A Project Report

On

Screening and Characterization of Bacterial Laccase and Asparaginase

Submitted by

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CERTIFICATE

This is to certify that the project entitled "**SCREENING AND CHARACTERIZATION OF BACTERIAL LACCASE AND ASPARAGINASE**" pursued by Chetna (131553) and Snigdha Ghosh (131554) in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology, Jaypee University of Information Technology, Waknaghat, has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other university or institute for the award of any degree or appreciation.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS	ABBREVIATIONS
LB	Luria Broth
OD	Optical density
pH	Potential of hydrogen
°C	Degree Celsius
g	Gram
µl	Microliter
µg	Microgram
ml	Milliliter
mg	Milligram
RPM	Rotation per minute
%	Percentage
nm	Nanometers
BSA	Bovine Serum Albumin

CHAPTER 1

REVIEW OF LITERATURE

1.1 LACCASE

1.1.1 Introduction

Laccases are oxidase which have multiple copper ions and is one of the lignin-degrading enzymes that have been studied since 18th century [1]. It comes under the group of blue oxidases which helps to catalyze the oxidation of phenols, aromatic amines, polyphenols and different non-phenolic substrates by using molecular oxygen from the atmosphere as an electron acceptor. [2]Laccases, when it is enzymatically active can be either in dimer or timer form. The name Laccase is derived from a Chinese lacquer tree from which this enzyme was first studied, by Gabriel Bertrandin in 1894.

1.1.2 Mechanism of action

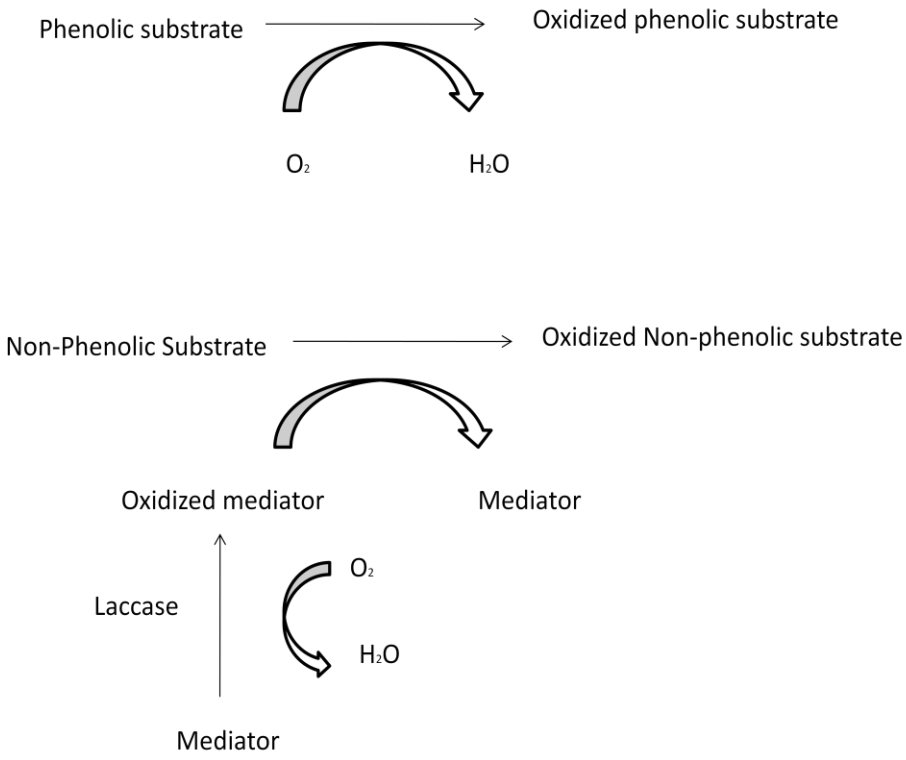


Figure 1: Action of laccase enzyme to oxidize phenolic and non-phenolic compounds

Laccase mediated catalysis occurs with reduction of oxygen to water, accompanied by the oxidation of substrate. Laccases can oxidize a wide range of compounds including polyphenols, methoxysubstituted phenols, aromatic diamines [3]. Therefore this enzyme has many use in

industrial areas such as the removal of textile dyes and waste detoxification, since it does not have the substrate specificity.

1.1.3 Structure of Laccase enzyme

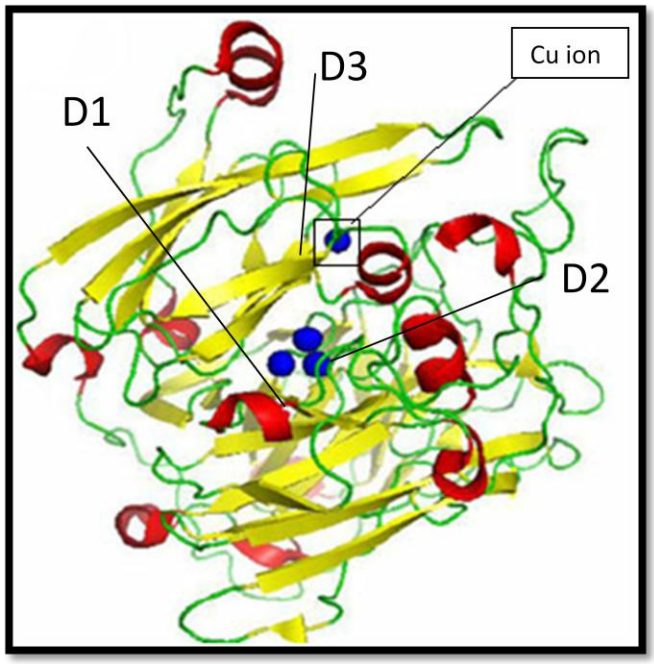


Figure 2: Overall structure of laccase enzyme where yellow denotes β -strands, red denotes α -helixes, and four copper ions in the structure are depicted as blue spheres. D1, D2 and D3 are the three domains.[16]
PubMed ID: 12118243

The copper ion forms the oxygen reduction site and generates the free radicals which oxidize the substrate. There are three domains (D1, D2, and D3) and 4 copper ions T1, T2 and two T3 ions. D1 and D2 are near the three copper ions and D3 is near the fourth copper ion. The three copper ions are distinguished from each other as they have unique and distinguishable spectroscopic properties. The three copper ions forms the catalytic site which does reduction part of a reaction. This three copper ions together are also called a trinuclear cluster. The other copper ion which is the fourth one, performs one electron oxidation of various organic substrates. These ions play an important role which can be proved by the inhibition property of cyanides, fluorides, halides and azides, which bind to the T2 and T3 copper ions and decrease the activity of the enzyme. The sites are mainly coordinated by amino acid residues like histidine and cysteine.

1.1.4 Source:

Laccase is generally found in **higher plants** (Cabbages, Apples, Beets, Asparagus, Peaches, Potatoes, Pears, Turnips, sycamore, tobacco and various other vegetables), **Fungi** (Ascomycetes, wood rotting fungi and some basidiomycetes) and also present in **bacteria**.[12] But in recent years, bacterial laccases are now being studied for their efficiency in biodegrading environmentally important phenolic pollutants and also because of its ease for providing proper culture conditions.

Some of the bacteria producing laccases which are reported:

Pseudomonas maltophila	Isono and Hoshino, 1989
P. syringae	Cha and Cooksey, 1991
Azospirillum lipoferum	Cha and Cooksey, 1991
Xanthomonas campesteris	Lee *et al*., 1994)
B. sphaericus	Claus and Filip, 1997
Bacillus subtilis	Hullo *et al*., 2001
Escherichia coli	Kim *et al*., 2001; Roberts *et al*., 2002
Streptomycetes sp.	Arias *et al*., 2003; Endo *et al*., 2003
B. halodurans Lbh-1	(Ruijssenaars and Hartmans, 2004)
Thermus thermophilus TTC1370	Miyazaki, 2005
Marinomonas mediterranea	Kalme *et al*., 2007

1.1.5 Applications:

1) As Additives in Food and Beverage Processing:

a) Baking: Laccases have gained interest in baking industry because of their ability to cross-link biopolymers. More will be the cross-links more firm will be the product. This enzyme also has additional features such as increasing the softness of baked products and improving the crumb structure.[15]

b) Stabilisation of Wine and beer: Laccases helps to remove polyphenols which when not removed could lead to destabilization of wine and beer. This can be done by binding of laccase with polyphenols and removing it out via filtration. This removal of polyphenols is a necessity as the polyphenols increases the probability of occurrence of the hazing effect which means that the beer or wine will not be clear, This will also enhance the lifetime of beer and can be stored for longer durations.[16]

c) Improving the Food Sensory Parameters: Different uses of laccase have been used to control parameters such as odor control, taste enhancement, or reduction of undesired products in several food products, Sometimes there are certain compounds which needs to be removed from the food products which otherwise causes contamination or toxicity in the food when consumed. As mentioned earlier since laccase has broad range of substrates to bind upon this enzyme can be used to treat out these compounds by binding to it and removing it out.[16]

d) Bioremediation of Food Industry Wastewater: The presence of phenolic compounds in places where the resources are meant for public consumption such as drinking water represents a significant health and/or environmental hazard. Laccase enzyme will therefore bind to the phenolic compounds and use it as substrate to convert to other compounds or completely use it up [14].

2) Pulp and paper industry: The industrial preparation of paper requires separation and degradation of lignin in wood pulp. The current environment issues targets to replace the conventional chlorine-based delignification and bleaching procedures. Hence to overcome this problem, laccase based delignification can be used. Bleaching using laccase will not lead to production of any harmful or toxic byproducts. It can be said that it is a natural way of delignification [15].

3) Textile industry: In this industry various dyes are used which are mainly of synthetic origin. These dyes remain in the effluents and create lot of pollution. Other methods that are employed to decolorize the dye has been failed, so laccases seem a good solution as they can degrade dyes of diverse chemical structure. Laccases of different sources are documented which can decolourize the dye without releasing any byproducts. Lacaases are reported to use the dye as a substrate to degrade it and hence clear the dye from a particular dye solution [15].

4) Nanobiotechnology: Laccase can be used as biosensors to detect phenolic compounds as they are able to catalyse electron transfer reactions. Since laccase can oxidize phenols, so the amount of oxidation can be detected and hence the amount of phenols can be detected and also can be quantified [15].

1.2 ASPARAGINASE

1.2.1 Introduction

Asparaginase is an enzyme which catalyzes the process of hydrolysis of asparagine into aspartic acid and ammonia. These enzymes are expressed and produced by many organisms and have applications in various fields.

1.2.2 Mechanism of action

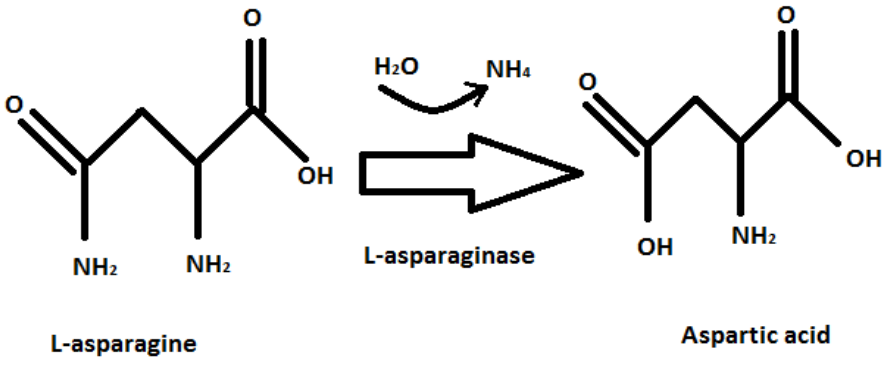


Figure 3: Action of asparaginase enzyme on L-asparagine

L-asparaginase catalyzes the reaction by using oxygen from the water molecule and breaks down into aspartic acid and ammonia [16].

1.2.3 Structure of L-asparaginase enzyme

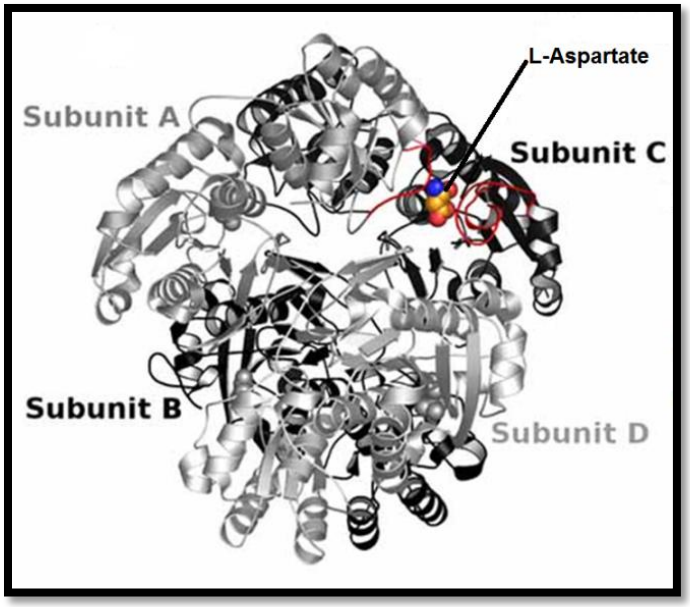


Figure 4: Structure of L-asparaginase isolated from *Escherichia coli* with L-aspartate bound together. It is a tetrameric assembly which shows the L-aspartate as spheres. Subunit A and C represent one dimer and subunit B and D represent the other dimer.

PubMed ID: 12595697

The structure is a homotetramer where each monomer is about 330 amino acids with a two domain fold which are alpha / beta class. This active, tetrameric form has four identical subunits which are denoted as A, B, C and D. There are six pair wise interactions which is possible among the subunits. Out of these six, interactions among A and B and interactions among C and D are most extensive. These interactions forms two intimate pairs of subunit and hence the dimer dimer association.

There are four active sites present in the tetramer. The location of these active sites is in between the subunits and each single dimer has two active sites [17].

1.2.4 Source

L-asparaginase has been isolated from variety of sources such as bacterial cells, yeast cells, fungal cells, plant cells and microbial sources from soil [18].

For clinical purposes L-asparaginase has been isolated *Escherichia coli* (EcAII), *Erwinia chrysanthemi* (ErA) and *Erwinia carotovora*. Estuarine bacteria is one of the best sources of L-asparagine found till date. These bacteria have a halophilic nature and this property leads its exploitation in the industrial production.

The production of the enzyme L-asparaginase is also done from filamentous organism such as *Aspergillus tamarii* and *Aspergillus terreus*. *A. terreus* has the highest L-asparaginase production which is reported.

For the usage in baking industries Asparaginases has been isolated from *Aspergillus oryzae* and *Aspergillus niger*[19]. This enzyme has been also isolated from Actinomycetes namely *Streptomyces tendae.*

Its isolation is even possible from plant sources such as tamarind (*Tamarindu sindica*), Green chillies (*Capsicum annum L*.) and *Lupinusangusti folius.* High specificity of enzyme has been reported in *Withania somnifera* [18] [19].

1.2.5 Application

1) Chemotherapeutic agent: The hydrolytic activity of asparaginase was first observed by Lang in the year 1904 and this activity was detected in bovine's tissues. These results were then confirmed again in the year 1910 by Furth and Friedmann who observed this activity in horse and pig organs. Because of these hydrolytic activities L-asparaginase has been used as a chemotherapeutic agent and is used to treat lympho-proliferative and lymphoma diseases.

This enzyme is used to treat patients with acute lymphoblastic leukemia and Hodgkin's disease. It works by converting the available circulating L-asparagine into aspartic acid and hence the

tumor cells do not get sufficient amount of L-asparagine to sustain them and eventually die off. Cancerous cells do not have the ability to produce L-asparagine by themselves. Hence when the already available amount of L-asparagine is also not available due to the conversion factor by the enzyme, the cancerous cell are not able to survive [19].

2) Food industry: Studies have confirmed that baked (specially fried potato) and fried food contain a significant amount of acrylamide. Acrylamide is a carcinogenic toxicant which is formed by the reaction of asparagine with the reducing sugars available. To get rid of this acrylamide, potato slices and bread dough are treated with asparaginase before frying or baking which prevents acrylamide formation. When asparaginase is treated with the food, the available asparagine will be converted by L-asparaginase into ammonia and aspartic acid. Hence asparaginase will not be able to react with the reducing sugars and the formation of acrylamide will be prevented [19].

3) Biosensors: This enzyme is also used for designing a diagnostic biosensor meant for clinical purposes. This is based on the principle that the amount of ammonia produced by asparaginase is in direct proportion to the level of L-asparagine in a patient's blood [19].

1.3 Rationale

Laccase and asparaginase are the two enzymes that were chosen because of their existing problems.

The asparaginase that has been isoloated from various sources (*Escherichia coli*, *Erwinia chrsanthemi*) has glutaminase activity associated with them which causes side effects. Moreover these enzymes have low stability and a reduced half-life in the blood which requires multiple dosages to be administered in the body for effective treatment. Hence research is being carried out for different sources of asparginases.

The other enzyme under study laccase have the issue of stability, therefore further investigations for finding a novel source of stable laccase is required.

1.4 Objective

- Screening of soil sample for industrially important enzyme producing halophilic microorganisms from Gumma, H.P.
- Analysis of cultures obtained from cell repository for Laccase and Asparaginase production.
- Identification of potential microbial strains using morphological, biochemical characterization.
- Optimization, Characterization and purification of enzymes (Laccase, Asparaginase).

CHAPTER 2

MATERIALS AND METHODS

2.1 ISOLATION OF HALOPHILIC MICROORGANISMS FROM A HALOPHILLIC SITE (GUMMA, H.P)

- Soil sample was collected in a sterilized reagent bottle from the halophillic site (Gumma, H.P).
- To 10g of soil sample 10 ml of saline water was added.
- 50 ml of LB broth was prepared in 7 flasks each having different concentration of salt (0%, 5%, 10%, 15%, 20%, 25%, 30%) and autoclaved at 121 ^{0}C at 15 psi for 15 minutes.
- LB agar plates were also prepared for each of the salt concentration.
- The soil sample was inoculated in 50 ml LB broth having 0% salt concentration (no addition of salt), and incubated at 37 ^{0}C overnight.
- Next day 2.5 ml of the turbid broth (0%) was pippetted to 50 ml of LB broth having 5% salt concentration and incubated at 37 ^{0}C overnight.
- Also the respected agar plate was streaked from the liquid culture (0% LB broth to 0% Agar plate) and incubated at 37 ^{0}C.
- This process was repeated till 15% of salt concentration (after which there was no growth).
- Individual colonies were identified and subcultured to get pure cultures.

2.2 REVIVAL OF GLYCEROL STOCKS

- LB media was prepared and poured in 5 test tubes (10 ml each) and then autoclaved at 121 ^{0}C at 15 psi for 15 minutes.
- The cultures N, L, Fc, SS, *Streptococcus thermophillus* maintained as glycerol stocks at -80 ^{0}C were collected from the cell repository.
- Cultures were kept on ice for an hour to bring them to 4 ^{0}C and kept at room temperature for culturing.
- Tubes of LB media were inoculated with 200 µl of glycerol stock for respective organism.
- Cultured test tubes were incubated at 37 ^{0}C at 140 RPM for 24 hours.

- Tubes were observed after incubation.

a. TO CHECK CONTAMINATION IN THE REVIVED CULTURES

- LB Agar media was prepared and autoclaved.
- After the media was solidified the culture was streaked on each plate.
- The plates were then incubated at 37^0C for 24 hours and observed thereafter.

b. SUBCULTURE OF THE REVIVED COLONIES AND PREPARATION OF GLYCEROL STOCKS OF THE ORGANISM (UNDER STUDY) FOR LONG TERM STORAGE

- The above cultures were sub cultured.
- 30% of glycerol stock was prepared and autoclaved.
- LB broth media was prepared and 10 ml was poured in each test tubes (for all the microorganisms isolated and revived) and autoclaved.
- From the above prepared plates small inoculums was taken and inoculated to the respective test tubes and incubated at 37 ^{0}C for 24 hours.
- After growth in seed culture media, 500 µl of culture was taken and pipetted in 1.5 ml of centrifuge tube and equal volume of glycerol (30%) was added and sealed using parafilm and stored at -80 ^{0}C.

2.3 QUALITATIVE ANALYSIS OF LACCASE AND ASPARAGINASE ACTIVITY (PLATE ASSAY)

a. LACCASE (using guaicol as a substrate)

Media used: Guaicol 0.02%
Yeast Extract 1%
Agar 2%

pH was set at 6.8

Theory: Guaicol acts as a substrate to laccase enzyme. The enzyme degrades the substrate to produce a brown colour pigment which gives the positive test.

Protocol:

- Using the above composition the media was prepared and autoclaved.
- The media was poured into the petri plate.
- The plates were inoculated with all the pure cultures obtained from the soil sample, N, Fc, SS, L and 7culture.
- Plates were incubated at 37 ^{0}C for 5 days and observed after incubation.

b. ASPARAGINASE

Media used: Quantity given in g/L

$Na_2HPO_4.2H_2O$ 6
KH_2PO_4 3
NaCl20. 5
L-asparagine 5
$MgSO_4.7H_2O$0. 5
$CaCl_2.2H_2O$ 0.15
Glucose 2
Agar 20
2.5 ml 3% (w/v) stock solution of phenol red in ethanol was added to media as pH indicator.

The pH was set at 7.

Theory: L-asparaginase converts L-asparagine into Aspartic acid and Ammonia which results into an alkaline environment majorly, which changes the color of the media to dark red (phenol red as a pH indicator is used) which is the positive result of the test.

Protocol:

- Using the above composition the media was prepared and autoclaved.
- The media was poured into the petri plate.
- The plates were inoculated with all the pure cultures obtained from the soil sample, *Steptococcus thermophillus* and 7culture.
- Plates were incubated at 37 ^{0}C for 5 days and observed after incubation.

2.4 QUANTITATIVE ANALYSIS OF ENZYME ACTIVITY

a. LACCASE

Chemicals used: 0.5 mM ABTS (prepared in sodium citrate buffer), 0.1 M Sodium citrate buffer (pH4.5), 1.5M TCA.

Theory: Laccase is a copper containing phenoloxidase. The substrate for this enzyme can include long range of substrates. One of them is ABTS which is oxidized to a stable cation radical, ABTS·+ which then accumulates and can be detected at 420 nm.

Activity Assay:

- Respective cultures were grown for organism in guaicol media for 72 hours in duplets.
- Cultures were collected in 2 ml centrifuge tubes and centrifuged at 5000 RPM for 10 minutes at 4 ^{0}C.
- The supernatant was collected in new 2 ml centrifuge tubes, marked accordingly and kept in ice.
- The pellet of sample 2C1 was also collected and 200 µl of lysis buffer was added to it.
- 2 µl of lysozyme was added to it and left for incubation at room temperature for 40 minutes.
- The sample were sonicated and then centrifuged at 12500 RPM for 20 minutes.
- The supernatant was collected and following steps were followed for both extracellular and intracellular samples.
- Both controls were taken, Enzyme control (enzyme + buffer), Substrate control (substrate + buffer) and incubated for 10 minutes at 37 ^{0}C.
- Further protocol was followed according to the table.

Tube no.	Enzyme (ml)	Substrate (ml)	Buffer (ml)	Incubate for 10 minutes at 37 °C	TCA (ml)	OD was taken at 420 nm
S_c	0	0.8	0.2		0.1	
N_c	0.2	0	0.8		0.1	
N_1	0.2	0.8	0		0.1	
N_2	0.2	0.8	0		0.1	
Fc_c	0.2	0	0.8		0.1	
Fc_1	0.2	0.8	0		0.1	
Fc_2	0.2	0.8	0		0.1	
L_c	0.2	0	0.8		0.1	
L_1	0.2	0.8	0		0.1	
L_2	0.2	0.8	0		0.1	
SS_c	0.2	0	0.8		0.1	
SS_1	0.2	0.8	0		0.1	
SS_2	0.2	0.8	0		0.1	
$2C1_c$	0.2	0	0.8		0.1	
$2C1_1$	0.2	0.8	0		0.1	
$2C1_2$	0.2	0.8	0		0.1	

(Note: marking goes as follows – S_c substrate control, $_c$ is control which corresponds to the enzyme control, $_1$ and $_2$ are enzymes in duplicates)

- Similar protocol was followed for different days as shown in the results and for the intracellular assay.

b. ASPARAGINASE

Chemicals used: 50 mM L-Asparagine (in water), 50 mM phosphate buffer (pH 7), 1.5 M TCA, Nessler's reagent, distilled water.

Theory: The reaction of the substrate (L-asparagine) and the enzyme generates ammonia which when binds to nessler's reagent produces a compound which is detected at 480 nm.

a. Standard curve of Ammonium Sulfate

Protocol was followed according to the table:

Tube no	$(NH_4)_2SO_4$ µl	Buffer (mL)	Incubation for 10 minutes at 37°C	TCA (µl)	Centrifuge for 10 minutes at 5000 RPM	dH_2O (mL)	N.R. (mL)	S/N (mL)	OD was taken at 480 nm
1	0	1.9		100		7	1	0.5	
2	50	1.850		100		7	1	0.5	
3	100	1.800		100		7	1	0.5	
4	150	1.750		100		7	1	0.5	
5	200	1.700		100		7	1	0.5	
6	250	1.650		100		7	1	0.5	
7	300	1.600		100		7	1	0.5	

b. Activity assay:

- Cultures were grown for organism in asparagine media for 72 hours in duplets.
- Cultures were collected in 2 ml centrifuge tubes and centrifuged at 5000 RPM for 10 minutes at 4 ^{0}C.
- The supernatant was collected in new 2 ml centrifuge tubes, marked accordingly and kept in ice.
- The pellets of samples were also collected and 200 µl of lysis buffer was added to each.
- 2 µl of lysozyme was added to each and left for incubation at room temperature for 40 minutes.

- The samples were sonicated and then centrifuged at 12500 RPM for 20 minutes.
- The supernatant was collected and following steps were followed for both extracellular and intracellular samples.
- Both controls were taken, Enzyme control (enzyme + buffer), Substrate control (substrate + buffer) and incubated for 10 minutes at 37 ^{0}C.
- Further protocol was followed according to the table.

Tube no	Enzyme (mL)	Substrate (mL)	Buffer (mL)	Incubation for 10 minutes at 37°C	TCA (μL)	Centrifuge for 10 minutes at 5000 RPM	dH_2O (mL)	N.R. (mL)	S/N (mL)	OD was taken at 480 nm
Blank	0	0	1.9		100		7	1	0.5	
Cs	0	0.5	1.4		100		7	1	0.5	
E1	0.5	0.5	0.9		100		7	1	0.5	
E1c	0.5	0	1.4		100		7	1	0.5	
E2	0.5	0.5	0.9		100		7	1	0.5	
E2c	0.5	0	1.4		100		7	1	0.5	
E3	0.5	0.5	0.9		100		7	1	0.5	
E3c	0.5	0	1.4		100		7	1	0.5	
E4	0.5	0.5	0.9		100		7	1	0.5	
E4c	0.5	0	1.4		100		7	1	0.5	
E5	0.5	0.5	0.9		100		7	1	0.5	
E5c	0.5	0	1.4		100		7	1	0.5	
E7	0.5	0.5	0.9		100		7	1	0.5	
E7c	0.5	0	1.4		100		7	1	0.5	
ES	0.5	0.5	0.9		100		7	1	0.5	
Esc	0.5	0	1.4		100		7	1	0.5	
I1	0.1	0.1	0.7		100		7	1	0.5	
I1c	0.1	0	0.8		100		7	1	0.5	
I2	0.1	0.1	0.7		100		7	1	0.5	
I2c	0.1	0	0.8		100		7	1	0.5	
I3	0.1	0.1	0.7		100		7	1	0.5	
I3c	0.1	0	0.8		100		7	1	0.5	

Tube no	Enzyme (mL)	Substrate (mL)	Buffer (mL)	Incubation for 10 minutes at 37°C	TCA (µL)	Centrifuge for 10 minutes at 5000 RPM	dH_2O (mL)	N.R. (mL)	S/N (mL)	OD was taken at 480 nm
I4	0.1	0.1	0.7		100		7	1	0.5	
I4c	0.1	0	0.8		100		7	1	0.5	
I5	0.1	0.1	0.7		100		7	1	0.5	
I5c	0.1	0	0.8		100		7	1	0.5	
I7	0.1	0.1	0.7		100		7	1	0.5	
I7c	0.1	0	0.8		100		7	1	0.5	

(Note: Marking goes as Cs-subtrate control. E stands for extracellular; I stands for intracellular 1, 2, 3, 4, 5, 7, S are codes for organisms (S-*Streptococcus thermophillus,* c donates controls for respective organisms.)

- Similar protocol was followed on 5^{th} day of the culture grown.

2.5 PROTEIN ESTIMATION USING BRADFORD METHOD

Chemicals used: Bradford reagent, BSA standard solution (1mg/ml)

Theory: The assay is based on the observation that the absorbance maximum for Comassie Brilliant Blue G-250 (an acidic solution) shifts from 465 nm to 595 nm when it binds to the protein. Hydrophobic and ionic interaction leads to stabilization of the anionic form of dye

a. Standard curve for Bradford using BSA

- Components were added as shown in the table and procedure

Tube no.	BSA concentration (µg/ml)	BSA volume (µl)	dH_2O (µl)	Amount of Sample added to microtiter plate (µl)	Bradford reagent (µl)	Incubate for 5 minutes at room temperature	OD was taken at 595 nm
1	0.2	200	800	10	200		
2	0.4	400	600	10	200		
3	0.6	600	400	10	200		
4	0.8	800	200	10	200		
5	1	1000	0	10	200		

b. Protein estimation of unknown samples of both the assays (laccase and asparaginase)

- Cultures were grown in respective media.
- 2 ml of culture was collected in centrifuge tubes from each sample and was centrifuged.
- Then following reaction was prepared according to the table and procedure followed henceforth.

Tube no.	Sample volume (µl)	Bradford reagent (µl)	Incubate for 5 minutes at room temperature	OD was taken at 59 nm
Control(media)	10	200		
Sample	10	200		

2.6 BIOCHEMICAL TESTS OF CULTURE 2C1

a. Gram staining

- Gram staining was performed on the culture and observation was noted down

b. Gelatin hydrolysis test

- A heavy inoculum is stabbed in nutrient gelatin medium (12% gelatin in Nutrient broth) in a culture tube.
- It was then incubated at 37 ^{0}C for 2 days.
- The culture tubes after incubation was kept at 4 ^{0}C for 15-20 minutes.
- The observation was noted that whether the medium was liquefied or not.

Positive result: partial liquification

Negative result: no liquification

c. Indole test

- The culture was grown in peptone broth (1% peptone) for 48 hours at 37^{0}C.
- After that few drops of Kovac's reagent was added to it and observations were noted down.

Positive result: red colour ring formation at the interface.
Negative result: no red colour ring formation.

d. Methyl red test and Voges-Proskauer test (MR-VP)

Methyl Red (MR) test:

- The culture was grown in MR-VP medium for a period of two days and incubated at 37 ^{0}C.
- Few drops of methyl red was added to the plate and observation was noted down.

Positive result: colour changes to red

Negative result: no colour change

Voges-Proskauer test:

Baritt'sA reagent: Alpha-naphthol 5% in absolute ethyl alcohol

Baritt's B reagent: Potassium Hydrooxide (40%) in water

- Culture was grown in MR-VP medium for 2 days at 37 ^{0}C.
- Culture was grown in MR-VP medium for 2 days at 37 ^{0}C.
- After incubation 10 drops of Baritts A reagent and Baritt's B reagent each, was added and observation was noted down.

Positive result: color changes to red after 15 minutes
Negative result: no color change

e. Urease test

- 9.5 ml of Urea broth base is prepared in test tube and autoclaved.
- To this 500 µl of filtered Urea solution (40%) is added aseptically.
- The medium is inoculated and kept for 2 days at 37 ^{0}C and observations were noted down.

Positive result: color changes to bright pink
Negative result: no color change

f. Starch hydrolysis test

- The culture was streaked on starch agar media (Nutrient agar with 2.5% starch) for 2 days at 37 ^{0}C.
- After incubation iodine is flooded on the plates and observation is noted down.
  After adding iodine:
  Positive result: zone of clearance, no blue colour near the colonies.
  Negative result: plate covered with blue color.

2.8 TEST FOR ENZYME ACTIVITY AT VARYING TEMPERATURES:

- The cultures 7 and 2C1 was grown in respective media at 37 ^{o}C. (culture 7 for asparaginase activity and culture 7 and culture 2C1 for laccase activity)
- The activity assay was performed at different incubating temperatures (25 ^{o}C, 37 ^{o}C, 45 ^{o}C, 55 ^{o}C, 65 ^{o}C and 75 ^{o}C)

For Laccase:

Tube no.	Enzyme (ml)	Substrate (ml)	Buffer (ml)	Incubate for 10 minutes at 25 ^{o}C, 37 ^{o}C, 45 ^{o}C, 55 ^{o}C, 65 ^{o}C and 75 ^{o}C	TCA (ml)	OD was taken at 420 nm
S_c	0	0.8	0.2		0.1	
Ec 7	0.2	0	0.8		0.1	
E1 7	0.2	0.8	0		0.1	
Ec 2C1	0.2	0.8	0		0.1	
E1 2C1	0.2	0	0.8		0.1	

For Asparaginase:

Tube no	Enzyme (mL)	Substrate (mL)	Buffer (mL)	Incubation for 10 minutes at 25 ^{o}C, 37 ^{o}C, 45 ^{o}C, 55 ^{o}C, 65 ^{o}C and 75 ^{o}C	TCA (µL)	Centrifuge for 10 minutes at 5000 RPM	dH_2O (mL)	N.R. (mL)	S/N (mL)	OD was taken at 480 nm
Cs	0	0.5	1.4		100		7	1	0.5	
E1	0.5	0.5	0.9		100		7	1	0.5	
E1c	0.5	0	1.4		100		7	1	0.5	

2.9 AMMONIUM PRECIPITATION

- Cultures of the selected organisms that showed highest activity were taken and grown in guaicol, wheatbran and aspargine media respectively.
- Cultures were taken in 50 mL centrifuge tubes and centrifuged at 8000 RPM for 10 minutes and supernatant was collected in new centrifuge tubes.
- From the table of ammonium sulfate precipitation (Table No. 3 in annexure) the required cut amount (30%, 60%, 90%) of ammonium sulfate was calculated.
- Ammonium sulfate was then slowly added pinch by pinch to each media and was continuously stirred at 4°C.
- Ammonium sulfate was mixed completely and the media was kept at 4°C for overnight precipitation.
- Next day the samples were centrifuged at 8000 RPM for 10 minutes and then the supernatant was collected in a new centrifuge tube and the pellet was again suspended in respective buffer and activity assay was performed by the above mentioned protocol.

2.10 DIALYSIS OF PROTEIN OBTAINED FROM AMMONIUM PRECIPITATION

- Dialysis membrane was dipped in cold water for some time.
- One end of the bag was carefully tied using a thread.
- It was then filled with protein sample and its other end was tied.
- Dialysis bag was then dipped in respective buffers and left undisturbed at 4 °C.
- After 8 hours the buffer was replaced with fresh buffer and the bag was again left undisturbed for the next 8 hours.
- Dialysis solution was take out in a vial and was centrifuged at 7000 RPM for 10 minutes.
- Pellet and supernatant were collected in separate vials.
- Activity assay was performed.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 BIOCHEMICAL TEST RESULT OF 2C1 CULTURE

Gram staining

COLONY NAME	Gram	Shape	Organization of cells
2C1	+	Coccus	Chains

Other biochemical tests

Biochemical tests	Results
Amylase test	+
MR-VP test	-
McConkey Agar test	-
Indole test	-
Urease test	-
Gelatin liquification	-

Amylase test:

It may not be *Corynebacterium, Clostridium, Bacillus, Bacteroides, Fusobacterium,* and members of *Enterococcus*.

Methyl red test:

MR-negative organisms are *Klebsiella pneumoniae* and *Enterobacter aerogenes* whereas organisms such as *Escherichia, Salmonella, Proteus* are methyl red positive.

VP test:

Organisms that give negative VP test are *Vibrio* species, *Yersinia*, *Citrobacter* sp., *Shigella, Edwardsiella*, *Salmonella, Proteus mirabilis, Escherichia coli.*

McConkey agar test:

These organisms are gram positive organisms and do not have the ability to ferment lactose.

Indole test:

Bacteria which give negative results for the indole test include: *Actinobacillus* sp., *Serratia* sp., *Aeromonas salmonicida, Mannheimia haemolytica*, most *Klebsiella* sp., most *Haempophilus* sp., *P.penneri*, *Enterobacter* sp., most *Bacillus* sp., *Bordetella* sp., *Lactobacillus* sp., *Alcaligenes* sp., *Neisseria* sp., *Pasteurell aureae, Proteus mirabilis, Pseudomonas* sp., *Salmonella* sp., *Yersinia* sp.

Urease test:

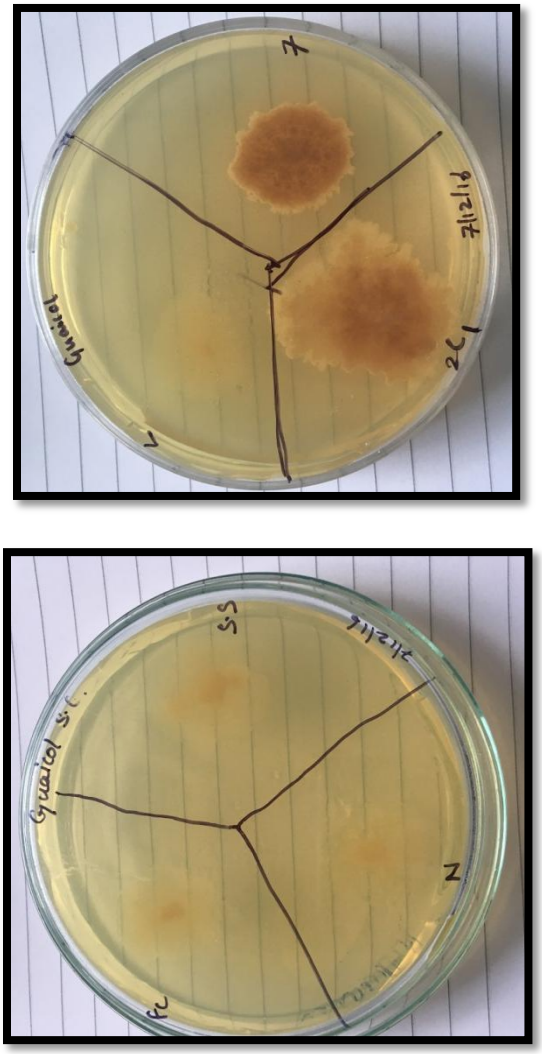
Urease test is positive for several genera and species of *Enterobacteriaceae* including *Proteus, klebsiella,* and *Yersinia* species. Negative test shows it might not be these species.

Gelatin liquification:

S. epidermidis, some species of *Enterobacteriaceae*, *E. coli* shows positive results. It might not be *Bacillus* species, *proteus, clostridium, S. aureus* and *serratia.*

3.2 LACCASE:

3.2.1 Qualitative analysis:



Qualitative screening of laccase (2C1, N, S.S, Fc, L)

This is the result for qualitative screening of laccase. Culture 7 and culture 2C1 are showing highest activity as the pigmentation of the culture grown is of brown colour. Other cultures are showing little activity as the cultures are light brown pigmented. The change in colour of colonies (brown colour) shows positive result for laccase.

3.2.2 Quantitative analysis of enzyme activity:

After performing the assay the following results were obtained:

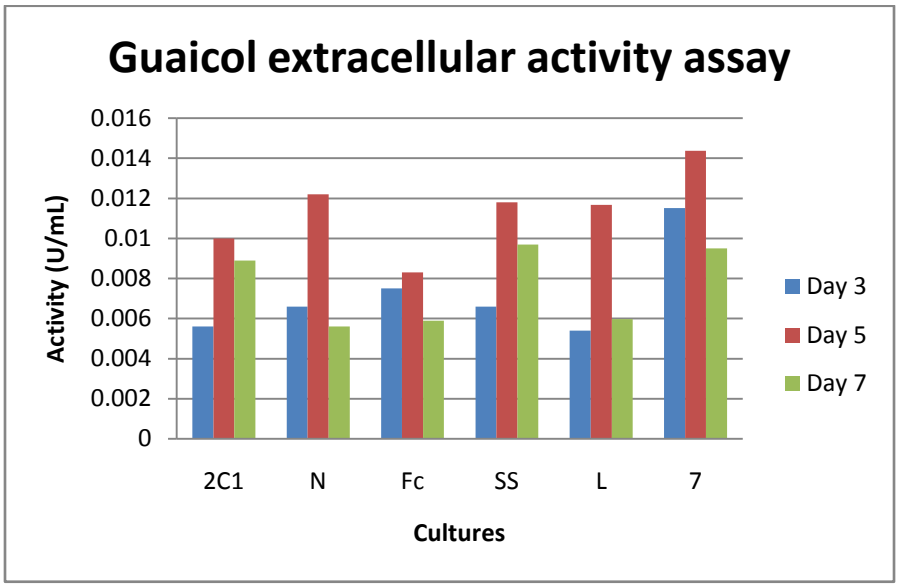


Chart 1: Comparative analysis of Guaicol extracellular activity of cultures 2C1, N, Fc, SS, L, 7 for different days

- From the bar graph in chart 1 it can be easily inferred that culture 7 has the highest activity (0.0143 U/mL) on the 5th day of its growth using guaicol as the substrate.

Enzyme activity (U/mL): (Change in extinction of light (min^{-1}) * Total volume measures) / (Molar Absorption coefficient of ABTS (M^{-1} cm^{-1}) * length of light travelled (cm) * Volume of enzyme stock solution)

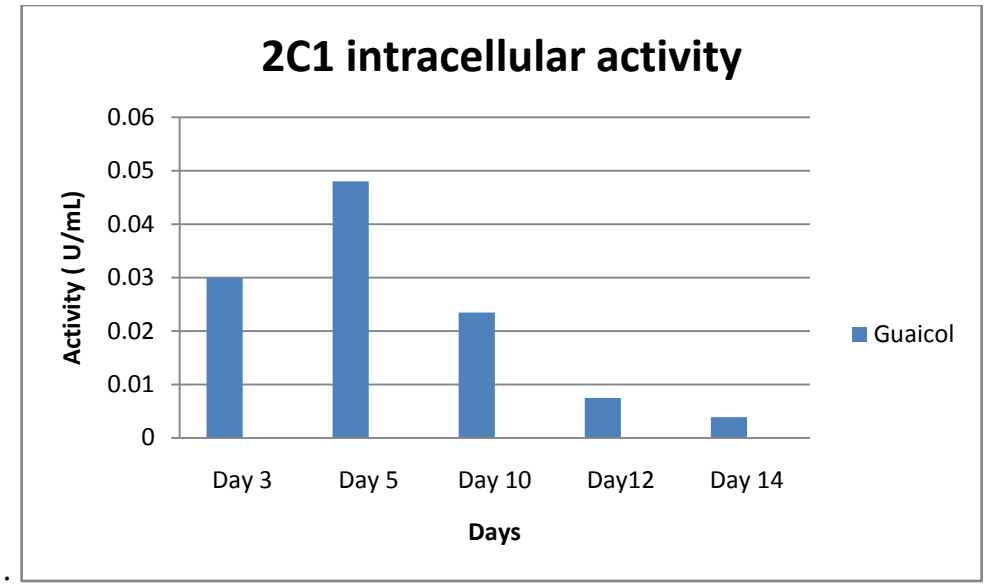


Chart 2: Comparative analysis of 2C1 intracellular activity with guaicol media

- From chart 2 it can be observed that the intracellular enzyme activity of 2C1 in guaicol medium is showing the highest activity on the 5th day of its growth which is 0.048 U/mL..

3.2.3 Protein estimation using bradford method:

a. Standard Graph of BSA:

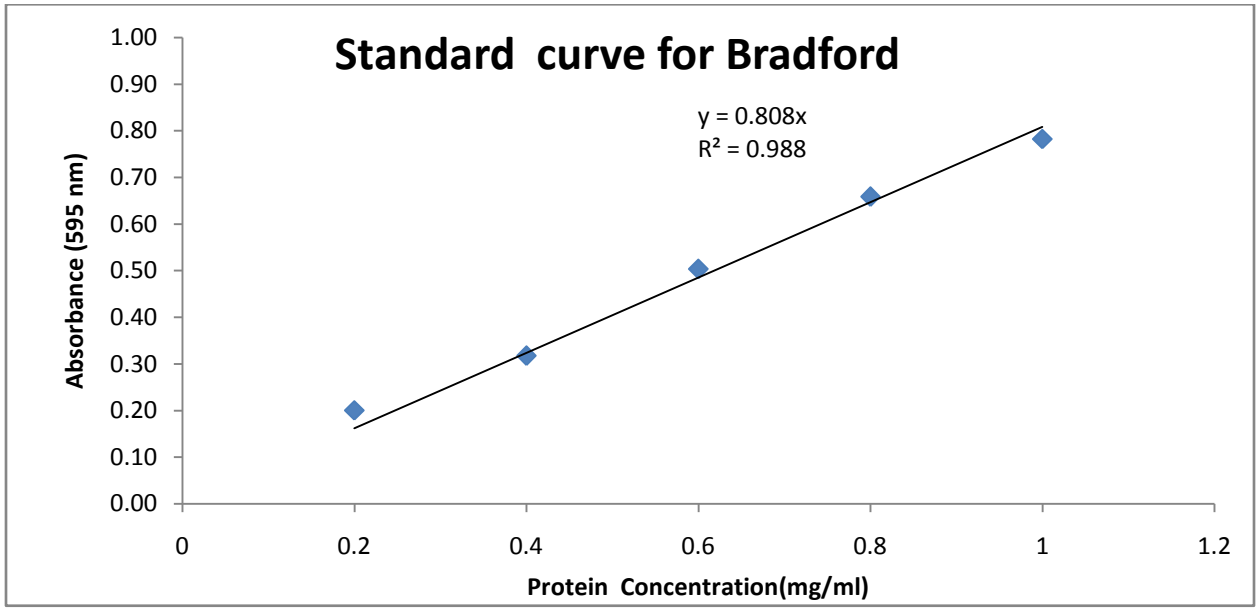


Chart 3: Standard curve of BSA for protein estimation

b. Protein estimation of unknown samples:

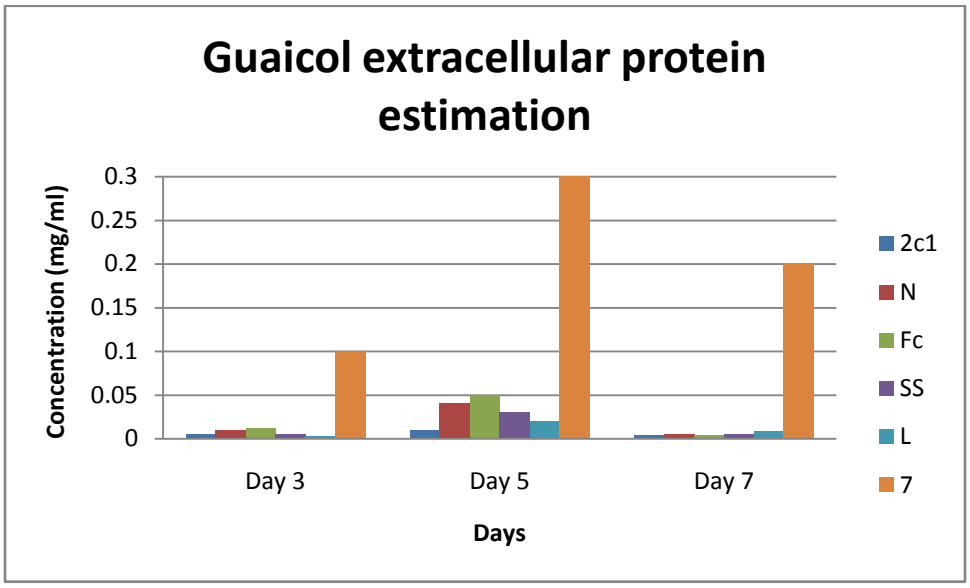


Chart 4: Guaicol extracellular protein estimation

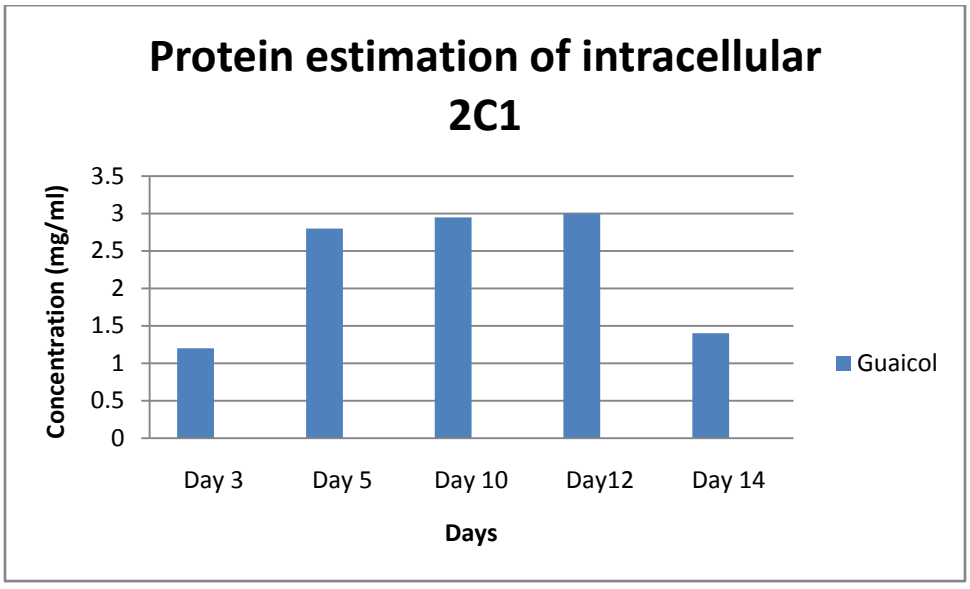


Chart 5: Protein estimation of intracellular assay of 2C1 with guaicol media

7 culture has the highest extracellular protein concentration in guaicol media on day 5 of its growth which can be observed from chart 4.

2C1 has highest intracellular protein concentration on 12th day with guaicol as the media as shown in chart 5.

3.2.4 Activity assay at varying temperatures:

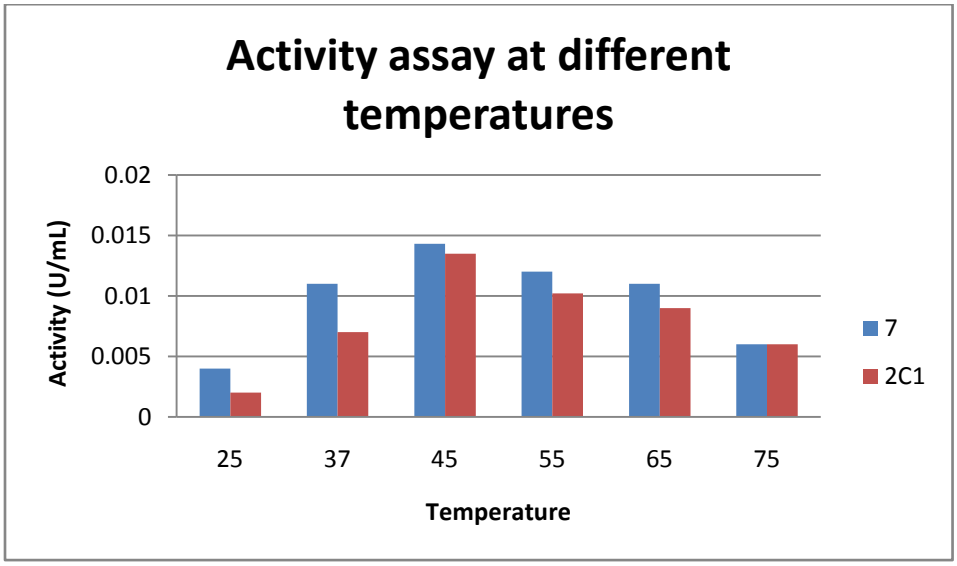


Chart 6: Activity assay at varying temperatures for culture 7, 2C1

Highest activity of Laccase was found at temperature 45 ^{o}C for both sample 7 and sample 2C1. (0.0143 U/mL and 0.0135 U/mL respectively)

3.2.5 Ammonium sulfate precipitation

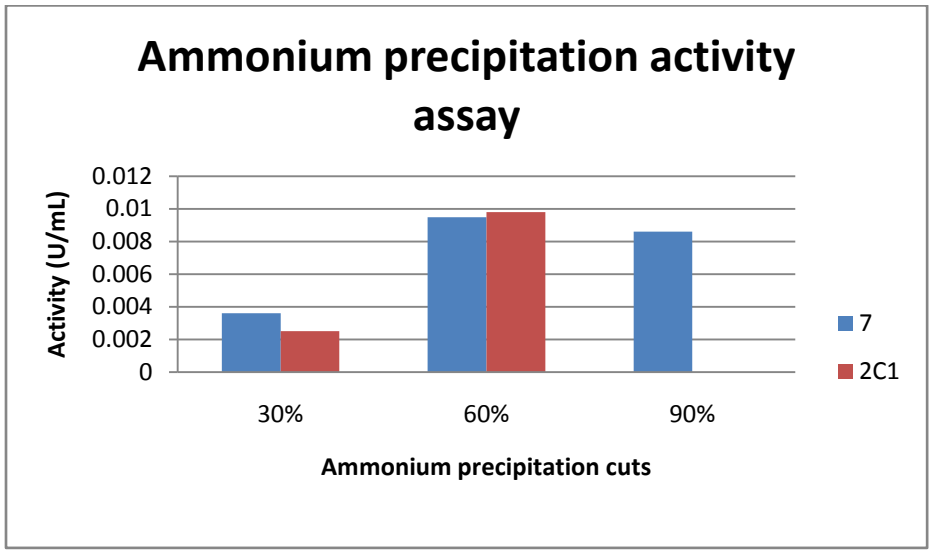


Chart 7: Activity assay of samples after Ammonium precipitation

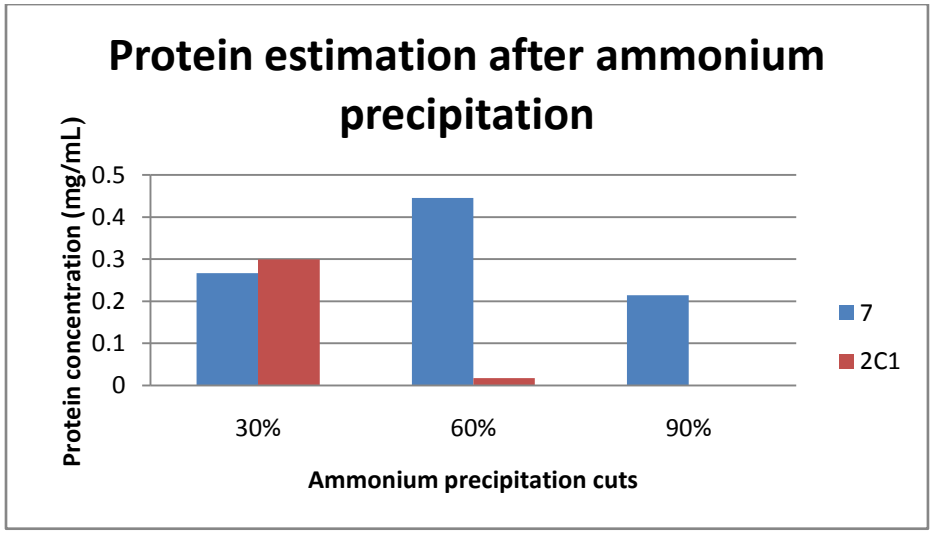


Chart 8: Protein estimation of samples after ammonium precipitation

The highest activity for 7th culture is 0.0095 U/mL which is obtained at 60% ammonium sulfate cut and the protein concentration for the same is 0.445 mg/mL and the highest activity for 2C1 culture is 0.0098 which is obtained at 60% ammonium sulfate cut and the protein concentration for the same is 0.017 mg/mL.

Purification table (for sample 7):

Sample	Total E.A (IU)	Total protein conc(mg/ml)	Specific activity (IU/mg)	Purification fold	Purification yield
Crude	0.0143	0.357	0.04	0.00	100.00
30%	0.0036	0.267	0.01	0.34	25.17
60%	0.0095	0.445	0.02	0.53	66.43
90%	0.0086	0.2141	0.04	1.00	60.14

Purification table (for sample 2C1):

Sample	Total E.A (IU)	Total protein conc(mg/ml)	Specific activity (IU/mg)	Purification fold	Purification yield
Crude	0.0135	0.343	0.04	0.00	100.00
30%	0.0025	0.299	0.01	0.21	18.52
60%	0.0098	0.017	0.58	14.65	72.59

Purification fold = Specific activity of purified enzyme/ specific activity of crude enzyme
Purification yield= Enzyme activity of purified enzyme/ Enzyme activity of crude enzyme

3.2.6 Dialysis:

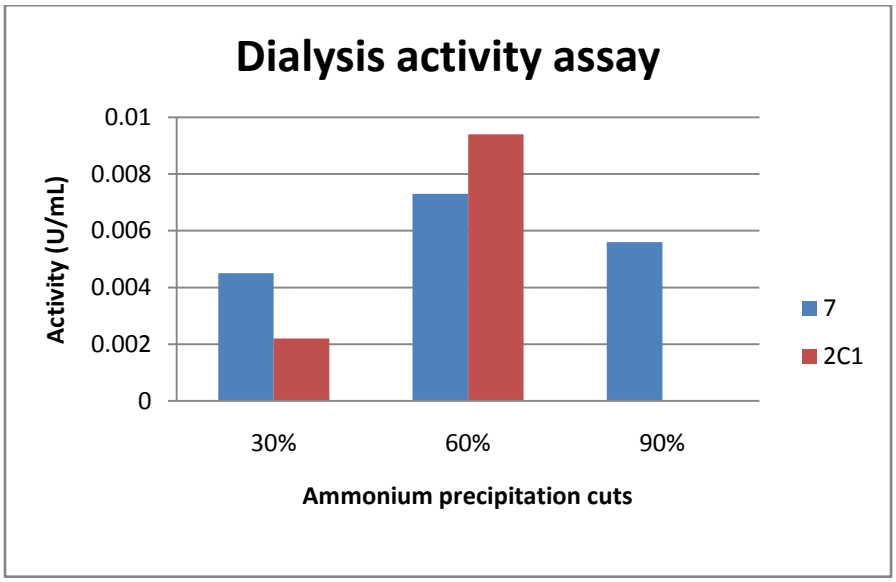


Chart 9: Activity assay of samples after dialysis

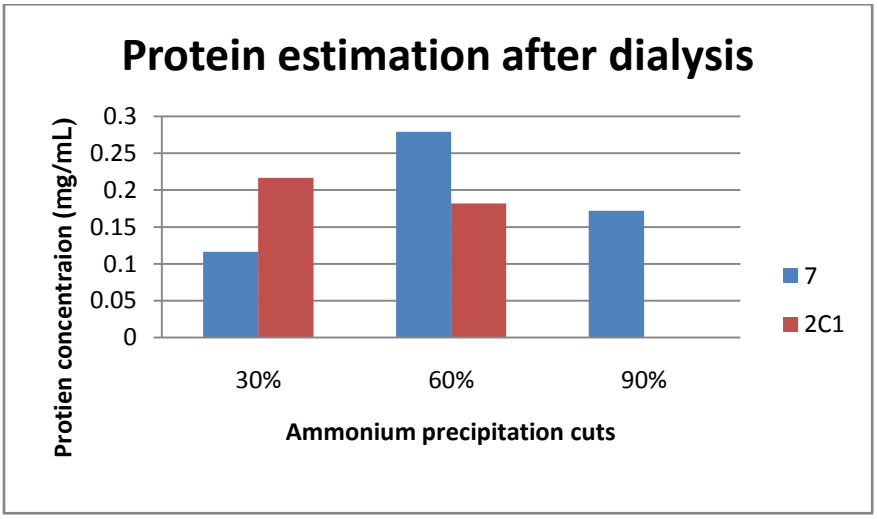


Chart 10: Protein estimation of samples after dialysis

The highest activity for 7th culture is 0.0071 U/mL which is obtained at 60% ammonium sulfate cut and the protein concentration for the same is 0.27 mg/mL and the highest activity for 2C1 culture is 0.0097 which is obtained at 60% ammonium sulfate cut and the protein concentration for the same is 0.18 mg/mL.

Purification Table (for sample 7):

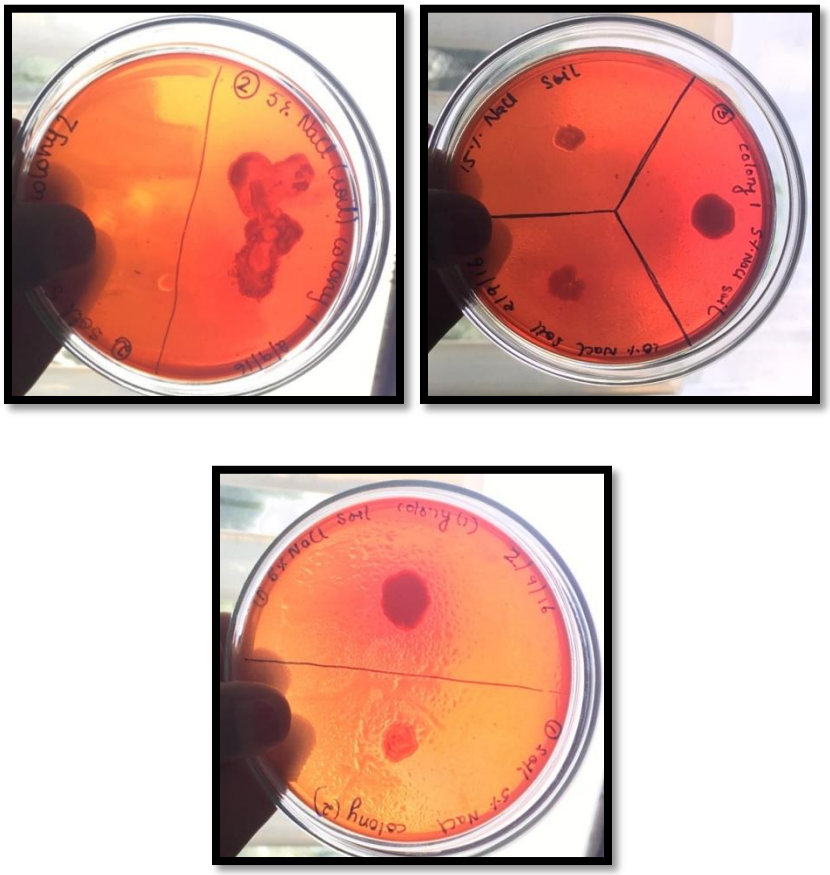
Sample	Total E.A (IU)	Total protein conc(mg/ml)	Specific activity (IU/mg)	Purification fold	Purification yield
Crude	0.0143	0.357	0.04	0.00	100.00
30%	0.0045	0.116	0.04	0.97	31.47
60%	0.0073	0.279	0.03	0.65	51.05
90%	0.0056	0.172	0.03	0.81	39.16

Purification table (for sample 2C1):

Sample	Total E.A (IU)	Total protein conc(mg/ml)	Specific activity (IU/mg)	Purification fold	Purification yield
Crude	0.0135	0.343	0.04	0.00	100.00
30%	0.0022	0.216	0.01	0.26	16.30
60%	0.0094	0.1819	0.05	1.31	69.63

3.3 ASPARAGINASE:

3.3.1 Qualitative analysis:



Qualitative screening of asparaginase (1C1, 1C2, 2C1, 3C1)

This is the result for qualitative assay for asparaginase where cultures 1C2 and 3C1 showed the highest activity as the color changed from orange to reddish pink was observed the most. The color changes because there is a change in ph from acid to alkaline conditions because of the production of asparaginase enzyme. Other cultures are showing little activity as less color change is observed.

3.3.2 Quantitative assay

Standard graph of ammonium sulfate:

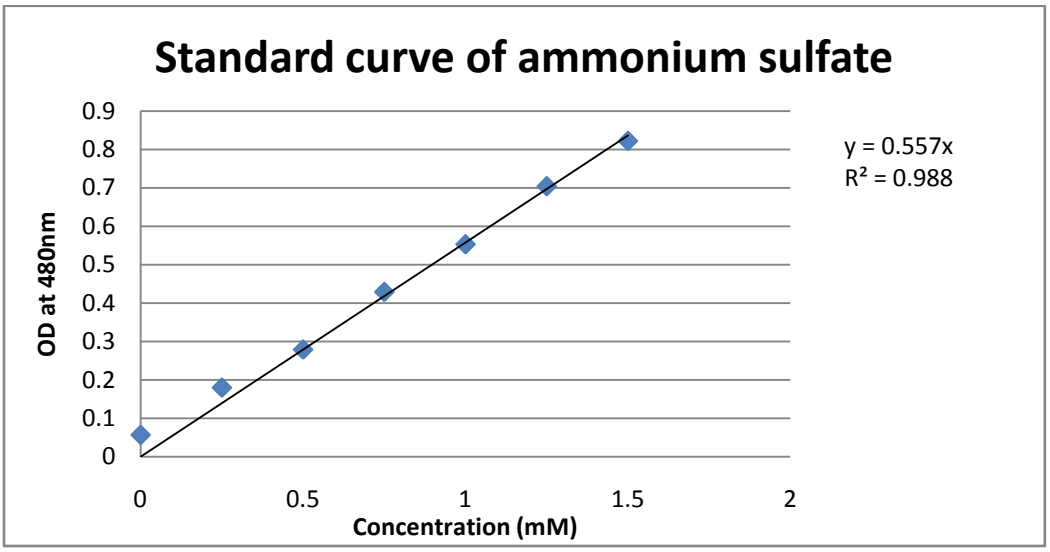


Chart 11: Standard graph of ammonium sulfate

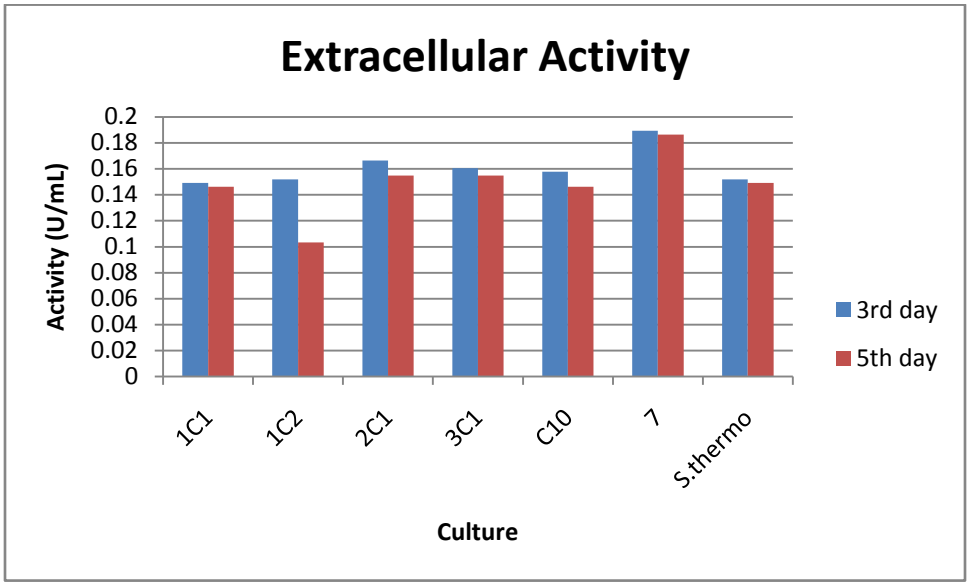


Chart 12: Extracellular activity assay graph for 1C1, 1C2, 2C1, 3C1, C10, 7, *S.thermophilus* cultures

The highest activity in the extracellular assay has been obtained in 7th culture for 3rd day as well as 5th day which is 0.189 U/mL and 0.186 U/mL respectively.

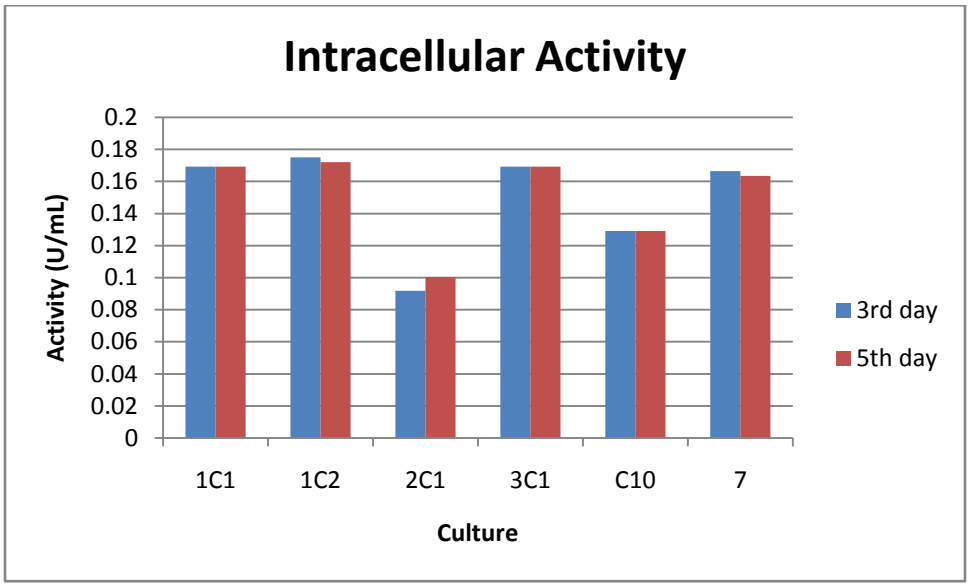


Chart 13: Intracellular activity assay graph for 1C1, 1C2, 2C1, 3C1, C10, 7 cultures

The highest activity in the intracellular assay has been obtained in 1C2 culture for 3rd day as well as 5th day which is 0.174 U/mL and 0.172 U/mL respectively.

Enzyme activity (U/mL): (µmole of ammonia released * volume of the reaction) / (Time * Volume of the enzyme used)

3.3.3 Protein estimation using bradford method:

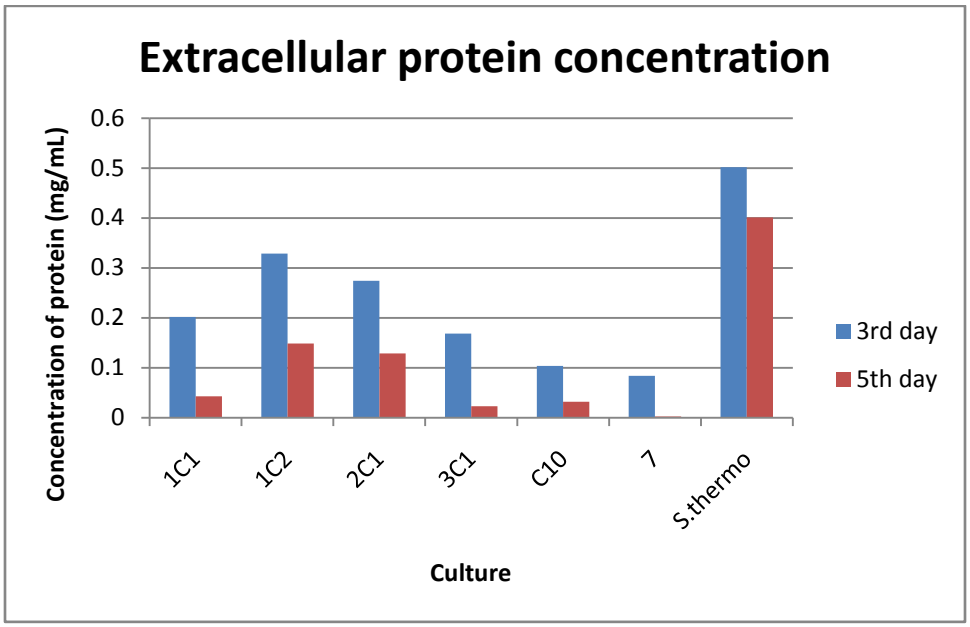


Chart 14: Extracellular protein concentration for 1C1, 1C2, 2C1, 3C1, C10, 7, *S. thermophilus* cultures

The highest protein concentration in the extracellular assay has been obtained in Streptococcus thermophilus culture for 3rd day as well as 5th day which is 0.501 mg/mL and 0.401 mg/mL respectively.

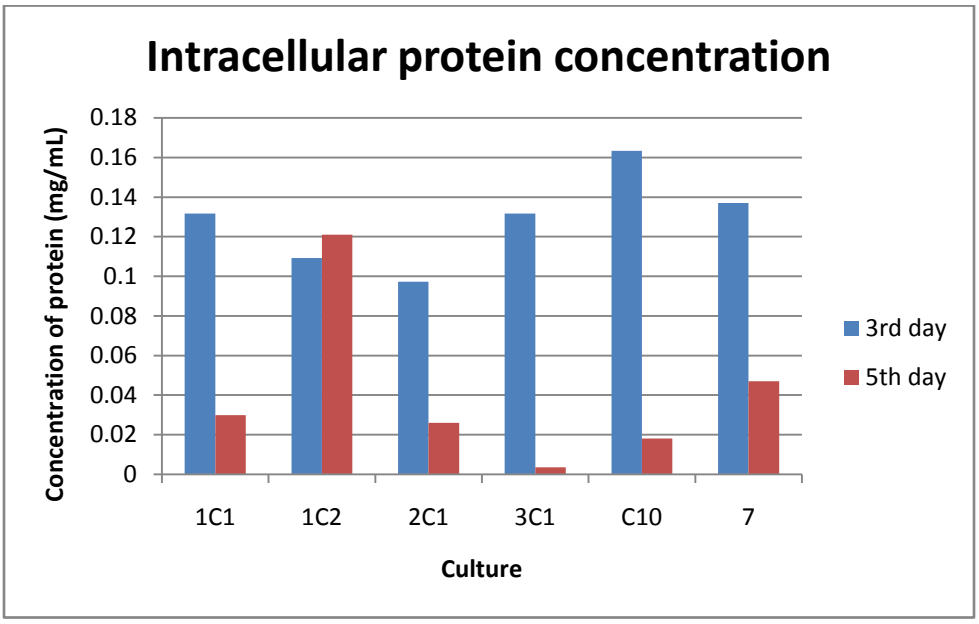


Chart 15: Intracellular protein concentration for 1C1, 1C2, 2C1, 3C1, C10, 7 cultures

The highest protein concentration in the intracellular assay has been obtained in C10 culture for 3rd day which is 0.163 mg/mL and for 5th day, 1C2 culture has the highest protein concentration which is 0.121 mg/mL.

3.3.4 Activity assay at varying temperatures:

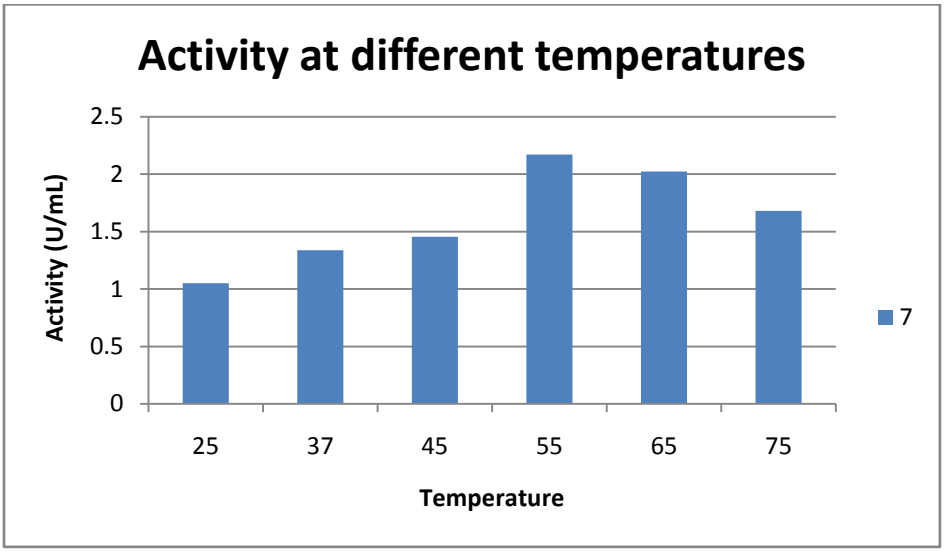


Chart 16: Activity assay at varying temperatures for culture 7

Highest activity of asparaginase was found at temperature 55 ^{o}C which is 2.171 U/mL

3.3.5 Ammonium sulfate precipitation:

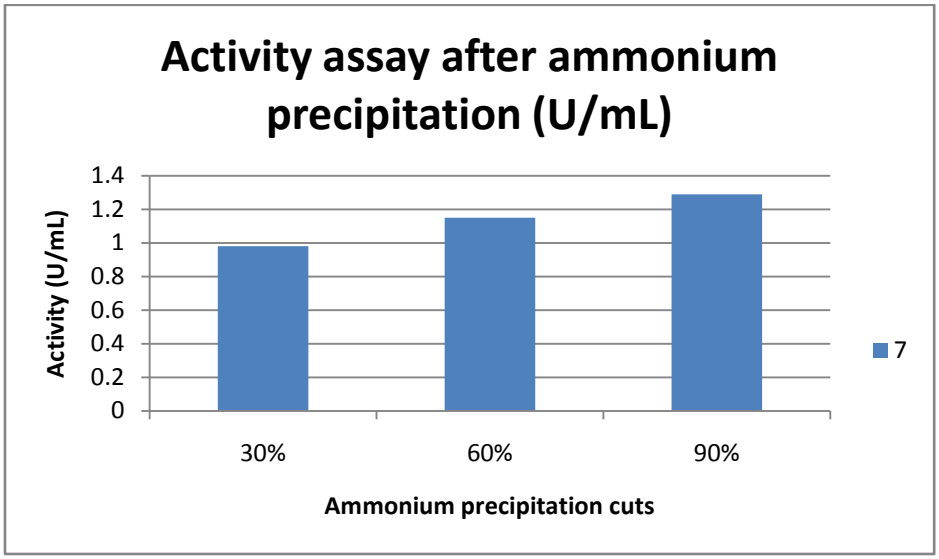


Chart 17: Protein estimation of samples after ammonium sulfate precipitation

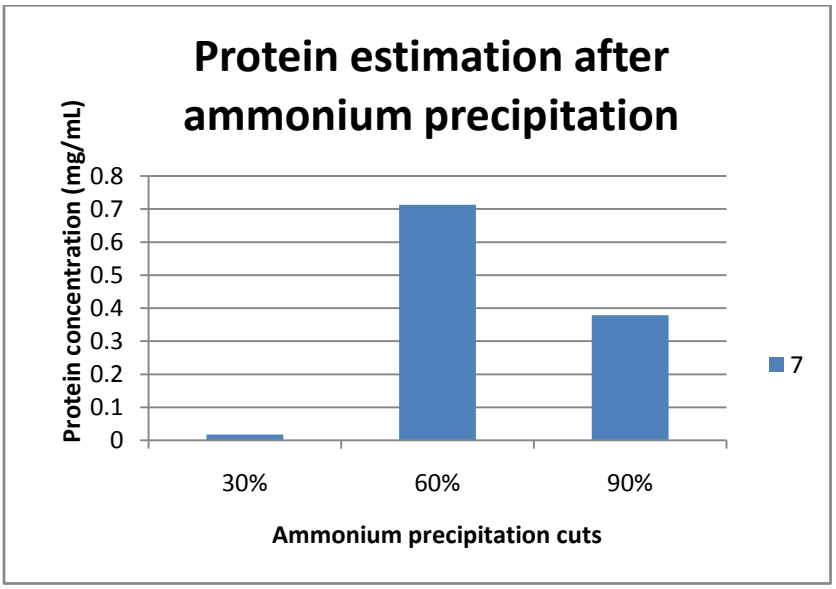


Chart 18: Activity assay of samples after ammonium sulfate precipitation

The highest activity is 1.29 U/mL which is obtained at 90% ammonium sulfate cut and the protein concentration for the same is 0.378 mg/mL.

Purification Table (for sample 7):

Sample	Total E.A (IU)	Total protein conc(mg/ml)	Specific activity (IU/mg)	Purification fold	Purification yield
Crude	2.17	0.084	25.83	0	100
30%	1.64	0.017	96.47	3.73	75.57
60%	1.8	0.713	2.52	0.09	82.94
90%	2.05	0.378	5.42	0.2	94.47

3.3.6 Dialysis:

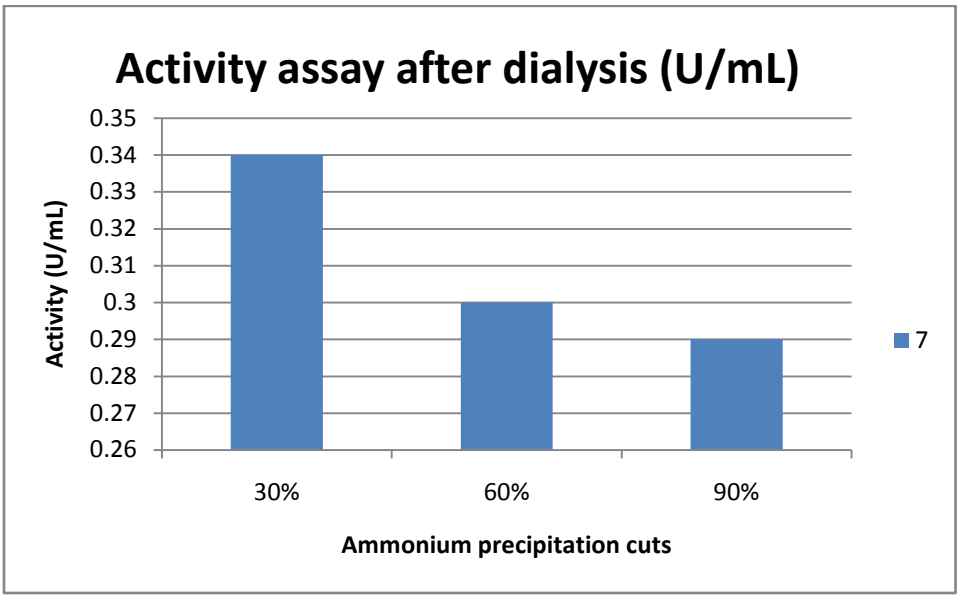


Chart 19: Activity assay of samples after dialysis

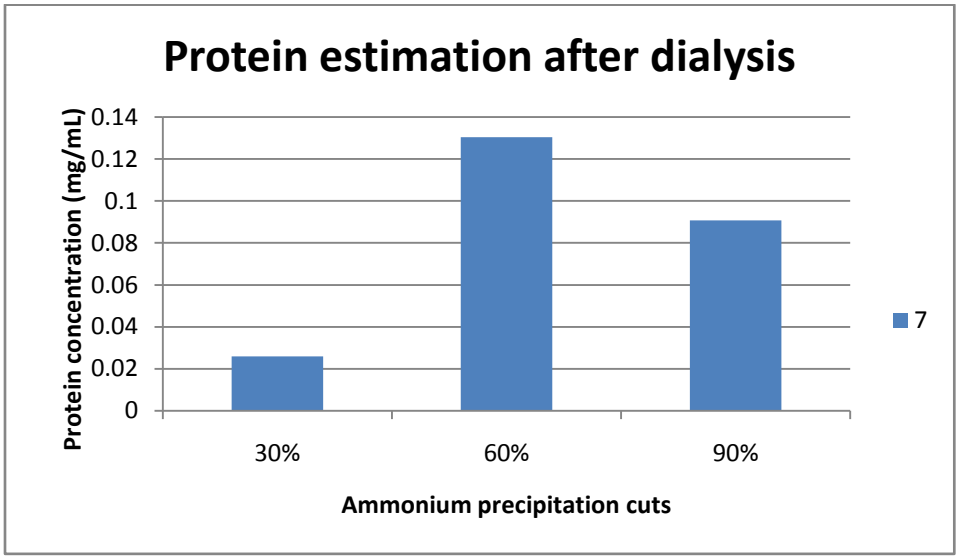


Chart 20: Protein estimation of samples after dialysis

The highest activity is 0.34 U/mL which is obtained at 30% ammonium sulfate cut and the protein concentration for the same is 0.024 mg/mL.

Purification Table (for sample 7):

Sample	Total E.A (IU)	Total protein conc(mg/ml)	Specific activity (IU/mg)	Purification fold	Purification yield
Crude	2.17	0.084	25.83	0	100
30%	0.34	0.025	13.6	0.52	15.66
60%	0.3	0.13	2.3	0.08	13.82
90%	0.29	0.09	3.22	0.12	13.36

3.4 Discussion

After isolating different cultures and taking control from the cell repository it was found that the halophillic organism 2C1 was showing moderate activity and the control organism 7 which is a thermophilic organism, was showing a higher activity after performing series of Laccase activity assays. Although the protein extracted after dialysis was significant with few impurities at different stages of ammonium precipitation and dialysis.

The culture 7 showed the highest activity when asparaginase activity assay was performed. Subsequently the purification fold increased after performing dialysis.

Further experimentation has to be done, such as verifying the purity of the protein using SDS. Identifying the unknown halophillic culture 2C1 which can be identified by performing 16s rRNA sequencing.

CHAPTER 4: REFERENCES AND ANNEXURE

4.1 REFERENCES

1)Verma.Ambika et al;" Purification and Characterization of Thermostable Laccase from Thermophilic *Geobacillus thermocatenulatus* MS5 and its applications in removal of Textile Dyes"

2)More.S.Sunil et al;" Isolation, Purification, and Characterization of Fungal Laccase from Pleurotus sp.";2011

3) P.Baldrian;" Fungal laccases-occurrence and properties".;*FEMSMicrobiol Rev*; 30;215-242;2016

4) P.Sharmaet al;"Bacterial laccases";*World J Microbiol Biotechnol*;23;823-832;2007

5)S.Sonica et al;"Purification and Characterization of an Extracellular, Thermo-Alkali-Stable, Metal Tolerant Laccase from *Bacillus tequilensis* SN4";*journal.pone*

6)Gonzalez.C.Juan et al;"Production of *Trametes pubescens* Laccase under Submerged and Semi-Solid Culture Conditions on Agro-Industrial Wastes"

7) Sole Magali et al;"Differential Regulation by Organic Compounds and Heavy Metals of Multiple Laccase Genes in the Aquatic Hyphomycete *Clavariopsis aquatic"*

8) B.Viswanathet al;" Screening and assessment of laccase producing fungi isolated from different environmental samples";*African Journal of Biotechnology*;7;1129–1133;2008

9) M.Bailey et al;"xpression of Melanocarpus albomyces laccase in Trichoderma reesei and characterization of the purified enzyme"; *Microbiology*; 150;3065-74;2004

10)*"*copper induction of laccase isoenzymes in the ligninolytic fungus Pleurotus ostreatus"

11)K.Rao.Koteswara et al;" Purification and characterization of laccase from Monocillium indicum Saxena"; *Applied Microbiology and Biotechnology*;37;321–323;1992

12)B.S.Pointing et al;"Decolorization of azo and triphenylmethane dyes by Pycnoporus sanguineus producing laccase as the sole phenoloxidase"; *World Journal of Microbiology and Biotechnology*;16;317–318;2000

13)Osma.F.Johann et al." Uses of Laccases in the Food Industry";*enzyme Res*;2010

14) "Industrial and biotechnological applications of laccases: A review";*biotechnology advance*;2006

15) ‘‘Crystal Structure of Laccase From Basidiomycete Pm1 (Cect 2971)”

16)Ehrman.Mark et al;”L-AsparaginaseII of *Escherichia coli* studies on the enzymatic mechanism of action”;*The Journal of Biological Chemistry*;246; 88-94;1971

17)Jaskolski.Mariusz et al;”Structure, Dynamics and Electrostatics of the L-Asparaginase Catalytic Centre:Implications for Reaction Mechanism”

18)Kumar.Kuldeep et al;”The Various Sources & Application of L-Asparaginase”;*Asian Journal of Biochemical and Pharmaceutical Research*;3;2012

19)Bansal.S et al;”Structural Stability and Functional Analysis of L-Asparaginase from Pyrococcus furiosus”;*Biochemistry (Moscow)*;75;375-381;2010

20)Gupta.Sonika et al;”A diverse group of halophilic bacteria exist in Lunsu, a natural salt water body of Himachal Pradesh, India”;*SpringerPlus*;4;274;2015

21)Batool.Tahira et al;”A Comprehensive Review on L-Asparaginase and Its Applications”;*ApplBiochem Biotechnol*;178;900–923;2016

22)Shirazian.Pejman et al;”The potential of halophilic and halotolerant bacteria for the production of antineoplastic enzymes: l-asparaginase and l-glutaminase”;*EXCLI Journal*;15;268-279;2016

23)Azhar.Mohsin et al;”Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India”;*International Journal of Current Microbiology And Applied Sciences*;3;753-760;2014

4.2 ANNEXURE

1. LB media composition

Composition	Quantity
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Distilled water	1 L
pH	7.0

2. Sodium Phosphate buffer

Composition	Concentration needed	Added to final buffer
Mono basic dihydrogen phosphate(1)	1 M	
Dibasic mono hydrogen phosphate(2)	1 M	
Sodium phosphate buffer	0.1 M	42.3mL (1) + 57.7mL (2)
Distilled water		900mL

3. Ammonium sulfate precipitation chart

Initial concentration of ammonium sulphate, % saturation	Final concentration of ammonium sulphate—% saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulphate to add to 100 ml of solution																
0	10·6	13·4	16·4	19·4	22·6	25·8	29·1	32·6	36·1	39·8	43·6	47·6	51·6	55·9	60·3	65·0	69·7
5	7·9	10·8	13·7	16·6	19·7	22·9	26·2	29·6	33·1	36·8	40·5	44·4	48·4	52·6	57·0	61·5	66·2
10	5·3	8·1	10·9	13·9	16·9	20·0	23·3	26·6	30·1	33·7	37·4	41·2	45·2	49·3	53·6	58·1	62·7
15	2·6	5·4	8·2	11·1	14·1	17·2	20·4	23·7	27·1	30·6	34·3	38·1	42·0	46·0	50·3	54·7	59·2
20	0	2·7	5·5	8·3	11·3	14·3	17·5	20·7	24·1	27·6	31·2	34·9	38·7	42·7	46·9	51·2	55·7
25		0	2·7	5·6	8·4	11·5	14·6	17·9	21·1	24·5	28·0	31·7	35·5	39·5	43·6	47·8	52·2
30			0	2·8	5·6	8·6	11·7	14·8	18·1	21·4	24·9	28·5	32·3	36·2	40·2	44·5	48·8
35				0	2·8	5·7	8·7	11·8	15·1	18·4	21·8	25·4	29·1	32·9	36·9	41·0	45·3
40					0	2·9	5·8	8·9	12·0	15·3	18·7	22·2	25·8	29·6	33·5	37·6	41·8
45						0	2·9	5·9	9·0	12·3	15·6	19·0	22·6	26·3	30·2	34·2	38·3
50							0	3·0	6·0	9·2	12·5	15·9	19·4	23·0	26·8	30·8	34·8
55								0	3·0	6·1	9·3	12·7	16·1	19·7	23·5	27·3	31·3
60									0	3·1	6·2	9·5	12·9	16·4	20·1	23·9	27·9
65										0	3·1	6·3	9·7	13·2	16·8	20·5	24·4
70											0	3·2	6·5	9·9	13·4	17·1	20·9
75												0	3·2	6·6	10·1	13·7	17·4
80													0	3·3	6·7	10·3	13·9
85														0	3·4	6·8	10·5
90															0	3·4	7·0
95																0	3·5
100																	0