PROJECT TITLE

CLONING OF YEAST GLYCEROL-3-PHOSPHATE PHOSPHATASE (GPP1) IN *ESCHERICHIA COLI*

Dissertation/ Project report submitted in partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

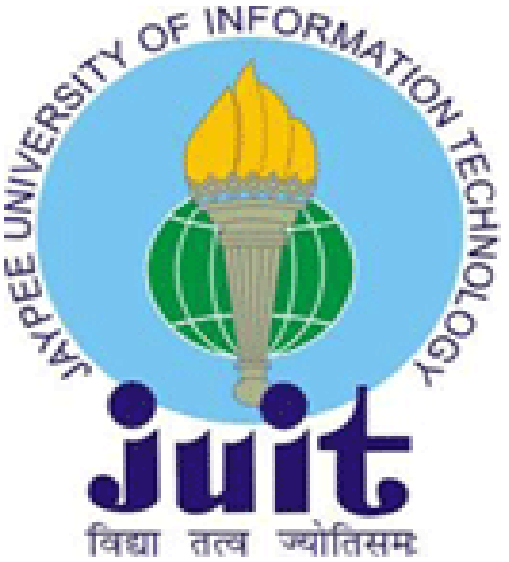
BIOTECHNOLOGY

By

AAKRITI BISHT

UNDER THE GUIDANCE OF

DR. SAURABH BANSAL



DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

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DECLARATION

I hereby declare that the work reported in the B-Tech thesis entitled **"CLONING OF YEAST GLYCEROL-3-PHOSPHATE PHOSPHATASE (GPP1) IN *ESCHERICHIA COLI*"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Saurabh Bansal**. I have not submitted this work elsewhere for any other degree or diploma.

Aakriti Bisht (141806)

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Date:

CERTIFICATE

This is to certify that the work titled "**Cloning of yeast glycerol-3-phosphate phosphatase GPP1 in *Escherichia coli***" submitted by "**Ms. Aakriti Bisht**" for the degree of B. Tech. of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor

Dr. Saurabh Bansal

Assistant Professor

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I am thankful to Jaypee University of Information Technology for funds to carry out my research work.

Signature of the student

Aakriti Bisht

141806

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	*Abbreviations*
G3PP	Glycerol-3-phosphate phosphatase
G3P	Glycerol-3-phosphate
HAD	Haloacid dehydrogenase
ATP	Adenosine triphosphate
Asp	Aspartate
GPP1	Glycerol-1-phosphate phosphohydrolase 1
GPP2	Glycerol-1-phosphate phosphohydrolase 2
LDL	Low density lipoproteins
HDL	High density lipoproteins
T2D	Type 2 diabetes
PGP	Phosphoglycolate phosphatase
HADSF	Haloacid dehydrogenase superfamily
Asp	Aspartic acid
HOG	High Osmolarity Glycerol
MAPK	Mitogen-activated protein kinase
GPD	Glycerol-3-phophate dehydrogenase
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GL/FFA	Glycerolipid/free fatty acid
TG	Triglycerides
$MgCl_2$	Magnesium chloride
$HClO_4$	Perchloric acid
MtGPP	Mycobacterium tuberculosis GPP
ALS	Alkaline Lysis Solution
RT	Room temperature

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ABSTRACT

G3PP, being a member of HAD superfamily, has been recently identified as a potential target for metabolic diseases, cardiovascular and neurological disorders, even cancer. Not just for treatment, it may also prove useful in fermentation and food industry. It occurs in all three superkingdoms – archaea, prokaryotes and eukaryotes. Its omnipresence yet varied purposes in all the organisms makes this enzyme quite unique, like glycerol production and several other metabolites. In this report, RNA of *S. cerevisiae* has been isolated and corresponding cDNA formed in order to clone GPP1 (which is similar to G3PP) gene into pET28a vector of *E. coli*. This can further be used to study about this enzyme and promote its commercialisation.

CHAPTER 1

INTRODUCTION

The HAD superfamily is a broad domain of enzymes that are omnipresent in all entities of life [1]. They are majorly involved in dephosphorylation of carbohydrates, lipids, metabolites, DNA and serine-, threonine- or tyrosine phosphorylated proteins in humans [2]. They were considered to be a part of housekeeping genes, until now. It has been seen that impairment of some HAD phosphatases cause congenital diseases, such as cancer, cardiovascular, metabolic and neurological disorders [1]. Eya phosphatases in breast cancer [3], G3PP in T2D and cardiometabolic disorders [4] are some of the examples.

PGP is one of the time-worn enzymes whose functioning has evolved along with its residing species in accordance to the organism demands. In bacteria, PGP breaks down phosphoglycolate generated while restoring DNA damage. On the other hand, in higher eukaryotic organisms, PGP function has also evolved to glycerol production from glucose [4], during fermentation in yeast or while acclimatising to environmental stresses like hypertonia, asphyxia and oxidative stress [6].

G3PP belongs to HAD family that hydrolyses glycerol-3-phosphate into glycerol and other byproducts like 2-phosphoglycolate, 4-phosphoerythronate and 2-phospholactate [5]. It is said to be involved in glycolysis, gluconeogenesis, lipid synthesis, lipolysis, fatty acid oxidation, cellular redox, and mitochondrial energy metabolism in β-cells and hepatocytes, as well as glucose-induced insulin secretion and the response to metabolic stress in β-cells, and in gluconeogenesis in hepatocytes [6]. Hence it can be targeted for metabolic disorders, T2D, cardiovascular diseases and cancer.

G3PP overproduction in rats showed decreased triglyceride levels in liver and enhanced HDL/LDL ratio in the laboratory, indicating its physiological importance and future prospects as a curative target for metabolic syndrome, obesity and T2D [5]. It may also

prove beneficial in the fermentation industry by increasing the natural sweetness in the fermented products and thus enhancing the taste.

In this report, G3PP gene (namely RHR2) was isolated from *Saccharomyces cerevisiae* and its cDNA amplified.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1. HAD SUPERFAMILY

HAD are a class of hydrolases portrayed in the proteomes of organisms belonging to all domains of life that play their roles in phosphorylation, dephosphorylation, dihydroxylation etc. HAD phosphatases make up approximately one-fifth of all human phosphatase catalytic subunits [2]. The enzymes in this superfamily are majorly involved in phosphate transfer.

2.1.1 CLASSIFICATION OF HAD SUPERFAMILY

HADSF members belong to one of these five classes [2]:

- Haloalkanoate dehalogenases (C−C bond hydrolysis)
- Phosphonoacetaldehyde hydrolases (P−C bond hydrolysis)
- Phosphonate monoesterases (P−O−C bond hydrolysis)
- ATPases (P−O−P bond hydrolysis)
- Phosphomutases (P−O−C bond cleavage, with intramolecular phosphoryl transfer).

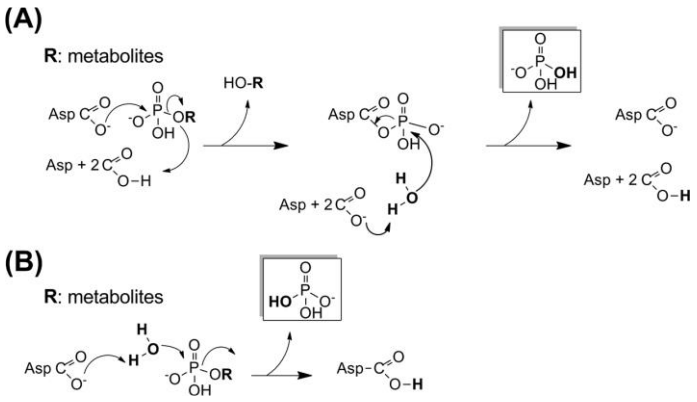
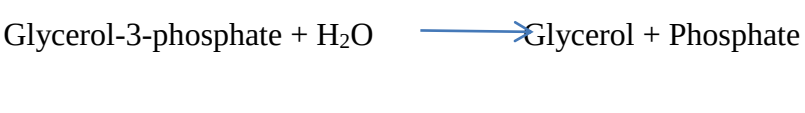


Fig.2.1. Proposed Mechanisms for Hydrolysis of Glycerol 3-Phosphate Catalyzed by MtGPP (Source: GL Maumus et al.)

2.2. G3PP

G3PP hydrolyses G3P into glycerol and phosphate, thereby regulates cellular levels of G3P, an intermediate of glucose, lipid and energy metabolism [5].



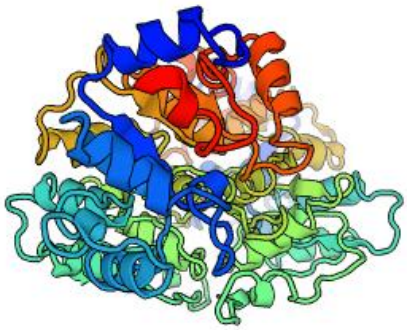


Fig 2.2: Structure of G3PP (Source: Expasy Swissmodel)

2.3. EVOLUTION OF G3PP

G3PP is an omnipresent enzyme in all life forms and has evolved in each of them. It continues to perform several tasks simultaneously in each of them.

2.3.1. BACTERIA

Rv1692 has been identified as a G3PP enzyme of HAD family in *Mycobacterium tuberculosis* which is involved in recycling of glycerophospholipid polar heads [7].

2.3.2. YEAST AND FUNGI

In yeast, two G3PP isoforms, namely GPP1/RHR2 and GPP2/HOR2 have been identified that are 95% identical whose expression was triggered under increased osmotic pressure [8]. Glycerol formation and environmental stress resistance (oxidative, osmotic, and anaerobic) are their major roles [9].

2.3.3. PLANTS

AtGpp1 & AtGpp2, isoforms of *A. thaliana* G3PP have been identified with 95% identity, similar to that of yeast. But their expression remained unaffected by osmotic, ionic, or oxidative stress. In spite of this, genetically modified plants showed increased AtGpp2 activity, and increased resistance to environmental stresses [10].

2.3.4. VERTEBRATES

Two possible G3PP enzymes that are similar to G3PP of the algae *Dunaliella salina* [11] and of the *Mycobacterium tuberculosis*, were proposed to have a glycerol-3-phosphatase activity in *Osmerus mordax* [12].

PGP was recognised originally in RBCs and was assumed to modulate 2, 3-bisphosphoglycerate levels which determines hemoglobin affinity to oxygen [13]. Although this supposition could not be proven *in vitro*, recent studies direct its involvement in other processes.

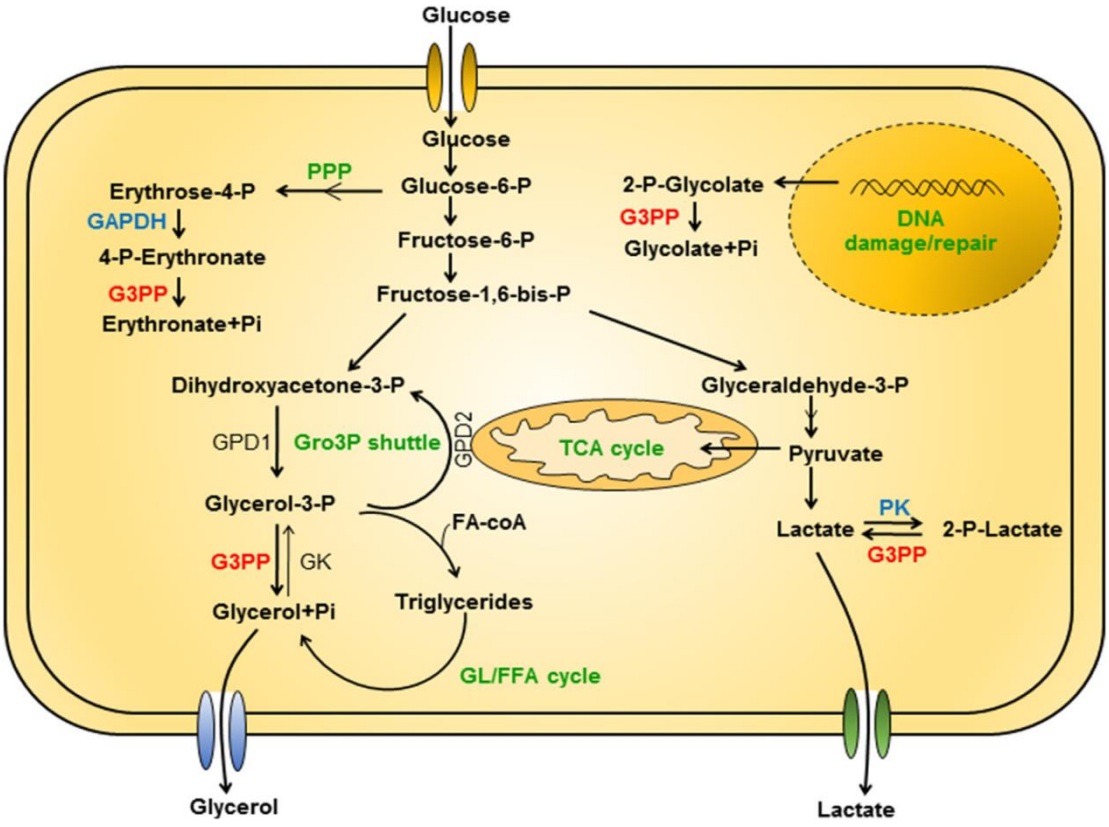


Fig. 2.3: Biochemical and metabolic functions of G3PP/PGP. (Source: E Possik et al.)

2.4. GPP IN YEAST

Glycerol is required in yeast *Saccharomyces cerevisiae* for osmoregulation and anaerobic redox regulation. It synthesizes glycerol in order to make up water loss in external hypersaline conditions through HOG1 MAPK pathway [9]. In order to maintain redox equilibrium, glucose fermentation to ethanol during anoxic state is channelized to glycerol production which is a more reduced output [9].

Dihydroxyacetone phosphate from glycolysis is converted to glycerol-3-phosphate by two different forms of GPD, GPD1 and GPD2. GPD1 is induced during osmotic stress whereas GPD2 in anoxic conditions. These enzymes mainly regulate glycerol production to overcome these environmental stresses [9].

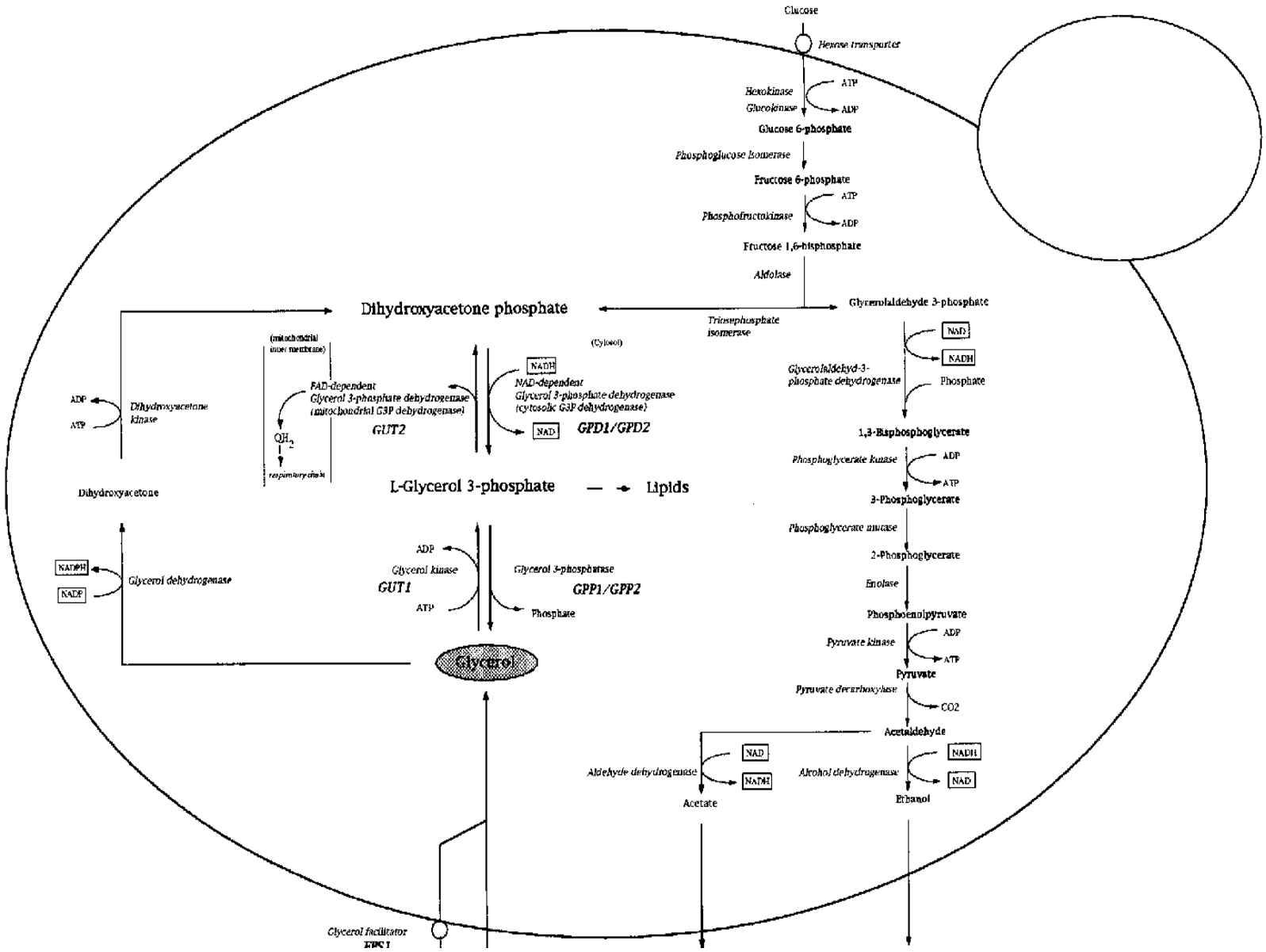


Fig. 2.4: Important pathways of glycerol metabolism in *S. cerevisiae*. (Source: E Nevoigt et al.)

2.5. ABOUT GPP1 AND GPP2

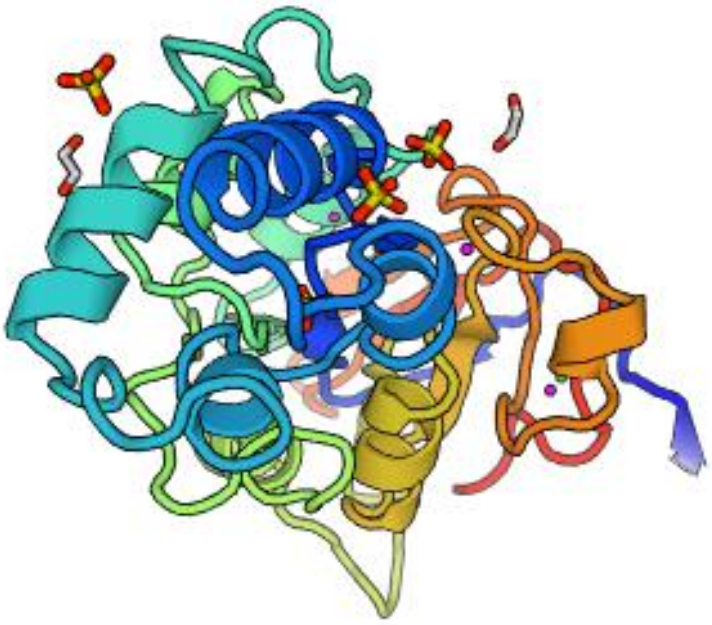


Fig. 2.5: Crystal structure of GPP1 from *S. cerevisiae* (Source: Expasy Swissmodel)

Location: Chromosome IX – 255115 to 255867

GPP1 protein sequence (250 amino acids)

MPLTTKPLSLKINAALFDVDGTIIISQPAIAAFWRDFGKDKPYFDAEHVIHISHG
WRTYDAIAKFAPDFADEEYVNKLEGEIPEKYGEHSIEVPGAVKLCNALNALPKE
KWAVATSGTRDMAKKWFDILKIKRPEYFITANDVKQGKPHPEPYLKGRNGLGF
PINEQDPSKSKVVVFEDAPAGIAAGKAAGCKIVGIATTFDLDFLKEKGCDIIVKN
HESIRVGEYNAETDEVELIFDDYLYAKDDLLKW

mRNA sequence

ATGCCTTTGACCACAAAACCTTTATCTTTGAAAATCAACGCCGCTCTATTCG
ATGTTGACGGTACCATCATCATCTCTCAACCAGCCATTGCTGCTTTCTGGAG
AGATTTCGGTAAAGACAAGCCTTACTTCGATGCCGAACACGTTATTCACATC
TCTCACGGTTGGAGAACTTACGATGCCATTGCCAAGTTCGCTCCAGACTTTG

CTGATGAAGAATACGTTAACAAGCTAGAAGGTGAAATCCCAGAAAAGTACG
GTGAACACTCCATCGAAGTTCCAGGTGCTGTCAAGTTGTGTAATGCTTTGAA
CGCCTTGCCAAAGGAAAAATGGGCTGTCGCCACCTCTGGTACCCGTGACAT
GGCCAAGAAATGGTTCGACATTTTGAAGATCAAGAGACCAGAATACTTCAT
CACCGCCAATGATGTCAAGCAAGGTAAGCCTCACCCAGAACCATACTTAAA
GGGTAGAAACGGTTTGGGTTTCCCAATTAATGAACAAGACCCATCCAAATC
TAAGGTTGTTGTCTTTGAAGACGCACCAGCTGGTATTGCTGCTGGTAAGGCT
GCTGGCTGTAAAATCGTTGGTATTGCTACCACTTTCGATTTGGACTTCTTGA
AGGAAAAGGGTTGTGACATCATTGTCAAGAACCACGAATCTATCAGAGTCG
GTGAATACAACGCTGAAACCGATGAAGTCGAATTGATCTTTGATGACTACTT
ATACGCTAAGGATGACTTGTTGAAATGGTAA

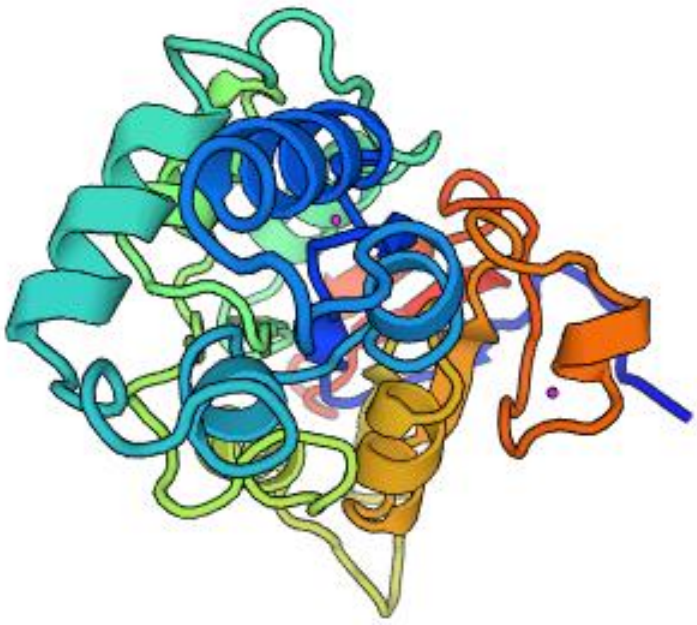


Fig. 2.6: Structure of GPP2 in *S.cerevisiae* (Source: ExpaSy Swissmodel)

Location: Chromosome V – 279930 to 280682

Protein sequence (250 amino acids)

MGLTTKPLSLKVNAALFDVDGTIIISQPAIAAFWRDFGKDKPYFDAEHVIQVSH
GWRTFDAIAKFAPDFANEEYVNKLEAEIPVKYGEKSIEVPGAVKLCNALNALPK

EKWAVATSGTRDMAQKWFEHLGIRRPKYFITANDVKQGKPHPEPYLKGRNGL
GYPINEQDPSKSKVVVFEDAPAGIAAGKAAGCKIIGIATTFDLDFLKEKGCDIIV
KNHESIRVGGYNAETDEVEFIFDDYLYAKDDLLKW

mRNA sequence

ATGGGATTGACTACTAAACCTCTATCTTTGAAAGTTAACGCCGCTTTGTTCG
ACGTCGACGGTACCATTATCATCTCTCAACCAGCCATTGCTGCATTCTGGAG
GGATTTCGGTAAGGACAAACCTTATTTCGATGCTGAACACGTTATCCAAGTC
TCGCATGGTTGGAGAACGTTTGATGCCATTGCTAAGTTCGCTCCAGACTTTG
CCAATGAAGAGTATGTTAACAAATTAGAAGCTGAAATTCCGGTCAAGTACG
GTGAAAAATCCATTGAAGTCCCAGGTGCAGTTAAGCTGTGCAACGCTTTGA
ACGCTCTACCAAAAGAGAAATGGGCTGTGGCAACTTCCGGTACCCGTGATA
TGGCACAAAAATGGTTCGAGCATCTGGGAATCAGGAGACCAAAGTACTTCA
TTACCGCTAATGATGTCAAACAGGGTAAGCCTCATCCAGAACCATATCTGA
AGGGCAGGAATGGCTTAGGATATCCGATCAATGAGCAAGACCCTTCCAAAT
CTAAGGTAGTAGTATTTGAAGACGCTCCAGCAGGTATTGCCGCCGGAAAAG
CCGCCGGTTGTAAGATCATTGGTATTGCCACTACTTTCGACTTGGACTTCCT
AAAGGAAAAAGGCTGTGACATCATTGTCAAAAACCACGAATCCATCAGAGT
TGGCGGCTACAATGCCGAAACAGACGAAGTTGAATTCATTTTTGACGACTA
CTTATATGCTAAGGACGATCTGTTGAAATGGTAA

2.6. POTENTIAL PHYSIOLOGICAL ROLES

2.6.1. REMOVAL OF SURPLUS GLUCOSE AND FFA

It was seen that G3PP levels in β-cells regulates the amount of glucose in the blood and therefore can be targeted for T2D and cardiovascular disorders [14]. In the similar way, G3PP also regulates glucolipotoxicity, hence proves beneficial in overcoming metabolic stress. Also, insulin production due to increased glucose levels can also be maintained by overexpressing G3PP, which makes it possible to avoid hyperinsulinemia [4].

G3PP overexpression was also related to reduced weight gain in transgenic rats and refine HDL/LDL levels in blood [4]. This also indicates its roles in cardiovascular and metabolic disorders.

2.6.2. COMBATING HARMFUL METABOLITES

Modern studies have revealed that G3PP enzyme converts certain unsafe compounds into non-toxic. These harmful metabolites are generally produced during increased levels of alternative substrates of some enzymes. For example, GAPDH produces 4-phosphoerythronate and pyruvate kinase produces 2-phospholactate [15]. Both of these metabolites hamper pentose phosphate pathway. PGP breaks down both these substances to harmless erythronate and lactate [15].

2.6.3. ROLE IN DEVELOPMENT

Embryonic termination of rat embryos was observed in PGP-inactivated rats, probably due to reduced ability to fix DNA injury [16]. Hence it can be said to be involved in some developmental roles.

2.7. POTENTIAL ROLE IN DISEASES

G3PP overexpression is being linked to enhanced glycerol levels, FFA and reduced TG [17]. G3PP leads G3P conversion to glycerol and fatty acyl-coenzyme A to FFA. G3PP has also been related to reduced glucose synthesis and increased oxidation of fatty acids, decreased LDL levels [4]. Therefore G3PP could provide protection against cardiac aberrations by reducing glucose levels in the blood.

Cancer cells are capable of utilizing glucose and convert it into lactate during glycolysis even under anoxic conditions. This is also known as Warburg effect [18]. Therefore, enhanced G3PP activity can reduce glucose availability to cancer cells and hence retard their growth and disrupt metabolic processes.

2.8. G3PP ACTIVITY ASSAY

The activity of G3PP is measured by the amount of phosphates released when G3P gets dephosphorylated to glycerol. The reaction mixture consists of –

- 20 mM tricine buffer (pH 7.0)
- 1 M glycerol-3-phosphate
- 5 mM $MgCl_2$
- 200 mL of extract

The reaction mixture is allowed to react at 37˚C for 30 min, and the reaction is ended by adding 300 mL of 50% $HClO_4$. Vanadium molybdate reagent is added and the absorbance of mixture is recorded spectrophotometerically at 415 nm. G3PP activity is defined as the amount of enzyme that causes liberate per micromole inorganic phosphorus per minute at 37˚C and pH 7.0 [19]. G3PP activity is calculated according to formula,

$$U = \frac{(C_s - C_c)V_t}{V_e T}$$

Fig.2.7: Formula to calculate G3PP activity

Here,

C_s: Concentration of inorganic phosphorus produced in reaction mixture (nmol/mL)

C_c: Concentration of inorganic phosphorus produced in control (nmol/mL); Vt is total reaction mixture volume (mL)

V_e: Enzymes extract volume in sample (mL)

T: Reaction time (min)

Units of specific enzyme activity (U/mg) are expressed as micromoles per minute per milligram of protein.

2.9. PRIMER DESIGN

Primers are short oligonucleotide sequences synthetically synthesized that are complementary to a certain specific DNA region. They are generally used to amplify a

specific DNA/gene region or to identify the sequence of DNA/gene complementary to that of the primer. Primer designing requires finding an equilibrium between –

- Specificity - frequency with which a primer binds to non-specific regions
- Efficiency - how close a primer pair is able to amplify a product to the theoretical optimum of a twofold increase of product for each PCR cycle [20]

Factors affecting primer designing are-

a. Primer length

Oligonucleotides length between 18 and 24 bases are said to be highly sequence specific. Short oligonucleotides of 15 bases or less tend to bind non-specifically. Longer primers, on the other hand, are not preferred because of increased chances of secondary structure formation within the primer [20].

b. 3' end nucleotide in the PCR primer

The 3'-terminal position in the primer is important for controlling misguided priming. Generally G/C is preferred as the terminal nucleotide as it promotes more strength to primer binding [20].

Also, primer complementarity should be avoided, particularly at 3' end because it causes unwanted primer dimer formation which hampers desired amplification.

c. Rational GC content and T_m

PCR primers should maintain a reasonable GC content. G+C content higher than 40% and T_m greater than 50˚C with similar or nearby values with difference not greater that 5˚C is preferred [20].

2.10. *SACCHAROMYCES CEREVISIAE*

Fig. 2.8: *S. cerevisiae* streaked on YPD agar

S. cerevisiae, a species of the fungi kingdom, is a unicellular eukaryotic model organism which has found its diverse applications in varied fields like industrial, food, pharmaceuticals, agriculture and research.

Scientific classification

Kingdom	Fungi
Phylum	Ascomycota
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Saccharomyces*

2.11. APPLICATIONS OF *S. CEREVISIAE*

2.11.1. *S. CEREVISIAE* AS A MODEL ORGANISM

Similarities of yeast to both bacteria and higher organisms makes it an intermediate between these two kingdoms and link them to each other. Its complete genome being already sequenced helps a lot to define these linkages and understand phylogeny [21].

2.11.2. FOOD INDUSTRY

Its ability to produce ethanol by utilizing glucose has enabled its usage in making several alcoholic beverages, breads, cheeses and many traditional foods whose variety depend on the species of the yeast [22].

2.11.3. RESEARCH

Easy genome manipulation offered by yeast helps in identifying the enzymes responsible for a certain metabolic process and its effect on the organism overall. Introducing a foreign gene into the genome of yeast helps in gaining knowledge of eukaryotic proteins in a much less complex organism [23].

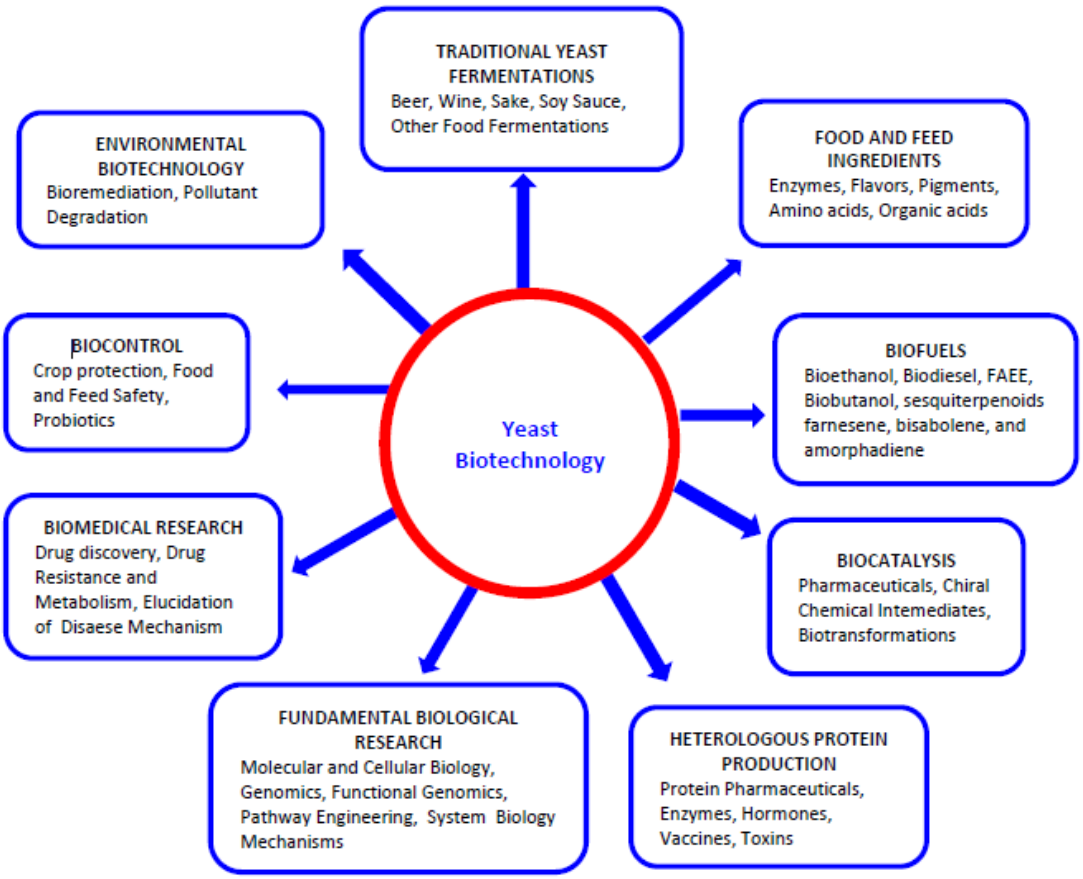


Fig. 2.9: Modern applications of yeasts [22]

2.12. CLONING AND EXPRESSION

Cloning and expressing a foreign gene into a suitable expression system is quite a common technique used nowadays to study a specific protein and the effect of surroundings in order to increase its overall yield. Cloning of a eukaryotic gene (specifically cDNA) basically involves the following steps:

1) Cells/tissue sample from which the target DNA has to be cloned is grown in sufficient amounts
2) RNA is extracted from the sample

3) cDNA is synthesized from mRNA of the target protein using RT-PCR
4) PCR product is then amplified and inserted into a suitable vector
5) The vector is then transformed into the expression system
6) The desired protein is allowed to synthesize and its expression checked through assays [24]

In this project, the target gene GPP1 is to be inserted into pET28a vector and then transformed into *E. coli*.

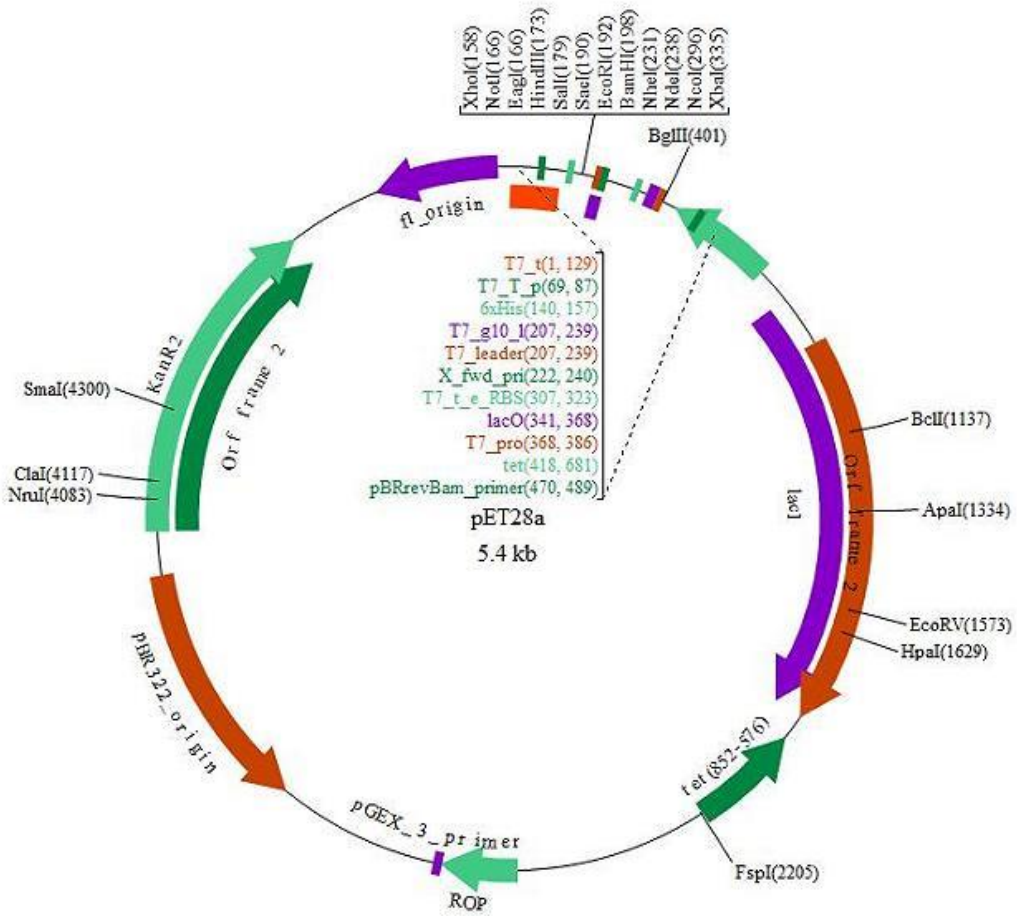


Fig. 2.10: pET28a (+) vector map

There are several many types of expression system whose usage depend upon the characteristics of the recombinant protein, like, bacterial expression system (*Escherichia coli*), yeast expression systems (*Saccharomyces*, *Pichia pastoris*) and of that of the higher organisms (Baculovirus, CHO), each with their own pros and cons.

Here, *E. coli* is being aimed for expressing GPP1 recombinant protein. The reason to choose *E. coli* in this case is that it is very easy to handle and transform colonies in good amounts. Researchers have been targeting *E. coli* even for expressing eukaryotic proteins like human insulin [25], firefly luciferase [26], murine interleukin-I [27], human serum albumin [28], murine tumor necrosis factor [29], human IFN-γ inducing factor [30] and many other such proteins belonging to the organisms at higher order. These experiments have proven to be quite successful in achieving their goals, and therefore this approach can help in cost-effective large-scale production of such valuable enzymes.

OBJECTIVES

- To extract RNA of *Saccharomyces cerevisiae* and corresponding cDNA formation
- To design primers for GPP1 gene
- To clone GPP1 gene into pET28a vector into *E.coli*

CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. MICROORGANISM

G3PP gene (GPP1) was isolated from *Saccharomyces cerevisiae* donor and expressed in *Escherichia coli* for vector transformation.

3.1.2. MEDIUM COMPOSITION

YPD agar and a secondary media were used for the isolation of pure colonies of *S. cerevisiae*.

3.1.3. INSTRUMENTS USED

- Laminar air flow cabinet
- Vertical autoclave
- Centrifuge
- Spectrophotometer
- Vortex
- -80˚C freezer
- Electrophoresis apparatus
- pH meter
- Incubator
- Weighing balance

3.2. METHODOLOGY

The steps mentioned below are to be followed:

1. Isolation of pure culture of microorganism

⬇

2. Total RNA isolation from yeast

⬇

3. mRNA conversion to cDNA

⬇

4. Amplification of GPP1 cDNA using gene-specific primers

⬇

5. Cloning of GPP1 into pET28a vector

⬇

6. Transformation of *Escherichia coli*

⬇

7. Expression analysis of GPP1 using G3PP assay

3.2.1. Isolation of pure culture of microorganism

a. Commercially available *Saccharomyces cerevisiae* was streaked on YPD agar plate supplemented with 0.7% NaCl [9] and incubated at 30˚C for 48 hours.
b. Pure colony was then verified by Gram's staining and through microscopy.
c. The pure culture was then transferred to the secondary media whose composition has been mentioned earlier.
d. The cells were incubated at 30˚C for 48 hours.
e. Glycerol stocks of pure *S. cerevisiae* were also prepared in 30% glycerol.

3.2.2. Total RNA isolation from yeast

Total RNA was extracted from yeast as mentioned by Schmitt and Brown [31].

3.2.3. Primer designing for GPP1

a. Two restriction sites from MCS of pET28a vector were chosen in such a way that they were not common in GPP1 gene. NdeI and BamHI were chosen for forward and reverse primer respectively.

b. Primers were designed keeping in mind the properties that they must have for high specificity and affinity.

The primers thus defined are as follows-

Forward primer: 5'- GGCG CAT ATG CCT TTG ACC ACA AAA CC -3'

Reverse primer: 5'- CCGC GGA TCC TTA CCA TTT CAA CAA GTC ATC CTT AGC -3'

Table 3.1: Properties of primer designed

Primers	*Length (gene specific)*	*Tm (°C)*	*GC content (%)*
Forward primer	20	54	45
Reverse primer	27	55.5	37

3.2.4. cDNA from total RNA

a. cDNA synthesis PCR reaction was prepared in a vial in the given amounts.

Table 3.2: cDNA synthesis reaction mixture composition

cDNA synthesis buffer	4 µl
DNTPs	2 µl
Oligo Dt	1 µl
Reverse transcriptase enhancer	1 µl
Reverse transcriptase enzyme	1 µl
RNA template	2 µl
Nuclease free water	9 µl
Total	20 µl

b. For first strand synthesis, PCR vial was placed in the thermocycler with the mentioned settings.

Table 3.3: Reaction conditions for first cDNA strand synthesis

Stages	*Temperature (˚C)*	*Time (min)*
Stage 1	42	30
Stage 2	95	2
Stage 3	4	∞

c. For second strand synthesis, following reaction mixture was prepared.

Table 3.4: PCR reaction for second cDNA strand synthesis

PCR buffer	1.25 µl
dNTPs	0.25 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Template (first cDNA strand)	1 µl
Taq polymerase	0.125 µl
Water	8.8 µl

d. Following reaction condition was set.

Table 3.5: Reaction conditions for second cDNA strand synthesis

Stages	*Temperature (˚C)*	*Time*
Initial denaturation	94	4 min
Denaturation	94	30 sec
Annealing	55	30 sec
Extension	72	50 sec
Final extension	72	7 min
	4	∞

3.2.5. Plasmid (pET28a vector) isolation from *E. coli* DH5α strain by alkaline lysis method

a. 2 ml fresh *E. coli* culture was transferred to a microcentrifuge tube.
b. Sample was centrifuged at 8,000 rpm for 7 minutes.

c. Supernatant was discarded.
d. 100 µl of ALS I solution (50 mM glucose, 25 mM tris Cl pH 8, 10 mM EDTA pH 8) was added to the pellet and vortexed.
e. Sample was incubated at RT for 5 minutes.
f. 200 µl of ALS II solution (0.2N NaOH, 1% SDS) was added. The solution was mixed by inverting the tube.
g. The sample was again incubated at RT for 5 minutes.
h. 500 µl of ALS III solution (3M potassium acetate pH 5.5) was added and mixed by inversion of the tube.
i. The sample was kept in ice for 5 minutes.
j. Equal volumes of phenol: chloroform (24:1) was added to the contents of tube.
k. Sample was centrifuged at 10,000 rpm, 4˚C for 5 minutes.
l. Supernatant was collected in a fresh microcentrifuge tube.
m. Equal volumes of isopropanol were added to the supernatant and mix properly.
n. Solution was incubated for 30 minutes and then centrifuged at 4,000 rpm, 4˚C for 30 minutes.
o. The pellet after removing the supernatant was twice washed with ethanol.
p. Pellet was then air-dried to remove ethanol completely.
q. Dried pellet was resuspended in 40 µl TE buffer and stored at -20˚C.

CHAPTER 4

RESULTS AND DISCUSSION

1. Pure culture isolates of *S. cerevisiae* were obtained on YPD agar plate and their 30% glycerol stocks were prepared and stored at -80˚C.

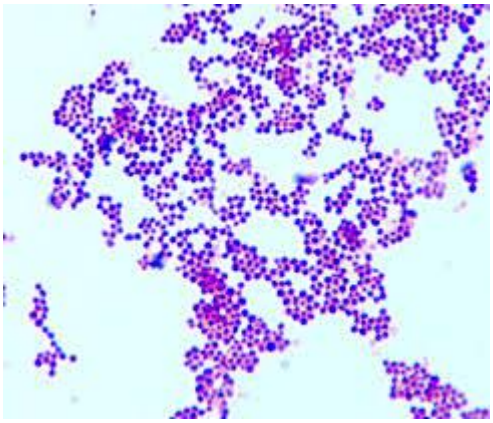


Fig. 4.1. *S. cerevisiae* in Gram's stain at 100x

2. Total RNA isolation was successfully performed.

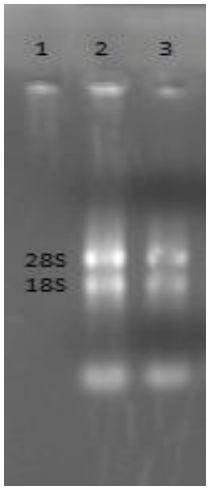


Fig. 4.2: Yeast total RNA bands on 1.5% agarose stained with EtBr

Table 4.1: Total RNA gel description

Lane 1	Empty
Lane 2	Clear bands of 28S and 18S rRNA
Lane 3	Clear bands of 28S and 18S rRNA

3. cDNA of GPP1 gene was successfully prepared.

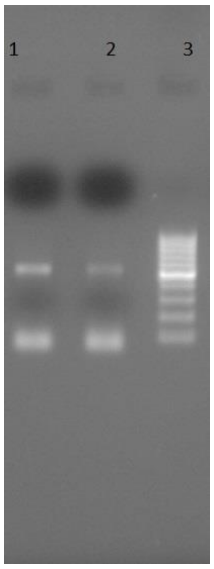


Fig. 4.3: GPP1 cDNA bands observed on 1% agarose gel stained with EtBr.

Table 4.2: cDNA gel description

Lane 1	cDNA band (~750 bp) observed
Lane 2	cDNA band (~750 bp) observed
Lane 3	DNA ladder of size 1 kb

4. pET28a vector was also isolated from *E. coli* DH5α strain.

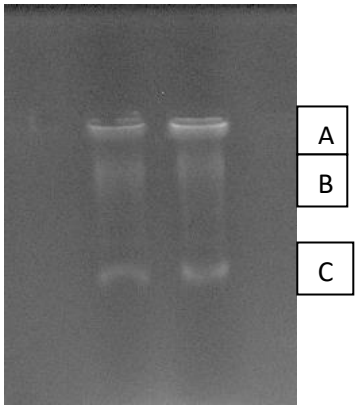


Fig. 4.4: Plasmid isolated and visualised on 1% agarose gel stained with EtBr.

Table 4.3: Plasmid gel description

A	DNA
B	Partially digested plasmid
C	pET28a vector

CHAPTER 5

CONCLUSION

Initially, pure cultures of *S. cerevisiae* were isolated and screened through Gram's staining. These cultures were grown in YPD agar at first, then later subculture to secondary media whose composition stimulates production of GPP1 enzyme in hyperosmotic conditions. RNA of yeast growing under such conditions was isolated and stabilized by converting it into cDNA. Furthermore, pET28a vector was also isolated from its host – *E. coli* DH5α strain successfully.

Although cloning and expression of the enzyme could not be established, it does not influence its potential therapeutic and industrial uses in the near future.

FUTURE WORKS

The enzyme GPP has become one of the newer enzymes to enter the research area because of its newly discovered capabilities. Not only will it help in solving the health issues of obesity, T2D and cancer around the globe, but its presence in yeast itself can be utilized by us in the food and beverage industry. Also further research for the exact mechanism of action would also help in its commercialisation.

Also its close linkage to that of G3PP in humans can also be targeted in order to boost its large scale production by making the process cost-effective.

CHAPTER 6

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APPENDIX

A.1. YPD agar medium

Components	*Quantity (g L^{-1})*
Yeast extract	1%
Peptone	2%
Dextrose	2%
Agar	2%

A.2. Secondary growth media

Components	*Quantity (g L^{-1})*
Sucrose	20
Yeast extract	10
Ammonium sulphate	1
Magnesium sulphate	0.75
Potassium hydrogen phosphate	3.5

A.3. TE buffer pH 8

Components	*Quantity (g L^{-1})*
Tris base	1.21
EDTA	0.37

A.4. ALS I pH 8

Components	*Quantity (g L-1)*
Tris base	6.06
EDTA	3.72

A.5. ALS II

Components	*Quantity (L-1)*
NaOH	8 g
SDS	50 ml

A.6. ALS III pH 5.5

Components	*Quantity (L-1)*
Potassium acetate	294.5 g
Glacial acetic acid	~110 ml