods and beverages such as tea, coffee, yerba mate, guarana, yaupon, yoco, colanuts, and cocoa(Matissek 1997; Saldaña et al. 2000). The caffeine content of different popular foods and beverages and drugs is listed in Table A. Caffeine is the most common psychoactive substance in the world, consumed at a rate of 120,000 tonnes annually, according to estimates. Drinking excessive amounts of caffeine does not cause the effects of withdrawal and devotion such as vomiting, drowsiness, headache, and nausea. It is also linked to a number of health issues, including increased cardiac output, cardiac arrhythmias, irregular muscular activity, and adrenal stimulation, but it also causes symptoms like drowsiness, vomiting, headaches, and nausea(Nehlig 1999; Quinlan et al. 2000). Adenosine monophosphodiesterase is inhibited, osteoporosis develops, and excessive caffeine consumption prevents DNA repairs and inhibition of adenosine mono phosphodiesterase mutation, malformation of foetus during pregnancy which may reduce fertility rates(Mazzafera et al. 1996; Kalmar and Cafarelli 1999; Mazzafera 2002).

Source of Caffeine	**mg/serving**	**mg/OZ**
Coffee		
Decaffinated (10 OZ)	4-15	0.4-1.5
Instant (10 OZ)	9-216	0.9-21.6
Plain, brewed (10 OZ)	128-250	12.8-25
Espresso (5 OZ)	150-450	30-90
Tea		
Tea, brewed (10 OZ)	80-120	8-12
Icea tea (12 OZ)	65-75	5.4-6.3
Green (8 OZ)	30-50	3.8-6.3
Black (8 OZ)	25-110	3.1-13.8
Yerba Mate (8 OZ)	65-130	8-16
Chocolate		
Hot cocoa (5 OZ)	4	0.8
Chocolate milk (6OZ)	4	0.7
Milk chocolate (1.5 OZ)	9	6
Chocolate bar (1.5 OZ)	30	20
Soft Drinks		
Coca Cola (12 OZ)	35	2.9
Pepsi (12 OZ)	40	3.3
Dr. pepper (12 OZ)	40	3.3
Mountain Dew (12 OZ)	55	4.6
Energy Drinks		
Monster (16 OZ)	160	10
Rockstar (16 OZ)	160	10
Red Bull (8.3 OZ)	80	9.6
Amp (8.3 OZ)	75	8.9
Drugs		
Anacin (2 tablets)	32	N/A
Esedrin (1 tablet)	65	N/A

Source: Table adapted from (Summers 2011)

Table A: Concentration of caffeine in various beverages and drugs

1.3. Caffeine degradation and its necessity

Now-a-days the use of decaffeinated food products has increased because of their many negative effects on human health. Decaffeination is a necessary step in coffee processing to reduce the caffeine content in food products and also for the treatment of caffeine containing effluents that are toxic to environment. Solvent extraction, water decaffeination and carbon dioxide oxidation were earlier used expensive methods for decaffeination(Dufresne and Farnworth 2000). In water decaffeination method, first caffeine is extracted in water and then removed from water by solvent extraction process. Caffeine laden solvent has been made free from caffeine by various processes which include evaporation, charcoal addition, filtration, centrifugation and crystallization. Sometimes supercritical fluids have also been used as solvents. But these conventional methods are toxic and non-specific to caffeine. Furthermore, waste products of toxic solvent in convectional decaffeination method require additional treatment. To overcome these problems, a biocompatible and efficient technique is required. Nanoparticle-based, adsorption-mediated, or microorganism-mediated decaffeination methods are becoming more popular because of their efficiency and low cost(Gokulakrishnan et al. 2005). A number of microbial strains have been identified for microorganism-based decaffeination process. The potential use of micro-organisms and enzymes obtained from microbial system for developing biological decaffeination techniques offer a much attractive alternative to the present existing techniques(Ogunseitan 1996; Mazzafera et al. 1996).

1.4. Environmental relevance in caffeine degradation

Apart from health effects, caffeine degradation is important from environmental point of view. Caffeine is one of the major industrial wastes in the coffee and tea industries. Release of the caffeine containing wastes like coffee pulp, husk and tea waste in the soil and later into

the nearby water sources (rivers and lakes) pollutes the surrounding landmass and water bodies. The presence of caffeine in soil affects soil fertility by inhibiting seed germination and growth of seedlings(Friedman and Waller 1983; Fernandes et al. 2017). Disposal of coffee and tea processing industrial wastes into water bodies makes drinking water non-potable (Buerge et al. 2003; Moore et al. 2008; Chen et al. 2008). Even, this causes damage to the aquatic life. Reports showed that caffeine is toxic to most of the aquatic organisms like *Ceriodaphnia dubia* (water flea) and coral algae. It has also been observed that no zebrafish embryos could survive and exhibited significantly reduced tactile sensitivity frequencies of touch-induced movement when the water body is polluted with caffeine (Schwimmer et al. 1971). Moreover, coffee and tea pulps are rich in nutritional compounds such as carbohydrates and proteins and thus have good biotechnological potential. But due to the presence of anti-nutritional factors like caffeine, tannins, etc. they could not be used as animal feed or for the production of organic fertilizers and biogas. Therefore, decaffeination of the by-products becomes a very necessary step in treatment of coffee and tea wastes.

1.5. General catabolism of caffeine

Caffeine (1,3,7-trimethylxanthine) is the most common naturally occurring alkyl purine, followed by theobromine (3,7-dimethylxanthine). Other intermediates of caffeine catabolism found in nature include theophylline (1,3-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, and 7-methylxanthosine.However, removing the three methyl groups in caffeine metabolism is extremely difficult, resulting in xanthine formation. Since then, a number of tracer experiments using ^{14}C-labeled purine alkaloids(Vitória and Mazzafera; Ashihara et al. 1997; Mazzafera 1998) have been reported, demonstrating that the major metabolic pathway of caffeine is caffeine - theophylline - 3-methyxanthine - xanthine - uric acid - allantion -

allantoic acid - purine catabolic pathway and is metabolised to CO2 and NH4 (Stasolla et al. 2003; Zrenner et al. 2006).

1.6. Degradation of caffeine using microbial method

Studies on caffeine degradation by micro-organisms were not reported till 1970 probably because caffeine was regarded as toxic to bacteria(Kihlman 1974). Microbial decaffeination, mainly including bacteria and fungi, was first reported in the early 1970s(Kurtzman and Schwimmer 1971). Since then researchers from all over the world are in search of microbes capable of breaking caffeine and utilizing the same as a source of energy It has been observed that some bacteria from the genera of *Pseudomonas* and *Serratia* and the fungi from the genera of *Aspergillus, Penicillium, Rhizopus* and *Stemphyllium* have the potential to metabolize the caffeine by enzymatic conversion(Hakil et al. 1999; Brand et al. 2002; Tagliari et al. 2003; Gutiérrez-Sánchez et al. 2004; Yu et al. 2008; Zhou et al. 2018). The characteristic and mechanism of caffeine biodegradation were investigated in many *Pseudomonas* strains which were isolated from the waste water, normal agricultural land, soil of tea garden and others(Dash and Gummadi 2006; Gokulakrishnan et al. 2007).

In spite of identification of several caffeine catabolising fungal strains, caffeine catabolic pathway in fungi is not elucidated in details. However, more studies have been done on bacterial caffeine degradation pathway. Under aerobic conditions, caffeine is catabolised in bacteria via two major pathways – N-demethylation and C-8 oxidation. In the demethylation pathway, as seen in *Pseudomonas*, caffeine is initially converted into theobromine and paraxanthine by demethylases. There is evidence that there are unique and substrate specific enzymes involved in the caffeine demethylation pathway of bacteria. Odafe Sideso et al.(Sideso et al. 2001) have studied the characterization and stabilization of a caffeine

demethylase enzyme complex from *P. putida*(Summers et al. 2012). Very recently, Summers et al. have identified and characterized novel, highly specific N-Demethylases (NdmA, NdmB and NdmC) isolated from *P. putida* CBB5 that enable the bacteria to live on caffeine and other related purine alkaloids. Yu et al have described coexpression of two distinct N demethylation pathways for caffeine and theophylline degradation in *P. putida* CBB5(Yu et al. 2008, 2009). Madhyastha and Sridhar have observed the catabolism of caffeine by oxidative pathway in a mixed culture consortium containing *Klebsiella* and *Rhodococcus* (Siddharth* et al. 2012). They have also isolated, purified and partially characterized a novel caffeine oxidase enzyme from the cell-free extract of the mixed culture consortium.

1.7. Degradation of caffeine by enzymatic method

For many years, researchers have discussed bio-decaffeination of coffee and tea using whole microbial cells or enzymes(Kurtzman and Schwimmer 1971; Sideso et al. 2001). *Pseudomonas putida* CBB5 can completely decaffeinate coffee and tea extracts, and *Pseudomonas* sp. CBB1 can decaffeinate tea extracts as well(Yu et al. 2009; Summers et al. 2015). In terms of relative efficacy, strain CBB5 used the N-demethylation pathway to degrade more caffeine in less time than strain CBB1 used the C-8 oxidation pathway. Additionally, tea extract was decaffeinated using C-8 oxidation using both batch and continuous processes using an immobilized mixed culture of *Klebsiella* sp. and *Rhodococcus* sp. (Summers et al. 2015). Overall, it appears that the N-demethylation pathway is more efficient than C-8 oxidation for use in microbial decaffeination of coffee. However, due to the possibility of endotoxin release, the use of bacterial cells for bio-decaffeination of beverages may not be feasible. Alternatively, the use of purified caffeine-degrading enzymes (either soluble or immobilized) may provide a viable alternative to eliminating endotoxin problems (Beltrán et al. 2006). caffeine oxidase with a molecular weight of 85kD (Madyastha et al.

1999) purified from a mixed culture of *Klebsiella* sp. and *Rhodococcus* sp. Mohapatra and colleagues (2006) discovered a caffeine oxidase with a molecular weight of 65 kDa in *Alcaligenes* sp(Mohapatra et al. 2006). Both caffeine oxidase enzymes were inactive against theobromine, theophylline, and a few other theobromine analogues. In *Pseudomonas* sp. CBB1, a heterotrimeric caffeine dehydrogenase (Cdh) enzyme was discovered, which catalyzed the C-8 oxidation of caffeine to form trimethyl uric acid(Yu et al. 2008). This 158-kDa protein was a novel quinone-dependent oxidoreductase (EC 1.17.5.2) with no NAD(P)+ activity.

1.8. Application of caffeine degradation

The study of caffeine-degrading micro-organisms began nearly four decades ago, and research in this field is limited. There have been very few studies that have isolated potential caffeine degrading microbial strains. Organisms that can grow successfully on tea and coffee plants have an ability to degrade caffeine. Apart from providing advantages in decaffeination, caffeine degradation by microbial enzymes or bacteria is useful in the production of useful by-products. Understanding the enzymes involved in microbial caffeine degradation could lead to the development of several new biotechnological applications. Among these are biological decaffeination of coffee, tea, and caffeinated plant matter, environmental remediation of high caffeine concentration soils and waters, synthesis of alkylxanthines and alkyl uric acids for use as chemicals or pharmaceuticals, and development of a rapid diagnostic test for caffeine and related methylxanthines. Numerous potential biotechnological applications are now possible thanks to the recent discovery of caffeine degrading enzymes involved in the metabolism of caffeine via both the N-demethylation and C-8 oxidation routes. The production of chemicals, pharmaceuticals, animal feed, and biofuels may all

benefit greatly from the use of these novel genes and enzymes, as well as the remediation of environments that have been contaminated with caffeine.

1.9. Kombucha tea

Kombucha tea is a fermented tea beverage produced by fermenting sugared black tea with Kombucha culture i.e. a consortium of yeasts and acetic acid bacteria for about 7-14 days (Jayabalan et al. 2014). It is composed of two portions- a floating cellulose pellicle layer and the sour liquid broth(Dutta and Gachhui 2007). This beverage is widely consumed in parts of the erstwhile Soviet Union and Central Asia, and has become popular even in Europe and the USA. Kombucha tea is claimed to have many beneficial effects to human health and some of its therapeutic effects have already been proven(Banerjee et al. 2010; Bhattacharya et al. 2011; Aloulou et al. 2012).

1.10. Preparation of Kombucha tea

Black tea and white sugar are the best substrates for the preparation of Kombucha tea, although green tea can also be used. First, tea leaves are added to boiling water and allowed to infuse for about 10 minutes after which the leaves are removed. Sucrose (10%) is dissolved in the hot tea and the preparation is left to cool. This is then acidified by the addition of 10% of old soup. The Kombucha culture is laid on the tea surface, and the jar is carefully covered with a clean cloth and fastened properly. The preparation is allowed to incubate at right temperature for 7-14 days. At the right temperature, the Kombucha culture multiplies continuously. It first spreads over the entire surface of the tea, and then thickens(Dufresne and Farnworth 2000). The sucrose was hydrolyzed to glucose and fructose by yeast invertase and ultimately ethanol is produced. Acetic acid bacteria utilize ethanol to produce acetic acid

and glucose to produce gluconic acid. Part of the glucose was also directed towards biosynthesis of cellulose(Dufresne and Farnworth 2000). Tea provides all the additional components and growth factors required by the Kombucha culture. The stimulating components, caffeine and theophylline, belong to the purine groups which are used as building blocks for nucleic acid synthesis. It could be assumed that the micro-organisms of Kombucha culture use these components as a source of nitrogen, thereby degrading these purine alkaloids. Moreover, earlier studies reported a reduction of caffeine by about 25% during the first two weeks of fermentation of black tea by Kombucha culture(Villarreal-Soto et al. 2018). This beverage is composed of sugars, gluconic, glucuronic, lactic, acetic, malic, tartaric, malonic, citric, and oxalic acids, as well as ethanol, amino acids, water soluble vitamins, antimicrobial matters and some hydrolytic enzymes. It has been reported that the Kombucha beverage helps digestion, gives relief from arthritis, acts as a laxative, prevents microbial infection, helps in combating stress and cancer and vitalizes the physical body, etc.

1.11. Yeast in Kombucha tea

The majority of the bacterial species in Kombucha tea are Acetic Acid Bacteria (Marsh et al. 2014; Jayabalan et al. 2014; Chakravorty et al. 2015, 2016), Lactic Acid Bacteria, and species of *Enterobacter* and *Komagataeibacter* (Marsh et al. 2014; Chakravorty et al. 2015, 2016). The fermentation is mainly carried out by a few key bacteria, *Acetobacter* and *Gluconobacter*. Comparatively, the yeast population is more diverse in Kombucha (Marsh et al. 2014; Chakravorty et al. 2016; Coton et al. 2017). Table B shows the yeast that has been identified from Kombucha tea.

Yeast	Reference
Species belonging to genus *Saccharomyces, Saccharomycodes, Schizosaccharomyces, Zygosaccharomyces, Brettanomyces, Candida, Torulospora, Koleckera, Pichia, Mycotortula and Mycoderma*	(Teoh et al. 2004)
Saccharomyces cerevisiae	(Herrera et al.,1989; Liu et al. 1996)
Saccharomycoides ludwigii	(Markov et al., 2001; Ramadani et al., 2010)
Zygosacchamyces rouxii and Zygosaccharomyces bailii	(Herrera et al., 1989; Liu et al., 1996)
Tortula, Torulopsis, Torulaspora delbrueckii, Mycoderma, Pichia Kloeckera apiculata and Kluyveromyces africanus	(Teoh et al., 2004; Herrera et al., 1989. Markov et al., 2001)
Saccharomyces bisporus	(Markov et al., 2001)
Candida guilliermondi, Candida Colleculosa, Candida kefyr and Candida krusei	(Ramadani et al., 2010)
Candida famata, Candida obutsa and Candida guilliermondii	(Kozaki et al. 1972; Teoh et al. 2004)
Brettanomyces intermedius	(Liu et al. 1996)
Brettanomyces bruxellensis and B. claussenii	(Teoh et al. 2004; Jayabalan et al. 2008)
Zygosaccharomyces Kombuchaensis sp.	(Kurtzman et al. 2001)
Dekkera bruxellansis, dekkera anomala, Zygosaccharomyces balili and Hanseniaspora valbyensis	(Jayabalan et al. 2014)
Dekkera, Candida, Koleckera, Mycoderma, Mycotorula, Pichia, Saccharomyces, saccharomycodes, Schizosaccharomyces, torulospora and zygosaccharomyces	(Coton et al. 2017)
Candida sp., Lachancea sp.	(Chakravorty et al. 2016)

Table B: lists of names of yeast identified from Kombucha tea

This thesis describes the caffeine-degrading yeast *Pichia* sp. strain CD1 isolated and identified from kombucha tea.

1.12. Aims and objective

Now-a-days the use of decaffeinated products has increased due to many negative effects on human health and environment. However, the conventional methods of caffeine removal are expensive, toxic and non-specific to caffeine. Hence from an economic point of view, it is necessary to investigate alternative modes of decaffeination. Microbial caffeine degradation methods are better solution to this problem. Therefore, it is important to investigate new species of microbes that are naturally capable of degrading caffeine from both health and environmental perspective.

The following research objectives were developed:

1. Isolation, characterization and identification of caffeine degrading yeast from Kombucha tea.
2. Optimization of physiological factors affecting caffeine degradation.
3. To study on the production, purification and characterization of a novel thermostable caffeine dehydrogenase from *Pichia manshurica* strain CD1 isolated from kombucha tea.
4. Investigating the influence of heat treatment on conformational properties of the novel thermostable caffeine dehydrogenase isolated from *Pichia manshurica* CD1.

CHAPTER II

Isolation and identification of caffeine degrading yeast CD1 from Kombucha tea

2.1. Introduction

Kombucha, a popular beverage, is a sugared black tea fermented by a consortium of yeast and acetic acid bacteria for about 7-14 days (Dufresne and Farnworth 2000; Dutta and Gachhui 2007). It has been reported that green tea contains about 3-6% caffeine and black tea about 3-4%(Chacko et al. 2010; Khan and Mukhtar 2013). Caffeine (1, 3,7-trimethylxanthine), a purine alkaloid, is one of the major components of both black tea and green tea(Heckman et al. 2010). Coffee pulp wastes and tea wastes are generated in large quantities during the processing of tea and coffee in these industries. Most of the coffee pulp and tea wastes remain underutilized due to the presence of antinutritional factors like caffeine, tannins and polyphenols and thus causing severe environmental pollution(Fernandes et al. 2017). Moreover, chronic ingestion of caffeine through beverages can have adverse effects on health. In recent times, the use of decaffeinated food products has increased because of a number of adverse impacts on human health(Lovallo et al. 2005). Thus from both health and environmental perspective, it is important to explore new species of microbes that are naturally capable of degrading caffeine. Further, the isolation, purification and identification of new caffeine-degrading microorganisms will open new avenues for caffeine degradation. Earlier studies reported a reduction of caffeine by about 25% during the first two weeks of fermentation of black tea by Kombucha culture(Villarreal-Soto et al. 2018). Therefore, it could be assumed that caffeine degrading micro-organisms might be present in this fermented tea and the participation of the microbes of Kombucha culture has resulted in this change in caffeine content during the course of fermentation.

In this study, to know whether the Kombucha culture can feed on caffeine solution and utilize it as carbon as well as nitrogen source, caffeine was inoculated with Kombucha culture and it was observed that, Kombucha biofilms were formed in caffeine media supplemented with some trace elements. Spectrophotometric and HPLC analyses were done for quantitative

measurement of caffeine degradation. To investigate the microorganism(s) which were degrading caffeine further isolation was attempted on caffeine agar medium. In this study, we have isolated and identified yeast *Pichia manshurica* capable of degrading caffeine from Kombucha tea. We encountered yeast (CD1) which grows on media that have no source of carbon and nitrogen except caffeine. A comparison of morphological analysis (SEM) and phylogenetic properties ensured that the strain CD1 that belongs to the genus *Pichia*. To best of our knowledge this is the first report of this study that investigating the presence of caffeine degrading yeast in the kombucha tea.

2.2. Materials and methods

2.2.1. Composition of medium

A. Fermentation medium

Caffeine ─ 1g/L

Na_2HPO_4 ─ 0.12g/L

KH_2PO_4 ─ 1.3g/L

$CaCl_2$ ─ 0.3g/L

$MgSO_4$ ─ 0.3g/L

$FeSO_4$ ─ 0.2 g/L

The salt solutions were prepared separately and autoclaved, caffeine stock was filtered sterile. It was prepared by adding caffeine and each of five salts in the above mentioned concentrations inside the laminar hood to prepare the media.

B. Caffeine agar medium

Caffeine ─ 1g/L

Na_2HPO_4 ─ 0.12g/L

KH_2PO_4 ─ 1.3g/L

$CaCl_2$ ─ 0.3g/L

$MgSO_4$ ─ 0.3g/L

$FeSO_4$ ─ 0.2 g/L

Agar ─ 1.8%

Double distilled water

pH = 5

C. YPD agar medium

Peptone ─ 2%

Yeast extract ─ 1%

Dextrose ─ 2% (separately added from 50% sterilized stock solution)

Agar ─ 1.8%

Double distilled water

pH= 5

D. Antibiotics used

Ampicillin [stock-100mg/mL]

E. Buffers and solutions for chromosomal DNA isolation

TE Buffer	10 mM Tris(pH 8), 1mM EDTA (pH 8)
Lysis buffer	1 M tris- Cl (pH 8), 10% SDS, Triton X, 5 M NaCl, 0.5 M EDTA (pH 8) Volume was made up to 1 mL with miliQ water.
RNase A	10 mg/mL
SDS solution	10%.

F. Buffers and solution for DNA gel electrophoresis

1% agarose (20 mL)	0.2 g agarose, 18 mL single distilled water, 2 mL TASE buffer (10X).
6X DNA gel loading buffer	20% Ficoll 400 0.1M Na_2EDTA, pH 8 1% Sodium dodecyl sulfate 0.25% Bromophenol blue 0.25% Xylene Cyanol
TASE buffer 10X stock solution (per litre)	Tris base –48.4g Sodium EDTA-6.7g Sodium acetate-14.12g pH was adjusted to 7.8 by Glacial acetic acid.

G. Solutions and buffers for plasmid DNA isolation

Solution I	50mM glucose 10mM EDTA 25mM TrisHCl (pH 8)
Solution II	0.2N NaOH 1% SDS
Solution III	3 M potassium-acetate, pH 4.8 glacial acetic acid
RNase A	10mg/mL

H. Buffers and solutions for SDS-PAGE

Acrylamide : bis-acrylamide solutions	30 gm acrylamide, 0.8 gm bis-acrylamide dissolved in water (de-ionized) and volume made up to 100mL.
Resolving gel buffer	1.5 M Tris-HCL pH 8.8
Stacking gel buffer	1 M TRis-HCL pH 6.8
SDS-PAGE running buffer	14.4 gm glycine/L, 3 gm Tris/L 0.1% SDS
Polyacrylamide gel staining solution	0.1% Coomassie blue R-250 solution in 50:10:40 methanol: acetic acid: water
De-staining solution	20:10:70 methanol: acetic acid: water

I. Instruments used

Electronic pH meter	Global digital pH meter, DPH 500]
UV-Visible spectrophotometer	JASCO V-530
HPLC analyser	Shimadzu, Japan
Cold centrifuge	Eppendorf 5810 R
Incubator	EYELA LTI-700
PCR machine	Eppendorf Mastercycler

2.2.2. Fermentation of Kombucha culture with caffeine

The fermentation media (caffeine + salts) was inoculated with Kombucha culture from previously fermented Kombucha tea. This was stored in glass beaker with their mouth covered with autoclaved cheese cloth and tightly tied with rubber bands. After that, the beaker was kept at room temperature for 10 days to ferment.

2.2.3. Caffeine degradation by kombucha culture

After 10 days of fermentation, the fermented medium (caffeine + salts) was centrifuged at 10,000 rpm for 10 minutes. Then the caffeine degradation was monitored by High Pressure Liquid Chromatographic (HPLC) analyses after extracting it in dichloromethane.

At first, a 25 mL aliquot was taken from each fermented and unfermented media. This aliquot was placed into a separating funnel and 25 mL of dichloromethane (DCM) was added.

The caffeine was extracted by inverting the funnel thrice. The DCM layer was collected in a clean flask and allowed to dry.

2.2.3.1. HPLC (High-performance liquid chromatography) analysis

Then Caffeine was measured by HPLC equipped with a PDA detector (SPD-M20A) using a C-18 column (100A$^{\circ}$ column 30 X 20mm Phenomenex, USA) with methanol/water (70:30 v/v) as mobile phase. The flow rate of the mobile phase was maintained at 1mL/min at 28°C. Detection of caffeine was monitored at 272 nm because it gives its maximum absorption at this wavelength. The caffeine content in the media was calculated by standard curve of caffeine.

2.2.4. Isolation and identification of caffeine degrading yeast *Pichia manshurica* strain CD1 from Kombucha tea

2.2.4.1 Isolation of CD1 from Kombucha tea

Kombucha is a symbiosis of several yeast strains and acetic acid bacteria. During ferment ation, a thin cellulose film was formed where a cell mass of bacteria and yeast was attached. It continuously multiplies and spreads over the entire surface of the tea, and then thickens. Kombucha biofilm was first washed gently and then thoroughly teased in sterile water. It was then kept in the shaker for 45 minutes. The biofilm was discarded and the soup was centrifuged at 5000 rpm for 10 minutes. The pellet was resuspended in caffeine broth (0.12 g/L Na_2HPO_4, 1.3g/L K_2HPO_4, 0.3 g/L $MgSO_4$, 0.3g/L $CaCl_2$, 0.2g/L $FeSO_4$ and 1g/L caffeine) and spread on caffeine agar plates after proper dilution. Plates were incubated at 30ºC for 3 days. Several rounds of restreaking on the caffeine agar plate was done and a yeast was isolated that could grow on the caffeine agar plate. Purification of the strain by streaking was done on YPD agar plates containing 125µg mL^{-1} ampicillin.

2.2.4.2. Morphological characteristics of the isolated yeast Strain CD1

The yeast isolated from kombucha tea was characterized on the basis of growth (colony size, shape and appearance) and microscopic observation. Colony morphology was observed on caffeine agar plate and YPD plate. Microscopic observation was done by compound microscope and scanning electron microscopy (SEM).

2.2.4.3. Gram staining techniques

A thin smear of the freshly grown culture was prepared on clean grease- free slides, fixed by passing over gentle flame. The primary stain- crystal violate was applied few drops on the

smear and was kept for 1 minute. Then the mordant-Gram's iodine was applied and kept for 45 seconds. Then the smear was washed with 95% ethanol until the violet colour disappears. After this a few drops of safranin was applied on the smear and kept for 45 seconds and finally rinsed with water, then allowed to air dry. Then the slide was observed under microscope.

2.2.4.4. Scanning electron microscopy (SEM)

Scanning electron microscopy was performed to investigate the cell morphology of the isolated yeast strain CD1. After solvent evaporation in vacuum and up to critical point drying, electronic microscopy of strain CD1 was performed. To reduce the sample charging, dried samples were coated with a thin gold layer of before electron microscopy. ZEISS EVO-MA 10 scanning electron microscope with 15 kV accelerating voltage was used to examine the samples at 10 KX magnification.

2.2.4.5. Genomic DNA isolation

The isolated strain obtained from caffeine agar plate was transferred to YPD broth; the grown cells in YPD after 2 days were harvested and lysed for genomic DNA isolation. The grown cells were centrifuged washed with distilled water and pellet was dissolved in 100 µl TE buffer. 250 µl lysis buffer was added to the cell suspension. Cells were then mechanically disrupted by vortexting with glass beads in ice condition. The aqueous phase was collected by centrifugation at 10,000 rpm for 10 minutes at 4 °C and treated with RNase at 37 °C for 30 minutes and extracted with chloroform. 0.1 volume of 3M sodium acetate (pH 7.2) and 2.5 volume of 100% ethanol was added to it and was preserved in -20˚C for overnight. The genomic DNA was precipitated by centrifugation at 10,000 rpm for 10 minutes at 4 °C. The

pellet was taken and dissolved in 1 mL 70% ethanol and again centrifuged in 10,000 rpm for 10 minutes at 4 °C. Then the supernatant was discarded and it was air dried. Finally it was dissolved in 50 µl nuclease free water.

The quality of the isolated DNA was determined by ethidium bromide staining after run on 1% agarose gel. The concentration of DNA was measured spectrophotometrically using UV-vis spectrophotometer (JascoV-530).

2.2.5. Sequencing of 18S rDNA, D1-D2 region and ITS region

2.2.5.1. PCR amplification

The rDNA regions (18S, D1-D2 and ITS) were then PCR amplified using standard primers (Table 1). The PCR reaction mixture contains 100 ng template DNA, 0.2 mM dNTPs, 50 pmol of each primer, 1X PCR buffer, and 2 units of Dream Taq DNA Polymerase (Thermo Scientific). The reaction conditions for 18S was three cycles each of initial denaturation at 96ºC for 4 minutes, annealing at 55ºC for 4 minutes, and extension at 72ºC for 4 minutes, followed by 27 cycles of denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 2 minutes. The final extension was done at 72ºC for 10 minutes.

Region	Primer	Sequence (5'-3')
18S	P108	ACCTGGTTGATCCTGCCAGT
18S	M3989	CTACGGAAACCTTGTTACGACT
D1-D2	NL-1	GCATATCAATAAGCGGAGGAAAAG
D1-D2	NL-4	GGTCCGTGTTTCAAGACGG
ITS	pITS-F	GTCGTAACAAGGTTAACCTGCGG
ITS	pITS-R	TCCTCCGCTTATTGATATGC

Table 1: Primers used for PCR amplification of rDNA regions of CD1

The initial denaturation time for the D1D2 region was 3 minutes at 95°C. 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, followed by a 10-minute extension step at 72°C. 3 minutes at 95°C, for ITS region the initial denaturation was 30 cycles of 30 seconds at 95°C, 30 seconds at 57°C, and 45 seconds at 72°C, with a final extension step of 10 minutes at 72°C.The PCR products were run in 1% Agarose gel and corresponding bands were excised and eluted using 'Nucleo-pore SureExtract PCR Clean-up/Gel Extraction Kit' according to the protocol. The PCR amplified products were run on 1% agarose gel, gel purified by QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. The gel purified DNA was ligated to PTZ57R/T vector using InsTAclone PCR Cloning Kit (Thermo Scientific, USA). The ligation mixture was prepared by adding 6 µl DNA (400ng), 1 µl DNA vector (55ng), 1 µl T4 DNA ligase (5U), 2 µl 5X ligation buffer. The ligation mixture was kept overnight at 4 °C.

2.2.5.2. Transformation

The transformation of the ligated mixture was carried out in JM107 *E. coli* according to the manufacture's protocol. In brief, a single colony of JM107 *E. coli* was inoculated into 1.5 mL of C medium and incubated for approximately 2.5 hours at 37°C shaker. For 5 minutes, the culture was centrifuged at 4500 rpm. The pellet was gently resuspended in 0.3 mL of ice cold T solution and incubated on an ice for 5 minutes. The suspension was centrifuged again for 5 minutes at 4500 rpm. The pellet was resuspended in 0.12 mL of ice cold T solution and incubated for 5 minutes in the ice. The suspension was centrifuged again for 5 minutes at 4500 rpm. The pellet was resuspended in 0.12 mL of ice cold T solutions and incubated for 5 minutes in the ice. 60 µl of the suspension was mixed to the ligation mixture and incubated for 30 minutes in an ice conditions. Then, 44µl IPTG-X gal (40µl X-gal + 4µl IPTG) was

spread on each ampicillin^{+} LB agar plate. The entire suspension was spread on that plate and these plates were incubated in dark at 37ºC for 16 hours.

2.2.5.3. Plasmid DNA isolation

Recombinant plasmids were isolated from the white colonies that appeared on the plate following the protocol of the alkaline lysis method described by Brinboim and DOlly et. al (Bimboim and Doly 1979). 3 mL of the overnight grown culture cells were was pelleted at 4 °C. The pellet was resuspended in 100 µl of ice cold solution I was added to the pellet and stored in ice for 5 minutes. Then 200 µl of freshly prepared solution II was added and mixed properly by inverting for 2/3 times. Sample was stored at room temperature for 5 minutes. Then ice cold solution III was added stored in ice for 15 minutes. After incubation, the solution was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was moved to a new sterile tube. RNaseA was added at a final concentration of 2 mg/mL, and the mixture was incubated for 20 minutes at 37°C. Then phenol: chloroform: isoamyl alcohol was added in 25:24:1 ratio and all the sample was mixed by inverting the tubes. The phase separation was obtained after centrifugation at 10000 rpm for 5 minutes. Then 2.5 volume of absolute ethanol was added to precipitate the plasmid DNA. The tube was kept at 20 °C for 30 minutes to allow for complete DNA precipitation. The mixture was then centrifuged at 12000 rpm for 15 minutes at 4°C, and the DNA pellet was washed twice with 70% ethanol. Finally, the plasmid DNA was suspended in 50 µl nuclease free water and kept at 4 °C.

2.2.5.4. Restriction enzyme digestion of isolated plasmid DNA

The presence of inserts within the isolated plasmid DNAs was determined by restriction digestion with the enzyme. (Thermoscientific, USA).The digestion mixture (Plasmid DNA

(300ng), digestion buffer (1X), Enzyme (*Sal*I- 2U))was incubated at 37°C for overnight and the resulting product was run on 1% agarose gel to check the presence of insert. Therefore, the plasmids containing the correct inserts were sequenced at Chromous Biotech India by ABI PRISM method with standard primers M13F & M13R.

2.2.6. Phylogenetic analysis of *Pichia manshurica* strain CD1

Various closely related 18S rRNA gene sequences (Fig.3), sequences specific for the D1/D2 domain of the 26S rRNA gene (Fig. 4), and sequences for the ITS region (Fig. 5) were recovered from GenBank and were compared with *Pichia* species and aligned using Clustal W(Roy et al., 2018). The design of the phylogenetic tree was developed and analysed using MEGA, Version 7 (USA) software (Kumar et al. 2016) with other *Pichia* species in the cladogram. The model used in the nucleotide substitution and phylogenetic trees was constructed using the neighbor-joining method. With each algorithm, confidence levels for individual branches within the tree were checked by repeating the MEGA 7 analysis with 1000 bootstraps (Ibrahim et al. 2016).

2.2.7. Statistical Analysis

All the values are expressed as mean ± SD (n=3). Significant differences between means were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the Origin Pro 2021b software. A difference was considered significant at the $P < 0.05$ level

2.3. Results and discussion

2.3.1. Biofilm formation in caffeine fermentation media

After 10 days of fermentation biofilm formation was observed in the caffeine fermentation media (Fig. A). The results showed that the biofilm formed in medium was considerably dense and biofilm formation was indicating that the microorganisms could utilize caffeine. Moreover in the fermentation media where there was no other carbon or nitrogen source except caffeine, it may be assumed that, Kombucha culture could fed on caffeine and utilize it as both carbon and nitrogen source.

Fig. A. Growth of Kombucha culture in caffeine fermentation media

2.3.2. Caffeine degradation by kombucha culture (HPLC analysis)

The caffeine degradation by Kombucha culture, performed with HPLC analysis of both unfermented and fermented medium. Fig. 1 shows that caffeine was degraded by the Kombucha culture by about 32.7%. This result encouraged us to investigate the presence of any caffeine degrading microorganism in this fermented tea product or in Kombucha biofilm.

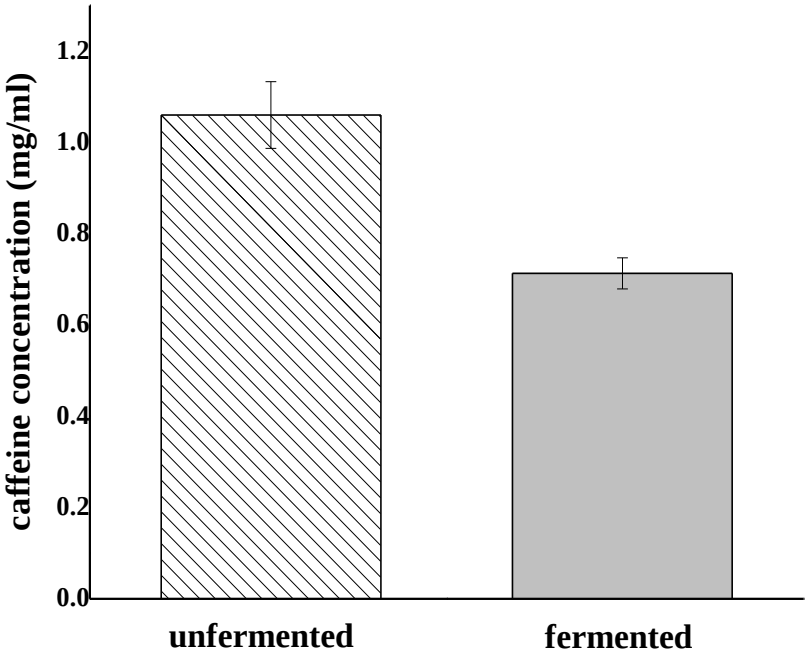

Fig. 1: Caffeine concentration in unfermented black tea and Kombucha tea. Values are expressed as mean ±SD (n=3).

2.3.3. Morphology of yeast CD1

2.3.3.1. Gram staining

The yeast isolated from kombucha tea on a caffeine agar medium was assigned the strain CD1. The cells are mostly spherical or round-shaped and occur singly or in bunch. Colonies appeared cream white and sticky on YPD plates, whereas on caffeine agar plates they are smaller and white. The morphological results of CD1 cells are shown in Table 2.

Sample	Gram character	Cell shape	Cell arrangement	Cell morphology
10^{-4} dilution plate	Gram positive	Mostly round	Single as well as bunch	Round, large, Coccus

Table 2: Gram character and cell morphology of the isolated yeast CD1

2.3.3.2. Scanning electron microscopy (SEM)

The morphology of the isolated yeast strain was analyzed by SEM, and the results are shown in Fig. 2. The strain CD1 seemed to have a round shape, and some budding yeast cells are also present in the SEM photograph.

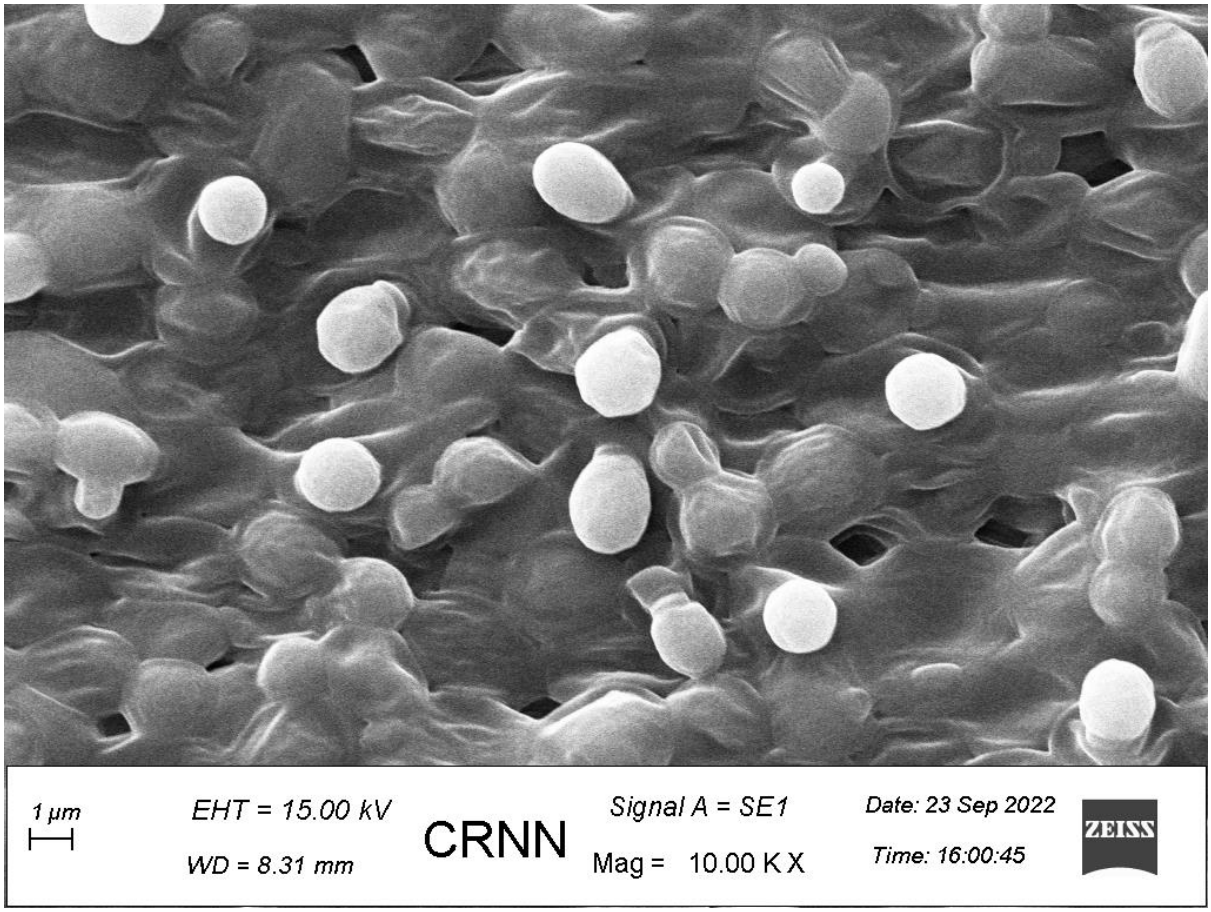

Fig. 2: Scanning electron microscopy (SEM) Of the yeast strain CD1

2.3.4. Isolation and identification of the yeast strain *Pichia manshurica* strain CD1

One yeast strain was isolated from Kombucha tea that could grow on caffeine agar medium and was designated as strain CD1. For molecular identification of the isolate, a 1084-bp fragment of the 18S rRNA gene was amplified from CD1 DNA (GenBank accession number **KY799109**) that showed close similarity (99.5%) to *P. manshurica* CBS 209. The DNA fragment specific for D1/D2 domain of the 26S rRNA gene (GenBank accession number **KY828209**) was 597 bp long and was similar (99%) to *P. manshurica* CBS 209. A 402 bp fragment of the ITS region (GenBank accession number **KY828210**), amplified using the primers pITS-F and pITS-R showed close similarity (98%) to *P. manshurica* CBS 209.

2.3.5. Phylogenetic analysis of *Pichia manshurica* strain CD1 (18S rRNA, 26S rRNA, and ITS region)

The phylogenetic tree (Fig. 3) was constructed using the sequences of the 18S rRNA gene of CD1 DNA (**KY799109**), showing the similar (more than 98%) genetic relationship with different type strains of *Pichia* spp. The evolutionary analyses of sequences specific for D1/D2 domain of the 26SrRNA gene (**KY828209**) showed close similarity with *P. membranifaciens* (97%), *P. manshurica* CBS 209 (99%) and several other *Pichia* species (Fig. 4). Additionally, the phylogenetic tree constructed using the sequences for fragment of ITS region (**KY828210)** showed a close phylogenetic relationship to *P. manshurica* CBS 209 (98%), a close similarity to *P. membranifaciens* (97%), and other *Pichia* species (Fig. 5).

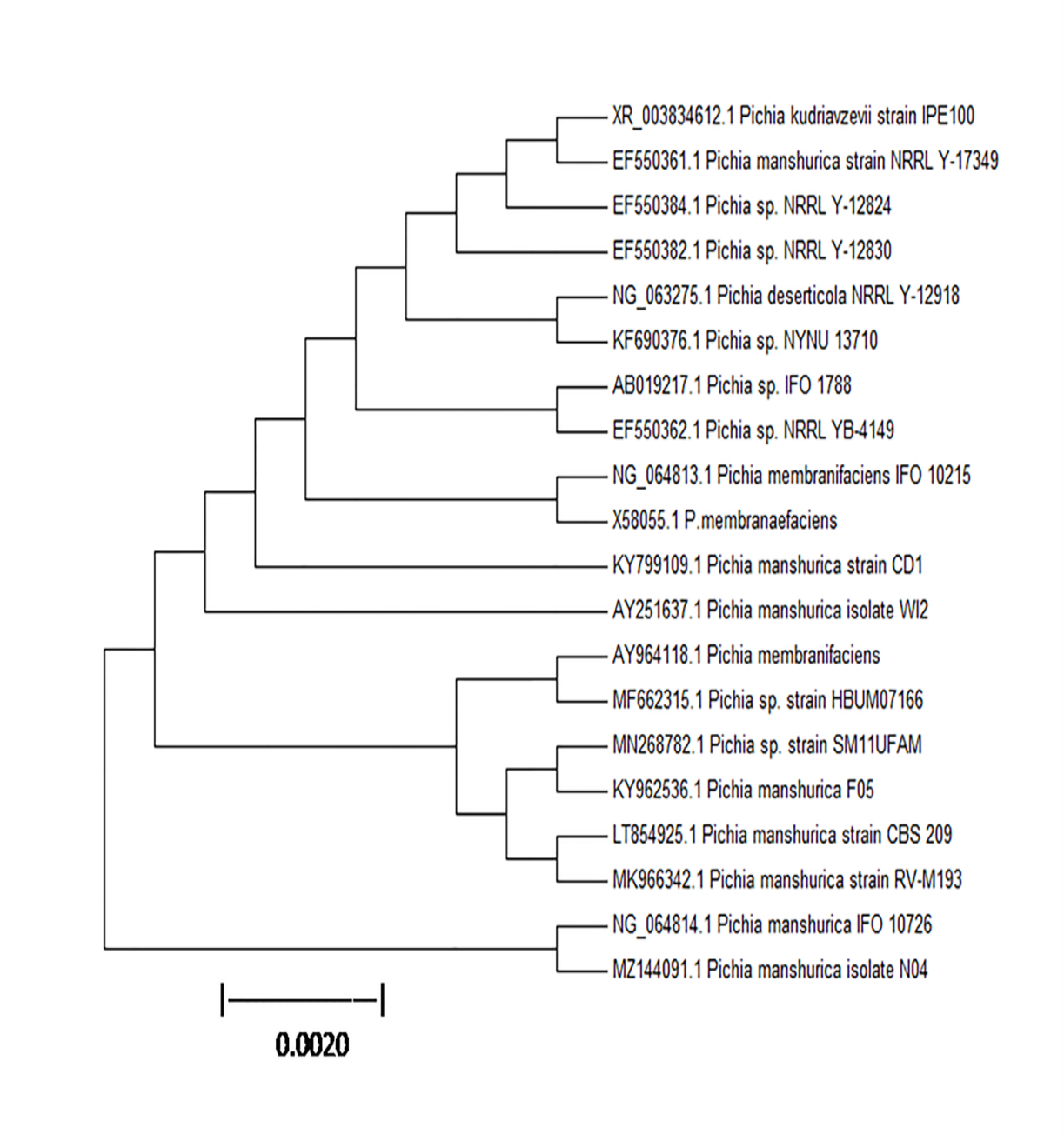

Fig.3: Neighbor-joining phylogenetic tree based on the 18S rRNA gene sequences (1084 bases), showing the relationship between *Pichia manshurica* strain CD1 and other close relatives of the genus *Pichia*. Bar indicates 0.002 nucleotide substitutions per site

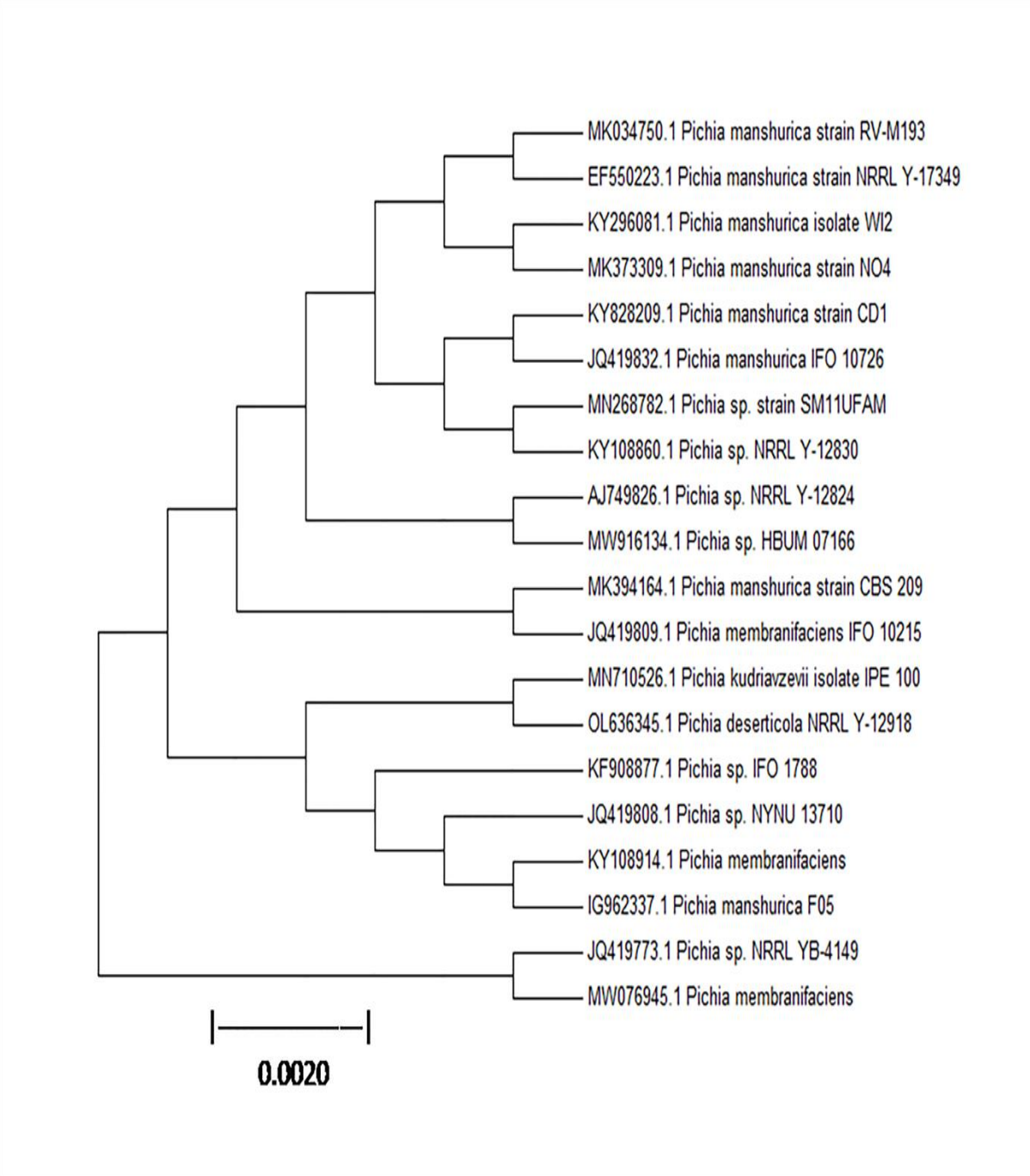

Fig. 4: Neighbor-joining phylogenetic tree based on the D1/D2 domain of the 26S rRNA gene sequences (597 bases), showing the relationship between *Pichia manshurica* strain CD1 and other close relatives of the genus *Pichia*. Bar indicates 0.002 nucleotide substitutions per site.

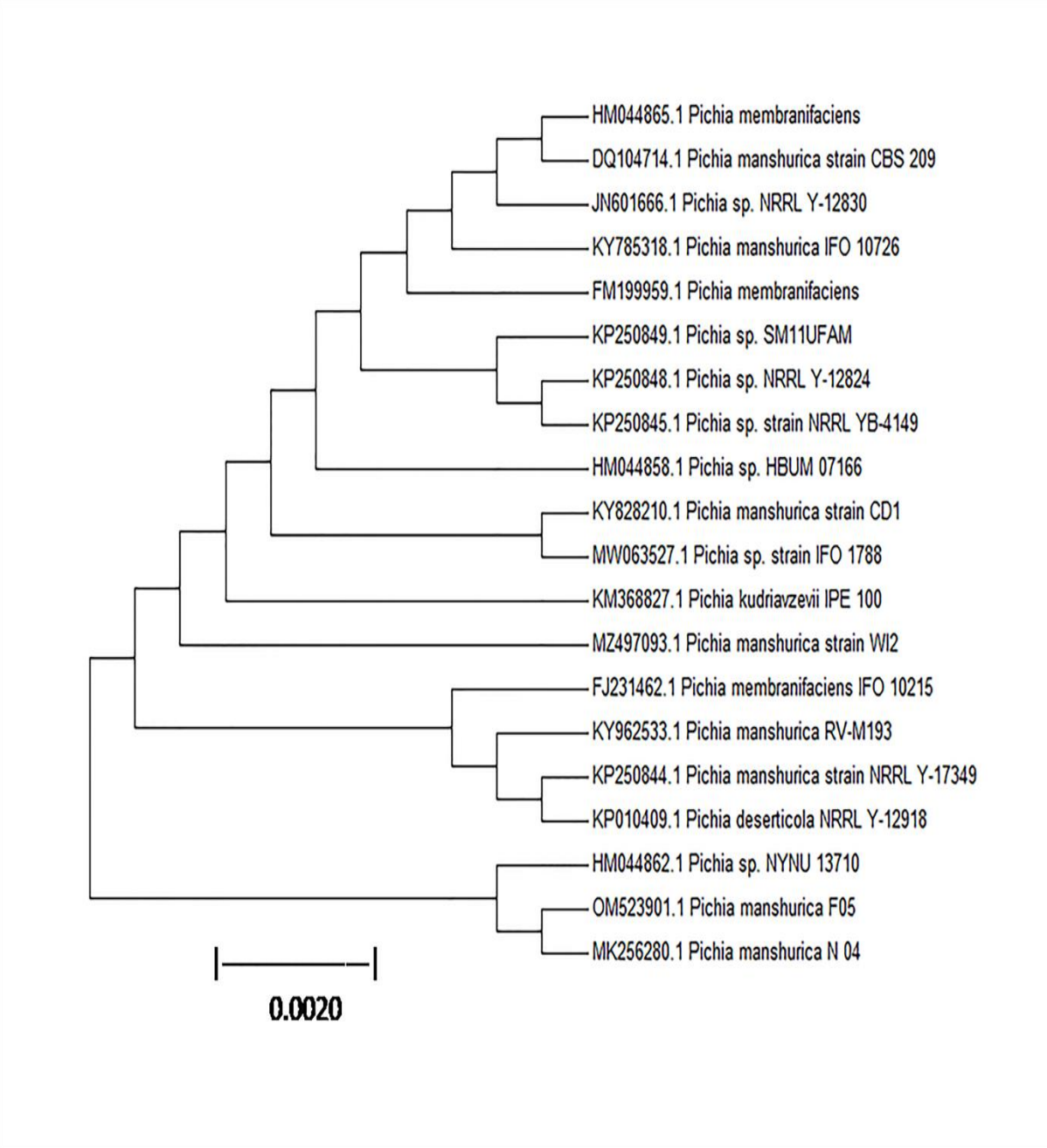

Fig.5: Neighbor-joining phylogenetic tree based on the nucleotide sequences of the ITS region (402 bases), showing the relationship between *Pichia manshurica* strain CD1 and other close relatives of the genus *Pichia*. Bar indicates 0.002 nucleotide substitutions per site.

2.4. Conclusions

In the present study, we described the isolation and identification of caffeine degrading yeast strain from Kombucha tea. This study also reported that *Pichia manshurica* isolated from Kombucha tea exhibited the ability to degrade caffeine and use it as the sole source of carbon and nitrogen. Phylogenetic analysis based on 18S rDNA, D1/D2 region and ITS region gene sequence analysis which is a reliable alternative option for conventional identification. D1/D2 domain sequence showed that CD1 belongs to genus *Pichia* and is closely related to the type of strain *P. manshurica* CBS 209 and will be referred as *Pichia manshurica* CD1 in this study.

CHAPTER III

Purification and characterization of a novel thermostable caffeine dehydrogenase from *Pichia manshurica* strain CD1

3.1. Introduction

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring chemical found in many plant species e.g., tea, coffee, cola, and cocoa (Heckman et al. 2010). It boosts our alertness and energy level due to its central nervous system stimulant activity. Although it has many health benefits but consuming too much of it can be dangerous. The global consumption of caffeine ranges from 80 mg to 400 mg per person per day through various other sources like caffeinated soft drinks, chocolates and desserts (Reyes and Cornelis 2018). Furthermore, excessive caffeine consumption can lead to anxiety, mutation, cardiovascular disease, liver disease and infertility (Lovallo et al. 2005). Caffeine contaminated solid wastes cause's great environmental burdens. The presence of caffeine in soil affects soil infertility, inhibits seed germination, kills microbes and insects (Fernandes et al. 2017) and also affects the aquatic life. For this adverse effects of caffeine, that may leads to the development of a decaffeination process.

Caffeine contaminated water bodies have a pernicious influence on the aquatic life and are also unsafe for drinking purposes (Chen et al. 2008; Gummadi et al. 2011). Consequently, demand for decaffeination of both food as well as these waste products is gradually increasing day by day.

In recent times, microbes with caffeine-degrading capacity have drawn considerable attraction in the decaffeination process (Gummadi et al. 2011; Summers et al. 2015). To date, a large number of bacteria and fungi have been reported to be capable of growing in the presence of caffeine and degrading the alkaloid (Zhou et al. 2018). From the aspects of enzymology, caffeine is generally catabolised by microbes via two major pathways - demethylation and oxidation involving demethylase and dehydrogenase or oxidase enzymes respectively (Dash and Gummadi 2006). Although there are many researches regarding the

characteristic and mechanism of caffeine biodegradation in bacteria (Summers et al. 2011), little is known about decaffeination by yeasts. Moreover, most of the caffeine degrading microorganisms were mainly isolated from soil (Mazzafera et al. 2004). Identification of caffeine degrading microbes from other sources are not much explored. A recent study has demonstrated the caffeine degrading role of a fungi *Aspergillus sydowii* isolated from Pu-erh tea that is produced by natural solid-state fermentation (Zhou et al. 2018).

In this study, we have isolated the enzyme caffeine dehydrogenase from the yeast *Pichia manshurica* CD1 capable of degrading caffeine from Kombucha tea. In addition, we describe the production, purification and characterization of the caffeine degrading enzyme from the yeast. To the best of our knowledge, the present research is the first study investigating the caffeine degrading enzyme from the kombucha yeast *Pichia manshurica*. The biochemical properties of caffeine dehydrogenase from *Pichia manshurica* will provide novel features such as thermostability, pH range, substrate specificity and kinetic parameters when compared to with other caffeine degrading enzymes. The findings of the current study will also be useful for industry-focused applications.

3.2. Materials and methods

3.2.1. Culture conditions and caffeine dehydrogenase production

For amplifying the caffeine tolerant strain and inducing the caffeine degrading enzyme, the yeast was grown in liquid amplifying media (LAM), that was prepared by adding 0.12 g/L Na_2HPO_4, 1.3g/L K_2HPO_4, 0.3 g/L $MgSO_4$, 0.3g/L $CaCl_2$, 0.2g/L $FeSO_4$, 2g/L dextrose and 4g/L yeast extract and 2g/L caffeine. The pH of the media was maintained at 5. The induced yeast cells were harvested by centrifugation at 10000 rpm for 12 min at 4°C. Then the cells were washed and suspended in 50mM potassium phosphate buffer (pH 7.5). They were lysed

by glass bead disruption and the unbroken cells and debris were removed from the lysate by centrifugation at 12000 rpm for 15 min at 4°C. The supernatant was designated as cell extract and was used for further experiments. The protein concentration of the cell extract was quantitated by Bradford assay using BSA as a standard (Bradford 1976).

3.2.2. Caffeine degradation by the yeast *Pichia manshurica* CD1

3.2.2.1. HPLC analysis

First, the yeast strain CD1 was grown in 50 mL caffeine enriched broth medium at 30°C with shaking at 110 rpm. After 4 days the media was centrifuged at 10,000 rpm for 10 minutes. Then, the caffeine degradation was monitored by spectrophotometric and High Pressure Liquid Chromatographic (HPLC) analyses after extracting it in dichloromethane. Identification of caffeine was established by comparing the retention time with standard caffeine solution. After centrifugation 25mL soup of both cultured (CD1) and uncultured media were taken and placed into a separating funnel. Then equal volume of dichloromethane (DCM) was added to the each funnel and the caffeine was extracted by inverting the funnel thrice. The DCM layer was collected in a clean flask and allowed to dry.

3.2.2.2. Spectrophotometric determination of caffeine degradation assay

Spectrophotometric determination of both dichloromethane extracts of caffeine in cultured (CD1) and uncultured media were 100 times diluted with dichloromethane and the OD was measured at a range of 240 -400 nm wavelength. The blank was set with only dichloromethane.

Caffeine degradation ability (%) =

$$\frac{O.D_{275} \text{ of Caffeine broth media (0.57189)} - O.D_{275} \text{ of CD1 grown caffeine media (0.31793)}}{\text{O.D of caffeine broth media (0.57189)} \times 100}$$

3.2.3. Growth curve of *Pichia manshurica* strain CD1

Pichia manshurica strain CD1 was grown in a caffeine medium at 30°C with shaking at 110 rpm. Cell density was monitored by measuring the optical density at 600nm (OD_{600}) at 7hr interval until 60hr from the time of inoculation.

3.2.4. Caffeine dehydrogenase assay

3.2.4.1. Qualitative assay of caffeine dehydrogenase

A qualitative assay of Caffeine degrading enzyme was carried out by using caffeine agar plate method. The caffeine agar plates contained 0.12 g/L Na2HPO4, 1.3g/L K2HPO4, 0.3 g/L MgSO4, 0.3g/L CaCl2, 0.2g/L FeSO4 ,18g/L Agar with 2g/L caffeine as the substrate, with a pH of 5. A loop of full-grown CD1culture from YPD medium was streaked out on the caffeine agar plates and incubated at 30°C for 4-5 days.

3.2.4.2. Quantitative assay of caffeine dehydrogenase

For the caffeine dehydrogenase enzyme assay (Yu et al. 2008), the assay mixture contained an appropriate amount of enzyme, 0.5mM caffeine as a substrate and 0.5mM NBT as the electron acceptor in 50mM potassium phosphate buffer (pH 7.5). The assay mixture was incubated at 100 °C for 1 h and the enzyme activity was determined by monitoring the rate of

increase in the absorbance at 566nm due to formazan formation with a UV/VIS spectrophotometer (Jasco). A non-enzymatic reaction containing caffeine and NBT was also run as a control in which potassium phosphate buffer was used instead of enzyme solution. Value, if any obtained, was used to set "auto-zero" for the respective experiments. One unit of caffeine dehydrogenase activity was defined as the amount of enzyme that produces 1nmol of formazan per min.

3.2.5. Effect of different nitrogen and carbon sources on the growth of strain CD1

The effects of different nitrogen sources (peptone, tryptone, ammonium sulphate, sodium nitrate) and carbon sources (fructose, lactose, starch, sucrose and maltose) were studied on strain CD1 growth and caffeine dehydrogenase activity. 25% (v/v) seed culture was inoculated in each 250mL flask containing 100mL of liquid amplifying medium with individual supplement of nitrogen (4g/L) and carbon (2g/L) sources instead of yeast extract and glucose respectively. The pH of the medium was kept at 5. The culture was incubated at 30°C for 4 days with shaking at 110rpm. After incubation, the cells were harvested by centrifugation, the cell mass was measured and the cells were subjected to lysis and the cell lysate was used for enzyme activity assay.

3.2.6. Purification of caffeine dehydrogenase

3.2.6.1 Activity staining

Activity staining was one of the method used to determine the enzyme activity of certain enzymes. Activity staining was done using 2% caffeine on native polyacrylamide gel (i.e

resolving gel) at the room temperature condition. After electrophoresis, the gel was incubated in 2mM NBT solution for 24h at 37ºC. The range where the dark blue band occurred was identified using a pre staining marker. The gel contained caffeine as a substrate for crude caffeine dehydrogenase enzyme, and the blue band indicated the enzyme's action site. The action site range of enzyme was determined by pre-stained marker (puregene 4 color protein marker cat#PG-PMT0782). According to the mechanism, blue colored formazon was produced on the gel as a result of caffeine dependent reduction of NBT by caffeine dehydrogenase.

3.2.6.2. Spehadex G- 100 column chromatography

The cell free extract was considered as a crude protein sample and was subjected for enzyme purification. The crude extract (0.2 mL) was loaded onto a 3 mL (bed volume) Sephadex G-100 resin, pre-equilibrated with 50mM potassium phosphate buffer (pH 7.5) containing 0.15M NaCl. Equilibration buffer was passed through the column to separate the protein and elute it from the column. Fractions were collected at a flow rate of 1 mL/min. Enzyme activity was assayed (NBT assay) on each fraction containing protein as determined by spectrophotometric assay at 280nm and the most active fraction (P1) was pooled and stored at -20ºC for further purification.

3.2.6.3. Purification from polyacrylamide gel by sonication extraction

The pooled protein fraction (P1) was further separated by running on 10% native PAGE at 100V for 90min at 4ºC. Once the electrophoresis was complete, the area of interest (The action site range of enzyme was determined by activity staining result) was cut out of the gel and washed thrice with 2 mL of 250mM Tris buffer (pH 7.4) followed by distilled

water. Then the gel slices were homogenized and 1 mL of 20 mM tris buffer (pH7.4) containing 0.1 % SDS was added. The samples were then stored at -20ºC for 1hr and sonicated for 3 min in an ice bath with a (Sartorius LABSONIC® M) sonicator. After sonication, the samples were centrifuged at 12000g for 30 min at 4ºC and the supernatant was collected (Retamal et al. 1999). Total protein concentration in the supernatant was measured by Bradford reagent using BSA as standard (Bradford 1976) and the enzyme activity was assayed.

3.2.7. Determination of molecular mass

The purity of the enzyme was judged by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE) and determined the homogeneity and molecular mass according to the method of Laemmli (Laemmli 1970). After completion of electrophoresis the gels were stained with silver by standard method (Chevallet et al. 2006). Pre-stained protein marker (puregene 4 color protein marker cat#PG-PMT0782) was used as molecular weight markers to identify the relative molecular mass of protein.

3.2.8. Effect of pH and temperature on caffeine dehydrogenase activity

To determine the optimum pH, caffeine dehydrogenase assay was carried out with 0.5mM NBT and 0.5mM caffeine at different pH (5,6,7.5 10), using 50 mM citrate buffer for pH 5 & 6, potassium phosphate buffer for pH 7.5 and tris HCL for pH 10. Then, the assay mixture was incubated at 100°C for 1 hour and enzyme activity was determined for each pH value under the standard assay method. In addition a control experiment was done where buffer was added instead of enzyme for each pH separately. Value, if any obtained, was used to set "auto-zero" for the respective experiments.

For optimum temperature determination, the enzyme reaction was carried out with 0.5mM NBT and 0.5mM caffeine in 50mM potassium phosphate buffer (pH 7.5) at different temperatures (4°C, 25°C, 30°C, 37°C, 55°C, 65°C, 80°C and 100°C) for 1 hour. Then enzyme activity was determined at each temperature under the standard assay method. Control experiment was performed where 50 mM potassium phosphate buffer (pH 7.5) was used instead of enzyme solution for each temperature separately. Value, if any obtained, was used to set "auto-zero" for the respective experiments.

3.2.9. Effect of pH and temperature on the stability of caffeine dehydrogenase

The effect of pH on the stability of the purified caffeine dehydrogenase enzyme was studied by pre-incubating the enzyme at different pH (5, 6, 7.5 and 10) for 20 min at 4 °C. Then enzyme activity was determined at 100°C after 1 hour incubation under the standard assay method. The activity at optimal pH was taken as 100%**.** Control experiment was done where appropriate amount of buffer was added instead of enzyme. Value, if any obtained, was used to set "auto-zero" for the respective experiments.

The effect of temperature on the stability of the purified enzyme was studied at different temperatures (37°C, 55°C, 65°C, 80°C and 100°C). For thermostabilty, the purified enzyme solution was pre-incubated for 20 min at each temperature. Then the enzyme solution was withdrawn at 5 min intervals and the enzyme activity was determined at 100°C after 1 hour incubation under the standard assay method. The 0 min value was 100**.** Control experiment was done where appropriate amount of 50mM potassium phosphate buffer (pH 7.5) was added instead of enzyme. Value, if any obtained, was used to set "auto-zero" for the respective experiments.

3.2.10. Substrate specificity

Caffeine dehydrogenase enzyme activity with different substrates of caffeine analogues (caffeine, xanthine, theobromine and theophylline), were determined at 0.5mM concentration by the standard assay method. The specific activity of purified enzyme towards caffeine was taken as control.

3.2.11. Determination of K_m and V_{max}

K_m and V_{max} values of purified caffeine dehydrogenase were evaluated by using different substrate concentrations of caffeine ranging from 0.01mM to 0.5mM under the optimized pH and temperature conditions.

3.2.12. Effects of different metal salts, additives and solvents on caffeine dehydrogenase enzyme activity

The effects of various metal salts ($K_2Cr_2O_7$, $CuSO_4$, $Pb(NO)_3$, $ZnSO_4$, $NiCl_2$, $HgCl_2$ & $MgCl_2$) on enzyme activity were studied at a concentration of 1mM. The enzyme activity was also determined in presence of EDTA (1mM), SDS (1%), β-mercaptoethanol (5%), Benzene (5%), methanol (5%), isopropanol (5%).The purified enzyme was incubated with each of the metal salts and chemical agents separately for 15 minutes at 100ºC and the activity was assayed. Activity in absence of any metal salts and chemical agents was taken as 100%.

3.2.13. Statistical analysis

All the values are expressed as mean ± SD (n=3). Significant differences between means were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple

comparison test using the Origin Pro 2021b software. A difference was considered significant at the $P < 0.05$ level.

3.3. Results and discussion

3.3.1. Caffeine degrading ability of *Pichia manshurica* CD1

3.3.1.1. HPLC analysis

Caffeine degrading ability of strain CD1 was studied by measuring the caffeine content in caffeine broth medium before and after the growth of strain CD1 for 4 days by HPLC analysis. Results showed that caffeine concentration was decreased from 0.948 mg/mL to 0.439 mg/mL suggesting 53.69% of the caffeine was utilized and degraded by the yeast (Fig. 6).

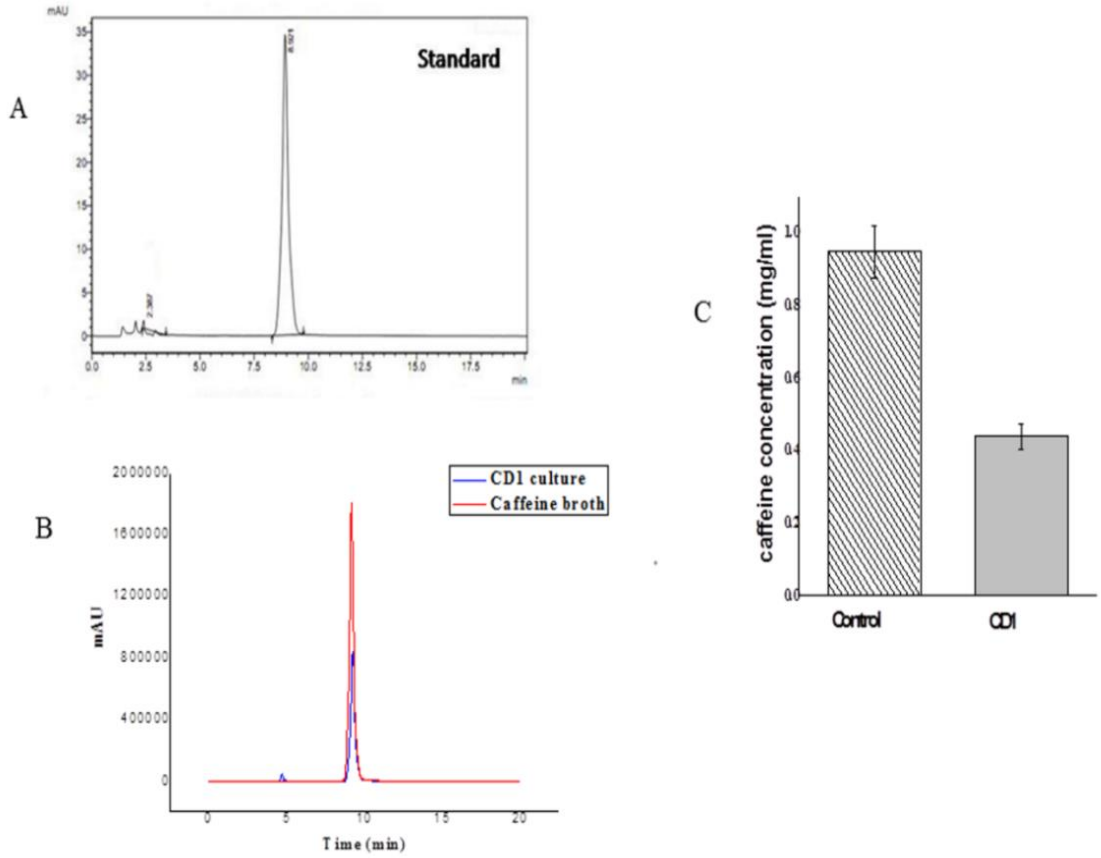

Fig. 6: Caffeine degradation assay by HPLC. (A) Peak heights of standard caffeine. (B) Comparison of peak heights of caffeine broth (control) and CD1. (C) Caffeine concentration in caffeine media before and after the growth of CD1 for 4 days. Control denotes media before growth of CD1 and CD1 denotes media after growth of the yeast for 4 days. Values are expressed as mean ± SD (n=3).

3.2.1.2. UV-Visible spectrophotometric determination

After 4 days of incubation, calculating the caffeine content in caffeine media before and after CD1 growth the results of UV-Visible spectrophotometric analysis revealed that the strain CD1 is able to degrade caffeine by about 44.4%. The results are shown in Fig 7. The maximum absorbance of standard caffeine was found to be 275 nm (Fig. 7B). Fig. 7A shows that the peaks of caffeine were dropped significantly after the growth of CD1.The findings indicated that the strain CD1 could both degrade and utilize caffeine.

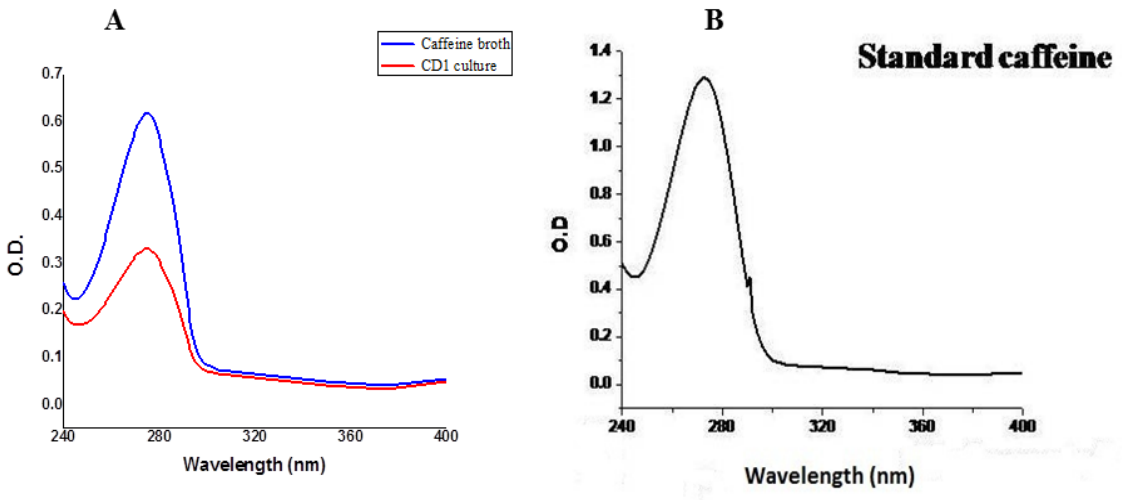

Fig. 7: UV-Visible spectrophotometric analysis of caffeine degradation by the yeast CD1. (A) Comparison of wavelength scans for Caffeine broth and CD1 culture. (B) Wavelength scan for standard caffeine.

3.3.2. Growth curve

The yeast was then grown in caffeine enriched medium and the growth curve was determined (Fig. 8).The OD value of CD1 at 600nm was found to be 0.2099±.018 at 50 hours of growth, after which it reached the stationary phase. The growth of CD1in caffeine enriched medium without any carbon and nitrogen source indicated that the yeast strain can degrade and utilize caffeine as the sole source of carbon and nitrogen.

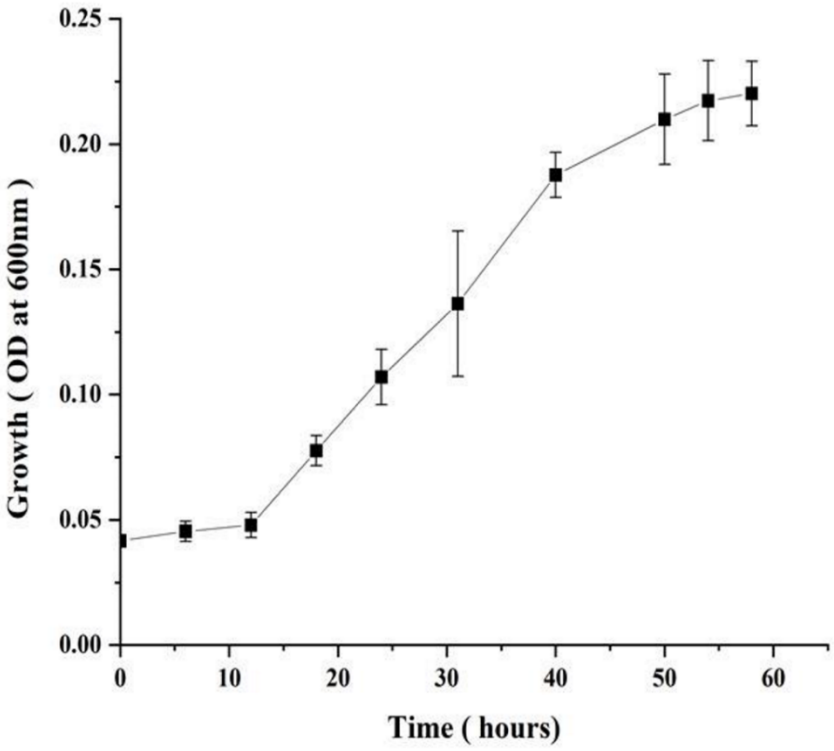

Fig. 8: Growth curve of CD1

3.3.3. Caffeine dehydrogenase production on solid media

Caffeine dehydrogenase activity of *Pichia manshurica* CD1 in solid media is shown in Fig. 9. Appearance of the small colonies with no zones indicated the production of intercellular caffeine dehydrogenase enzyme. Here, the only source of carbon and nitrogen was caffeine.

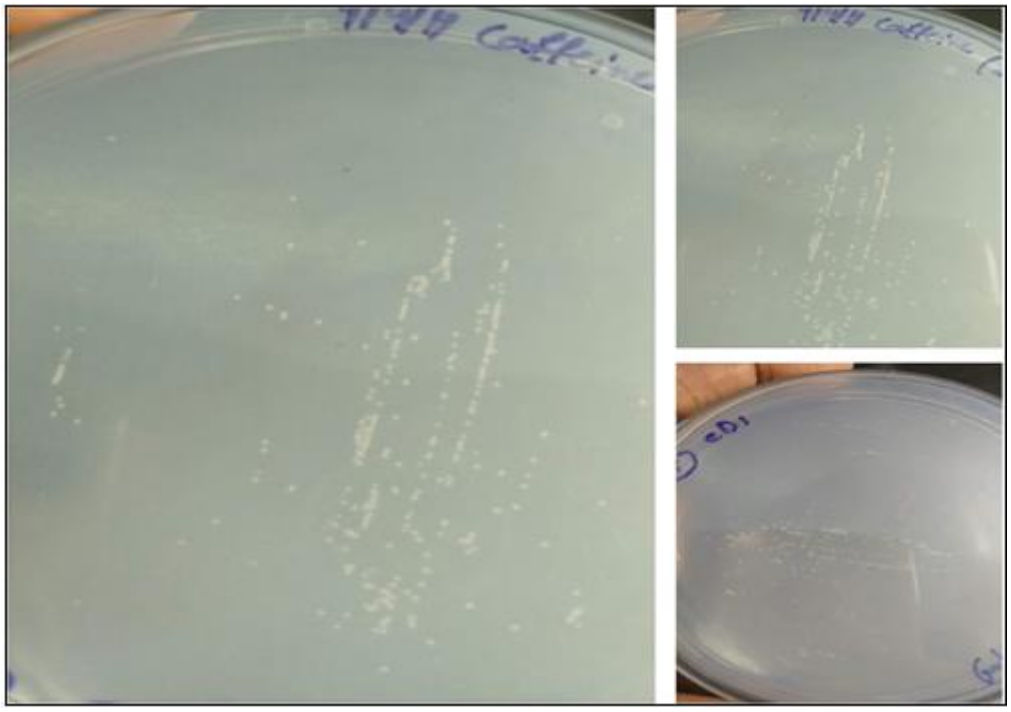

Fig. 9: Caffeine dehydrogenase activity on caffeine agar plate

3.3.4. Effect of different nitrogen and carbon sources on CD1 cell growth and caffeine dehydrogenase production

Different nitrogen and carbon sources were found to modulate many enzyme productions in microbes by induction and repression mechanism (Dhar and Kaur 2009). Previous studies have demonstrated that the enzymatic pathways of caffeine catabolism in microbes are inducible (Madyastha and Sridhar 1998; Madyastha et al. 1999; Mohapatra et al. 2006). External nitrogen and carbon sources were found to have regulatory effects on enzymes for caffeine degradation in many microbial species (Madyastha et al. 1999; Mohapatra et al. 2006; Asano et al. 2014). Therefore, in this study CD1 growth and enzyme production were monitored in presence of different nitrogen (Table 3) and carbon sources (Table 4).

Nitrogen source (0.4%)	Biomass[a] (g/100mL)	Caffeine dehydrogenase activity[a] (nmol/(min mg))
Yeast extract	1.29±0.07	19.25±0.62
Peptone	0.80±0.04	4.08±0.64
Tryptone	0.76±0.025	3.61±0.41
Ammonium sulphate	0.61±0.02	9.4±0.40
Sodium nitrate	0.52±0.016	7.44±0.51

[a]Values are means ± standard deviation for three independent measurement

Table 3: Influence of different nitrogen sources on the biomass and caffeine dehydrogenase activity of *Pichia manshurica* (CD1)

Five different organic and inorganic nitrogen sources (peptone, tryptone, ammonium sulphate, sodium nitrate) were used separately (0.4%) into the amplifying medium instead of yeast extract to check any improvement in the production of caffeine dehydrogenase. Among all the nitrogen sources tested, the maximum growth of CD1 (1.29gm/100) as well as the

optimum activity of caffeine dehydrogenase (19.25 nmol/(min mg)) was obtained with yeast extract as the nitrogen source. However, the enzyme production was found to be hindered in the presence of peptone (4.08 nmol/(min mg)) and tryptone (3.61 nmol/(min mg)). The inorganic nitrogen compounds also supressed the enzyme production to some extent (Table 3).

Carbon source (0.2%)	**Biomass(g/100mL)[a]**	**Caffeine dehydrogenase activity[a] (nmol/ (min mg))**
Glucose	1.27±0.07	18.57±0.51
Fructose	1.36±0.04	13.13±0.6
Lactose	1.60±0.07	5.62±0.32
Starch	1.18±0.01	3.82±0.25
Sucrose	1.36±0.05	6.73±0.42
Maltose	1.11±0.04	4.61±0.35

[a] Values are means ± standard deviation for three independent measurement

Table 4: Influence of different carbon sources on the biomass and caffeine dehydrogenase activity of *Pichia manshurica* (CD1)

We have also investigated the effects of different carbon sources on CD1 cell growth and caffeine dehydrogenase production. Five carbon sources namely fructose, lactose, starch, sucrose and maltose were used at a concentration of 0.2% in amplifying medium instead of glucose (0.2%). The result (Table 4) illustrated that the tested carbon sources do not show any profound effect on cell growth. However, the activity of caffeine dehydrogenase was found to be maximum with glucose as the carbon source (18.57 nmol/ (min mg)), followed by fructose (13.13 nmol/(min mg)). A low level of enzyme synthesis was observed in the presence most of the disaccharides (lactose, sucrose). Furthermore, starch had a repressible effect on the enzyme production. These results are in accordance with previous studies

(Mohapatra et al. 2006), suggesting that different nitrogen and carbon sources might have a regulatory effect on caffeine dehydrogenase biosynthesis by CD1.

3.3.5. Purification of caffeine dehydrogenase enzyme

Initially, based on the result of the activity staining gel of crude caffeine dehydrogenase, the action site range of the enzyme was determined (Fig. 10-I). By help of activity staining result the work of enzyme purification was progressed. The caffeine dehydrogenase enzyme was purified from CD1 cell extracts in a two-step procedure resulting in a 25-fold purification of the enzyme, relative to the activity in cell extracts. The crude enzyme was first subjected to sephadexG-100 column chromatography (Fig. 10-II) followed by purification from polyacrylamide gel by sonication depending on the result of activity staining. The purified protein fraction was found to have a specific activity of 485 nmol/(min mg). A summary of the purification steps was presented in Table 5.

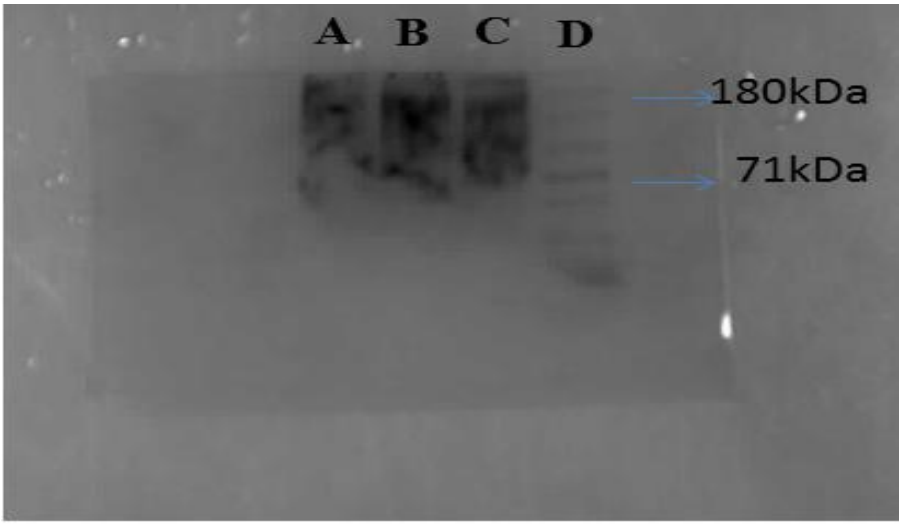

Fig. 10-I: Activity staining gel of crude caffeine dehydrogenase. Lane A, B and C = crude caffeine dehydrogenase; Lane D = protein marker

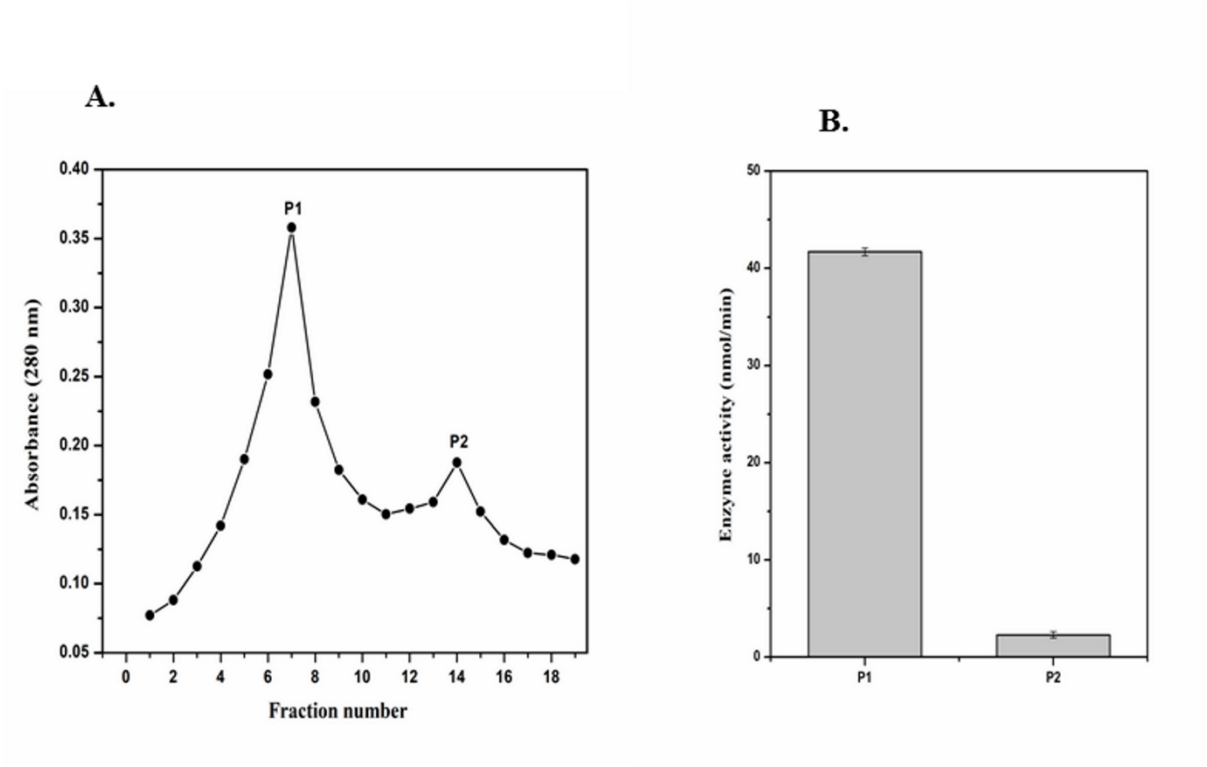

Fig. 10-II: (A) Sephadex G-100 gel filtration chromatogram; (B) Enzyme activity at 566nm of the peak material (P1&P2).

Steps	Total enzyme activity (nmol/min)	Protein concentration(mg)	Specific activity (nmol/(min mg))	Yield (%)	Purification fold
Crude	77	4	19.25	100	1
Sephadex G-100 column	37.35	1.27	29.4	48	1.5
Polyacrylamide gel sonication	4.85	0.01	485	6.29	25

Table 5: Summary of the purification procedures of caffeine dehydrogenase from *Pichia manshurica* CD1

3.3.6. Determination of purity and molecular mass of enzyme

The purity of the protein fraction having caffeine dehydrogenase activity was determined by 10% SDS – PAGE. The purified fraction also showed a single band (approximately 85kDa) (Madyastha et al. 1999) and caffeine dehydrogenase enzyme was discovered as a single sub unit enzyme (Fig. 11).

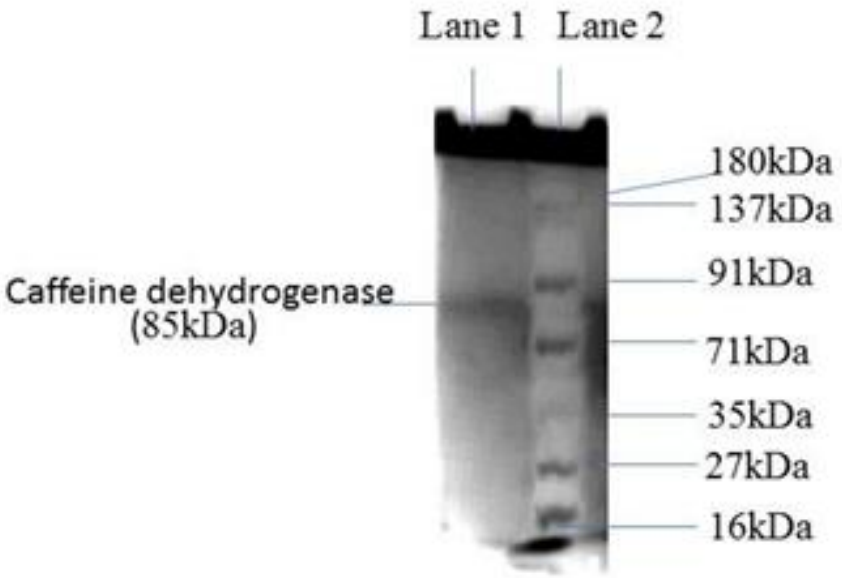

Fig. 11: SDS-PAGE analysis of purified caffeine dehydrogenase. Lane 1, the purified enzyme; Lane 2, the protein marker

3.3.7. Effect of pH and temperature on caffeine dehydrogenase activity

Activity of an enzyme depends on certain physical and chemical properties which may induce its reducing activity. pH & temperature are important physical parameters and changes in both play a major role in enzyme activity (da Silva et al. 2012). The optimum pH of the purified enzyme caffeine dehydrogenase isolated from CD1 was found to be 7.5 (Fig. 12A). The activity increased steadily from pH 5 to 7.5 after which it decreased and reached to its minimum value at pH 10. Caffeine dehydrogenase enzyme obtained from other sources like *Alcaligens* speices, mixed consortium of *Klebsiella* and *Rhodococcus* speices and

Pseudomonas sp. strain CBB1 were also found to have pH optima within the range 7 to 7.5 (Madyastha and Sridhar 1998; Madyastha et al. 1999; Mohapatra et al. 2006; Yu et al. 2008).

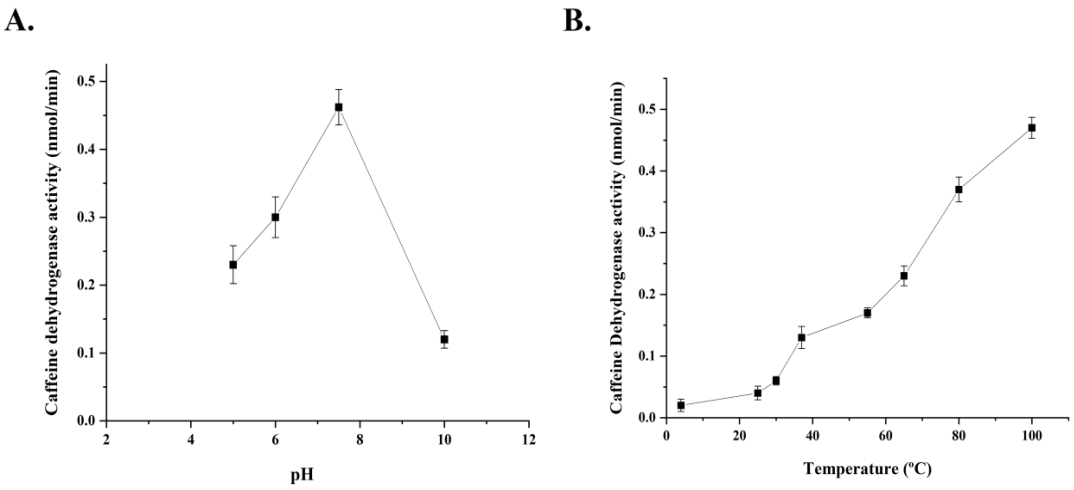

Fig. 12: (A) The effects of different pH conditions on the activity of purified Caffeine dehydrogenase. (B) The effects of different temperature conditions on the activity of purified Caffeine dehydrogenase.Values are expressed as mean ± SD (n=3).

Next, we assayed the enzyme activity at various temperatures and observed that the purified caffeine dehydrogenase activity increased linearly from 4°C to 100°C (Fig. 12B), which was the highest temperature assessable in the laboratory. This is an interesting observation. It is already known about certain enzymes that are found in mesophilic micro-organisms like trimethylamine *N*-oxide reductase of *Escherichia coli* (Jean et al. 1999) and chlorate reductase of *Pseudomonas chloritidis mutans* (Wolterink et al. 2003) which have optimal temperatures of 80°C and 75°C respectively. Moreover, Yu et al (Yu et al. 2008) have also reported that the caffeine dehydrogenase enzyme purified from *Pseudomonas* sp. strain CBB1 exhibited increased activity at high temperature. They showed that the enzyme activity

increased as the temperature was increased from 25 to 66°C, which was the highest temperature they measured. However, the optimum temperature of caffeine oxidase (or caffeine dehydrogenase) isolated from mixed cultures of *Kelbsiella* and *Rhodococcus* species (Madyastha and Sridhar 1998; Madyastha et al. 1999) and from *Alcaligens* species (Mohapatra et al. 2006) was found to be much lower (~35°C). These results suggested that there might be more than one isoform of the caffeine dehydrogenase enzyme with two different temperature optima.

3.3.8. Effect of pH and temperature on stability of caffeine dehydrogenase

The purified enzyme was incubated at different pH (5, 6. 7.5 and 10) and temperatures (37ºC, 55 ºC, 65 ºC, 80 ºC and 100 ºC) for 20 min to determine the pH stability and thermostability by calculating the percentage of relative activity. As shown in Fig. 13A, the enzyme retained almost 53% and 36% of its initial activity when incubated at pH 5 and pH 10, respectively, and remained stable over a narrow pH range from 6 to 7.5. The enzyme caffeine oxidase isolated from *Alcaligens* species (Mohapatra et al. 2006) shows quite similar results.

The result of thermostability in Fig. 13B shows that the enzyme retained 80% of its initial activity when incubated at 37 ºC for 20 min and about 71% when incubated at 55 ºC. Furthermore, after 20 min of incubation at 100 ºC, the enzyme retained 54% of its activity. The thermostability of caffeine dehydrogenase is considered a significant and helpful criteria for industry focused property for bio-decaffeination, as the result demonstrated that the enzyme is stable over a wide range of temperatures.

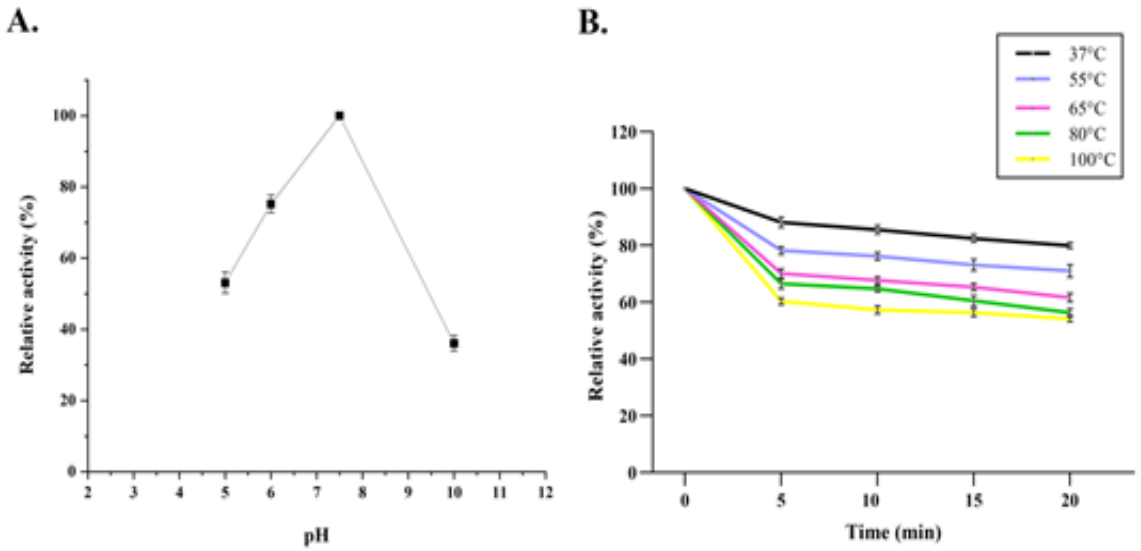

Fig. 13: pH & Temperature stability of caffeine dehydrogenase. (A) pH stability of caffeine dehydrogenase. Purified enzyme was pre-incubated in different buffers from pH 5 to 10 for 20min at 4 °C; the relative activities were measured at 100 °C for 1 h, under the standard assay condition. The maximum value was 100 %. (B) Temperature stability of caffeine dehydrogenase. Purified enzyme was incubated in absence of substrate for 20 min at 37, 55, 65, 80, and 100 °C respectively. Samples were taken at 5 min intervals and relative activities were measured at 100 °C for 1 h, under the standard assay method. The 0 min value was 100. Values are expressed as mean ± SD (n=3).

3.3.9. Substrate specificity

The relative activities of purified enzyme with different substrates are represented in Table 6. The enzyme showed high specificity for caffeine (100%) followed by theobromine (31.23%), theophylline (27.66%) and very less specificity for xanthine (2.33%) respectively. Similarly, caffeine oxidase from *Klebsiella* and *Rhodococcus* species (Madyastha et al. 1999) and from *Alcaligenes* species(Mohapatra et al. 2006) showed high specificity towards caffeine with almost no specificity towards xanthine.

Substrate(0.5mM)	**Relative activity[a] (%)**
Caffeine	100
Xanthine	2.33±0.25
Theophylline	27.66±0.71
Theobromine	31.23±0.53

[a] Values are means ± standard deviation for three independent measurement

Table 6: Substrate specificity of purified caffeine dehydrogenase from *Pichia manshurica* (CD1)

3.3.10. Determination of K_m and V_{max}

The K_m and V_{max} of purified caffeine dehydrogenase with caffeine as the substrate at pH 7.5 and temperature 100ºC were found to be 11.2 µM and 0.372 nmol/(mL min) respectively from Lineweaver–Burk plot (Donald Voet, Judith G. Voet 2016) (Fig. 14).

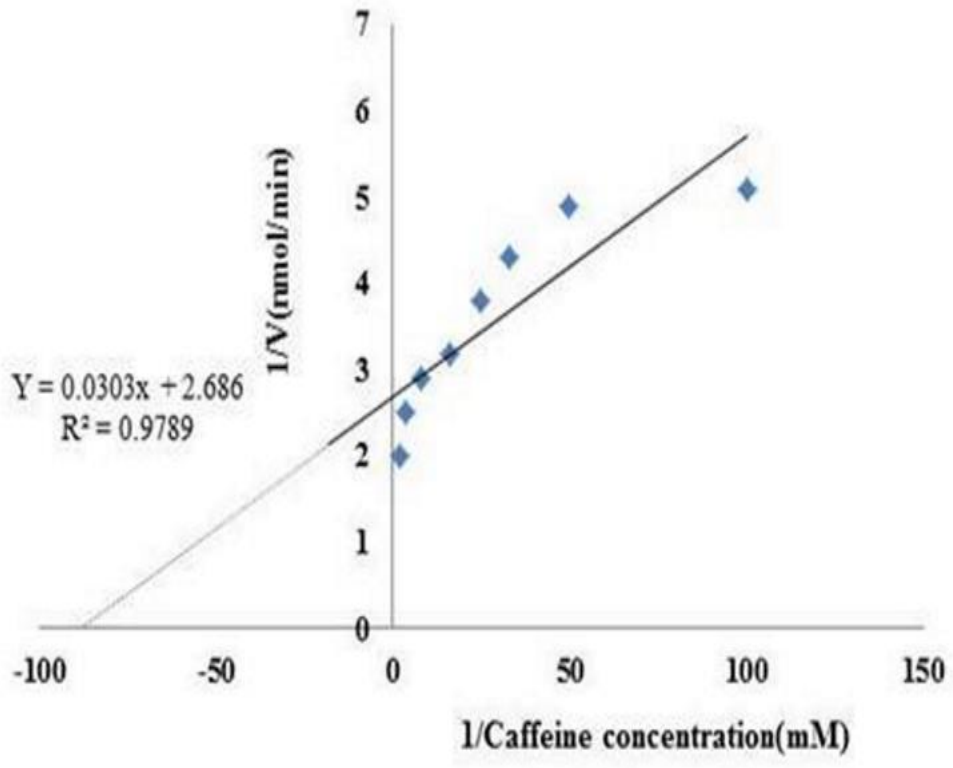

Fig. 14: Lineweaver-Burk plot of purified caffeine dehydrogenase against the caffeine concentrations. S= caffeine concentration; V= caffeine dehydrogenase activity.

3.3.11. Effects of different metal salts, additives and solvents on caffeine dehydrogenase activity

The relative activities of the purified enzyme after treatment with different metal salts and chemical agents are represented in Table 7.

Metal salts and Chemical agents	Relative activity[a] (%)
$K_2Cr_2O_7$	13.00±0.45
$CuSO_4$	0
$Pb(NO)_3$	17.10±0.62
$ZnSO_4$	14.00±0.41
$NiCl_2$	15.01±0.71
$HgCl_2$	6.50±0.36
$MgCl_2$	11.00±0.53
EDTA	12.50±0.49
SDS	8.00±0.42
DTT	18.07±0.57
β-mercaptoethanol	0
Benzene	71.00±2.51
Methanol	78.00±3.07
Isopropanol	65.22±2.21

[a] Values are means ± standard deviation for three independent measurement

Table 7: Effect of different metal salts and chemical agents on activity of purified Caffeine dehydrogenase from *Pichia manshurica* CD1

All the metal ions were found to inhibit caffeine dehydrogenase activity. However, complete inhibition was observed in case of Cu^{2+}. According to previous reports, the catalytic activity of caffeine dehydrogenase isolated from other sources was mostly affected by various metal ions (Mohapatra et al. 2006).

The enzyme activity was also inhibited in presence of EDTA, SDS, DTT, β-mercaptoethanol, methanol, isopropanol and benzene. The inhibition of caffeine dehydrogenase activity by EDTA suggests that it might be a metalloenzyme. Inhibition by SDS, DTT, β-mercaptoethanol, benzene, methanol and isopropanol might be due to the denaturation of the enzyme by these agents, which leads to the loss of enzyme activity.

3.4. Conclusions

In the present study, we described the isolation of a caffeine degrading yeast strain from Kombucha tea. To the best of our knowledge, this study reported for the first time that *Pichia manshurica* isolated from Kombucha tea exhibited the ability to degrade caffeine and use it as the sole source of carbon and nitrogen. The caffeine degrading ability of the yeast strain is attributed to its intracellular caffeine dehydrogenase enzyme. The enzyme was purified in a two-step procedure resulting in a 25-fold purification and was thoroughly characterized. A unique feature of this enzyme is that it remains highly active and stable over a wide range of temperatures (even at 100ºC), making it a promising enzyme for industrial application of decaffeination. Furthermore, we determined the variety of activity profile of enzyme caffeine dehydrogenase, including optimum pH, substrate specificity, kinetic parameters and effect of metal salts, additives and solvents. This study, therefore, provides knowledge on the important industry-oriented properties of caffeine dehydrogenase on caffeine degradation and potential biotechnological utilization of the enzyme in waste treatment and biosensor.

CHAPTER IV

Temperature dependent conformational changes of thermostable caffeine dehydrogenase from *Pichia manshurica* CD1

4.1. Introduction

Caffeine dehydrogenase (E.C.1.17.5.2) is an intracellular enzyme that is also known as caffeine oxidase in the scientific literature. The enzyme is a member of oxidoreductase family(Yu et al. 2008). The enzyme is most well-known for its potential ability to directly oxidize caffeine(Mohapatra et al. 2006). The enzyme is caffeine-specific, has less affinity for theobromine, and has no affinity for xanthine (Mohanty et al. 2012). Caffeine enzymatic catabolism in microbes has been reported to be performed by *N*-demethylation and oxidation pathways with demethylases and dehydrogenase or oxidase enzymes, respectively(Dash and Gummadi 2006). Caffeine degrading enzymes can be used to treat caffeine in the agro-industrial wastes such as coffee pulps and husks, as well as in biotechnological processes such as waste treatment and biosensor development (Mohapatra et al. 2006; Shanmugam et al. 2021).

A Caffeine dehydrogenase enzyme was isolated and purified from *Pseudomonas* sp. strain CBB1(Yu et al. 2008). They found that the enzyme activity increased as the temperature increased from 25 °C to 66 °C. Caffeine oxidase was also isolated and purified from mixed cultures *of Klebsiella and Rhodococcus* species (Madyastha et al. 1999) and *Alcaligenes sp.* CF8 species showed the maximum enzyme activity at 35 °C (Mohapatra et al. 2006).

Thermostable enzymes are highly specific; they have significant advantages in applications where high temperatures are required to increase the bioavailability or substrate solubility to reduce the viscosity (Collins et al. 2005.). Thus, thermostable enzymes are important for the potential use in many industrial applications, such as optimizing reactions in food and paper industries, detergents, drug and waste treatment(Temsaah et al. 2018; Sharma et al. 2019). As a result, discovering enzymes that can work at high temperatures is important(Gutarra et al. 2009; Vaseekaran et al. 2010; Suresh and Anil Kumar 2012; Bhatt et al. 2020).

Thermostability is an important property of enzymes because it increases industrial productivity(Shao et al. 2020; Karray et al. 2021; Okpara and Okpara 2022). Therefore, both in basic research and industrial applications, it is necessary to identify a thermostable caffeine-degrading enzyme with better enzymatic activity. Enzyme characterization is also essential for a better understanding of the structure and function of the enzyme. Although biochemical studies and kinetic data of the caffeine degrading enzymes (caffeine dehydrogenase/caffeine oxidase) have been reported previously, the impacts of high temperature on stability and conformational changes of the enzyme caffeine dehydrogenase have not been clearly elucidated.

In this study, Caffeine dehydrogenase was identified as an intracellular enzyme of *Pichia manshurica* strain CD1. The purified caffeine dehydrogenase showed its maximum activity at 100 ºC and retained 56% of its stability. The present study was the first to investigate the influence of heat treatment on the activity and stability of caffeine dehydrogenase based on circular dichroism and fluorescence spectroscopy. Both the experimental results corroborate with the activity result of the enzyme caffeine dehydrogenase with increased temperature. The enzyme can maintain its conformation to remain catalytically active at high temperatures. The findings of the current study will also be helpful for industry-focused applications.

4.2. Materials and methods

4.2.1. Organism, culture condition and preparation of cell lysate

The caffeine degrading enzyme producing yeast *Pichia manshurica* CD1 (GenBank accession number **KY799109**) isolated from kombucha tea (Parvin et al. 2023) was grown in 100 mL caffeine broth media (0.12 g/L Na_2HPO_4, 1.3g/L K_2HPO_4, 0.3 g/L $MgSO_4$, 0.3g/L $CaCl_2$, 0.2g/L $FeSO_4$ and 1g/L caffeine). The culture condition of this strain was pH 5 for 3 days at 30°C with constant shaking at 110 rpm. The grown yeast cells from caffeine media were pelleted and transferred into caffeine-enriched media containing 0.12 g/L Na_2HPO_4, 1.3g/L K_2HPO_4, 0.3 g/L $MgSO_4$, 0.3g/L $CaCl_2$, 0.2g/L $FeSO_4$, 2g/L dextrose and 4g/L yeast extract and 2g/L caffeine (pH 5). After 4 days, the maximum caffeine dehydrogenase production was found. The induced yeast cells were collected by centrifugation at 12000 *g* for 12 min at 4 °C. Then the yeast cells were suspended in a 50 mM potassium phosphate buffer (pH 7.5) and lysed by glass bead disruption. The cell lysate was collected after centrifugation at 16000 *g* for 15 min at 4 °C and stored at -20 °C for enzyme purification.

4.2.2. Caffeine dehydrogenase activity assay

The caffeine dehydrogenase activity of a purified enzyme was determined by incubating an appropriate amount of the enzyme with 0.5 mM caffeine and 0.5 mM NBT in 50 mM potassium phosphate buffer (pH 7.5) for 1 h at 100 °C. In the caffeine-dependent NBT reduction assay, when NBT was used as the electron acceptor, the enzyme activity was determined by using a UV/VIS spectrophotometer (Jasco V-530) to monitor the increasing absorbance at 566 nm. A non-enzymatic reaction containing caffeine and NBT was also run as a control in which potassium phosphate buffer (pH 7.5) was used instead of enzyme solution. Value, if any obtained, was used to set "auto-zero" for the respective experiments.

Under the standard activity assay conditions, one unit of caffeine dehydrogenase activity was defined as the amount of enzyme that released 1 nmol of formazan per min.

4.2.3. Effects of temperature on purified caffeine dehydrogenase enzyme

To determine the effect of temperature on enzyme activity, the purified enzyme (Parvin et al. 2023) was incubated at 30 °C, 37 °C, 55 °C, 65 °C, 80 °C, and 100 °C for 1 hour. Then enzyme activity was determined at each temperature under the standard assay method (Chapter 4, section 4.2.2.). Control experiment was performed where 50 mM potassium phosphate buffer (pH 7.5) was used instead of enzyme solution for each temperature separately. Value, if any obtained, was used to set "auto-zero" for the respective experiments.

The thermostability of the enzyme was determined by pre-incubating the enzyme at various temperatures ranging from 30 °C to 100 °C for 15 min. Then the enzyme activity was determined at 100°C after 1 hour incubation under the standard assay method (Chapter 4, section 4.2.2.). The 0 min value was 100. Control experiment was done where appropriate amount of 50mM potassium phosphate buffer (pH 7.5) was added instead of enzyme. Value, if any obtained, was used to set "auto-zero" for the respective experiments. All reactions were carried out in triplicate.

4.2.4. Thermodynamic parameters of caffeine dehydrogenase

The purified caffeine dehydrogenase activity was determined over a temperature range of 30 °C to 100 °C, as described in the assay system. From the temperature activity profile result, the activation energy *(E_a)* of purified caffeine dehydrogenase from *Pichia manshurica* CD1 was measured using the Arrhenius equation (1).

$$k = Ae^{-Ea/RT} \quad (1)$$

$$ln\ k = ln\ A - E_a / RT$$

$$2.303\ log\ k = -E_a / RT + 2.303\ log\ A$$

$$log\ k = -E_a / 2.303RT + log\ A$$

$$log\ k = log\ A - E_a / 2.303RT$$

The Arrhenius equation is rearranged as y = c+mx, equation of straight line. Where, y = *log k*, x = *1/T*, slope = m = $-E_a / 2.303R$ and intercept = *log A*

The activation energy was calculated by finding the slope of the plot of *log k* vs *1/T* (Fig. 16).

Also, using the equations below (2, 3 & 4), the thermodynamic parameters (*ΔH, ΔG & ΔS*) of purified caffeine dehydrogenase were determined.

$$\Delta H = E_a - RT \quad (2)$$

$$\Delta G = -RT\ ln(k\ h / k_B\ T) \quad (3)$$

$$\Delta S = \Delta H - \Delta G / T \quad (4)$$

In the Arrhenius equation, k (s^{-1}) is the reaction rate constant at temperature T (K), A represents the frequency at which atoms and molecules collide, E_a (kJ mol^{-1}) is the activation energy of the reaction, and R is the universal gas constant (8.314 J mol^{-1} K^{-1}). *ΔH* (kJ mol^{-1}) is the change in enthalpy, *ΔG* (kJ mol^{-1}) is the change in Gibbs free energy, *ΔS* is the change in entropy (kJ mol^{-1}), h is the Planck constant (6.63×10^{-34} J s), and k_B is Boltzmann constant (1.38×10^{-23} JK^{-1}). *ΔS* denotes the change in entropy (kJ mol^{-1}).

4.2.5. Circular dichroism (CD)

Circular Dichroism (CD) spectroscopy studies were carried out on a JASCO J-1500 spectrophotometer equipped with a peltier to investigate the effect of temperatures on the secondary structure of the protein caffeine dehydrogenase. The far-UV CD spectra were monitored in the range of 190-250 nm at a protein concentration of 6 µg /mL using a quartz

cuvette with a 1 mm path length. Near-UV CD spectra were taken in the range of 250-350 nm using a quartz cuvette with a 1 cm path length at a protein concentration of 12 μg/mL. The spectra were obtained in the far/near UV range with a scanning speed of 100 nm/min. The CD spectrum of native protein was first recorded, and the temperature of the samples in both the near-UV and far-UV ranges were gradually increased and recorded at 30 °C, 50 °C and 100 °C. Each CD spectrum represents the average of three scans. The calculation of individual secondary structure components was done using BeStSeL software (Micsonai et al. 2015, 2021) from the spectra obtained in the far UV range of 190-250 nm.

4.2.6. Tryptophan fluorescence spectroscopy

The tryptophan fluorescence emission of purified caffeine dehydrogenase was studied in its native state as well as at temperatures of 30 °C, 50 °C, and 100 °C. The protein was pre-incubated for 15 minutes at the above temperatures to study its effect on fluorescence spectra. The excitation wavelength was set at 295 nm to excite the tryptophan residues of the protein, with a bandpass of 5 nm. The emission spectra of protein solutions were obtained at wavelengths between 305-400 nm with a bandpass of 5nm. The scanning was done at a rate of 200 nm/min. Each fluorescence spectra represents an average of three scans.

4.2.7. Statistical analysis

All the values are expressed as mean ± SD (n=3). Significant differences between means were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the Origin Pro 2021b software.

4.3. Results and discussion

4.3.1. Effects of temperature on caffeine dehydrogenase activity and stability

The activity of purified caffeine dehydrogenase was measured at temperatures ranging from 30 °C to 100 °C, and the results are shown in Fig. 15A. We found a remarkable observation that the enzyme activity linearly increased even at 100 °C, which was the highest temperature we could reach in the laboratory. Thus, the results conclude that the enzyme caffeine dehydrogenase is active over a wide temperature range. Many studies on the effect of temperature on caffeine dehydrogenase enzyme, isolated from various environments, have been published in the literature(Madyastha et al. 1999; Mohapatra et al. 2006; Yu et al. 2008).

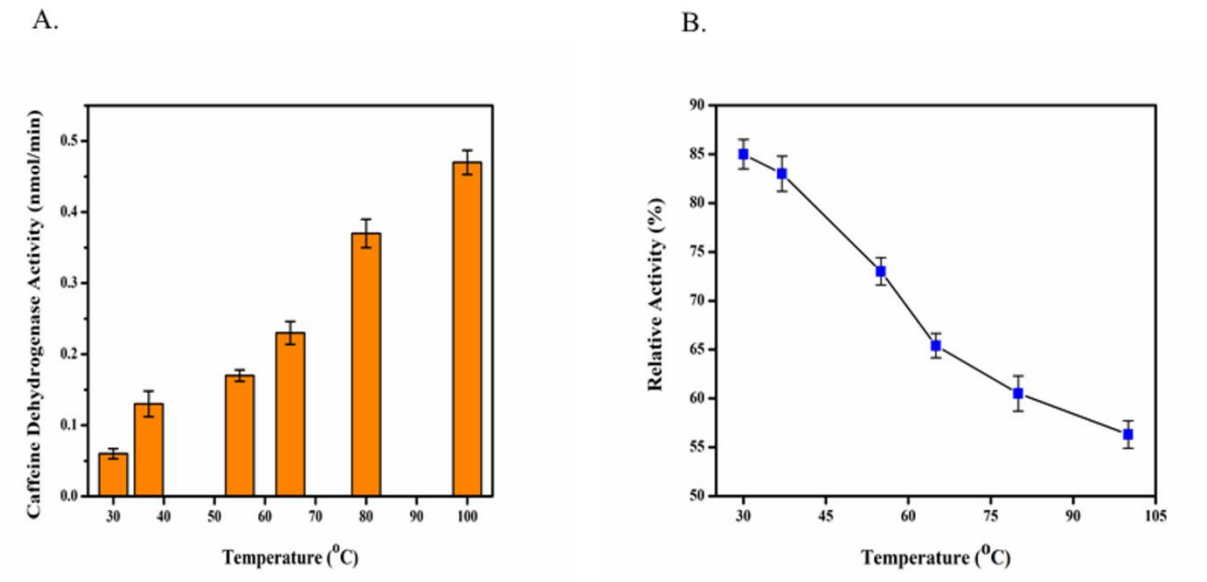

Fig. 15: The effects of temperature on Caffeine dehydrogenase enzyme. (A) Activity of purified Caffeine dehydrogenase at different temperatures (30 °C to 100 °C). (B) Thermostability of purified Caffeine dehydrogenase. Enzyme was pre-incubated in absence of substrate for 15 min at respective temperatures and relative activity was measured at 100 °C for 1 h, under standard assay condition. Values are expressed as mean ± SD (n=3).

Fig. 15B shows that the residual activity of caffeine dehydrogenase after 15 min of heating at 100 °C remained around 56% of the control sample activity, indicating that the enzyme was stable up to 100 °C. However, the enzyme retained 73 % activity at 55°C, 65.4% at 65°C and 60.5% activity at 80°C for 15 min incubation at respective temperatures. Moreover it is already reported that the enzyme is called stable when its residual activity is greater than 50 % (Jayanthi et al. 2019). The thermostability of caffeine dehydrogenase isolated from *Pichia manshurica* strain CD1 is an important and helpful criterion for industrial use.

4.3.2. Thermodynamic parameters of caffeine dehydrogenase

The thermodynamic parameters of purified caffeine dehydrogenase were analyzed and are represented in Table 8. The minimum energy required by reactants to transform into products is known as activation energy (E_a). In our experiments, the activation energy (E_a) of purified caffeine dehydrogenase was found to be 27.05±0.8 kJ mol^{-1} (Fig. 16).

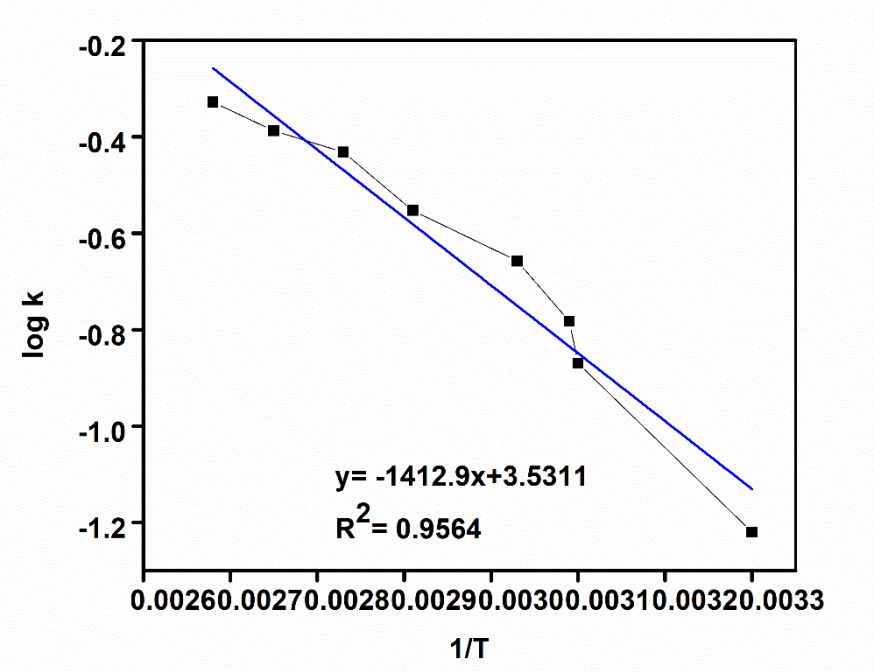

Fig. 16: Graph of log k vs 1/T plot with slope – E_a / 2.303RT

We also discovered that as temperature increased, *ΔH* decreased from 24.53 to 23.95 kJ mol^{-1}. The positive value of the change in enthalpy (*ΔH)* suggested that the enzymatic reaction is endothermic. . Moreover, increasing the temperature caused a less reduction in the *ΔH* value, which indicates that the variations were significant and increased the activation efficiency of the enzyme substrate complex (Silva et al. 2019; Soares da Silva et al. 2018). However, as the temperature increased, the change in Gibbs free energy (*ΔG)* value increased from 81.31 to 94.39 kJ mol^{-1}. The positive *ΔG* values suggest that the enzymatic reaction cannot proceed spontaneously; the reaction requires energy input. Also, the increased *ΔG* value indicates greater resistance to denaturation and, as a result, greater thermostability (Soares da Silva et al. 2018).On the other hand, the negative values of *ΔS* indicate the occurrence of an aggregation phenomenon as temperature increases (Chafik et al. 2020; Gentile et al. 2021)**.** In fact, it has been reported that if the hydrophobic chains of an enzyme are close together, a negative entropy effect should be observed at higher temperatures as long as the enzyme active site retains its structure (Afzal et al. 2005; Soares da Silva et al. 2018).

Temperature (°C)	**k (min^{-1})**	**ΔH ($kJmol^{-1}$)**	**ΔG ($kJmol^{-1}$)**	**ΔS ($kJmol^{-1}$)**
30	0.06±0.007	24.53±0.7	81.31±0.18	-0.1873±0.0001
40	0.135±0.018	24.44±0.63	81.97±0.13	-0.1838±0.00015
50	0.165±0.008	24.36±0.44	84.18±0.12	-0.1852±0.00012
60	0.22±0.016	24.28±0.75	86.10±0.16	-0.1856±0.0001
70	0.28±0.01	24.20±0.61	88.03±0.21	-0.1860±0.00017
80	0.37±0.02	24.11±0.77	89.86±0.11	-0.1862±0.0002
90	0.41±0.029	24.03±0.57	92.16±0.12	-0.1876±0.00015
100	0.47±0.017	23.95±0.53	94.39±0.17	-0.1884±0.00011

Values of k, ΔH, ΔG, and ΔS are the means ± standard deviation for three independent measurements.

Table 8: Thermodynamic parameters of purified caffeine dehydrogenase

4.3.3. Far and Near UV circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy is a widely known method for characterizing the secondary structures of proteins(Greenfield 2006). The CD spectrum in the far-UV region of 190-250 nm corresponds to the presence of peptide bonds and can be analyzed to obtain secondary structural components of α–helix, β–sheet and random coils in a protein. The far ultraviolet wavelength region is useful for protein secondary structure quantification due to the CD peak sensitivity for the dihedral angles between adjacent amino acids. In contrast, the CD spectrum in the near-UV region of 250-350 nm can detect the changes in the environments of the aromatic amino acid side chains such as phenylalanine (250-270 nm), tyrosine (270-290 nm), and tryptophan (280-300 nm).

The CD spectra of the native protein in the far-UV range revealed a positive peak at ∼192 nm, whereas the negative peak was observed at ∼224. The CD peak intensities observed for the native protein exhibit the signature peaks for the α-helix (Murtaza et al. 2018), suggesting that the protein exists predominantly as α-helix (Fig. 17A). It was observed that initially the protein exhibits 50.6% α-helical character whereas the minor conformations of β-sheet were found to be 3.7%. The stability of the folded protein structure was analyzed after raising the temperature of the protein sample to 30 °C, 50 °C and 100 °C. With the incremental increase in temperature, a transition of α-helix to β-sheet was observed (Table 9). Interestingly, an inverse relationship was observed for the α-helix structure with an increase in temperature. The α-helix percentage decreased from 50.6% for native protein to 45.1%, 40.1%, and 35.5% at 30 °C, 50 °C, and 100°C respectively. In addition, an increase of β-sheet character with the increase in temperature was also observed. The β-sheet percentage did not differ significantly between the protein samples at 30 °C and the native protein. However, with further increase in temperature, a transition was observed at 50 °C with the β-sheet percentage increasing to 10.8%. Further increase of temperature resulted in an increase of β-sheet to 19.8% at 100 °C.

These results suggest that the protein predominantly adopts α-helical structure, which is not completely disrupted at even high temperatures of 50 °C and 100°C.

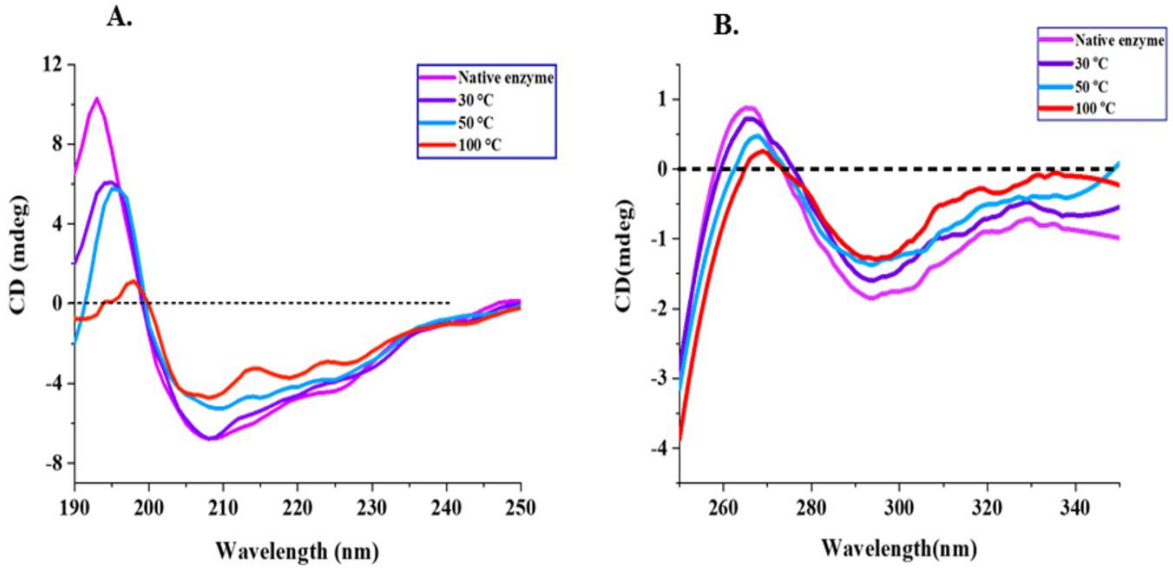

Fig. 17: Circular Dichroism spectra of native and thermally treated Caffeine dehydrogenase at different temperatures (A) Far-UV region (B) Near-UV region.

Temperature (°C)	α-helix[a] (%)	β-sheet[a] (%)
Native enzyme	50.6±0.58	3.7±0.07
30	45.1±0.49	3.1±0.055
50	40.1±0.66	10.8±0.039
100	35.5±0.74	19.8±0.033

[a] Values are means ± standard deviation for three independent measurement

Table 9: The secondary structure profile of the Caffeine dehydrogenase with increasing temperature

In addition, the tertiary structure of the enzyme caffeine dehydrogenase was assessed at different temperatures using near-UV CD measurements (Fig. 17B). The near-UV CD experiments were undertaken to study the local environment of the aromatic amino acid side chains of the protein of interest. The near-UV CD spectra of caffeine dehydrogenase revealed the presence of a defined tertiary structure mainly contributed by aromatic amino acids. With the increase in temperature to 30 °C, 50 °C, and 100 °C, the intensity of alpha-helix peaks decreased, which may be a result of the alpha-helix to beta sheet transition observed in the secondary structure of the protein. However, the changes are not sufficient to disrupt the tertiary structure of the protein, suggesting that the local environment of the folded protein is conserved at high temperatures. Thus, both the near and far-UV CD results indicate that the protein is not completely denatured at high temperatures and that the secondary and tertiary structures are altered to some extent but not completely disrupted. We can also conclude from the above results that the enzyme activity increases with increasing temperature, suggesting that the active site of this enzyme becomes more exposed as the temperature increases.

4.3.4. Tryptophan fluorescence spectroscopy

Fluorescence spectral analysis is a useful technique to investigate structural aspects of proteins, such as folding and conformation. The intrinsic tryptophan fluorescence of native and thermally treated protein was measured, and results are shown in Fig 18. The tryptophan fluorescence spectra of the native protein showed a maximum emission at wavelength 337 nm, while the fluorescence emission wavelength of thermally treated protein ranged from 338 nm (30 °C), 338.4 nm (50 °C) and 339 nm (100 °C). On increasing the temperature, the maximum emission peak of protein slightly (1-2 nm) red shifted, which could indicate that the enzyme caffeine dehydrogenase is not unfolded during thermal processing. When the temperature was increased, the fluorescence intensity decreased compared to that of native protein because the other non-radiative processes, such as internal conversion, increased the rate of photon emission (Möller and Denicola 2002)**.** Temperature-dependent dynamic quenching (Bushueva et al. 1978)may happen. Quenching in protein molecules is caused not only by the external quencher factors but also by groups of atoms capable of reducing the fluorescence of chromophore groups in close proximity. The most effective quenchers in proteins are the carbonyl group of the peptide bond and the side amino and carboxyl groups.

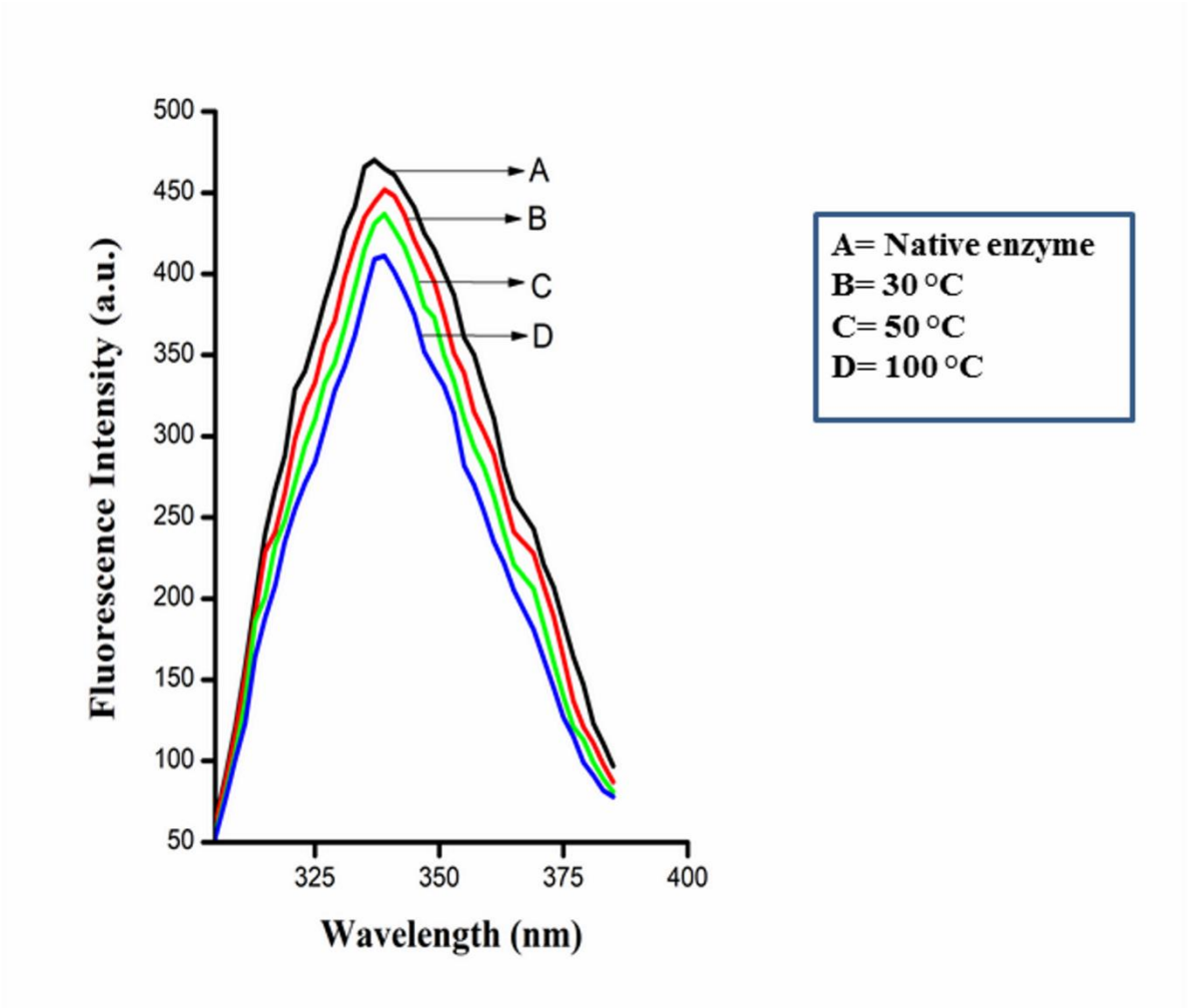

Fig. 18: Tryptophan emission fluorescence spectra of native and thermally treated Caffeine dehydrogenase at different temperatures. The excitation wavelength was 295 nm.

We hypothesized that the decrease in fluorescence intensity of caffeine dehydrogenase with temperature in the absence of an external quencher was caused by chromophore interaction with these close proximity protein groups also.

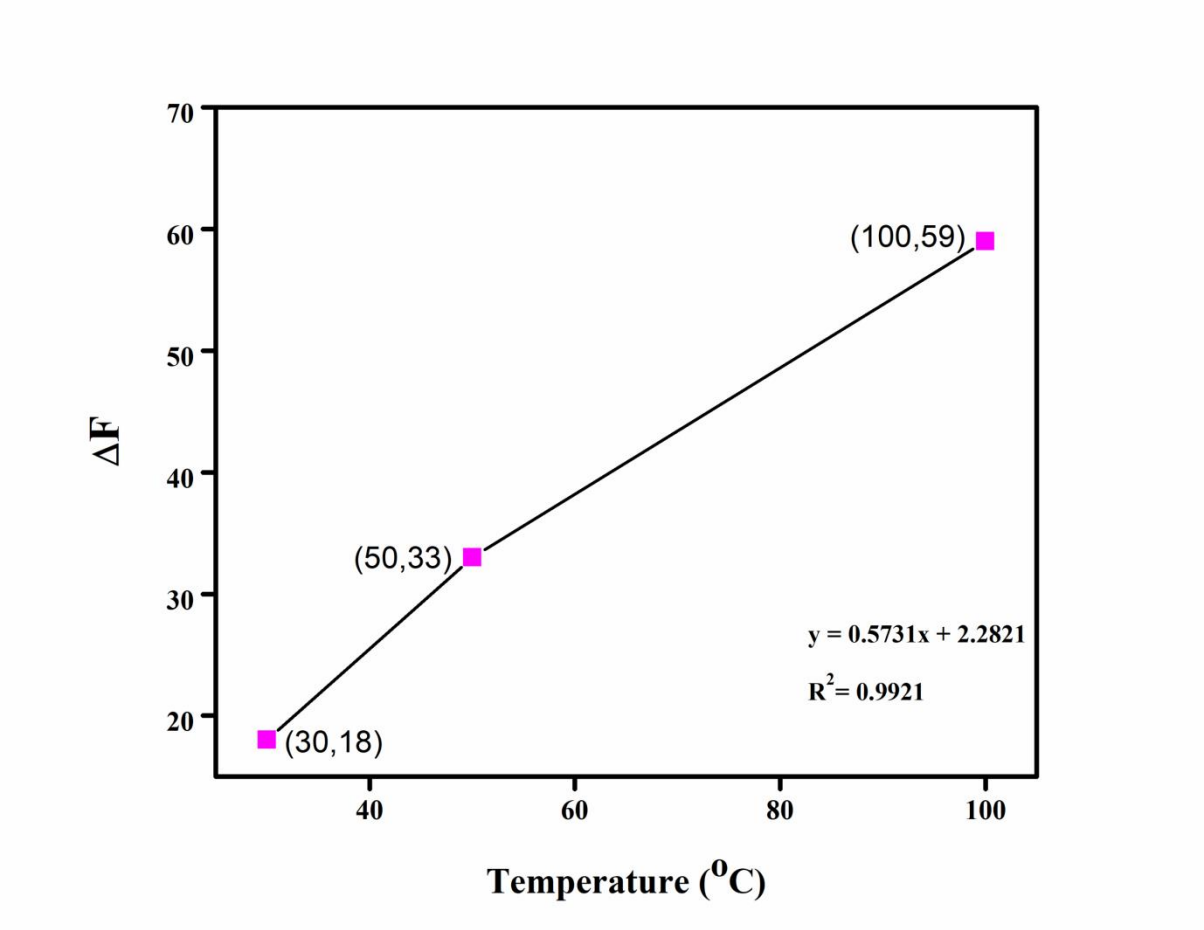

Fig. 19: Plot of ΔF vs temperature, where ΔF= (F0 – F), F0 and F are the fluorescence intensity of native and the thermally treated Caffeine dehydrogenase.

Also, the fluorescence intensity change is studied by the equation $\Delta F = F^0 - F$, where F^0 and F are the fluorescence intensity of the native protein and the thermally treated protein, respectively. When ΔF was plotted against temperature, the enzyme showed an increase in ΔF with increasing temperature. Thus, Fig. 19 indicates that as temperature increases, the degrees of freedom of the protein region around tryptophan increase, and excited energy dissipates due to vibrational and rotational motion. Therefore, fluorescence emission decreases consistently in a non-cooperative manner. As a result, the enzyme is able to maintain its conformation and remain catalytically active at all the temperatures studied.

4.4. Conclusions

In this study, caffeine dehydrogenase enzyme was found to be active at a wide range of temperatures (30 °C to 100 °C). The activity of the purified caffeine dehydrogenase enzyme was increased linearly with the increase of temperature (up to 100 °C) and remained stable (56% compared to control) at 100 °C. In addition thermodynamic parameters (ΔH, ΔG & ΔS) of purified caffeine dehydrogenase were studied and the activation energy was 27.05±0.8 $kJmol^{-1}$. The CD spectroscopy and fluorescence spectroscopy results showed that the enzyme caffeine dehydrogenase is remain catalytically active with increased temperature (up to 100 °C). The use of thermostable enzyme at higher temperatures in industrial processes also increases substrate and product solubility, decreases hydrolysis time, and reduces the risk of microbial contamination. For these reasons, thermostable enzymes are especially desirable as biocatalysts. Therefore, this enzyme might replace the conventional chemical method of decaffeination, thereby reducing the risk of environmental pollution due to the accumulation of toxic by-products. Moreover, from the economical point of view decaffeination by the use of thermostable caffeine dehydrogenase would be advantageous over the conventional methods. The findings of this study should be extremely beneficial for biotechnological processes such as waste treatment and the development of biosensors.

CHAPTER V

Conclusions

5. Summary

Chapter 1

In the first chapter of the thesis describes the introductory section about caffeine, caffeine degrading enzyme, caffeine degradation and its necessity. The chapter also describes kombucha tea and its microbial population.

Caffeine has been found in surface water, ground water, and wastewater effluents all over the world due to its widespread use in food, beverages, and medicines. A specific decaffeination process is required to solve these problems. Microbial bioprocessing is increasingly being recognized as an effective method for caffeine abatement in the environment. Some bacteria from genera *Pseudomonas* and *Serratia*, as well as fungi from the genera *Aspergillus*, *Penicillium*, *Rhizopus*, and *Stemphyllium*, have been shown to metabolize caffeine via enzymatic conversion. Caffeine-oxidizing enzyme can used to treat caffeine in agro-industrial wastes such as coffe plups and husks, groundwater, and wastewaters. They can also be used as a bio-sensing element for real-time monitoring of caffeine in natural waters. Because of the potential biotechnological applications of caffeine degrading enzymes, it is important to investigate this enzyme from various microbial sources.

Chapter II

Kombucha tea is a consortium of yeasts and bacteria (predominantly acetic acid ones). One caffeine-degrading yeast was isolated from kombucha tea, identified using 18S rRNA sequencing and phylogenetic analysis, and classified as *Pichia manshurica* CD1 (GenBank accession number **KY799109**). The DNA fragment specific for D1/D2 domain of the 26S

rRNA gene (GenBank accession number **KY828209**) was 597 bp long and was similar (99%) to *P. manshurica*. A 402 bp fragment of the ITS region (GenBank accession number **KY828210**), amplified using the primers pITS-F and pITS-R showed close similarity (98%) to *P. manshurica.* The strain was found to able not only to degrade caffeine, but also to use it as a sole source of carbon and nitrogen. To the best of our knowledge, this study reported for the first time that *Pichia manshurica* isolated from Kombucha tea exhibited the ability to degrade caffeine and use it as the sole source of carbon and nitrogen by producing some novel enzymes through the enzymatic pathway.

Chapter III

A novel thermostable caffeine degrading enzyme was isolated and identified as caffeine dehydrogenase enzyme from *Pichia manshurica* strain CD1. The present study was investigating the degradation of caffeine by Kombucha tea-derived *Pichia manshurica* with the help of the enzyme caffeine dehydrogenase. In such a caffeine medium the strain required about two days of incubation to reach maximum growth. For inducing the production of caffeine degrading enzyme, the yeast was grown in caffeine enriched media (**Media composition (g/L) :** Na_2HPO_4, 0.12; K_2HPO_4, 1.3; $MgSO_4$, 0.3; $CaCl_2$, 0.3; $FeSO_4$, 0.2; dextrose, 2.0; yeast extract, 4.0; and caffeine, 2.0 (pH= 5)) at 30°C with shaking at 110 rpm for 4 days. Then the cells were lysed by glass bead disruption and protein concentrations were determined by Bradford assay using BSA as a standard. An NBT-dependent (nitro blue tetrazolium chloride), caffeine dehydrogenase activity assay was established and the enzyme was purified. The enzyme was purified in a two-step procedure resulting in a 25-fold purification and was thoroughly characterized. It was found that the caffeine dehydrogenase was a monomer of approximately 85 kDa and had optimal activity at pH 7.5. Interestingly, the enzyme showed high activity and stability over a wide range of temperatures, with almost

71% of activity being retained after incubation at 55 °C for 20 min and 54% of its initial activity was retained even after incubation at 100 °C. A unique feature of this enzyme is that it remains highly active and stable over a wide range of temperatures (even at 100ºC), making it a promising enzyme for industrial application of decaffeination. The purified caffeine dehydrogenase had high substrate specificity towards caffeine (K_m 11.2µM and V_{max} 0.372 nmol/(mL min)) at 0.5mM concentration. The enzyme activity was partially inhibited in the presence of Cr^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+}, and Mg^{2+} at 1 mM, and completely inhibited by Cu^{2+}. The enzyme activity was also strongly suppressed by β-mercaptoethanol, SDS, DTT and EDTA. This study, therefore, provides knowledge on the important industry-oriented properties of caffeine dehydrogenase on caffeine degradation and potential biotechnological utilization of the enzyme in waste treatment and biosensor.

Chapter IV

A novel thermostable caffeine dehydrogenase enzyme was isolated from *Pichia manshurica* strain CD1. At increased temperatures, caffeine dehydrogenase exhibits a proportional increase in enzyme activity. The enzyme also exhibits high stability after the heat treatment and remains up to 56% stable at 100 ºC compared to its untreated condition. Thermodynamic parameters of the enzyme caffeine dehydrogenase (*E_a, ΔG, ΔH, and ΔS*) were also calculated and the activation energy was found to be 27.05±0.8 kJ mol^{-1}. In this study, the conformational changes of caffeine dehydrogenase in native and thermally treated states were investigated by CD spectroscopy and intrinsic tryptophan fluorescence spectroscopy. The far-UV CD spectra results showed that the protein was able to maintain its alpha-helical structure after the heat treatment and the near-UV CD spectra results showed that temperature had no such effect on the protein's tertiary structure. The results of tryptophan fluorescence spectroscopy of the enzyme showed a temperature-dependent dynamic quenching. The

fluorescence intensity of the protein was decreased as the degree of freedom in the tryptophan- region of the protein was increased with temperature. Thus, the enzyme does not undergo denaturation at high temperatures and can maintain its conformation to remain catalytically active.

The stability of the enzyme is a crucial factor in determining whether biocatalysis will be commercially successful. Thermostable enzymes can operate at high temperatures where more reagents and compounds are available that help increases industrial productivity. Because of the increased solubility of the thermostable enzymes, reactions at higher temperatures are more effective. The CD spectroscopy and fluorescence spectroscopy results showed that the active structural conformation of the caffeine dehydrogenase enzyme is maintained and remains catalytically active even at high temperatures (up to 100 °C). Therefore, it can be concluded that the thermal stability of caffeine dehydrogenase is an essential key factor in increasing commercial interest due to its high catalytic efficiency and specificity.

5.1. Scope and future prospects

In the present research caffeine degrading yeast was isolated and identified as *Pichia manshurica* strain CD1 from kombucha tea. The strain was found to exhibit the ability to degrade caffeine by producing a novel thermostable enzyme Caffeine dehydrogenase. The enzyme showed high activity over a wide range of temperatures even at 100 ºC. This is the first study that investigating the influence of heat treatment on conformational properties of the novel thermostable caffeine dehydrogenase isolated from *Pichia manshurica* CD1.

Caffeine degradation via microbial system is poorly characterized, there is still much work to done in order to completely understand the process. Little is known about the enzymology of caffeine degradation pathway and structure function of caffeine degrading enzyme. Thus the future work can lead to:

1. Determine the metabolic pathway of caffeine degradation by the yeast *Pichia manshurica* strain CD1. Also we try to identify the metabolites and reaction sequence by NMR.
2. The amino acid sequencing of the enzyme if obtained can be used to investigate the detailed secondary structure of the protein. There is also a scope of structural studies of caffeine dehydrogenase in detailed by X-ray crystallography so that conformational changes of the protein with high temperature can be studied.
3. Scale up of the caffeine dehydrogenase system for industrial applications.

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Publications

LIST OF PUBLICATIONS

Thesis related:

1. Rubia Parvin, Semantee Bhattacharya, Sutapa Som Chaudhury, Uttariya Roy, Joydeep Mukherjee, Ratan Gachhui. "Production, purification and characterization of a novel thermostable caffeine dehydrogenase from *Pichia manshurica* strain CD1 isolated from kombucha tea." Microbiology (Russian Federation) 92(2023):230–241. https://doi.org/10.1134/S0026261722601476

**Rubia Parvin and Semantee Bhattacharya contributed equally to the study

2. Rubia Parvin, Khushnood Fatma, Debbethi Bera, Jyotirmayee Dash, Joydeep Mukherjee, Ratan Gachhui. "The effect of temperature on the activity and stability of the thermostable enzyme caffeine dehydrogenase from *Pichia manshurica* CD1." Biologia(2023) 1–10. https://doi.org/10.1007/S11756-023-01473-9

Others:

3. Avishek Mukherjee, Soumyadev Sarkar, **Rubia Parvin**, Debbethi Bera, Uttariya Roy and Ratan Gachhui. "Remarkably high Pb2+ binding capacity of a novel, regenerable bioremediator *Papiliotrema laurentii* RY1: Functional in both alkaline and neutral environments." Ecotoxicology and Environmental Safety 195(2020):110439. https://doi.org/10.1016/j.ecoenv.2020.110439

4. Soumyadev Sarkar, Avishek Mukherjee, **Rubia Parvin**, Subhadeep Das, Uttariya Roy, Somdeep Ghosh, Punarbasu Chaudhuri , Tarit Roychowdhury ,Joydeep Mukherjee, Semantee Bhattacharya and Ratan Gachhui. "Removal of Pb (II), As (III), and Cr (VI) by nitrogen-starved *Papiliotrema laurentii* strain RY1." Journal of Basic Microbiology 59(2019):1016-1030. https://doi.org/10.1002/jobm.201900222

5. Debbethi Bera, Kunal Pal, Souravi Bardhan, Shubham Roy, **Rubia Parvin**, Parimal Karmakar, Papiya Nandy and Sukhen Das." Functionalised biomimetic hydroxyapatite NPs as potential agent against pathogenic multidrug-resistant bacteria." Advances in Natural Sciences: Nanoscience and Nanotechnology 10(2029):045017. https://doi.org/10.1088/2043-6254/ab5104

6. Sudipta Roy, **Rubia Parvin**, Subhadeep Ghosh, Somesankar Bhattacharya, Santanu Maity and Debdulal Banerjee." Occurrence of a novel tannase (tan BLP) in endophytic *Streptomyces* sp. AL1L from the leaf of *Ailanthus excelsa* Roxb." 3 Biotech 8(2018):33. https://doi.org/10.1007/s13205-017-1055-4

ISSN 0026-2617, Microbiology, 2023, Vol. 92, No. 2, pp. 230–241. © Pleiades Publishing, Ltd., 2023.

EXPERIMENTAL ARTICLES

Production, Purification and Characterization of a Novel Thermostable Caffeine Dehydrogenase from *Pichia manshurica* Strain CD1 Isolated from Kombucha Tea

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Received June 1, 2022; revised November 12, 2022; accepted November 15, 2022

Abstract—Kombucha tea is a consortium of yeasts and bacteria (predominantly acetic acid ones). One caffeine-degrading yeast was isolated from kombucha tea, identified using 18S rRNA sequencing and phylogenetic analysis, and classified as *Pichia manshurica* CD1 (GenBank accession number KY799109). The strain was found to able not only to degrade caffeine, but also to use it as a sole source of carbon and nitrogen. In such a caffeine medium the strain required about two days of incubation to reach maximum growth. An NBT-dependent (nitro blue tetrazolium chloride), caffeine dehydrogenase activity assay was established and the enzyme was purified. It was found that the caffeine dehydrogenase was a monomer of approximately 85 kDa and had optimal activity at pH 7.5. Interestingly, the enzyme showed high activity and stability over a wide range of temperatures, with almost 71% of activity being retained after incubation at 55°C for 20 min and 54% of its initial activity was retained even after incubation at 100°C. The purified caffeine dehydrogenase had high substrate specificity towards caffeine (K_m 11.2 μM and V_{max} 0.372 nmol/(mL min)) at 0.5 mM concentration. The enzyme activity was partially inhibited in the presence of Cr^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Hg^{2+}, and Mg^{2+} at 1 mM, and completely inhibited by Cu^{2+}. The enzyme activity was also strongly suppressed by β-mercaptoethanol, SDS, DTT and EDTA. These interesting industry-oriented properties of caffeine dehydrogenase isolated from *Pichia manshurica* CD1 may be useful for many biotechnological processes in the future.

Keywords: *Pichia manshurica*, Kombucha tea, caffeine dehydrogenase, purification, characterization, thermostability

DOI: 10.1134/S0026261722601476

Caffeine, a natural purine alkaloid (1,3,7-tri methyl xanthine), is one of the major components of many popular beverages, especially tea and coffee (Heckman et al., 2010). The global consumption of caffeine ranges from 80 to 400 mg per person per day through various other sources like caffeinated soft drinks, chocolates and desserts (Reyes and Cornelis, 2018). It is a well-known central nervous system stimulant. However, excessive consumption of caffeine may lead to occasional adverse effects including adrenal stimulation, increased blood pressure, anxiety, cardiac arrhythmias, etc. (Lovallo et al., 2005). Awareness of these side effects has increased the demand for decaffeinated food items.

Caffeine is also one of the major toxic products generated by several industries like food, beverages, and pharmaceuticals. Inappropriate disposal of these solid wastes causes great environmental burdens (Fernandes et al., 2017). The presence of caffeine in soil affects soil fertility, inhibits seed germination and causes toxicity to the soil insects and microorganisms (Fernandes et al., 2017). Caffeine contaminated water bodies have a pernicious influence on the aquatic life and are also unsafe for drinking purposes (Chen et al., 2008; Gummadi et al., 2011). Consequently, demand for decaffeination of both foodstuffs and these waste products is gradually increasing day by day.

In recent times, application of microorganisms with caffeine-degrading capacity in the decaffeination process attracted considerable attention (Gummadi et al., 2011; Summers et al., 2015). To date, a large number of bacteria and fungi have been reported to be capable of growing in the presence of caffeine and degrading the alkaloid (Zhou et al., 2018). From the point of view of enzymology, caffeine is generally catabolised by microbes via two major pathways—demethylation and oxidation involving demethylase and dehydrogenase or oxidase enzymes, respectively (Dash and Gummadi, 2006). Although there are many researches regarding the characteristics and

Biologia
https://doi.org/10.1007/s11756-023-01473-9

ORIGINAL ARTICLE

The effect of temperature on the activity and stability of the thermostable enzyme caffeine dehydrogenase from *Pichia manshurica* CD1

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Received: 27 March 2023 / Accepted: 27 June 2023

Abstract
A novel thermostable caffeine dehydrogenase enzyme was isolated from *Pichia manshurica* strain CD1. At increased temperatures, the enzyme caffeine dehydrogenase exhibits increased activity. The enzyme also exhibits high stability after the heat treatment and remains up to 56% stable at 100 ºC compared to its untreated condition. A Comprehensive analysis was carried out to monitor the thermodynamic parameters (E_a, ΔG, ΔH, *and* ΔS) of the enzyme. In this study, the conformational changes of caffeine dehydrogenase in native and thermally treated states were investigated by CD spectroscopy and intrinsic tryptophan fluorescence spectroscopy. The far-UV CD spectra results showed that the protein was able to maintain its alpha-helical structure after the heat treatment and the near-UV CD spectra results showed that temperature had no such effect on the protein's tertiary structure. The results of tryptophan fluorescence spectroscopy of the enzyme showed a temperature-dependent dynamic quenching. The fluorescence intensity of the protein was decreased as the degree of freedom in the tryptophan- region of the protein was increased with temperature. Thus, the enzyme does not undergo denaturation at high temperatures and can maintain its conformation to remain catalytically active. Therefore, it can be concluded that the thermal stability of caffeine dehydrogenase is an essential key factor in increasing commercial interest due to its high catalytic efficiency and specificity.

Keywords *Pichia manshurica* CD1 · Caffeine dehydrogenase · Thermostability · Secondary structure · Fluorescence spectroscopy

Introduction

Caffeine dehydrogenase (E.C.1.17.5.2) is an intracellular enzyme that is also known as caffeine oxidase in the scientific literature. The enzyme is a member of the oxidoreductase family(Yu et al. 2008). The enzyme is most well-known for its ability to directly oxidize caffeine (Mohapatra et al. 2006). The enzyme is caffeine-specific, has less affinity for theobromine, and has no affinity for xanthine (Mohanty et al. 2012). Caffeine enzymatic catabolism in microbes has been reported to be performed by *N*-demethylation and oxidation pathways with demethylases and dehydrogenase or

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Published online: 05 July 2023

Ecotoxicology and Environmental Safety 195 (2020) 110439

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Remarkably high Pb^{2+} binding capacity of a novel, regenerable bioremediator *Papiliotrema laurentii* RY1: Functional in both alkaline and neutral environments☆

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ARTICLE INFO

Keywords:
Papiliotrema laurentii RY1
Lead (Pb^{2+})
Adsorption
Neutral and alkaline pH
Kinetics
Equilibrium and thermodynamics study

ABSTRACT

The ability of *P. laurentii* strain RY1 to remediate lead (Pb^{2+}) from water was investigated in batch and column studies. The lead removal ability of non-viable biomass, non-viable biomass immobilised on agar-agar (biobeads) and agar-agar at different pH was compared in batch studies. It was found that among the three, biobeads have maximum ability to remove Pb^{2+} followed by biomass and agar-agar beads. Maximum and almost equal lead removal by biobeads was observed at both neutral and alkaline pH making it a novel and more applicable bioremediator as all other reported bioremediators have a single pH for optimum activity. Studies were performed to determine the optimum conditions for lead removal from aqueous solutions for biobeads. The physical and chemical characterization of the biobeads before and after Pb^{2+} biosorption was done by using S.E.M. and F.T.I.R. respectively. The adsorption of Pb^{2+} on biobeads obeyed the Langmuir adsorption isotherm and pseudo first order kinetics. These mean that the Pb^{2+} binding sites are identical, located on the surface of the adsorbant and the rate of Pb^{2+} removal from aqueous solution is directly proportional to the number of Pb^{2+} binding sites on the biobeads. The thermodynamics of the biosorption process is also investigated. The binding capacity of the biobeads in batch study was found to be 52.91mg/gm which is higher in comparison to other reported yeast bioremediators. The used biobeads can be desorbed using 0.1(M) $CaCl_2$. The desorbed biobeads can be used subsequently for several cycles of lead removal making it cost-effective. Column studies were also performed for biobeads with the help of Thomas model for examining its suitability for industrial application. Maximum specific lead uptake of the biobeads when applied in the column was found to be 58.26mg/gm which being promising makes it suitable for application in industries involved in the treatment of wastewater contaminated with high amounts of lead. The high mass transfer co-efficient indicate that small sized column can be used effectively to remove high amounts of lead which makes the bioremediation process by the biobeads more economical and advantageous for industrial application. Several factors like effectiveness of the biobeads in Pb^{2+}removal at both neutral and alkaline pH, reusability, high mass transfer co-efficient, regenerability and high binding capacity makes it a novel versatile, cost–effective and high utility bioremediator.

1. Introduction

Lead is a widely distributed toxic heavy metal which affects almost every organ of the human body (Wani et al., 2015). The major source of lead pollution is gasoline vehicle exhaust, lead paint, lead pesticide, natural sources (bedrock, soil) and industrial emission (Cheng and Hu, 2010). Industries producing lead as waste include pulp and paper industry, tannery, fertilizer and herbicide industries, battery manufacturing industries as well as mining and metallurgical industries (Asuquo et al., 2017).

☆ The authors declare that they have no conflict of interest.

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https://doi.org/10.1016/j.ecoenv.2020.110439
Received 2 September 2019; Received in revised form 13 February 2020; Accepted 3 March 2020
Available online 14 March 2020
0147-6513/

Received: 18 April 2019 | Revised: 11 July 2019 | Accepted: 25 July 2019

DOI: 10.1002/jobm.201900222

RESEARCH PAPER

Journal of Basic Microbiology

Removal of Pb (II), As (III), and Cr (VI) by nitrogen-starved *Papiliotrema laurentii* strain RY1

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Abstract

Heavy metals such as lead, chromium, and metalloid like arsenic dominate the pinnacle in posing a threat to life. Being environment-friendly, elucidating the mechanism by which microorganisms detoxify such elements has always been an active field of research hitherto. In the present study, we have investigated the capability of nitrogen-deprived *Papiliotrema laurentii* strain RY1 toward enhanced tolerance and neutralizing toxic elements. There were biosorption and bioprecipitation of lead and chromium at the cell surfaces. Bioprecipitation mechanisms included the formation of lead phosphates and pyromorphites from lead, grimaldite from chromium. Transcripts such as metallothionein, aquaporins, and arsenical pump-driving ATPase have been surmised to be involved in the detoxification of elements. Furthermore, activation of antioxidant defense mechanisms for the cells for each of the elements should contribute towards yeast's propagation. The efficiency of removal of elements for live cells and immobilized cells were high for lead and chromium. To the best of our knowledge, this is the first report of such high tolerance of lead, arsenic, and chromium for any yeast. The yeast showed such varied response under dual stress due to nitrogen starvation and in the presence of respective elements. The yeast possesses promising potentials in nitrogen deprived and enriched environments to aid in bioremediation sectors.

KEYWORDS

arsenic, chromium, lead, *Papiliotrema laurentii* strain RY1

1 | INTRODUCTION

Lead, arsenic, and chromium are pollutants to the environment and lethal to living organisms. Excessive amounts of these elements in the ecosystem are associated with several health hazards and thus a source of major concern [1]. Lead allowable range in the United States is 400–1,200 ppm in soil and 15 ppb in water (Advisory Committee on Childhood Lead Poisoning Prevention, 2012). The permissible arsenic range is 10 ppb in drinking water (Agency for Toxic Substances and Disease Registry, 2013). Environmental Protection Agency has a maximum acceptable range of 0.1 mg/L or 100 ppb for total chromium in drinking water. Often the

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IOP Publishing | Vietnam Academy of Science and Technology Advances in Natural Sciences: Nanoscience and Nanotechnology

Adv. Nat. Sci.: Nanosci. Nanotechnol. **10** (2019) 045017 (11pp) https://doi.org/10.1088/2043-6254/ab5104

Functionalised biomimetic hydroxyapatite NPs as potential agent against pathogenic multidrug-resistant bacteria

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Received 3 May 2019
Accepted for publication 13 September 2019
Published 14 November 2019

Abstract

The persistent dissemination of resistant bacterial strains is a grave contemporary global impediment in hospital-acquired infections which needs to be mitigated with immediate effect. In particular, infections from pathogenic multidrug-resistant (MDR) Gram-positive bacteria (like *Enterococcus faecalis*) which are resistant to conventional antibiotic therapy are attracting immediate global attention. Here we report the synthesis of nanoscale hydroxyapatites (HAPs), which are the well known biomimetic ceramic material having needle shaped morphologies. We have encapsulated vancomycin (VAN) within these nanoparticles and have conjugated the targeting ligand (folic acid) by a facile synthesis process in order to enhance the therapeutic efficacy against MDR *E. faecalis*. These functionalised HAPs are thoroughly characterised by employing field emission scanning electron microscopy (FESEM), powder x-ray diffraction (PXRD), ultraviolet–visible spectroscopy (UV-Vis) and dynamic light-scattering (DLS) techniques. Our results suggest that these functionalised HAPs could successfully transport vancomycin across the cell wall of MDR *E. faecalis* through endocytosis. The determination of selective antibacterial activity has been envisaged with the help of extensive *in-vitro* assays like the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the generation of reactive oxygen species (ROS). This study vividly establishes that this folic acid conjugated HAPs are promising antibacterial agents against MDR *E. faecalis* and related pathogenic resistant bacterial strains.

Keywords: hydroxyapatite nanoneedle, vancomycin, folic acid targeting, multidrug-resistant, enterococcus faecalis, antibacterial drug delivery
Classification numbers: 2.04, 2.05, 5.00, 5.08, 5.09

1. Introduction

The emergence of drug-resistant bacteria has posed a grave threat to the modern society. The antibiotic-resistant bacteria are the primary driving force behind millions of deaths that occur every year worldwide as mentioned in the previous reports [1, 2]. Furthermore an incessant decline in approved antibiotics in the past decade is responsible for aggravating this issue. This has given rise to the need of the development of alternate strategies that would help in overcoming the resistance in bacterial strains. Numerous active strategies including the development of novel antibiotics have been implemented for the treatment of multidrug-resistant (MDR) bacteria. The functionalisation of nanomaterials is a very effective process to develop antibacterial agents for the treatment of MDR bacteria. The fluorescent-carbon quantum

3 Biotech (2018) 8:33
https://doi.org/10.1007/s13205-017-1055-4

ORIGINAL ARTICLE

Occurrence of a novel tannase (*tan B_{LP}*) in endophytic *Streptomyces* sp. AL1L from the leaf of *Ailanthus excelsa* Roxb

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Received: 29 May 2017 / Accepted: 18 December 2017

Abstract
The tannase production ability by endophytic actinobacteria and the genetic identity of responsible tannase gene were determined. The studied strains were isolated from surface-sterilized leaf discs of *Ailanthus excelsa* Roxb. Four strains were found to hydrolyze tannic acid on solid media containing 0.4% tannic acid. The strain AL1L was found as *tan B_{LP}* indicating production of tannase with diverse of substrate affinity. The tannase production from the potential strain AL1L was performed in liquid tannic acid broth (0.4%, w/v). The strain was later identified as *Streptomyces* sp. AL1L on the basis of 16S rDNA homology. Highest enzyme activity was observed at 48 h of incubation at the exponential growth phase. The enzyme was purified by ammonium sulfate precipitation followed by dialysis (15 kD cut off). This enzyme, with molecular weight 180 kD shows highest catalytic activity at 35 °C, pH 6 with substrate concentration 0.1 g%. The purified enzyme possesses 1.4×10^{-3} K_m and 11.15 U/ml as V_{max}. The above study indicates high industrial prospective of endophytic actinobacteria as source of tannase of potential biotechnological applications.

Keywords Endophytes · *Streptomyces* · *Ailanthus* · Tannase · *Tan B_{LP}*

Introduction

Tannins are polyphenolic secondary metalolites that are extensively produced in many species of plants, where they execute the key roles in protection from microbial attacks, pests and mites also play crucial in plant growth regulation (Banerjee and Mahapatra 2012; Thorington and Ferrell 2006). Natural tannins are either galloyl esters or their derivatives, where the galloyl moieties or their derivatives are attached to different polyol, catechin, triterpenoid or others (Khanbabaee and Ree 2001). Tannin acyl hydrolase (EC 3.1.1.20) or tannase belonging to the super-family of esterase catalyze hydrolysis of galloyl ester bond of tannins and yield gallic acid as major. After its discovery in the early 1900s, this enzyme has established wide applications in food, feed, beverage, pharmaceutical, and chemical industries (Aguilar et al. 2007). De-esterification of tea polyphenol complexes, especially the gallated catechins are also carried out by this enzyme in preparation of instant tea (Lu et al. 2009). Tea leaves treated with tannase showed decreased level of esterified catechin content, whereas an increased non-galloylated catechin and gallic acid level. Tannase-treated tea leaves showed reduced protein-binding ability and decreased tea cream levels. The leaf extract also exhibited higher antioxidant ability than untreated tea leaves (Li et al. 2017; Liu et al. 2017). Application of tannase increases the aromatic content, maintains color, prevents any undesired turbidity and clarifies the beverage. It has high demand in homogenization of tannins in leather industry as well as in effluent treatment of water contaminated with tannins (Rodríguez-Durán et al. 2011). The gallic acid or their derivatives yielded by this enzyme also have astonishing pharmaceutical importance due to its anticancer, antimicrobial and antioxidation property (Ow and Stupans 2003). Gallic acid on the other hand is also employed in dye industries and even as photosensitive resin in semiconductor preparation (Rodríguez-Durán et al. 2011). Hence, there is an emergent interest on the basic and applied dimension of

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Published online: 26 December 2017