

Microbial cell factories for the production of recombinant human Interferon gamma

A THESIS

Submitted by

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For the award of the degree

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Department of Biosciences and Bioengineering

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***Dedicated to my beloved wife
and
my family***



**INDIAN INSTITUTE OF TECHNOLOGY
GUWAHATI**

**DEPARTMENT OF BIOSCIENCES AND
BIOENGINEERING**

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India under the supervision of Prof. Venkata Dasu Veeranki.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date:

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CERTIFICATE

It is certified that the work described in this thesis entitled “**Microbial cell factories for the production of recombinant human Interferon gamma**” carried out by Mr. Rajat Pandey for the award of the degree of Doctor of Philosophy is an authentic record of the results obtained from the research work performed under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India and this work has not been submitted elsewhere for a degree.

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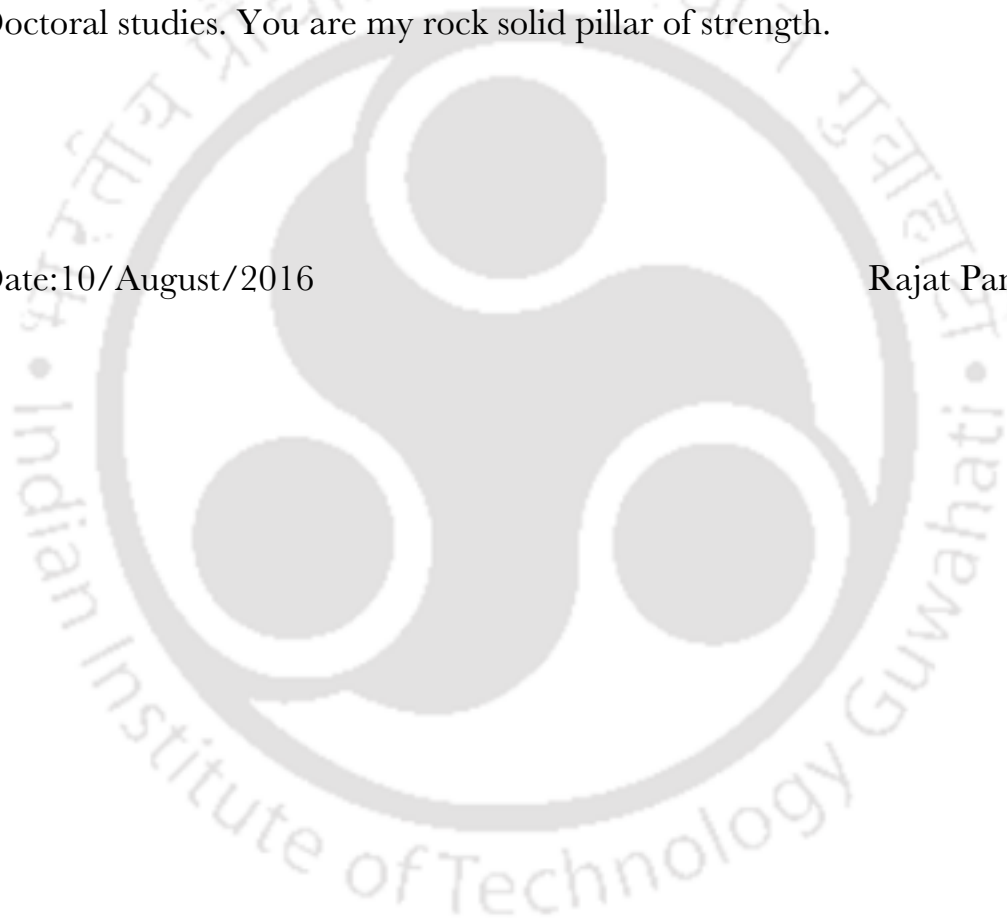
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ABSTRACT

IFN- γ is a dimerized, pleotropic cytokine and plays an important role in stimulating immune reactions. It is also critical for innate and adaptive immunity against viral and intracellular bacterial infections. The importance of IFN- γ in the human immune system is due to its ability to block viral replication directly and most importantly from its immunostimulatory and immunomodulatory effects. Additionally IFN- γ has shown promising indication in the treatment of tuberculosis/MDR-TB as well as of the other fatal diseases such as chronic myeloid leukemia, AIDS & oral sub-mucous fibrosis. IFN- γ has also been studied for the treatment of protozoan and fungal infections. Presently the interferon gamma is approved for the treatment of chronic granulomatous disease & severe malignant osteopetrosis. With its already approved FDA status for the treatment of two diseases and excellent indications and positive results in the treatment of Tuberculosis and MDR-TB and other diseases, IFN- γ has the potential of becoming a lifesaver drug. IFN- γ as a drug is available in majority of developed countries throughout the world; however it is not available in India. A cost effective method for the production of Recombinant Human IFN- γ can reduce the cost of treatment of chronic granulomatous disease and malignant osteopetrosis significantly which currently stands as \$50000/year.

In this study Recombinant Human IFN- γ was expressed in two separate expression systems a) *E. coli* strain GalG20 and b) *Kluyveromyces lactis* GG799.

E. coli GalG20 strain is newly developed strain (originally MG1655 strain) developed by metabolic engineering tools where *endA*, *recA* and *pgi* genes of metabolic pathway have been knocked out to redirect the carbon flow into the pentose phosphate pathway and to promote nucleotide biosynthesis. Originally this strain was developed for enhanced plasmid biosynthesis for the purpose of plasmid DNA vaccine. We hypothesized that it could be a hyper protein producer strain by the virtue of its enhanced plasmid producing capabilities. For the purpose of expressing IFN- γ in GalG20 strain, this plasmid producing strain was engineered into a protein producing strain by λ DE3 lysogenization technique. GalG20DE3 produced 230 ± 20 mg/l of IFN- γ i.e. 1.5 fold more IFN- γ as compared to BL21DE3, a most commonly strain used for protein production, and 3.3 fold more as compared to MG1655DE3. In GalG20DE3 only 5%, in BL21DE3 only 3% and in MG1655DE3 28% of total IFN- γ protein was present in soluble form. The only bottleneck observed was IFN- γ aggregation as inclusion bodies while expressing in GalG20DE3.

Kluyveromyces lactis, a “budding yeast” from phylum Ascomycota was developed as an alternative host to *Escherichia coli* GalG20DE3 for the production of IFN- γ . It is a generally regarded as safe (GRAS) organism. This research is the first to best of our knowledge of human IFN- γ expression and production in *K. lactis*. Five physical conditions (pH, rpm, incubation temperature and time, age of inoculum) for IFN- γ expression were optimized. After the optimization of physical parameters for IFN- γ expression a total increase of 2.2 fold in the expression level was observed. This study revealed that growth

medium pH 7, incubation temperature 30°C, agitation rate of 250 rpm and growth duration of 60 hours favors the highest IFN- γ production level. After 60 hours of growth IFN- γ concentration starts to reduce due to depletion of carbon source. Production medium was optimized by assessing the tolerance level of carbon source by *K. lactis* cells and substrate inhibition was observed over and above 80 gms/litre of lactose. Ethanol and acetate production was detected by high performance liquid chromatography (HPLC). Growth pattern of *K. lactis* showed a diauxic growth pattern, after first lag phase we observed a transient pause in the growth of organism which can be attributed to metabolic readjustments inside the cells, where organism prepares itself to consume ethanol for its growth and second lag phase is supported by ethanol as a carbon source. Response surface methodology was used for the optimization of complex growth medium. Artificial neural network supported by genetic and simulated annealing algorithms was also used for growth medium optimization for maximising biomass and IFN- γ . IFN- γ purification was performed using reverse micellar extraction. IFN- γ production in bioreactor yielded a total biomass of 25 ± 3 g/l and a 6 fold increase in IFN- γ protein (1100 ± 80 μ g/l) under optimized conditions.

Finally we can conclude that for the first time recombinant human IFN- γ protein was successfully expressed in *K. lactis* and also in a novel *E. coli* strain GalG20DE3.

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ABBREVIATIONS AND NOTATIONS

AIDS	Acquired Immune Deficiency Syndrome
<i>amdS</i>	Acetamidase
ANOVA	Analysis of variance
APCs	Antigen presenting cells
BEE	Back Extraction Efficiency
BL21DE3	<i>E. coli</i> protein expression strain
BSA	Bovine Serum Albumin
CCD	Central composite design
CGD	Chronic Granulomatous Disease
CTAB	Cetyl trimethylammonium bromide
DF	Degree of freedom
DH5 α	<i>E. coli</i> cloning strain
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immune Sorbent Assay
GalG20	<i>E. coli</i> cloning strain
GRAS	Generally Regarded As Safe
GuHCL	Guanidine Hydrochloride
HPLC	High Performance Liquid Chromatography
HPRD	Human Protein Reference Database

ABBREVIATIONS AND NOTATIONS

IFN- γ	Recombinant Human Interferon Gamma
IPA	Iso-propyl alcohol
IPTG	Isopropyl β -D-1-thiogalactopyranoside
<i>K. lactis</i>	<i>Kluyveromyces lactis</i>
KCl	Potassium Chloride
LAL	Limulus Amebocyte Lysate
LB	Luria Bertani
LioAc	Lithium Acetate
MDR-TB	Multiple Drug Resistance Tuberculosis
MHC	Major histocompatibility complex
MQ	Milli-Q water
MTCC	Microbial Type Culture Collection and Gene Bank
NaCl	Sodium chloride
NEB	New England Biolabs
NK cells	Natural killer cells
OD	Optical Density
OVAT	One Variable at a Time
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pET28a	<i>E. coli</i> protein expression vector
pI	Isoelectric Point
pKLAC2	Integrative expression vector for the yeast <i>K. lactis</i>

ABBREVIATIONS AND NOTATIONS

PMSF	Phenyl Methyl Sulfonyl Fluoride
pUC19	<i>E. coli</i> plasmid cloning vector
RNA	Ribose nucleic acid
RPM	Revolutions per minute
S/N	Signal to Noise ratio
SDS	Sodium Dodecyl Sulfate
Tc cells	Cytotoxic T cells
TCA	Trichloroacetic acid
TE BUFFER	<i>Tris-EDTA buffer</i>
Th cells	T helper cells
USFDA	United States Food and Drug Administration
YPGAL	Yeast Peptone Galactose
YPGlu	Yeast Peptone Glucose
YPLAC	Yeast Peptone Lactose
λ DE3	lambda DE3 expressing T7 RNA Polymerase

LIST OF UNITS

°C	Degree Centigrade
bp	Base Pair
g/l	Gram/litre
kb	Kilo Base pair
kDa	Kilo daltons
M	Molar
mg	Milli gram
ml	Milli litre
mM	Milli molar
ng	Nano gram
nm	Nanometer
µg	Micro Gram
µg	Microgram
µg/l	Micro gram/litre
µl	Micro litre



CHAPTER 1

INTRODUCTION

1.1 Cytokines

The term "cytokine" is derived from a combination of two Greek words "cyto" means cell and "kinos" meaning movement. Cytokines are cell signalling molecules that aid cell to cell communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection and trauma. Cytokines exist in form of protein and glycoprotein (proteins with a sugar attached). The cytokines are a large family of molecules that are classified in various different ways due to an absence of a unified classification system. Examples of cytokines include Interferons, Interleukins, Lymphokines, Tumour necrosis factor (TNF).

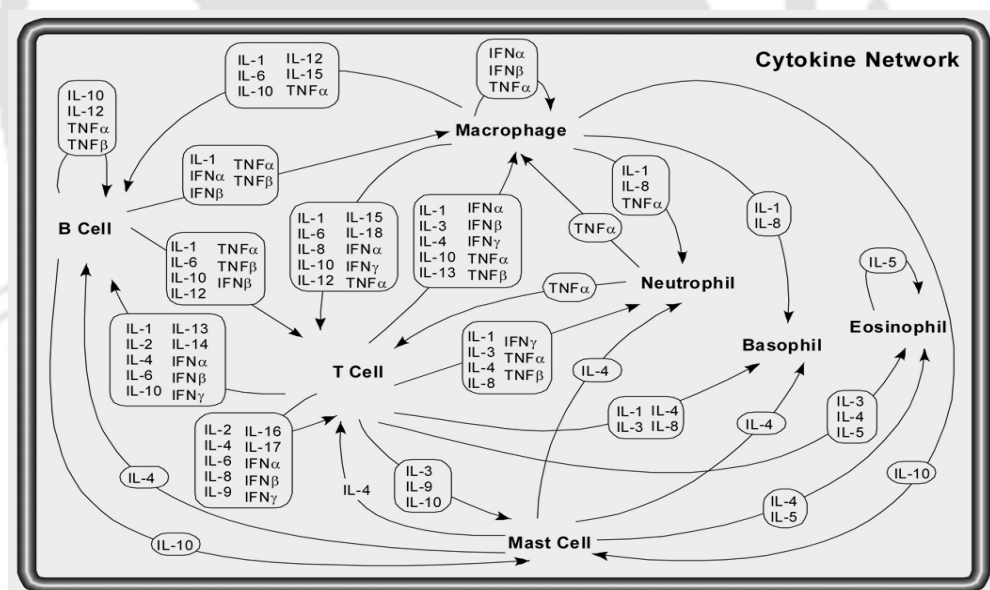


Figure 1.1: Schematic of Cytokines and their interactions (Zhang et al., 2007)

Cytokines are involved in regulating the immune system's response to inflammation and infection (Meager, 2013; Janeway CA Jr., 2001). Schematic

of Cytokines and their interactions, adopted from Zhang et al., 2007 is presented in figure 1.1.

1.2 Interferons

Interferons are described as means by virtue of which cells protect themselves from viral infection (Isaacs A & Lindenmann J, 1957) by affecting the overall life cycle of virus (Samuel CE, 1991). Interferons are also known to block viral translation through activation of 2, 5-oligoadenylate synthetase pathway and the eukaryotic protein synthesis pathway which ultimately leads to degradation of viral messenger RNA (Silverman, 2007).

Initially interferons were classified according to the secreting cell type but with ever evolving research, the Interferons have now been classified into type I & II according to the receptor, they bind and their sequence homology. The type I interferon group are further classified into four types: Interferon alpha (IFN- α), Interferon beta (IFN- β), Interferon omega (IFN- ω), and Interferon tau (IFN- τ). All the above mentioned types of Interferon are structurally related to each other and bind to a common heterodimeric receptor and are secreted by almost all cell types (Bach *et al.*, 1997). IFN- α and IFN- ω are secreted mainly by hematopoietic cells, whereas fibroblasts are a major cellular source of IFN- β (Bach *et al.*, 1997). IFN- α and IFN- β expression is stimulated by viral infection (Bach *et al.*, 1997; Jonasch & Haluska 2001). IFN- τ has only been reported in ruminants (Ott *et al.*, 1997).

In contrast, Type II interferon group is composed of only one member i.e. Interferon gamma (IFN- γ).

1.3 Interferon gamma (IFN- γ)

IFN- γ is a dimerized soluble pleotropic cytokine. Expression of IFN- γ is induced by immune and inflammatory stimuli. IFN- γ is produced when T lymphocytes are activated (Valente et al., 1992). IFN- γ plays an important role in stimulating immune reactions in spite of direct antiviral actions. It is also known for inducing cellular resistance to cytomegalovirus, herpes simplex virus, and adenovirus in vitro (Bolinger & Taeubel, 1992).

IFN- γ is required for macrophage activation (Held et al., 1999) but the exact mechanism of macrophage activation is unknown. However it seems to result from enhancement of oxidative metabolism in tissue macrophages (Nathan et al., 1983) as well as enhancement of antibody-dependent cellular cytotoxicity (Hibi et al., 1993) and natural killer cell activity (Weigent et al., 1983; Ueno et al., 1985). IFN- γ also enhances antigen processing and signaling through APCs by increasing the expression of MHC class II on the membranes of macrophages. Cellular responses to IFN- γ are activated when it interacts with its receptor (Pestka, Sidney et al. 1997), namely IFN- γ receptor 1 & 2 (Figure 1.2). JAK-STAT pathway is activated once IFN- γ binds to its receptors (Bach et al., 1997). In addition to receptor binding, IFN- γ binds to the glycosaminoglycan heparan sulfate (HS) at the cell surface (Jacob & Grimaud, 1991). Majority of heparan sulfate binding proteins gets a boost in their biological activity when they bind to heparan sulfate but the binding of IFN- γ to heparan sulfate inhibits its biological activity (HA, Young, 1996).

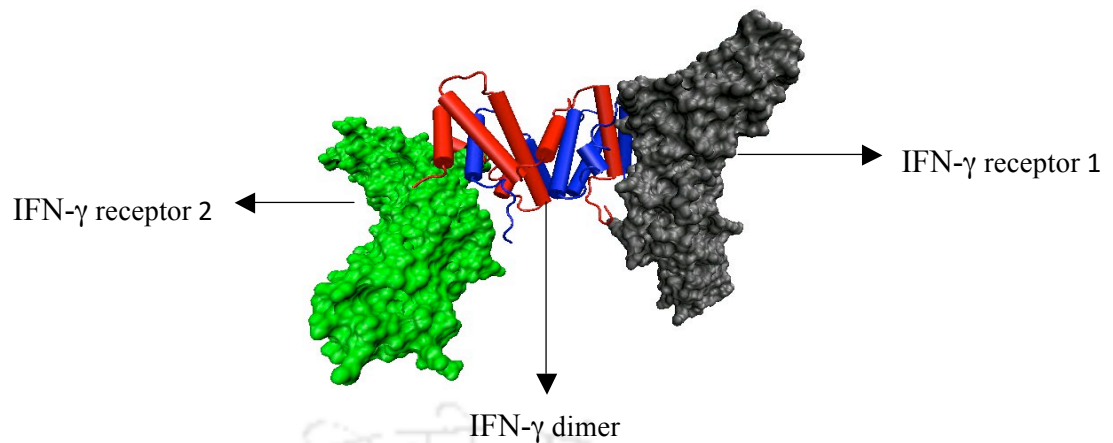


Figure 1.2: IFN- γ dimer interacting with two IFN- γ receptor molecules (DJ Thiela et al., 2000).

In contrast to interferon- α and interferon- β , which can be expressed by all cells, IFN- γ is secreted by Th1 cells, Tc cells, dendritic cells and NK cells. It is serologically distinct from Type I interferons and it is acid-labile, while the type I variants are acid-stable.

1.4 Structure of human Interferon gamma protein

The IFN- γ monomer consists of a core of six α -helices and an extended unfolded sequence in the C-terminal region. This is shown in the structural models below in figure 1.3. The α -helices in the core of the structure are numbered 1 to 6.

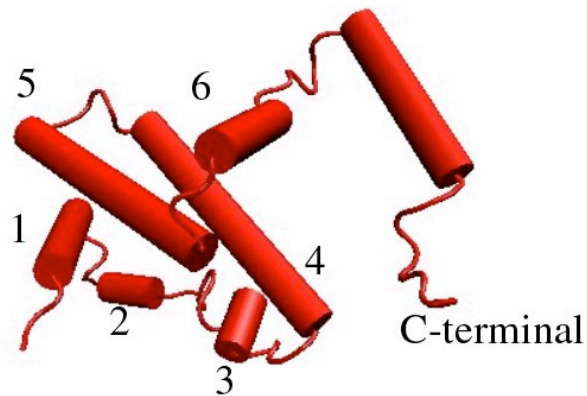


Figure 1.3: Representation of an IFN- γ monomer (DJ Thiela et al., 2000).

The biologically active dimer is formed by anti-parallel inter-locking of the two monomers as shown below in figure 1.4. In the model, one monomer is shown in red, the other in blue.

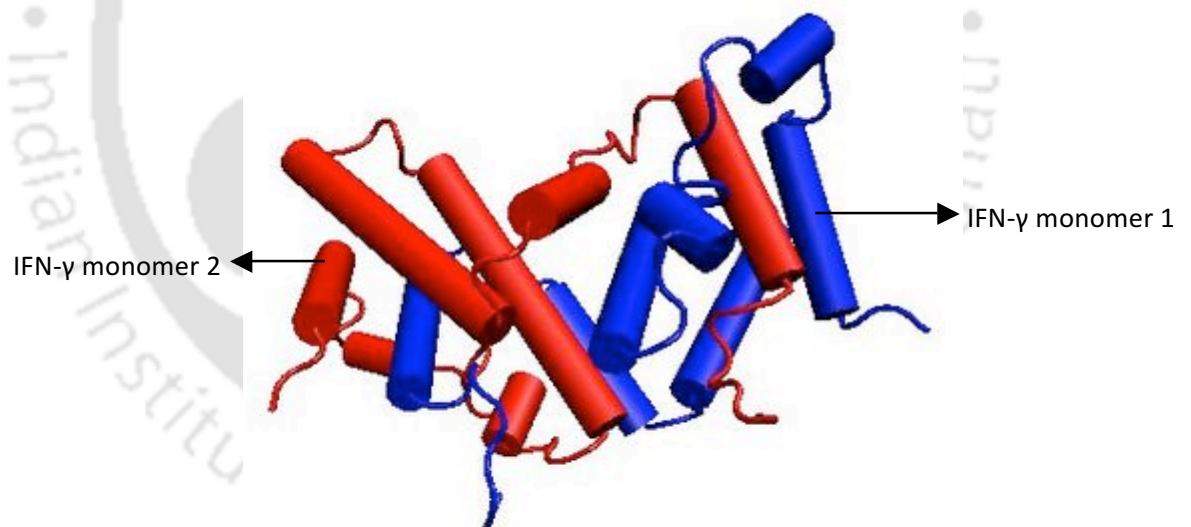


Figure 1.4: Representation of an IFN- γ dimer (DJ Thiela et al., 2000).

1.5 *Escherichia coli* for the production of Interferon gamma

Low cost and ease of cultivation made *Escherichia coli* the preferred choice for recombinant therapeutic protein production. With the progress made in recombinant DNA technology, cloning and expressing a heterologous gene in

Escherichia coli became very easy. Several reports pertaining to IFN- γ production in *E. coli* have been described (Rodriguez et al., 2008; Reddy et al., 2007; Petrov et al., 2009; Arbabi et al., 2003; Khanna et al., 1996; Geng et al., 2004).

However IFN- γ , when expressed in *E. coli* tends to form inclusion bodies due to its extreme hydrophobic nature which makes the recovery of active and soluble IFN- γ very difficult. Obtaining active and soluble protein from inclusion bodies is also a very difficult task. Many reports have been published where active IFN- γ recovery from inclusion body have been described (Reddy et al., 2007; Khanna et al., 1996; Geng et al., 2004). Despite of the availability of these reports, inclusion body solubilization and refolding remains a challenging and time consuming task. Moreover this method may not be economically viable when producing the IFN- γ at large scale in fermentor. Additionally the endotoxins¹ pose a threat while producing recombinant therapeutics in *E. coli*, and it is the reason *E. coli* has not been granted GRAS (Generally Regarded As Safe) status by regulatory authorities.

¹ Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. Endotoxin is invariably associated with Gram-negative bacteria whether the organisms are pathogenic or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, in bacteriology it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of Gram-negative bacteria.

1.6 *Kluyveromyces lactis*: A viable alternative to *Escherichia coli* for IFN- γ Production

Kluyveromyces lactis, a “budding yeast” from phylum Ascomycota is naturally present in cultured dairy processes where lactose concentration is very high. Wild type *K. lactis* isolates have exclusively been isolated from dairy processes such as yogurt, cheese and buttermilk. *K. lactis* has a history of fifty years in food industry applications (Bonekamp and Oosterom, 1994). *K. lactis* was one of the first yeasts to be transformed by foreign DNA in the laboratory (Hollenberg et. al 1982). Since then many proteins have been successfully expressed in *K. lactis* with the help of recombinant DNA technology. A thermostable xylanase, *xynB*, from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 was expressed in *K. lactis* (Yin T et al., 2010). Production and optimization of β -galactosidase in *K. lactis* (Goksungur & Dagbagli, 2008) was carried out. Human interferon alpha was cloned and expressed in *K. lactis* (Chen et al., 1992). Human interleukin-1 beta was produced in *K. lactis* (Fleer et al., 1991). However Human Interferon gamma has never been expressed in *Kluyveromyces lactis*.

Kluyveromyces lactis can be developed as an alternative host to *Escherichia coli* as it is a GRAS organism (Bonekamp and Oosterom, 1994). Completely sequenced genome of *K. lactis* makes it an attractive heterologous protein expression host which can be genetically manipulated very easily. *K. lactis* does not secrete endotoxins and grows to high cell density in bioreactor.

1.7 Interferon gamma in diseases

IFN- γ is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. Abnormal IFN- γ expression is associated with many diseases. The importance of IFN- γ in the human immune system is due to its ability to block viral replication directly and most importantly from its immunostimulatory and immunomodulatory effects.

Role of IFN- γ has already been well established in the treatment of chronic granulomatous disease (Marciano et al., 2004; Errante et al., 2008) & severe malignant osteopetrosis (Lyndon Key et al. 1995). Additionally several researches conducted throughout the world indicate its possible role in the treatment of tuberculosis/MDR-TB (Gao et al., 2001; Reljic R., 2001) as well as of other fatal diseases such as chronic myeloid leukemia (Russo D. et al., 1990; Kurzrock et al., 1987), AIDS (Fantini et al., 1993; Sarol et al., 2002) & oral sub-mucous fibrosis (Haque et al., 2001).

Presently the interferon gamma is approved for the treatment of chronic granulomatous disease & severe malignant osteopetrosis disease. Chronic granulomatous disease (CGD), an inherited disorder of phagocytic cells, results from an inability of phagocytes to produce bactericidal superoxide anions (O_2^-) (Von Rosenvinge et al., 2010). This leads to recurrent life-threatening bacterial and fungal infections.

Whereas in osteopetrosis, a disorder characterized by an osteoclast defect leading to bone overgrowth and deficient macrophage oxidative metabolism, IFN- γ mediated enhancement of superoxide production by phagocytes has

been observed in vitro. IFN- γ also has been shown to enhance osteoclast function in vitro and resulted in reduced risk of developing a serious infection. (Key LL et al., 1995, Shankar et al., 1997)

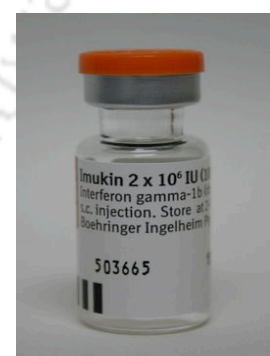
1.8 Interferon gamma as a drug

IFN- γ was thought to be a promising immunomodulatory drug in the early eighties. Initial clinical trials were focused on side effects and dosage. Later trials systematically determined its potential against cancers and infections. In the year 1983 American Biotechnology company Genentech started human clinical trials of Recombinant human IFN- γ targeting the treatment of patients with cancer and viral diseases. Preclinical studies at Genentech indicated that IFN- γ may have two types of anti-tumor activity: It appears to directly inhibit the growth of cancer cells and, more than other interferons, appears to stimulate the body's immune system to fight the cancer, such as chemotherapy agents which frequently suppress the immune system. Preclinical studies also indicated that IFN- γ offers potential in the treatment of viral infections by interfering with the replication of viruses. Eventually Genentech was granted license from Food and Drug Administration (FDA) for the treatment of chronic granulomatous disease under the trade name Actimmune (1). Till now recombinant human IFN- γ has been approved by the U.S. Food and Drug Administration for treatment of chronic granulomatous (CGD) and severe malignant osteopetrosis diseases in children and adults. Furthermore, clinical trials of IFN- γ for the treatment of Multi drug resistant tuberculosis, HIV, hepatitis, lung diseases and various cancers are ongoing (2). IFN- γ has also

shown promising results in the treatment of Cutaneous lymphoma (Kaplan et al., 1990), malignant melanoma (Hasse et al., 1987) and against autoimmune diseases, including rheumatoid arthritis (Machold et al., 1992) and multiple sclerosis (Lees et al., 2008). Boehringer Ingelheim is selling the IFN- γ -1b in most of the European and South American countries; Intermune has the exclusive rights to sell the drug in North America. Japan and China has their own products. Even an Iranian company (Exir Pharma) has started selling the drug in the Republic of Iran. Currently IFN- γ is available at worldwide level but not in the Indian sub-continent (Table 1.1 and Figure 1.5). As far as the Indian market is concerned, no company is manufacturing or selling the IFN- γ -1b. However Indian GMO Research Information System (IGMORIS) recommended clinical trials for Human IFN- γ -1b (IFNG) in the year 2009 with the permission of Drug Controller General of India (3). But till now the product has not been launched in the Indian market by any company. Since there are no national/international companies manufacturing or marketing IFN- γ -1b in India, importing the drug is the only option for Indian patients which costs around 37,152 USD and majority of Indians cannot afford such high cost of treatment.

Table 1.1: Current worldwide availability status of IFN- γ -1b

S. No.	Brand Name	Licensee Company	Countries covered
1	Gama	Wanbang Biochemical Pharmaceutical Company	China
2	Gamaferon	Farmagen	Bulgaria
3	Ingeron	Firma Ferment	USA
4	Lizhu Yindefu	Livzon Zhuhai	China
5	Shang Sheng Lei Tai	Shanghai Biological Products Research Institute	China
6	Imunomax-gamma	Shionogi Seiyaku	Japan
7	Actimmune	InterMune	USA
8	Immukin	Boehringer Ingelheim	United Kingdom, Hong Kong, Ireland, Malta
9	Immukine	Boehringer Ingelheim	Belgium, Luxembourg, Netherlands
10	Imukin	Boehringer Ingelheim	Argentina, Austria, Australia, Bahrain, Switzerland, Colombia, Czech Republic, Germany, Denmark, Finland, France, Greece, Hungary, Italy, Norway, New Zealand, Oman, Sweden, Taiwan, Spain
11	Ogamma	Otsuka Pharmaceutical	Japan
12	Gamma-immunex	Exir pharma	Iran

**Figure 1.5:** Variants of IFN- γ -1b drug.

1.9 Rationale of the study

Tuberculosis (TB) is a major public health problem in India. According to the data, presented by World Health Organization, India accounts for one-fifth of the global TB incident cases (4). Each year nearly 2 million people in India develop TB, of which around 0.87 million are infectious cases. It is estimated that annually around 330,000 Indians die due to TB (4). Currently a new type of tuberculosis referred to as MDR-TB or multi drug resistant tuberculosis is arising due to imprudent and improper use of antibiotics in the chemotherapy of drug-susceptible TB patients. India has the world's third largest population suffering from AIDS (5). Areca nut chewing is very common among Indian Population which gives rise to oral sub-mucous fibrosis which can be a new epidemic in the sub-continent. With its already approved FDA status for the treatment of two diseases, excellent indications and positive results in the treatment of Tuberculosis and MDR-TB and other diseases, IFN- γ has the potential of becoming a lifesaver drug. The proposed study will have two possible impacts:

a) Societal impacts:

A cost effective method for the production of Recombinant Human IFN- γ can reduce the cost of treatment of chronic granulomatous disease and malignant osteopetrosis significantly which currently stands as 37,152 US Dollars (Approx.). India is an agriculture based economy where a person earns as low as 1570 US Dollars per year (6). With this state of income, most of the Indian population cannot afford IFN- γ based treatment since it is extremely expensive

as one vial of Actimmune[®] costs 344 USD approximately. According to the guidelines of Actimmune, for the treatment of CGD, 50 mcg/m² of Actimmune, for the patients whose body surface area is greater than 0.5 m² and 1.5 mcg/m², for patients whose body surface area is equal to or less than 0.5 m², should be administered three times a week for 12 months (7). This corresponds to a total cost of 37,152 USD approximately for treatment of a single patient.

b) Monetary impacts:

Impending expiry of several patented blockbuster biopharmaceuticals and the increasing demand from patients, insurers, and government agencies to reduce drug costs have created numerous opportunities in the global biosimilars market. The biosimilars market is segmented into peptides, recombinant glycosylated proteins and recombinant non-glycosylated proteins. The main factors driving the demand in the biosimilars market include increasing user acceptance, low costs, large application area, increasing governmental initiatives, and the emerging Indian and Chinese economies. Asia's early commercialization and high absorption rate of biosimilars products made it the dominant market in 2008 with 34.1% share of the global biosimilars market.

Tuberculosis and its variants are one of the most commonly occurring diseases in Indian population and a well established role of IFN- γ in the diagnosis (IFN- γ release assay) and current ongoing clinical trials for treatment makes it a highly valuable therapeutic. Furthermore USFDA approved status of IFN- γ for the treatment of CGD and severe malignant osteopetrosis, increases the

value of the drug. A better and cost effective approach for the large scale production of Recombinant Human IFN- γ may prove to be helpful to treat several diseases among Indian Population.

1.10 Objectives of the study

Based on extensive literature survey, clinical importance of IFN- γ and need to produce IFN- γ in a cost effective manner, the present investigation was carried out with following objectives:

- Molecular cloning of human IFN- γ gene in *Escherichia coli* and *Kluyveromyces lactis*.
- Expression of recombinant human IFN- γ in *Escherichia coli* and *Kluyveromyces lactis*.
- Optimization of human IFN- γ expression in *Kluyveromyces lactis*.
- Medium optimization for the production of recombinant human IFN- γ from *Kluyveromyces lactis*.
- Purification of recombinant human IFN- γ produced from *Kluyveromyces lactis*.
- Production of recombinant human IFN- γ in batch bioreactor from *Kluyveromyces lactis*.

1.11 Organization of the thesis

The presentation of the work has been divided into four chapters. The current **Chapter 1** presents a general introduction, objective and scope of the present work. While the literature that supports the present work is presented in **Chapter 2**. **Chapter 3** includes the details of the materials and methods adopted in the present study. Details of the cloning, expression, purification of IFN- γ , optimization of medium components in shake flask and production at batch fermentation scale are presented in this chapter. It also provides technical information about the analytical methods adopted in the present work. **Chapter 4** contains the results and discussion, where the results of recombinant IFN- γ construction, purification, optimization of medium components for higher IFN- γ production and are presented and thoroughly discussed.



CHAPTER 2

REVIEW OF LITERATURE

2.1 Interferon gamma (IFN- γ)

Interferons were discovered because of their potent antiviral activity (Isaacs A, Lindenmann J, 1957; Nagano Y, Kojima Y, 1958). In addition to their hallmark ability to interfere with virus multiplication, the property from which the name *interferon* originates, they display several physiologically important activities (Samuel CE, 1991). IFNs constitute a multigene family of inducible cytokines. Among the key activities of IFN- γ are those that affect the innate host response to microbial pathogens.

Initially, Interferons were classified by the secreting cell type but are now classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs are comprised of multiple IFN- α (Interferon alpha) subtypes, IFN- β (Interferon beta), IFN- ω (Interferon omega), and IFN- τ (Interferon tau), all of which are structurally related and bind to a common heterodimeric receptor (IFNAR, comprised of IFNAR1 and IFNAR2 chains). Although type I IFNs are secreted at low levels by almost all cell types, hematopoietic cells are the major producers of IFN- α and IFN- ω , whereas fibroblasts are a major cellular source of IFN- β (Schreiber et al., 1997). IFN- β is also produced by macrophages under appropriate stimulus. Viral infection is the classic stimulus for IFN- α and IFN- β expression (Schreiber et al., 1997; Akira S, 2000). IFN- τ has only been reported in ruminants (Dinarello CA, 1999).

IFN- γ is a dimerized soluble cytokine that is the only member of the type II class of interferons (Gray & Goeddel, 1982). This interferon was originally called macrophage-activating factor, a term now used to describe a larger family of proteins to which IFN- γ belongs. In humans, the IFN- γ protein is encoded by the *ifng* gene which is located on p1205-qter region of human chromosome 12 (Gray et al, 1983).

2.2 Properties of Interferon gamma (IFN- γ)

IFN- γ belongs to the class of interferons, which are species-specific proteins produced in response to viruses as well as a variety of other natural and synthetic stimuli. As compared to the other interferons (α and β), IFN- γ is acid labile and binds to different cell surface receptors. Similar to interferon α and β , IFN- γ increases the expression of class I major histocompatibility complex (Gastl, Gunther et al. 1996), enhances the activity of NK cells (Carnaud C et al., 1999), interferes with viral replication (Ramana C V et al., 2000), and decreases cell proliferation. In addition, IFN- γ activates macrophages, increases expression of MHC class II (Gastl, Gunther et al. 1996), and stimulates formation of cytotoxic T lymphocytes. There are two types of IFN- γ receptors which differ in molecular weight, acid stability, and regulation of receptor expression. IFN- γ receptors have been found on epithelial and hematopoietic cells and fibroblasts (Bolinger & Taeubel 1992). Since IFN- γ is produced by activated T lymphocytes and regulates the activity of immune cells, it may be more appropriate to describe IFN- γ as a lymphokine of the

interleukin type. There is an evidence that IFN- γ interacts functionally with other interleukin molecules such as interleukin-2 (Kawano, Y., & Noma, T., 1996). The interaction of IFN- γ with interleukin-4 (Paludan, S. R., 1998) results in suppression of IgE levels as well as inhibition of collagen production in humans. IFN- γ also stimulates the release of interleukin-1 (Kakizaki, Y et al., 1993) which is important in the activation of T-lymphocytes. The initial discovery of IFN- γ was based on their ability to inhibit viral growth in target cells (Friedland 1996). Their biological effects *in-vivo* include antiviral activity, cell growth inhibitor and immunomodulator activity (Pitha, 2007; Dheda et al., 2005; Perez et al., 1990; Chen et al., 1992). The anti-proliferative effects make it a potentially useful against cancer (kidney cell carcinoma, colon cancer) and rheumatoid arthritis. The IFN- γ is secreted by human antigen stimulated T-lymphocytes in response to viral infection. The native human IFN- γ is composed of 143 amino acid residues with a total molecular mass of 20-25 kDa. It is glycosylated and does not contain cysteine residues (Khalilzadeh et al., 2004; Mobammadian-Mosaabadi et al., 2007). IFN- γ is a glycoprotein, the indirect evidence for this claim is based on its affinity for concanavalin A-Sepharose (Mizrahi et al., 1978). Further support comes from the structural and sequence analysis of cloned human IFN- γ cDNA that revealed two possible glycosylation sites and a single mature polypeptide consisting of 146 amino acids with a molecular weight of 17 kDa (Gray, 1982). In native form the IFN- γ protein was thought to exist as a dimer because when analyzed by SDS-PAGE, IFN- γ protein resolved into two species of 20 and 25 kDa (Yip et al., 1982). Kelker et al., 1983 performed a

glycosidase treatment of highly pure glycosylated IFN- γ protein and found that two species of 18.5 and 16 kDa of deglycosylated species of IFN- γ corresponding to 25 and 20 kDa of glycosylated species existed. Rinderknecht et al., 1984 produced human IFN- γ from human peripheral blood lymphocytes and found two active species of IFN- γ corresponding to 20 and 25 kDa as suggested by SDS-PAGE analysis. Both species were found to have identical amino acid sequence with a pyroglutamate residue at N-terminus, whereas in both cases six different COOH terminus were found. They also found 2 possible Asn-X-Ser/Thr glycosylation sites. In 25 kDa species both sites and in 20 kDa species only one site was glycosylated. The gene of *ifng* has been cloned in *E. coli* and monkey cells (Gray et al., 1982), insect, yeast and CHO cells (Mutsaers et al., 1986). Although each eukaryotic expression system produces a biologically active protein *in-vitro*, the structure of oligosaccharides added to the IFN- γ polypeptide during the glycosylation process varies significantly with cell type.

Both natural (Rindernecht et al., 1984) and CHO derived IFN- γ (Mutsaers et al., 1986) express complex biantennary N-linked oligosaccharides attached to either the Asn¹⁰⁰ residue only, and/or to the Asn²⁸ residue, yielding a mixture of fully and partially glycosylated proteins.

Initially, it was believed that CD4⁺ T helper cell type 1 (Th1) lymphocytes, CD8⁺ cytotoxic lymphocytes, and NK cells exclusively produced IFN- γ (Schreiber et al., 1997; Kumararatne et al., 2000). However, there is now

evidence that other cells, such as B cells, NKT cells, and professional antigen-presenting cells (APCs) secrete IFN- γ (Young HA, 1996). IFN- γ production by professional APCs such as monocyte/macrophage, dendritic cells (DCs) acting locally may be important in cell self-activation and activation of nearby cells (Gessani, S., Belardelli, F. 1998; Koyasu et al. 2001). IFN- γ secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN- γ in the adaptive immune response (Dougan et al. , 1999).

IFN- γ production is controlled by cytokines secreted by APCs, most notably interleukin-12 and interleukin-18. These cytokines serve as a bridge to link infection with IFN- γ production in the innate immune response (Hägglöf et al., 2011). Macrophage recognition of many pathogens induces secretion of IL-12 and chemokines e.g., macrophage-inflammatory protein-1 α (MIP-1 α) (Campbell D et al., 1996). These chemokines attract NK cells to the site of inflammation, and IL-12 promotes IFN- γ synthesis in these cells (Biron et al., 2000). In macrophages, NK and T cells, the combination of IL-12 and IL-18 stimulation further increases IFN- γ production (Darnell JE Jr, 1997; Levy et al., 1996). Negative regulators of IFN- γ production include IL-4, IL-10, transforming growth factor- β , and glucocorticoids (Howard JC. et al., 1997).

2.3 Historical perspective of Interferon gamma research

In the year 1965, eight years after the discovery of IFN- γ by Isaacs & Lindenmann, Wheelock EF (1965) reported the appearance of interferon-like

antiviral activity in supernatant fluid of cultures of fresh human leukocytes following incubation with the plant lectin phytohemagglutinin (PHA). Like virus induced interferon, the PHA-induced inhibitor was macromolecular (non-dialysable) but soluble (non sedimentable) and acted on human cells only.

Glasgow (1966) hypothesized that interferon production might take place and play an important role not only during primary infection by viruses, but also following re-infection. To validate his hypothesis, he immunized mice against Chikungunya virus and tested interferon production by their cultured peritoneal leukocytes following *in-vitro* challenge with the same virus. Leukocytes from immunized mice produced up to four times as much interferon-like antiviral activity as cells from non-immunized mice. Rebecca Falcoff (Falcoff 1972) characterized interferon induced by anti-lymphocyte immunoglobulin in cultured peripheral human lymphocytes and showed it to be acid-labile and to migrate differently from virus-induced interferon on gel filtration and on DEAE-cellulose chromatography and concluded that IFN- γ can be called as immune interferon.

Youngner and Salvin (1973) showed that BCG-infected (*Bacillus Calmette-Guérin*) mice inoculated intravenously with old tuberculin (OT) release a viral inhibitor into the circulation (up to 8000 units/ml). This inhibitor also had all basic properties of interferon, but differed from virus-induced mouse interferon by being less stable at pH 2 and at 56°C, by different host range

specificity and by resisting neutralization by an antiserum prepared against highly purified virus-induced mouse interferon. On the basis of this evidence, the authors termed this interferon as “Type II interferon”. Gray & Goeddel (1982) suggested the structure of human IFN- γ gene. At the same time Devos et al. (1982) successfully cloned and expressed human IFN- γ cDNA in eukaryotic cells.

Purification of IFN- γ started as early as in the year 1979 when Langford et al. (1979) produced human IFN- γ at large scale and also physicochemically characterized the protein as well. Efforts in purification continued and Billiau A, et al., 1980 used human leukocytes to induce IFN- γ by phytohemagglutinin P, concanavalin A (Con A) and staphylococcal enterotoxin A. They partially purified the protein by batch adsorption on controlled-pore glass (CPG) beads and desorption by ethylene glycol. Yip et al. (1981) developed a method for Human IFN- γ purification. They produced IFN- γ in lymphocyte cultures stimulated with a phorbol ester (12-O-tetradecanoylphorbol 13-acetate) and purified phytohemagglutinin and purified the protein by sequential chromatographic separations on controlled-pore glass, concanavalin A-Sepharose, and Bio-Gel P-200.

Scientific community established a clear role of interleukins in the regulation of IFN- γ . The ability of NK cells to produce IFN- γ was suggested in 1983 by Handa et al. who showed IFN- γ production by mouse splenocytes enriched in NK cells by growth in IL-2. Later studies confirmed that NK cell enriched lymphocyte populations produce IFN- γ in response to various exogenous and

endogenous stimuli, including the NK stimulating factors IL-12 (Young HA, et al., 1991) and IL-18 (Tanabe F, et al., 1995).

2.4 Role of human Interferon gamma in diseases

Human IFN- γ has already been approved by USFDA for the treatment of chronic granulomatous disease (CGD) & severe malignant osteopetrosis in children and adults. As per the data from <https://clinicaltrials.gov/> almost 300 clinical trials of Human IFN- γ are ongoing for the treatment of various diseases ranging from brain tumor to dry eye disease. In one of the earliest reports, Kurzrock et al., (1987) demonstrated therapy of Chronic myelogenous Leukemia (CML) with recombinant human IFN- γ . They reported IFN- γ to have significant clinical role in the treatment of CML.

2.4.1 Interferon gamma in Protozoan infections

Interferon gamma provides resistance against *Toxoplasma gondii*

In a report published in 1988, Suzuki et al. demonstrated that an antibody to IFN- γ can eliminate resistance to acute *Toxoplasma* infection in mice suggesting that this lymphokine plays an important role in host resistance to *Toxoplasma gondii*. Further evidence for the role of IFN- γ against the *Toxoplasma gondii* came yet again from the work of Yasuhiro Suzuki who in 2002 demonstrated that IFN- γ dependent, cell-mediated immunity plays the major role in resistance against development of toxoplasmic encephalitis (TE) in brain.

Leishmaniasis & Interferon gamma

Badaro et al., (1990) reported treatment of acute visceral leishmaniasis with IFN- γ in combination with conventional pentavalent antimony their research clearly indicated that six out of eight patients with visceral leishmaniasis (mean duration, 17 months) who had been unresponsive to multiple courses of pentavalent antimony responded to treatment with recombinant human IFN- γ (100 to 400 μg per square meter of body-surface area per day) in addition to pentavalent antimony (20 mg per kilogram of body weight per day) for 10 to 40 days. In one of the very recent reviews by Kima PE & Soong L (2013) our current understanding of some the host responses during *Leishmania* infections that are regulated by IFN- γ have been summarized.

2.4.2 Interferon gamma in Viral infections

Interferon gamma induced immunity against Human papilloma virus

Sikorski M and Zrubek H. (2003) reported that 20 women with a definite diagnosis of cervical intraepithelial neoplasia (CIN) grade I or II with coexistent high-risk Human papilloma virus (HPV) infection underwent four months observation followed by intracervical administration of IFN- γ in cases without spontaneous regression (17 cases). Human recombinant IFN- γ 1-b (Imukin) was administered intracervically four times in equal doses in two-day intervals to a total dose of $300\mu\text{g}/\text{m}^2$ of body surface area. The results of therapy were verified by punch biopsy evaluation and HPV-DNA testing two months after completion, and revealed a complete response in nine women (complete regression of CIN and remission of HPV infection in 53% of treated

cases) and partial response in four cases (lower grade of CIN or/and remission of HPV infection-23.5%).

Interferon gamma mediated immunity against Herpes simplex virus

Yu Z. et al. (1996) reported that CD4⁺ T cells orchestrate an inflammatory response with IFN- γ appearing to play the essential role in viral clearance. Similarly Decman et al. (2005) demonstrated that IFN- γ alone can block herpes simplex virus reactivation in some latently infected neurons, and they identified points of intervention in the life cycle of the reactivating virus.

Interferon gamma can inhibit human cytomegalovirus replication

Morris et al. (2005) reported that IFN- γ synergizes with the innate IFNs (IFN- α and IFN- β) to inhibit human cytomegalovirus replication *in-vitro*.

Interferon gamma and HIV

Kamoto et al. (2002) reported that IFN- γ induces CIITA through the JAK-STAT pathway and inhibits HIV-1 replication in latently infected cells. Its effect appears to be mediated through the reciprocal action of Tat and CIITA. With this beneficial effect, IFN- γ and its inducers can be considered as an adjunct to the currently available therapy.

2.4.3 Interferon gamma in fungal Infections

It has been demonstrated that IFN- γ is highly effective in augmenting the antifungal activities of effector cells—for example, *in-vitro* with IFN- γ and

tissue macrophage killing of *Blastomyces dermatitidis* (Brummer et al., 1985), *Paracoccidioides brasiliensis* (Brummer et al., 1988), and *Candida albicans* (Brummer et al., 1989), and with pulmonary macrophage killing of *Blastomyces* (Brummer et al., 1987), *Paracoccidioides* (Brummer et al., 1988), and *Histoplasma* (Brummer et al., 1991), species. It has also been reported that IFN- γ administration *in-vivo* would up-regulate killing of *Blastomyces* and *Paracoccidioides* species by pulmonary macrophages *ex vivo* (Brummer et al., 1988). It is also showed that neutrophils would also be up-regulated by IFN- γ , both *in-vitro*, with increased respiratory burst and killing of *Blastomyces* species (Morrison et al., 1987 & 1989), and *ex vivo*, after systemic administration for killing of *Blastomyces* species (Morrison et al., 1989).

2.4.4 Interferon gamma has the potential to cure oral submucous fibrosis

IFN- γ is a known anti-fibrotic cytokine. Haque et al. (2001) investigated, a) the effect of IFN- γ on collagen synthesis by arecoline-stimulated oral submucous fibrosis fibroblasts *in-vitro*, b) the effect of intra-lesional IFN- γ on the fibrosis of oral submucous fibrosis patients and c) the immunohistochemical analysis of pre- and post-treatment inflammatory cell infiltrates and cytokine levels in the lesional tissue. The results show that the increased collagen synthesis *in-vitro* in response to arecoline was inhibited in the presence of IFN- γ . Patients also reported reduced burning dysaesthesia and increased suppleness of the buccal mucosa. The post-treatment

immunohistochemistry showed a decreased amount of inflammatory cell infiltrate and an altered level of cytokines compared with the pre-treatment lesional tissue.

2.4.5 Interferon gamma induces resistance to Tuberculosis

Tuberculosis, a major health problem in developing countries, has reemerged in recent years in form of multiple drug resistant tuberculosis (MDR-TB), where presently available drugs are a failure for the treatment. Grahmann & Braun (2008) reported successful treatment of multiple drug resistance tuberculosis with Aerosolized IFN- γ . Four MDR-TB patients were treated with aerosolized recombinant IFN- γ and anti-tuberculosis drugs, twice weekly for 8 weeks. Before treatment, a patient with more than twenty years of history of TB had no γ - δ T-cells; these cells appeared during treatment. The proportion of natural killer (NK) cells was also enhanced and remained elevated. The proportion of CD4⁺/CD25⁺ T-cells in the blood rose after treatment and remained elevated at 2 and 10 months afterwards. The increased susceptibility of immunocompromised individuals to tuberculosis and many experimental studies indicate that T cell-mediated immunity plays an important role in resistance. The lymphokine IFN- γ is thought to be a principal mediator of macrophage activation and resistance to intracellular pathogens. Gao et al. (2001) evaluated the safety and efficacy of adjunctive therapy using IFN- γ for the treatment of pulmonary tuberculosis (TB).

2.5 *Escherichia coli* as an expression host for IFN- γ production

Escherichia coli (*E. coli*) has remained one of the most widely used heterologous protein expression system. The ease of designing cloning experiments and quantity of protein produced has made it the expression system of choice for recombinant protein production. Earlier, human cells were the only source for producing IFN- γ for scientific studies and clinical use, by exposing T lymphocytes to mitogenic stimuli (Nakayama, 1983; Bhayani et al., 1985) or by translating mRNA in oocytes (Vaquero, 1982). With the development of recombinant DNA technology, human IFN- γ cDNA sequence has been identified and cloned into a prokaryotic expression vector. It was then proved that the produced recombinant IFN- γ has the same immunological and chemical properties as authentic IFN- γ (Reddy et al., 2007). Moreover, procedures for mass production and purification of IFN- γ were simplified (Vassileva Atanassova et al., 2000; Zlateva et al., 1999; Arora and Khanna 1996; Kendrick et al., 1998; Blackburn et al., 2000; Xu et al., 1997; Tatsumi and Sata, 1997). Human IFN- γ has mostly been expressed and purified using *E. coli* system till date (Rodriguez et al., 2008; Reddy et al., 2007; Petrov et al., 2009; Arbabi et al., 2003; Khanna et al., 1996; Geng et al., 2004).

Human IFN- γ is a highly hydrophobic protein and that's the reason it tends to aggregate when expressed in *E. coli* expression system. Till date most of the reports pertaining to IFN- γ expression and purification from *E. coli* demonstrate that this protein forms inclusion bodies. Many reports have been published, where methods for IFN- γ denaturation, solubilization and refolding

have been described. Reddy et al. (2007) described a reversed phase chromatography (RPC) procedure using Source-30 matrix in the purification of IFN- γ from *E. coli* that resulted in a higher yield [40 mg/g of cell mass] than previously reported (Khalilzadeh et al., 2004) [0.3–14 mg/g of cell mass]. They isolated T-lymphocytes from human peripheral blood cells and induced IFN- γ mRNA with the help of phytohemagglutinin. Isolated mRNA was used to amplify IFN- γ which was subsequently cloned into pET21a vector and expressed in Rosetta (DE3) pLysS cells. Inclusion body solubilization was initiated with 4M urea. Inclusion bodies were solubilized with 6M guanidine hydrochloride. The purified IFN- γ monomer from the RPC column was refolded in Tris buffer. Optimal refolding occurred at protein concentrations between 50 and 100 μ g/ml. This method yielded greater than 90% of the dimer form with a yield of 40mg/g cell mass. Greater than 99% purity was achieved with further purification over a Superdex G-75 column. Khanna et al. (1996) described a strategy for improved refolding and purification of IFN- γ from *E. coli*. As the source of recombinant IFN- γ , they used an *E. coli* strain (BL-21) transformed with an expression plasmid bearing the human IFN- γ gene. Inclusion body preparation was initiated by adding 8M urea to sonicated cells and inclusion bodies were solubilized with 6M guanidine hydrochloride and refolded with Tris buffer containing 0.5 M arginine. Petrov et al., (2010) reported a method for refolding of IFN- γ using urea and ammonium chloride solution. IFN- γ protein was denatured by 1M urea and inclusion bodies were solubilized with 7.4M guanidine hydrochloride. Rodriguez et al. (2008) reported periplasmic expression and recovery of IFN- γ in *E. coli* BL21-SI

cells. IFN- γ gene was fused to SP1 and SP3, two Sec-dependent artificial signal peptides to transport the IFN- γ to the periplasm of *Escherichia coli* BL21-SI.

Geng et al., (2004) developed a new technology for renaturation of recombinant human IFN- γ with its simultaneous purification. Based on chemical equilibrium and molecular interactions, they described the principle of IFN- γ refolding by high performance hydrophobic interaction chromatography (HPHIC). They also showed that stationary phase is more important than mobile phase in the IFN- γ refolding. Extract containing the IFN- γ in guanidine hydrochloride solution was directly pumped into a unit of simultaneous renaturation and purification of proteins (USRPP) packed by small particle packings of hydrophobic interaction chromatography. Bioactivity of $8.7 \times 10(7)$ IU mg^{-1} of the IFN- γ was obtained. Renaturation by the USRPP increased the total bioactivity of IFN- γ by 62-fold.

2.5.1 *Escherichia coli* GalG20: An emerging protein expression platform

With the onset of 20th century, non viral gene therapy became an important area of biopharmaceutical research. The fact that naked plasmid DNA could mediate gene transfer and expression *in-vivo* (Wolff et al., 1990) was a major pillar behind the emergence of these researches and with that started the research on producing large quantities of plasmid DNA. Metabolic engineering has proven to be a very powerful tool for the novel strain

development and strain improvement programmes, especially in *E. coli*. Various strategies have been developed to identify constraints in metabolite production, engineering efficient metabolic pathways, improved production of metabolites and many more.

In 2013 Geisa et al. successfully developed an *E. coli* strain GalG20 (MG1655 Δ endA Δ recA Δ pgi) from the *E. coli* strain (MG1655 Δ endA Δ recA). The newly developed strain was capable of producing 25 fold more plasmid DNA than its parental strain. Five different key genes (*pykF*, *pykA*, *endA*, *recA* & *pgi*) of glycolytic pathway were knocked out from MG1655 strain to produce Gal G20 strain. *pykF* & *pykA* have already been shown to increase plasmid DNA yields (Cunningham et al. 2009a, b; Pablos et al. 2011). *endA* & *recA* mutations were made to avoid recombination and non-specific digestion of DNA (Phue et al. 2008; Summers 1998). *pgi* gene was knocked out to redirect carbon flow into the pentose phosphate pathway and promote nucleotide biosynthesis.

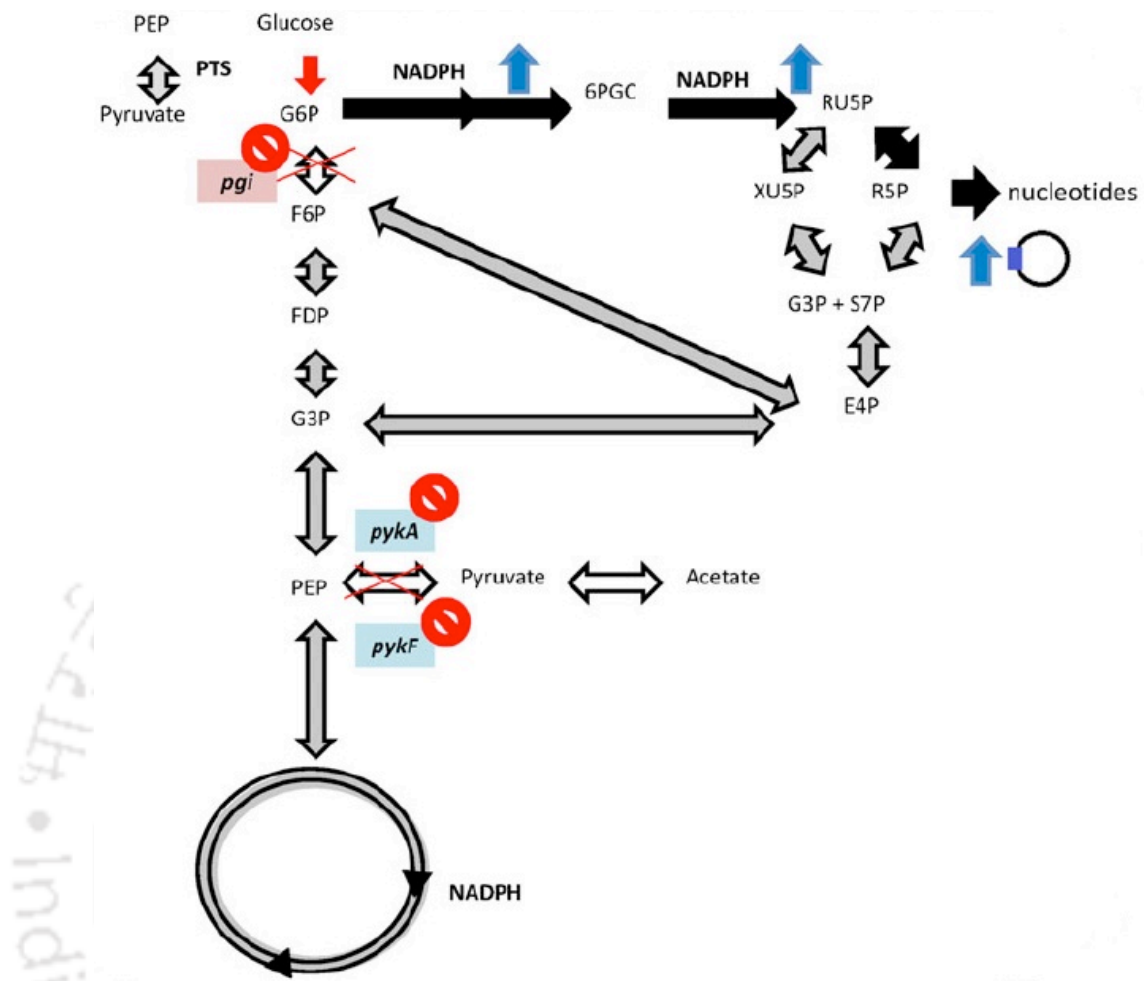


Figure 2.1: Gene knock-out strategy to improve plasmid DNA production in *E. coli*

MG1655 Δ endA Δ recA (adopted from Geisa et al., 2012).

2.6 *Kluyveromyces lactis*: a Generally Regarded as Safe (GRAS) eukaryote for recombinant protein production

Kluyveromyces lactis is a “Non-methylotrophic budding yeast” from the phylum Ascomycota. It is evolutionarily closely related to the well-known baker’s yeast *Saccharomyces cerevisiae*. In fact, *K. lactis* was originally classified as *Saccharomyces lactis*, but this taxonomic classification was changed in 1984 to *Kluyveromyces marxianus* var. *lactis*, with *K. lactis* being the accepted shortened notation (Walt J.P. van der and Johannsen E. 1984). Both organisms share many similarities and several key differences.

Both organisms are morphologically similar with *K. lactis* cells generally being smaller than *S. cerevisiae* cells. Both organisms divide by budding and can exist in either in a haploid or diploid state. Diploids of either organism can undergo sexual reproduction and generate four haploid ascospores, the products of meiotic segregation. Similar to *S. cerevisiae*, *K. lactis* haploids can have either a or α mating types. However, *K. lactis* haploids cannot mate with *S. cerevisiae* haploids. No other modes of genetic transmission for *K. lactis* have been described. In nature, *S. cerevisiae* and *K. lactis* have different ecological niches that primarily reflect physiological differences in how the two organisms metabolize sugars. *S. cerevisiae* thrives in rotting fruit where glucose concentration is high. It can also produce ethanol aerobically as a metabolic byproduct when glucose concentration is high, a process known as the “Crabtree effect” (Deken, 1966). In contrast, *K. lactis* possesses a highly expressed lactase that degrades milk sugar (lactose) to galactose and glucose.

This enzyme is absent in *S. cerevisiae*. Thus, *K. lactis* is a natural and indispensable component of cultured dairy processes where lactose concentration is very high. Wild type *K. lactis* isolates have exclusively been isolated from dairy processes such as yogurt, cheese and buttermilk.

Kluyveromyces lactis is one of the few yeast species that can utilize lactose as a sole source of carbon and energy (Parascandola et al. 2004 & 2006; Romano et al., 2010; Breunig et al., 2012). Its *LAC* genes allow for utilization of the inexpensive sugar lactose as a sole source of carbon and energy. Lactose efficiently induces the *LAC4* promoter, which can be used to drive regulated expression of heterologous genes.

2.6.1 Historical perspectives of *Kluyveromyces lactis*

Kluyveromyces lactis (*K. lactis*) has more than twenty years' history of safe use as an expression system in regulated food industry processes and has become an important genetic model organism in fungal genetics akin to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *K. lactis* and products derived from *K. lactis* have been safely used in food industry applications for almost fifty years (Oosterom et. al 1994). In notable examples, dried *K. lactis* powder was used as a dietary protein supplement in the 1960's and 1970's, a purified native lactase isolated from *K. lactis* has been (and still is) used to orally treat lactose intolerance since the 1980's, and recombinant bovine chymosin is produced from engineered *K. lactis* for use in cheese manufacturing (Van den Berg JA et al., 1990).

In the early 1980's, *K. lactis* became one of the first yeasts to be transformed by foreign DNA in the laboratory (Hollenberg et. al 1982). This led to the development of a host-vector expression system by the Dutch food company Gist-brocades BV (now DSM Food Specialties) that culminated with the production of the important food industry enzyme, recombinant chymosin (a cheese rennant). A large industrial fermentation and manufacturing process was established for production of this enzyme and in 1988, *K. lactis*-produced chymosin became the first recombinant protein approved for use in food applications. This *K. lactis* produced Lactose was also granted GRAS status by the FDA (5). However, due to the lucrative nature of the patents covering the use of the *K. lactis* host-vector system (for production of prochymosin), it remained exclusively a food industry technology.

2.6.2 Advantages of using *Kluyveromyces lactis*

Advantages of *K. lactis* as an expression system are that it grows to extremely high cell densities in bioreactor, it is a GRAS organism hence no endotoxin analysis is required, Additionally, the expression in *K. lactis* does not require the induction by methanol, and thus the explosion-proof equipment for methylotrophic yeasts such as *Pichia pastoris* is not required (Van Ooyen et al. 2006). It supports protein expression from both stable episomal vectors and integrative vectors, and it can be easily manipulated with genetic techniques and media originally devised for manipulation and growth of *S. cerevisiae*.

2.6.3 Heterologous protein expression efforts in *Kluyveromyces lactis*

Yin T et al. (2010) expressed an extremely thermostable xylanase, *xynB*, from the hyperthermophilic bacterium *Thermotoga maritima* MSB8 in *Kluyveromyces lactis*. A statistical approach, Response Surface Methodology, was applied to optimize the medium components for the production of XynB secreted by the recombinant *K. lactis*. They successfully achieved secretion level of 102 mg/l and enzyme activity of 49 U/ml of XynB in the optimized medium composed of yeast extract, lactose, and urea which was three fold higher than obtained in the original medium of yeast extract, lactose, and peptone (56 mg/l & 16 U/ml). In a similar kind of study, Goksungur & Dagbagli (2008) investigated the production and optimization of β -galactosidase enzyme in *Kluyveromyces lactis* NRRL Y-8279 in shake flask cultures. Response surface methodology was applied to investigate the effects of agitation speed, pH, initial substrate concentration and incubation time on β -galactosidase production. Results of the statistical analysis showed that the fit of the model was good in all cases. Maximum specific enzyme activity of 4218.4 U g⁻¹ was obtained at the optimum levels of process variables (pH 7.35, agitation speed 179.2 rpm, initial sugar concentration 24.9 g l⁻¹ and incubation time 50.9 hrs). Chen et al. (1992) inserted human interferon alpha secretion cassette into pE1 vector derived from pKD1. They demonstrated that the secretion signal sequence of the alpha factor (α -MF) of *Saccharomyces cerevisiae* was functionally active in *K. lactis*. The *K. lactis* transformants carrying expression cassette efficiently secreted IFN α into the growth medium. The total amount of IFN α released reached 1-2 mg/l of culture

supernatant. Fleer et al. (1991) reported the secretion of recombinant human interleukin-1 beta from *K. lactis*. High levels of protein were secreted into the growth medium when the structural gene was fused in-frame to a synthetic secretion signal derived from the 'pre'-region of the *K. lactis* killer toxin. Native human interleukin-1 beta is not glycosylated but *K. lactis* interleukin-1 beta was found to be glycosylated. This glycosylation led to a 95% loss of its biological activity. Removal of the glycosylation by endo-beta-N-acetylglucosaminidase H treatment fully restored the biological activity. The secretion level of recombinant human interleukin-1 beta from *K. lactis* was found to be independent of glycosylation status. Previous efforts related to recombinant heterologous protein production in *K. lactis* have been summarized in table 2.1.

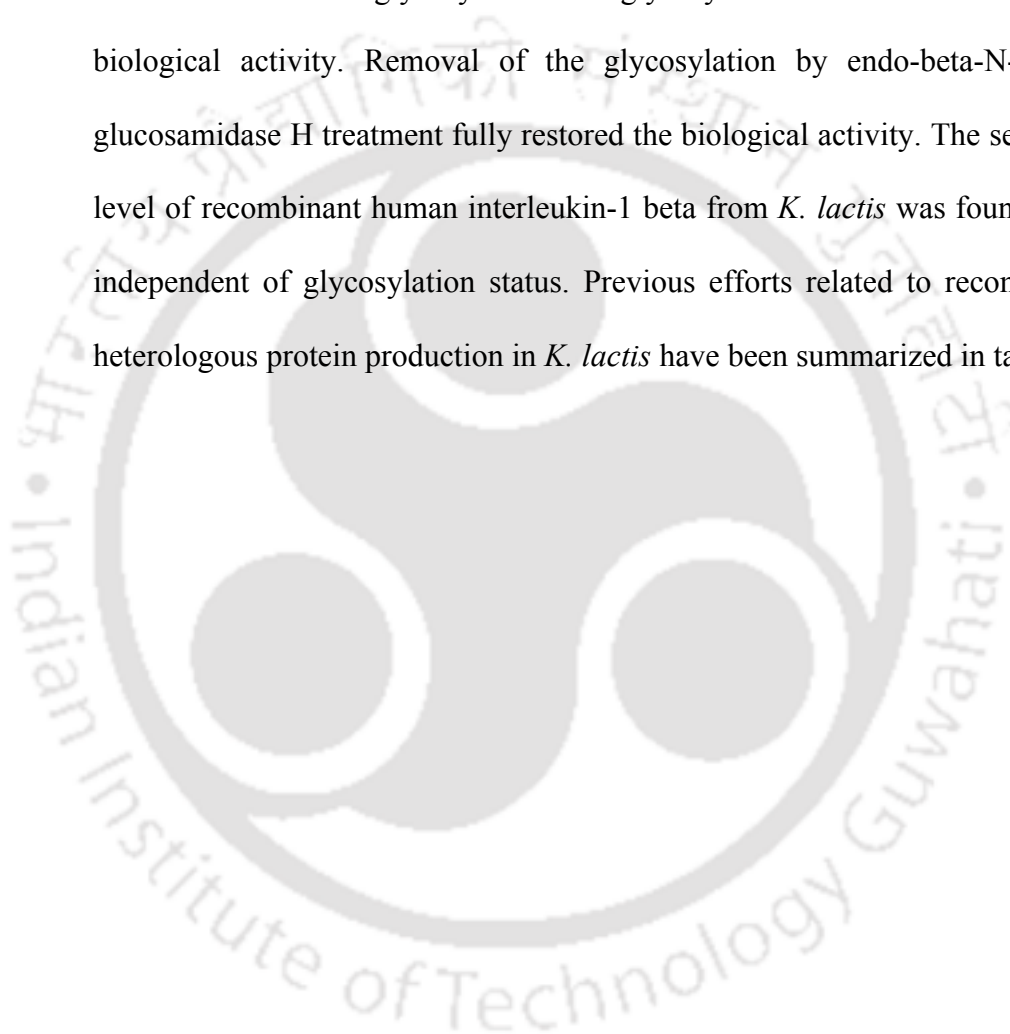


Table 2.1: Recombinant Protein production studies in *K. lactis*

Protein	Source	Expression Vector	Promoter	Leader Sequence	Yield	Reference
D-amino acid oxidase	<i>Trigonopsis variabilis</i>	pKD1	Sc GAL1	NA	150 U g ⁻¹	Gonzalez et al.(1997)
α -amylase	Mouse	pKD1	Sc PGK	KT	0.527 U mL ⁻¹	Tokunaga et al. (1997)
Foot protein three (Mcfp-3)	<i>Mytilus californianus</i>	Lac4 Int.	Kl LAC4	KL- α - factor	1 mg L ⁻¹	Platko et al., 2008
Endoxylanase	<i>Neocallimastix frontalis</i>	pKD1	Sc PGK	KT	110 U mL ⁻¹	Durand et al. (1999)
α -galactosidase	<i>Cyamopsis tetragonoloba</i>	pKARS	Sc GAL7	Sc pre- SUC2	2 mg L ⁻¹	Bergkamp et al.(1992)
Glucoamylase	<i>Arxula adeninivorans</i>	pKD1	Sc GAP	Native	101 nkat mL ⁻¹	Bui et al. (1996)
Glycoprotein	E2 Hepatitis C virus	TRP1 integration	Kl AAC	KT	1 mg L ⁻¹	Mustilli et al. (1999)
Insulin precursor	Pig	pKD1	ADH2	Sc α -factor	30 mg L ⁻¹	Feng et al. (1997)
Interferon alpha	Human	pKD1	PGK	Sc α -factor	2 mg L ⁻¹	Chen et al.(1992)
Interleukin-1beta	Human	pKD1	PGK	KT	80 mg L ⁻¹	Fleer et al. (1991)
Invertase	<i>Saccharomyces cerevisiae</i>	rDNA integration	Sc α -factor	Sc α -factor		Hsieh & Da Silva (1998)
Laccase	<i>Pleurotus ostreatus</i>	pKD1	Kl ADH4	Native	2.03 U mL ⁻¹	Piscitelli et al.(2005)
b-lactoglobulin	Sheep	pKD1	Sc PGK	Native	50 mg L ⁻¹	Rocha et al. (1996)
Lysozyme	Human	pKD1	PGK	Pre-HEW	100 mg L ⁻¹	Iwata et al. (2004)
Pancreatic trypsin inhibitor	Cow	pKD1	Kl LAC4	Sc α -factor	0.32mgL ⁻¹	Panuwatsuk & Da Silva (2003)
Serum albumin	Human	Plasmid/Inte grated	PGK	Pre-HSA	3 gL ⁻¹	Fleer et al. (1991)
scFv antibody4B2	Mouse	pKD1	Kl LAC4	Sc α -factor	1.3 mg L ⁻¹	Robin et al. (2003)
Luciferase	<i>Gaussia princeps</i>	LAC4 int.	Kl LAC4- PBI	KL- α - factor	1 mg L ⁻¹	Read et al., 2007
Xylanase	<i>Humicola insolens</i>	pKD1	Kl LAC4	Native		Muller et al. (1998)

2.7 Expression optimization of Interferon gamma

Heterologous protein production in yeasts could be enhanced by optimizing culture physical conditions (pH, temperature and agitation speed etc.) and culture media composition (carbon source, nitrogen source and trace elements etc.). Furthermore, systems biology and proteomics analysis during the recombinant protein expression could help to identify the different types of stresses caused to the cell by the exogenous gene. There is still a less amount of information regarding the proteomics of yeast cells during heterologous gene expression. Besides that, lab scale analysis may not correspond to the conditions faced by the microorganism in an industrial plant, where scale of operation dependent external conditions (local temperature shifts, osmolarity and local nutrient limitations) can also be stressful (Graf et al., 2009).

Although Human IFN- γ has never been expressed in *K. lactis*, several reports are available of its expression in *E. coli*. Being a highly hydrophobic protein this protein tends to form inclusion body when expressed in *E. coli* and researchers have tried to obtain IFN- γ in soluble fraction by tuning culture and expression conditions. Zhu et al. (2012) used small ubiquitin-related modifier fusion technology (SUMO) for the expression of IFN- γ in *E. coli*. They induced the expression of protein by 50 mM arginine and 1% glycerol and successfully expressed IFN- γ to a level of 62 mg of soluble protein.

Babaeipour et al. (2013) tuned expression conditions of recombinant human IFN- γ by optimizing amount of inducer, induction time and post induction duration in *E. coli* BL21DE3 expression system.

Hernández et al. (2008) reported production of human IFN- γ using a synthetic gene in *E. coli* BL21-SI. Protein expression was optimized by Response Surface Methodology (RSM) and a Box-Behnken design. They studied three process variables: temperature, biomass concentration at induction time and the NaCl concentration as inducer. A second order response function was developed as per the results obtained by the Box-Behnken design. The optimal expression conditions were a temperature of 32.6° C, induction biomass of 0.31 g/L and 0.3 M NaCl in minimal medium. The model prediction for the maximum IFN- γ production was 77.3 mg/L. Meetul kumar et al., 2014 reported simple yet robust purification approach for obtaining recombinant IFN- γ protein expressed as inclusion body in *E.coli*.

2.8 Production studies of Interferon gamma

Babaeipour et al. (2007) designed a simple fed-batch process using a modified variable specific growth rate feeding strategy for IFN- γ expression and reported highest productivity (2.57g/litre/hour). They expressed the protein in *Escherichia coli* BL21DE3. Glucose was used as the sole source of carbon and energy. *E. coli* cell density (dry weight) reached up-to 100 g/l.

In the variable specific growth rate fed-batch process, plasmid stability and specific yield of IFN- γ were greater than constant specific growth rate fed-batch process. The final specific yield and overall productivity of IFN- γ was 0.35 ± 0.02 g IFN- γ g⁻¹ dry cell wt and 0.9 ± 0.05 g IFN- γ l⁻¹ h⁻¹ in the variable specific growth rate fed-batch process, respectively.

Vaiphei et al. (2009) studied the product formation kinetics of IFN- γ in continuous cultures of *E. coli* and reported specific product yield ($Y_{p/x}$) reaching a maximum value of 182 mg/gm DCW.

Babaeipour et al. (2013) used *E. coli* BL21DE3 expression system to over-express human IFN- γ in an exponential fed-batch procedure with a maximum attainable specific growth rate of 0.55 h⁻¹. They achieved final concentration of biomass and IFN- γ as 127 g L⁻¹ (DCW) and 51 g IFN- γ /l after 17 h, and also the final specific yield and overall productivity obtained was 0.4 g (IFN- γ) g⁻¹ DCW and 3 g (IFN- γ) L⁻¹ h⁻¹, respectively.

Khalilzadeh et al. (2004) designed a simple fed-batch process with pre-determined exponential feeding strategy for expressing IFN- γ from *E. coli* BL21 (DE3). They used glucose and glycerol as the sole sources of carbon and energy. Acetate accumulation was not observed. The final cell density of 100 g l⁻¹ in case of glucose and 118 g l⁻¹ in case of glycerol was observed. The concentration of acetate was always maintained below toxic level. The specific yield of IFN- γ with glucose and glycerol was 93 and 92 mg g⁻¹ of dry cell mass, and the overall productivity of IFN- γ was 0.16 and

0.14 g l⁻¹ h⁻¹ for these two carbon sources, respectively. Preceding efforts of IFN- γ production have been summarized in table 2.2.

Table 2.2: Preceding efforts of IFN- γ Production from *Escherichia coli*

Carbon source	Biomass achieved (gDCW/l)	IFN- γ production level	Reference
Glucose	127.00	51 g/l	Maghsoudi et al. 2013
Glucose	-	77.3 mg/l	Rodríguez et al., 2008
Glucose	100.00	0.9 g l ⁻¹ h ⁻¹	Khalilzadeh et al., 2004
Glucose	115.00	42.5 g/l	Maghsoudi et al. 2007
Glucose	100.00	93 mg/g DCW	Maghsoudi et al. 2004
Glycerol	118.00	92 mg/g DCW	Rodríguez et al., 2010
-	-	182 mg/g DCW	Vaiphei et al. 2009



CHAPTER 3

MATERIALS & METHODS

3.1 Strain, vector and plasmid

Kluyveromyces lactis GG799 expression host and pKLAC2 vector were purchased from NEB, USA (*Kluyveromyces lactis* protein expression kit). *Escherichia coli* DH5 α was purchased from MTCC, Chandigarh, India. Plasmid pUC19 harboring the gene of human Interferon Gamma was kindly gifted by Dr. Howard A. Young, Lab. of Experimental Immunology, National Cancer Institute Frederick, NIH, USA. *Escherichia coli* GalG20 strain was provided by Dr. D. M. F. Prazeres, Department of Bioengineering, Instituto Superior Tecnico Lisbon, Portugal. λ DE3 lysogenization kit was purchased from Novagen USA.

3.2 Enzymes & Chemicals

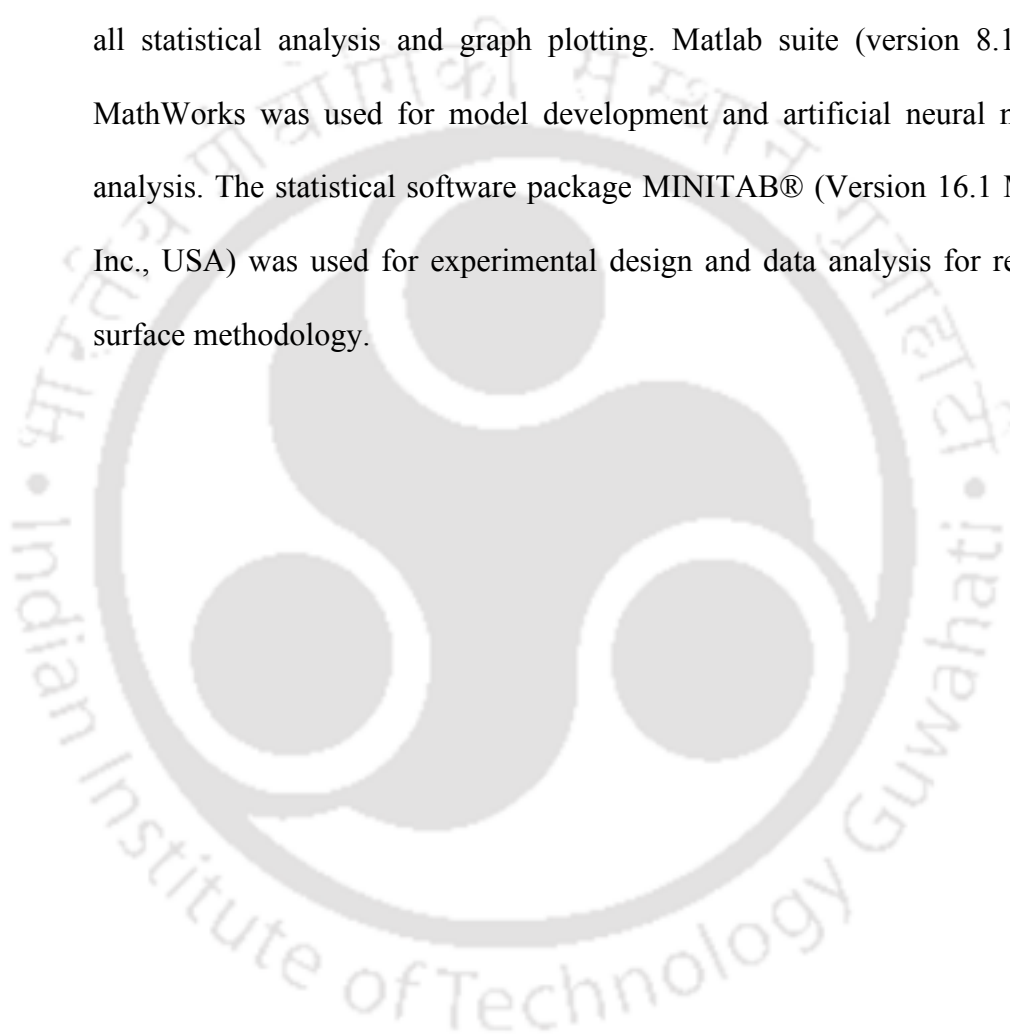
All the restriction and ligation enzymes were purchased from NEB, USA. High fidelity DNA polymerase enzyme (HF Taq) was purchased from NEB, USA. Plasmid isolation and purification kits are purchased from Qiagen & Roche. Protein & DNA Molecular ladders were purchased from NEB, USA and nzytech, Portugal. Other routinely used chemicals were purchased from Merck, Sigma, and Hi-Media, India.

3.3 Human IFN- γ detection and quantification

Human IFN- γ ELISA kit was purchased from eBioscience, Affymetrix USA.

3.4 Software suites

Vector NTI 10 from Invitrogen was used for all cloning related experiments and sequence analysis. Sigma plot 11 from Systat Software Inc was used for all statistical analysis and graph plotting. Matlab suite (version 8.1) from MathWorks was used for model development and artificial neural network analysis. The statistical software package MINITAB® (Version 16.1 Minitab Inc., USA) was used for experimental design and data analysis for response surface methodology.



3.5 Cloning and expression strategy of IFN- γ in *E. coli* GalG20DE3 system

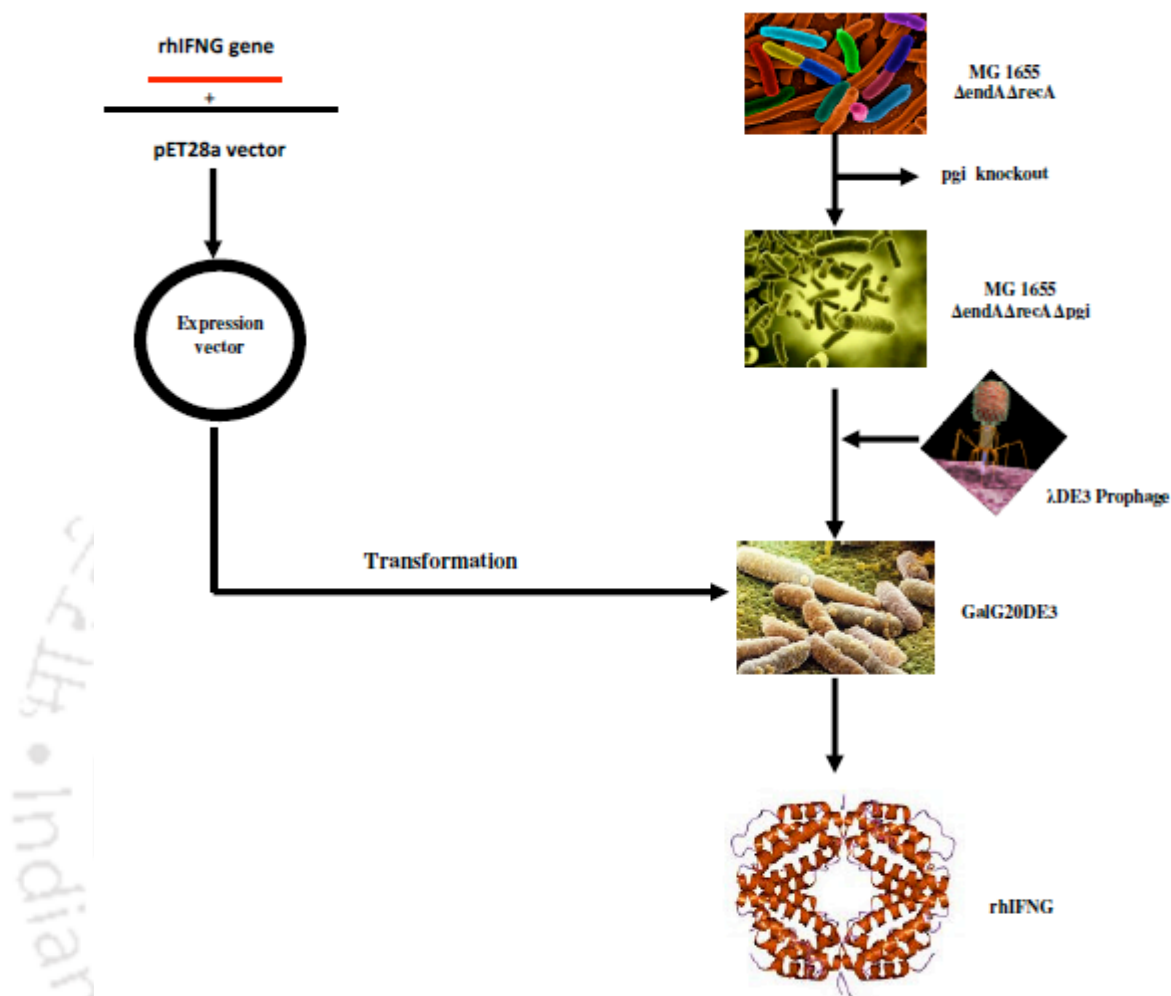


Figure 3.1: Strategy for construction of a novel *E. coli* GalG20DE3 strain for cloning and expression of IFN- γ .

3.6 Cloning of IFN- γ gene in pET28a

3.6.1 Primer designing

Full length Human *ifng* cDNA cloned in pUC19 vector was used as the DNA template for primer designing and gene extraction. Vector NTI 10 (Life technologies) was used for designing all primers. Primers were synthesized by Eurofins scientific, Bangalore, India.

3.6.2 *ifng* amplification

A 501 bp *ifng* gene was amplified from the template pUC19 plasmid harboring full length *ifng* cDNA. For this DH5 α cells were transformed with pUC19 plasmid harboring the Full length Human *ifng* gene. pUC19 was isolated by following the kit manufacturer's protocol and used to amplify the 501 bp *ifng* using designed primers (Table 3.1). Primer annealing temperature was optimized using gradient PCR method and based on the results of this experiment a final PCR (Table 3.2 and 3.3) was performed to generate *ifng* cDNA in sufficient quantity for the subsequent cloning steps.

Table 3.1: Primers used for amplification of *ifng* for cloning in pET28a vector. [Nhe1 (forward: Red) & Xho1 (reverse: Green) sites highlighted and underlined].

Forward primer	GGC <u>GCTAGC</u> ATGAAATATACAAGTTATATC
Reverse primer	GTG <u>CTCGAG</u> TTATTACTGGGATGCTCTTC

Table 3.2: (a) Gradient PCR reaction mix; (b) PCR reaction mix with optimized temperature.

S. No.	Reaction component	Quantity/ concentration
1	10 X buffer	1X
2	MgCl ₂	1.5 mM
3	Primer forward	0.2 μ M
4	Primer reverse	0.2 μ M
5	dNTP (10 mM)	0.5 mM
6	Taq Polymerase	1U/25 μ l
7	DNA template	100 ng

(a)

S. No.	Reaction component	Quantity/ concentration
1	10 X buffer	1X
2	Primer forward	0.2 μ M
3	Primer reverse	0.2 μ M
4	dNTP	0.5 mM
5	Phusion (HF)	1U/20 μ l
6	DNA template	100 ng

(b)

Table 3.3: Optimized PCR reaction conditions

Steps	Temperature ($^{\circ}$ C)	Time (seconds)	Number of cycles
Initial denaturation	94	300	1
Denaturation	94	60	30
Annealing	66	45	
Extension	72	45	
Final extension	72	300	1
Hold	4	hold	NA

The PCR product thus amplified was eluted and purified from agarose gel.

Purified DNA was stored and used for subsequent cloning experiments.

3.6.3 Enzymatic digestion of amplified *ifng* and expression vector pET28a

PCR amplified *hifng* cDNA and pET28a vector (Figure 3.2) was digested with NheI & XhoI restriction enzymes. Reaction was carried out at 37°C for 4 hours (Table 3.4 and 3.5).

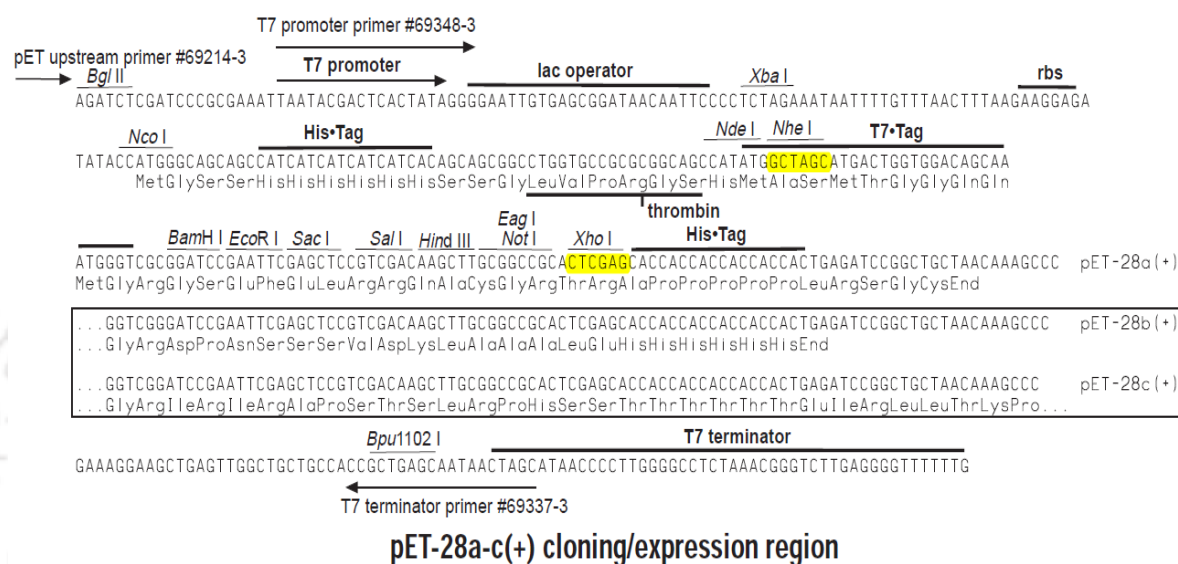


Figure 3.2: pET28a cloning/expression region with NheI & XhoI sites highlighted (adopted from Novagen's pET28a manual TB074).

Table 3.4: Restriction digestion reaction of *ifng*

S. No.	Reaction component	Quantity/ concentration
1	10X Buffer	1X
2	BSA 100X	1X
3	NheI	10 Units
4	XhoI	10 Units
5	DNA template	2 µg

Table 3.5: Restriction digestion reaction of pET28a

S. No.	Reaction component	Quantity/ concentration
1	10X Buffer	1X
2	BSA	1X
3	NheI	10 Units
4	XhoI	10 Units
5	DNA template	1 µg

3.6.4 Ligation of *ifng* and pET28a

Enzymatically digested *ifng* and vector pET28a were ligated at 4°C for 16 hours (Table 3.6). After the reaction the new recombinant plasmid carrying 501 bp *ifng* was designated as pVVDRP01.

Table 3.6: Ligation reaction mixture for *ifng* and expression vector pET28a

S. No.	Reaction component	Quantity/ concentration
1	Ligase buffer 10X	1X
2	Insert	200 ng
3	Vector	60 ng
4	T4 DNA Ligase	200 units

3.6.5 Transformation of recombinant pVVDRP01 into DH5 α

DH5 α competent cells were chemically transformed with recombinant pVVDRP01 plasmid and plated on LB-agarose (Luria Bertani-Agarose) petri plate with 30 $\mu\text{g ml}^{-1}$ kanamycin. Transformed cells were allowed to grow at 37°C for 16 hours. Transformed DH5 α cells were screened for the presence of pVVDRP01 by colony PCR.

3.6.6 Screening of transformed clones by colony PCR

Ten colonies from the population of transformed DH5 α cells were randomly selected and screened for the presence of pVVDRP01 plasmid by colony PCR (Table 3.7 and 3.8) with *ifng* gene specific primers.

Table 3.7: Colony PCR reaction mixture for screening of positive clones

S. No.	Reaction component	Quantity/concentration
1	10 X buffer	1X
2	MgCl ₂	1.5 mM
3	Primer forward	0.2 μM
4	Primer reverse	0.2 μM
5	dNTP	0.5 mM
6	Taq Polymerase	1U/25 μl

Table 3.8: Colony PCR reaction conditions

Steps	Temperature (°C)	Time (seconds)	Number of cycles
Initial denaturation	94	300	1
Denaturation	94	60	30
Annealing	66	45	
Extension	72	45	
Final extension	72	60	1
Hold	4	hold	NA

3.6.7 Recombinant plasmid isolation and sequence confirmation

Out of the ten transformants screened in colony PCR experiment, four positive clones were selected for pVVDRP01 plasmid isolation. 5 ml of L.B. media supplemented with 30 $\mu\text{g ml}^{-1}$ kanamycin was inoculated with single colony corresponding to each of the four selected clones. pVVDRP01 plasmid DNA was isolated following the protocol supplied by the kit manufacturer. All four plasmids were subjected to double restriction digestion by Nhe1 & Xho1. Two plasmids showing positive result were selected for nucleotide sequence and open reading frame confirmation from Eurofins scientific, Bangalore, India.

3.7 Development of a novel recombinant protein production host from *Δpgi* mutant *E. coli* GalG20, for production of IFN- γ

E. coli host strain GalG20 (MG1655 $\Delta\text{endA}\Delta\text{recA}\Delta\text{pgi}$) was lysogenized with the help of λDE3 prophage as per the kit manufacturer's protocol. Site specific integration of λDE3 prophage into GALG20 chromosome was accomplished in a three-way co-infection with λDE3 phage, a Helper Phage and a Selection Phage. λDE3 is a recombinant phage carrying the cloned gene for T7 RNA polymerase under *lacUV5* control. *E. coli* strain (MG1655 $\Delta\text{endA}\Delta\text{recA}$) from which the *E. coli* GalG20 (MG1655 $\Delta\text{endA}\Delta\text{recA}\Delta\text{pgi}$) has been developed was also lysogenized following the same protocol, in order to evaluate the effect of *pgi* knock-out on the protein production capability of GalG20DE3. After this experiment we obtained two strains, GalG20DE3 and MG1655DE3. Both of

these strains, along with a routinely used *E. coli* strain BL21DE3 were assessed for IFN- γ expression.

3.7.1 Lysogenigation protocol

- 1) GalG20 and MG1655 host strains were grown in LB supplemented with 0.2% maltose, 10 mM MgSO₄, and 30 $\mu\text{g ml}^{-1}$ kanamycin at 37°C to an OD₆₀₀ of 0.6.
- 2) 10⁸ pfu λ DE3 phage, 10⁸ pfu helper phage, and 10⁸ pfu selection phage was incubated with 20 μl cells at 37°C for thirty minutes.
- 3) Incubated cells were plated on L.B. plates and again incubated at 37°C for overnight.

3.7.2 Verification of λ DE3 Lysogens (GalG20DE3 and MG1655DE3)

λ DE3 lysogens (GalG20DE3 and MG1655DE3) were evaluated by their ability to support the growth of the T7 Tester Phage. T7 Tester Phage is a T7 phage deletion mutant that is completely defective unless active T7 RNA polymerase is provided by the host cell. The T7 Tester Phage makes very large plaques on authentic λ DE3 lysogens in the presence of IPTG, while much smaller plaques are observed in the absence of inducer.

Protocol:

- 1) GalG20DE3 and MG1655DE3 host strains were grown in LB supplemented with 0.2% maltose, 10 mM MgSO_4 , and $30 \mu\text{g ml}^{-1}$ kanamycin at 37°C to an OD_{600} of 0.6.
- 2) An aliquot of T7 Tester Phage was diluted in 1X Phage Dilution Buffer (Table 3.9) to a titer of 2×10^3 pfu/ml.
- 3) In duplicate tubes, $100 \mu\text{l}$ cells were mixed with $100 \mu\text{l}$ diluted T7 Tester phage.
- 4) Cells and T7 Tester phage mixture was incubated at room temperature for 10 min to allow phage to adsorb to host.
- 5) 3 ml molten top agarose (47°C) (Table 3.10) was added to each tube containing cells and T7 Tester phage. Contents of one duplicate tube was plated onto a plane LB plate and the other duplicate onto an LB plate supplemented with 0.4 mM IPTG (isopropyl-b-thiogalactopyranoside) to evaluate induction of T7 RNA polymerase.
- 6) Plates were allowed to sit undisturbed for a few minutes until the top agarose hardened and incubated inverted at 37°C at room temperature overnight.

Table 3.9: Phage Dilution buffer recipe

Phage dilution buffer
(100 ml of 10X buffer)
20 ml 1 M Tris-HCl, pH 7.4
20 ml 5 M NaCl
10 ml 1 M MgSO ₄

Table 3.10: Molten top agarose recipe

Molten top agarose
(100 ml)
1 gm tryptone
0.5 gm NaCl
0.6 gm agarose

In the absence of IPTG, observation of very small plaques with discrete edges is an indication that basal level expression of T7 RNA polymerase is low. Cells that have high uninduced levels of T7 RNA polymerase give plaques that are large and have diffuse edges or “halos”.

3.8 IFN- γ production from *E. coli* GalG20DE3, MG1655DE3 and BL21DE3 expression hosts

3.8.1 Transformation of pVVDRP01 in expression hosts GalG20DE3, MG1655DE3 and BL21DE3

GalG20DE3, MG1655DE3 and BL21DE3 expression strains were chemically transformed with pVVDRP01 plasmid and plated on LB (Luria Bertani) petri plate with 30 $\mu\text{g ml}^{-1}$ kanamycin. GalG20DE3 cells harboring pVVDRP01 were designated as GGVVDRP, MG1655DE3 cells harboring pVVDRP01 were designated as MGVVDRP and BL21DE3 cells harboring pVVDRP01

were designated as BLVVDRP. Cells were allowed to grow at 37°C for 16 hours. Only GalG20DE3, MG1655DE3 and BL21DE3 cells harboring pVVDRP01 plasmid could grow in the presence of antibiotic. Two colonies from each plate were used to inoculate 15 ml of LB media supplemented with 30 $\mu\text{g ml}^{-1}$ kanamycin for IFN- γ expression analysis.

3.8.2 GGVVDRP, MGVVDRP and BLVVDRP cells cultivation and induction of IFN- γ

Two flasks of 100 ml LB media supplemented with 30 $\mu\text{g ml}^{-1}$ kanamycin were inoculated with 1 ml of overnight culture of GGVVDRP, MGVVDRP and BLVVDRP expression host harbouring pVVDRP01 plasmid. Cells were grown at 37°C & 220 RPM, till the $\text{OD}_{600\text{nm}}$ reached 0.5. IFN- γ expression was induced by adding 1mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) in all the flasks and cells were incubated for further four hours. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes.

3.8.3 Sonication of *E. coli* biomass

Harvested cell pellet of GGVVDRP, MGVVDRP and BLVVDRP cells was resuspended in 20 ml buffer having composition 20 mM Tris, 1mM EDTA, 100 $\mu\text{g ml}^{-1}$ lysozyme, 1mM PMSF (phenylmethanesulfonyl fluoride) and pH 7.5. This suspension was transferred to ice chilled beaker and cells were disrupted by sonication with a programme of 5 seconds burst and 5 seconds off for a total duration of 10 minutes.

3.8.4 SDS-PAGE analysis of IFN- γ expressed in GGVVDRP, MGVVDRP and BLVVDRP cells

IFN- γ expression was checked on 12% SDS-PAGE gel (Shapiro et al. 1967) by loading 30 μ l of sample mixed with loading dye. Samples were incubated at 95°C for 15 minutes before loading in SDS-PAGE gel to completely denature the protein.

3.8.5 Western blot analysis of IFN- γ expressed in GGVVDRP, MGVVDRP and BLVVDRP cells

Confirmation of IFN- γ expressed in GGVVDRP, MGVVDRP and BLVVDRP strains was carried out by western blot experiment as described previously by Towbin et al. (1979). IFN- γ expressed as insoluble protein in all three strains was denatured by 8M Urea and 30 μ l samples of all three strains were used directly to run a 1 mm SDS-PAGE gel. A wet transfer of proteins was performed at 20 volts for overnight to transfer all the proteins on a nitrocellulose membrane. A primary antibody directed against human IFN- γ raised in sheep was used to probe recombinant IFN- γ protein. An anti sheep HRP conjugated Secondary antibody was used to detect the presence of IFN- γ protein on nitrocellulose membrane.

3.8.6 Washing and harvesting of IFN- γ inclusion bodies

The equal volume of lysis buffer containing 8M urea and 1mM PMSF was added to sonicated GGVVDRP, MGVVDRP and BLVVDRP cells. Cell

suspension was stirred for 45 minutes inside cold chamber and centrifuged at 12,000 rpm for 40 minutes. The supernatant was kept aside for SDS-PAGE analysis and pellet was resuspended in 500 ml of a buffer having composition 100 mM Tris pH 8.0, 1 mM EDTA, 1 M NaCl. Solution was mixed well and centrifuged at 12,000 rpm for 30 minutes. This step was repeated twice. Finally, inclusion body pellet was suspended in 400 ml of MQ and centrifuged again at 12,000 rpm for 30 minutes. Supernatant was discarded and inclusion body pellet was stored at -80°C (Arora & Khanna, 1996)

3.8.7 Solubilization of IFN- γ inclusion bodies

The inclusion body pellet was resuspended in 25 ml of solubilization buffer (6M GuanidineHCL, 100 mM Tris pH 8.0 & 200 mM EDTA pH 8.0) to yield a homogeneous solution. This solution was centrifuged at 20,000 rpm for 30 minutes at 4°C.

3.8.8 Renaturation of IFN- γ inclusion bodies

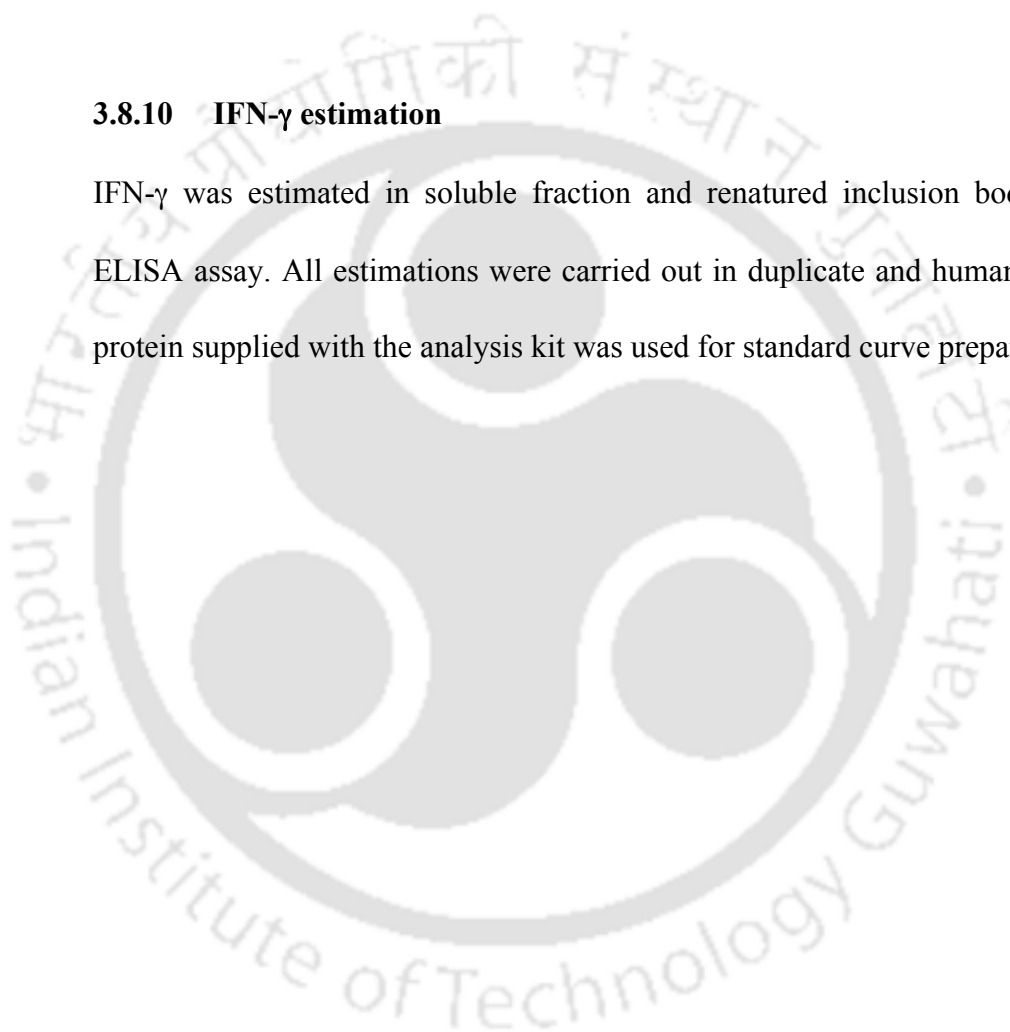
Renaturation was initiated by dilution of the denatured inclusion bodies in refolding buffer (0.1 M Tris pH 8.0 & 0.2 mM EDTA). As a routine, refolding buffer containing 0.5 M L-arginine was used. Buffer (1 L) at 10°C was stirred and three injections of inclusion body of 10 ml were added (not dropwise) into the refolding buffer at 2 h intervals. The solution was incubated for 36–48 h, without stirring at 10°C.

3.8.9 Dialysis of IFN- γ inclusion bodies

The renatured IFN- γ protein (in the presence of 0.5 M L-arginine) was dialysed against freshly prepared 20 mM Tris-HCl pH 8.0, containing 100 mM urea. The dialysis was carried out at 10°C until conductivity was between 3–3.7 mMhos. The solution was spun at 10,000 rpm for 10 min and the supernatant was collected.

3.8.10 IFN- γ estimation

IFN- γ was estimated in soluble fraction and renatured inclusion bodies by ELISA assay. All estimations were carried out in duplicate and human IFN- γ protein supplied with the analysis kit was used for standard curve preparation.



3.9 Cloning, expression and production strategy of IFN- γ in *Kluyveromyces lactis*

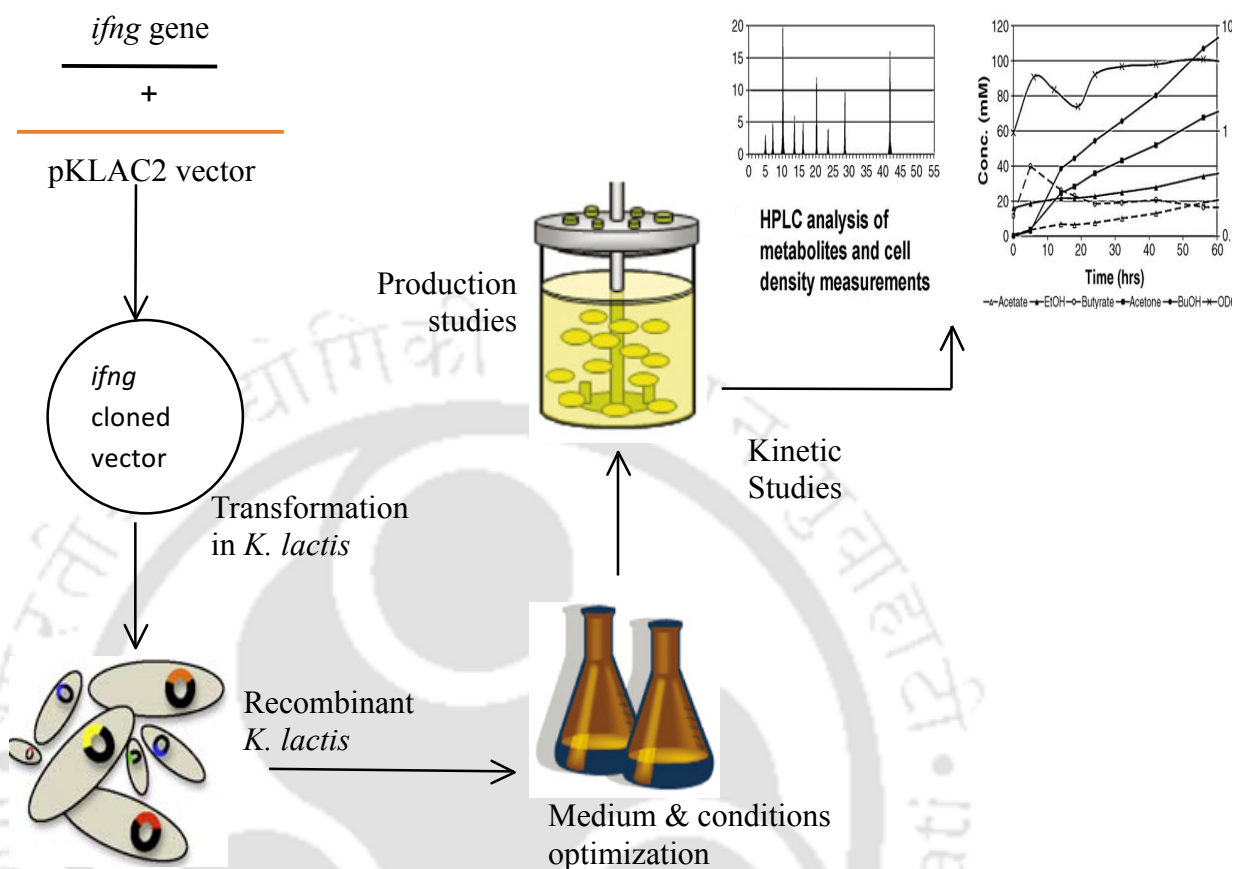


Figure 3.3: Strategy for *ifng* cloning and expression in *Kluyveromyces lactis*.

3.10 Cloning of human Interferon Gamma gene in *Kluyveromyces lactis*

Full length human IFN- γ gene and protein corresponds to 501 bp and 166 amino acids respectively out of which 66 bp corresponding to 22 amino acids towards the N-terminal constitute the native signal peptide sequence. In order to clone and express the gene correctly in the *K. lactis* expression system, the native signal sequence was removed from the gene. IFN- γ nucleotide sequence

was obtained from human protein reference database (HPRD ID: 00957) to design primers. Primers were synthesized by Eurofins scientific, Bangalore, India. IFN- γ gene was amplified with DNA primers (Table 3.11). A stop codon (TAA) was inserted in the reverse primer for proper termination of transcription process. A gene thus amplified was cloned in XhoI & BamHI sites of pKLAC2 vector and called as pVVDRP02. Resulting plasmid pVVDRP02 was used to transform *E. coli* DH5 α competent cells and plasmids were isolated from the positive clones. Isolated pVVDRP02 plasmids were digested & linearized with SacII restriction enzyme. Linearized IFN- γ -pKLAC2 expression cassette was used to transform *Kluyveromyces lactis* (GG799) ultra-competent cells.

Table 3.11: Primers used for amplification of *ifn γ* for cloning in pKLAC2 vector.

[XhoI (forward: Red) & BamHI (reverse: Green) sites highlighted and underlined].

Forward primer	GGC <u>CTCGAG</u> AAAAGACAGGACCCATAT
Reverse primer	GTG <u>GGATCCT</u> TATTACTGGGATGCTCTTC

3.10.1 Human IFN- γ gene amplification

A 432 bp *ifn γ* gene was amplified from the template pUC19 plasmid harboring full length *ifn γ* cDNA. DH5 α cells were transformed with pUC19 plasmid harboring the full length Human IFN- γ cDNA. pUC19 was isolated following the standard protocol supplied by the kit manufacturer. Isolated pUC19 was used to amplify the truncated IFN- γ gene (432bp) using gene specific primers described above. Primer annealing temperature was optimized using gradient

PCR (Table 3.12) and based on the results of this experiment an optimized PCR (Table 3.13 and 3.14) was performed to amplify *ifng* in sufficient quantity for the subsequent cloning steps. The PCR product thus amplified was eluted and purified from agarose gel and used for digestion and ligation with expression vector.

Table 3.12: Gradient PCR reaction mix.

S. No.	Reaction component	Quantity/concentration
1	(a) 10 X buffer	1X
2	MgCl ₂	1.5 mM
3	Primer forward(10 µM)	0.2 µM
4	Primer reverse (10 µM)	0.2 µM
5	dNTP (10 mM)	0.5 mM
6	Taq Polymerase	1U/25µl
7	DNA template	100 ng

Table 3.13 Final PCR reaction mix with optimized temperature.

S. No.	Reaction component	Quantity/concentration
1	10 X buffer	1X
2	Primer forward(10 µM)	0.2 µM
3	Primer reverse(10 µM)	0.2 µM
4	dNTP	0.5 mM
5	Phusion (HF) Polymerase	1U/20µl
6	DNA template	100 ng

Table 3.14: Optimized PCR reaction conditions

Steps	Temperature (°c)	Time (seconds)	Number of cycles
Initial denaturation	96	300	1
Denaturation	96	60	28
Annealing	61	50	
Extension	72	60	
Final extension	72	300	1
Hold	4	hold	NA

3.10.2 Enzymatic digestion of Insert (*ifng*) and Vector (pKLAC2)

PCR amplified *ifng* and pKLAC2 vector were digested with Xho1 & BamH1 restriction enzymes to generate sticky ends for the ligation with each other. Reaction (Table 3.15 and 3.16) was carried out at 37°C for 4 hours.

Table 3.15: Restriction digestion reaction of *ifng*

S. No.	Reaction component	Quantity/concentration
1	10X Buffer	1X
2	BSA 100X	1X
3	Nhe1	10 Units
4	Xho1	10 Units
5	DNA template	3 µg

Table 3.16: Restriction digestion reaction of pKLAC2

S. No.	Reaction component	Quantity/concentration
1	10X Buffer	1X
2	BSA	1X
3	Nhe1	15 Units
4	Xho1	15 Units
5	DNA template	2 µg

3.10.3 Ligation of restriction digested *ifng* and pKLAC2

Enzymatically digested *ifng* and vector pKLAC2 were subjected to ligation reaction at 4°C for 16 hours (Table 3.17). After the reaction the new plasmid created was called pVVDRP02.

Table 3.17: Ligation reaction mixture for *ifng* and expression vector pKLAC2.

S. No.	Reaction component	Quantity/concentration
1	Ligase buffer 10X	1X
2	Insert	300 ng
3	Vector	100 ng
4	T4 DNA Ligase	200 units

3.10.4 Transformation of DH5 α with pVVDRP02

DH5 α competent cells were chemically transformed with ligation product pVVDRP02 recombinant plasmid and plated on LB-agarose (Luria Bertani-Agarose) petri plate with 100 $\mu\text{g ml}^{-1}$ ampicillin. Cells were allowed to grow at 37°C for 16 hours. Only DH5 α cells harboring pVVDRP02 plasmid could grow in the presence of antibiotic.

3.10.5 Plasmid isolation and sequence confirmation

Four colonies were randomly selected for screening of the presence of pVVDRP02 recombinant plasmid and plasmid isolation. 5 ml of LB media supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin was inoculated with single colony corresponding to each of the four selected clones. Plasmid DNA was isolated following the protocol supplied by the kit manufacturer. All four plasmids were subjected to double restriction digestion experiment with Xho1 & BamH1. Two plasmids showing positive result were selected for nucleotide sequencing and open reading frame confirmation.

3.10.6 Linearization of the recombinant pVVDRP02 plasmid for transformation and integration in the genome of *Kluyveromyces lactis*

Out of the two sequence and open reading frame confirmed clones, one was linearized with SacII restriction enzyme (Table 3.18). Linearized expression cassette was used to transform *Kluyveromyces lactis* GG799 chemically competent cells.

Table 3.18: Restriction digestion and linearization of pVVDRP02 with SacII

S. No.	Reaction component	Quantity/concentration
1	10X Buffer	1X
2	BSA 100X	1X
3	SacII	10 Units
5	DNA template	4 µg

3.11 Transformation of *Kluyveromyces lactis* competent cells with linearized pVVDRP02

Kluyveromyces lactis GG799 chemically competent cells were transformed with linearized pVVDRP02 expression cassette harboring *ifng*. Yeast colonies were screened with acetamide for the selection of positive transformants.

pKLAC2 vector contains a fungal acetamidase gene (*amdS*) which helps in selection of yeast cells containing an integrated heterologous expression cassette by allowing their growth on nitrogen-free minimal medium containing acetamide. Only cells expressing acetamidase can break down acetamide to ammonia for use as a nitrogen source. An advantage of this selection method is that it enriches transformant populations for cells that have integrated

multiple copies of the heterologous expression cassette and therefore produce more recombinant protein.

3.11.1 Transformation protocol

Introduction of the linearized pVVDRP02 expression cassette into *K. lactis* cells was achieved by chemical transformation using the *K. lactis* GG799 competent cells and NEB Yeast Transformation Reagent (Figure 3.4). Transformants were selected by growth on Yeast Carbon Base (YCB) Agar Medium containing 5 mM acetamide. YCB medium contains glucose and all nutrients needed to sustain growth of *K. lactis* GG799 cells except a simple nitrogen source. Only transformed cells can utilize acetamide as a source of nitrogen after it is broken down to ammonia by acetamidase, the product of the *amdS* gene present in pKLAC2.

Transformation protocol

1. Thaw a tube of *K. lactis* GG799 Competent Cells on ice. Add 620 μ l NEB Yeast Transformation Reagent to the cells. Briefly shake or invert the tube until the solution is homogeneous. Do not vortex.
2. Add 1 μ g of linearized pVVDRP02 expression cassette containing the *ifng* gene to the cell mixture. Briefly shake or invert the tube to mix.
3. Incubate the mixture at 30°C for 30 minutes.
4. Heat shock the cell mixture by incubation at 37°C for 1 hour in a water bath.

5. Pellet cells by centrifugation at 7000 rpm for 2 minutes and discard the supernatant.
6. Resuspend the cell pellet in 1 ml sterile YPGlu medium.
7. Pellet cells by centrifugation at 7000 rpm for 2 minutes and discard the supernatant.
8. Resuspend the cell pellet in 1 ml YPGlu medium and transfer the cell mixture to a sterile culture tube. Incubate with shaking (250–300 rpm) at 30°C for 3–4 hours.
9. Transfer the cell mixture to a sterile 1.5 ml centrifuge tube. Pellet the cells by centrifugation at 7000 RPM for 2 minutes and discard the supernatant. Resuspend the cell pellet in 1 ml sterile 1X PBS.
10. Remove 10, 50 and 100 µl of the cell suspension to separate fresh sterile 1.5 ml centrifuge tubes, each containing 50 µl of sterile deionized water. Mix briefly and spread the entire cell mixture from each tube onto separate YCB agar medium plates containing 5 mM acetamide. Incubate plates inverted at 30°C for 3–4 days until colonies form.
11. Streak or patch 10–20 individual colonies onto fresh YCB Agar Medium plates containing 5 mM acetamide. Incubate at 30°C for 1–2 days.
12. Transformants were tested to verify that they have correctly integrated the pVVDRP02 expression cassette.

13. Correctly integrated transformants can be further screened to identify cells that have integrated multiple tandem copies of the pVVDRP02 expression cassette.

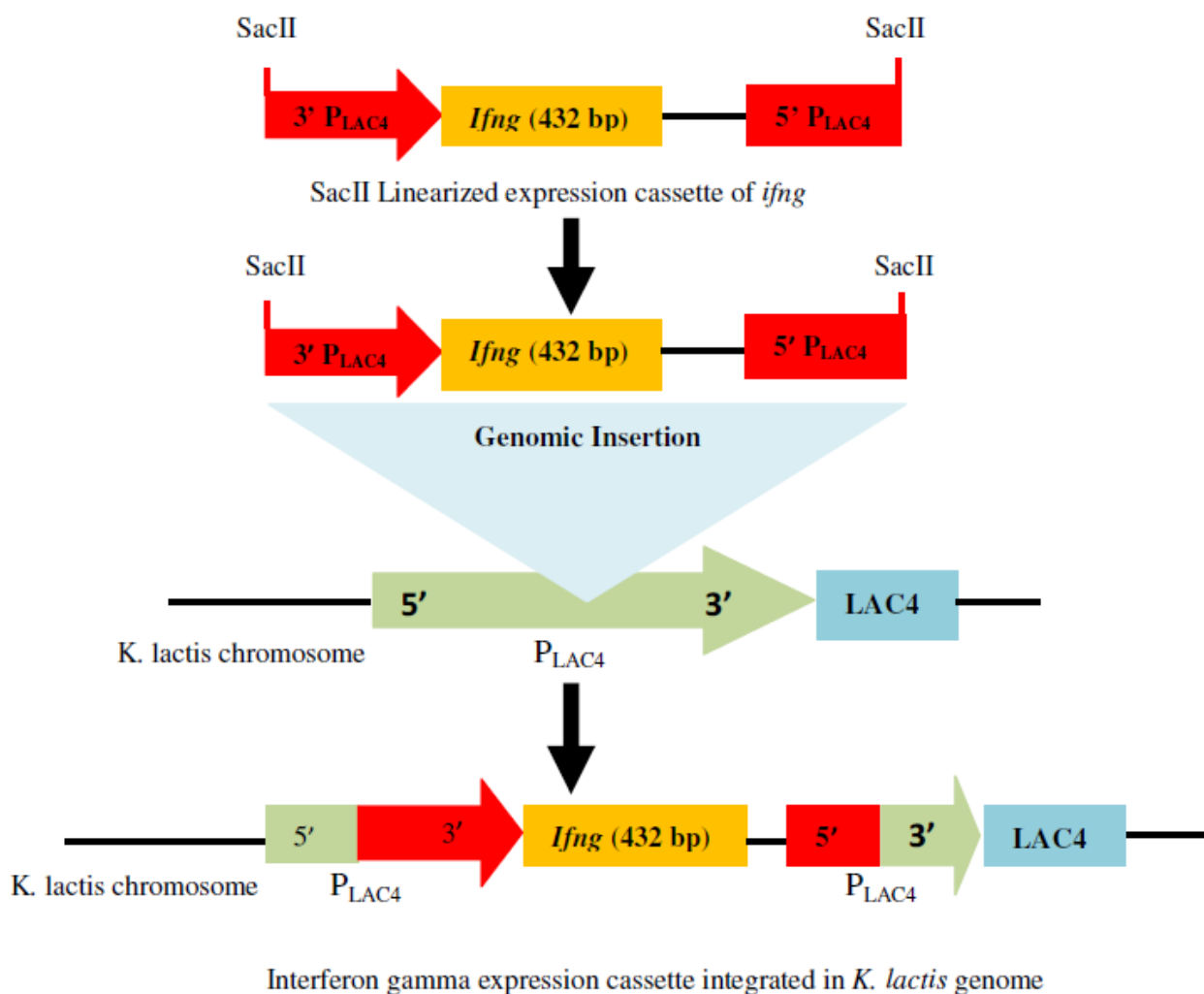


Figure 3.4: Strategy for *ifng* integration in *K. lactis* genome. (pKLAC2 harboring *ifng* was digested with SacII and introduced into *K. lactis* cells. The 5' PLAC4 and 3' PLAC4 sequences direct insertion of the cassette into the promoter region of the LAC4 locus in the *K. lactis* genome).

3.11.2 *Kluyveromyces lactis* genomic DNA isolation

The genomic DNA of *Kluyveromyces lactis* was isolated using LioAc-SDS mediated protocol as described previously by Kristjuhan et al. (2011). 0.2M Lithium acetate, 1% SDS solution and ethanol was used for the experiment.

Protocol:

1. Pick one yeast colony from the plate or spin down 100-200 μ L of liquid yeast culture (OD_{600nm} 0.4). Suspend cells in 100 μ L of 200mM LiOAc, 1%SDS solution.
2. Incubate for 5 minutes at 70°C.
3. Add 300 μ L of 96-100% ethanol, vortex.
4. Spin down DNA and cell debris at 15,000g for 3 minutes.
5. Wash pellet with 70% ethanol.
6. Dissolve pellet in 100 μ L of H₂O or TE, and spin down cell debris for 15 seconds at 15,000g.
7. Use 1 μ L of supernatant for PCR.

3.11.3 Confirmation of proper integration of the pVVDRP02 in the genome of the transformed *K. lactis* clones

The genomic DNA isolated was used to identify correctly integrated transformants by whole cell PCR method (Table 3.19) following a standard protocol supplied by the kit manufacturer. The transformants were screened for single or multiple copies of integrated expression cassette pVVDRP02 (Figure 3.5). Thermocycling consisted of 30 rounds (94°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minutes), followed by incubation at 72°C for 10 minutes.

Table 3.19: PCR reaction mix for confirmation of proper integration of the pVVDRP02 in the transformed *K. lactis* cells.

S. No.	Reaction component	Quantity/concentration
1	10X ThermoPol Buffer	1 X
2	10X Integration Primer 1	10 µl
3	10X Integration Primer 2	10 µl
4	dNTPs(10 mM)	0.5 mM
5	Taq DNA Polymerase	1 U/25µl

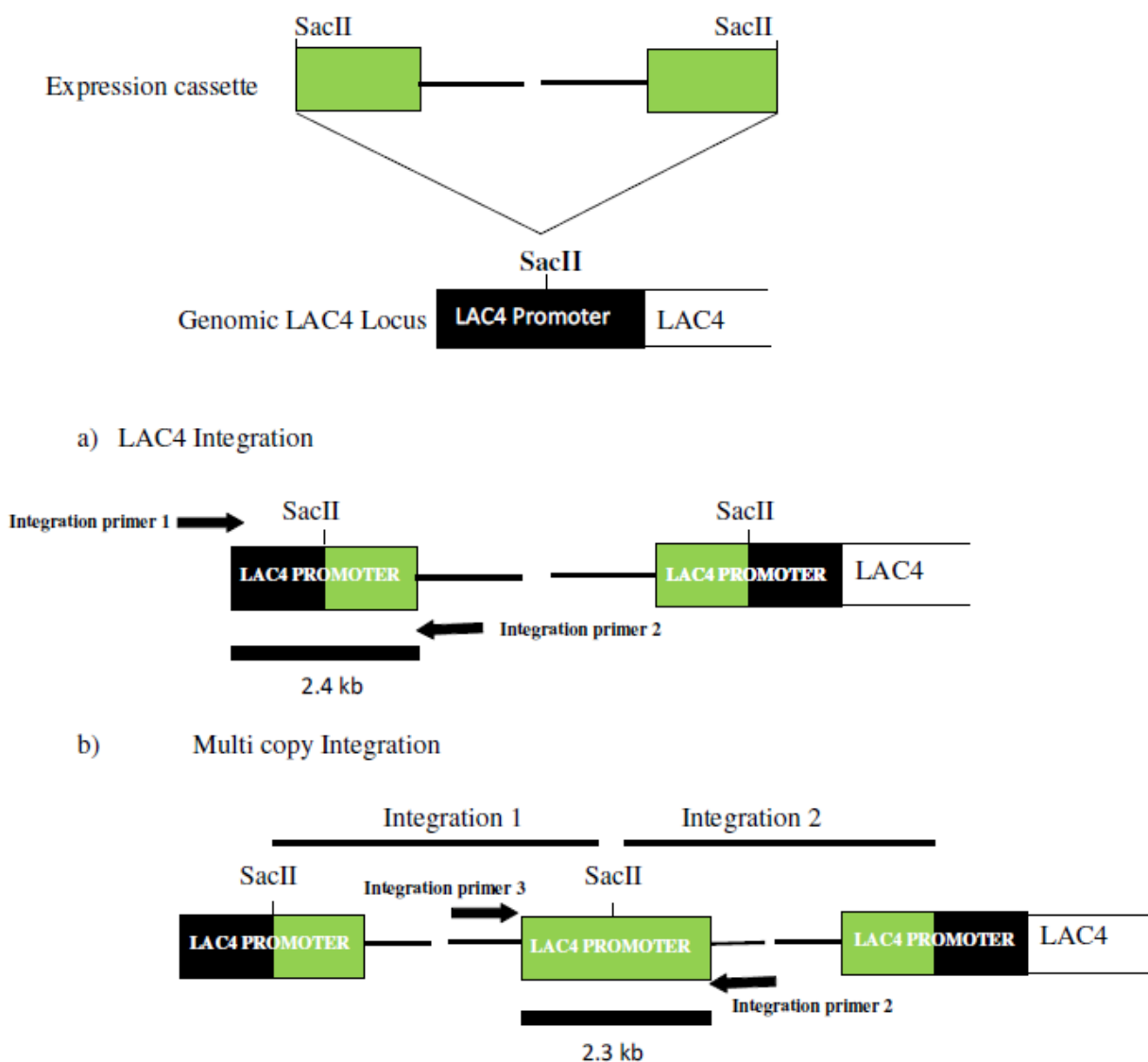


Figure 3.5: Strategy for the confirmation of single/multi copy integration of pVVDRP02 in *K. lactis* genome. [Upon transformation of *K. lactis* cells with the linearized expression cassette (figure 3.4), the fragment inserts into the promoter of the LAC4 locus. Single-copy integration at the LAC4 locus can be detected by PCR using Integration Primers 1 and 2 to amplify a 2.4 kb fragment (a), whereas multi-copy integration can be detected using Integration Primers 2 and 3 to amplify a 2.3 kb fragment (b)].

3.11.4 Identification and confirmation of multi-copy transformants

It is possible for up to ten copies of the expression cassette pVVDRP02 to get tandemly inserted into the genome during transformation (figure 3.5 b). *K. lactis* Strains harboring multiple integrations often produce more secreted protein. Multiply integrated cells were identified using whole-cell PCR with integration primers (Table 3.20) following the standard protocol supplied by the manufacturer.

Table 3.20: Integration primers used to identify single and multiple copies of pVVDRP02 integrated in *K. lactis* genome.

Integration Primer 1	5' ACACACGTAAACGCGCTCGGT 3'
Integration Primer 2	5' ATCATCCTTGTCAGCGAAAGC 3'
Integration Primer 3	5' ACCTGAAGATAGAGCTTCTAA 3'

3.12 Expression of recombinant IFN- γ

Constitutive expression of IFN- γ in *K. lactis* is driven by a mutant form of the strong *K. lactis* *LAC4* promoter ($P_{LAC4-PBI}$). Five *K. lactis* transformants with multiple copies of IFN- γ gene were selected for expression analysis. A single colony corresponding to each transformant was inoculated in 10 ml YPGAL medium and incubated at 30°C and 275 rpm.

3.12.1 Expression analysis of IFN- γ on SDS-PAGE

IFN- γ expression was checked on 12% SDS-PAGE gel (Shapiro et al. 1967) by loading 30 μ l of spent culture medium mixed with loading dye. Samples were incubated at 95° Celsius for 15 minutes to completely denature the IFN- γ . BSA served as the positive control and loaded in three wells in different quantity. Wild type *K. lactis* cells (without *ifng* gene) served as negative control. Gels were stained with Coomassie brilliant blue R250.

3.12.2 Silver staining of SDS-PAGE gel

Silver staining of SDS-PAGE gel was performed to detect IFN- γ . Staining was done using standard silver staining protocol (Sambrook et. al. 1989).

3.12.3 Enzyme linked immunosorbent assay (ELISA)

ELISA test was performed according to kit manufacturer's protocol using human IFN- γ specific primary antibody to detect and confirm the presence of IFN- γ . Five *K. lactis* transformants with multiple copies of pVVDRP02 selected for expression analysis were analyzed with ELISA assay for quantification of the secreted IFN- γ protein.

3.12.4 Western blot analysis of IFN- γ expressed in *K. lactis*

Confirmation of IFN- γ expressed in *K. lactis* GG799 strain was carried out by western blot experiment as described previously by Towbin et al. (1979). IFN-

γ expressed from clone 12 as secreted protein in fermentation broth was precipitated by TCA as described previously by Link & LaBaer 2011. 100 ml fermentation broth was precipitated and pellet was dissolved in 1 ml 1x PBS buffer. 30, 20 and 10 μ l samples were used to run a 1 mm SDS-PAGE gel. A wet transfer of proteins was performed at 20 volts for overnight to transfer all the proteins on a nitrocellulose membrane. A primary antibody directed against human IFN- γ raised in sheep was used to probe recombinant IFN- γ protein. An anti sheep HRP conjugated Secondary antibody was used to detect the presence of IFN- γ protein on nitrocellulose membrane.

3.12.5 Trichloro acetic acid precipitation of *K. lactis* culture broth

Trichloro acetic acid (TCA) was used for protein precipitation from the spent culture medium of clone 12 (Link & LaBaer 2011). 25 ml of 100% TCA was mixed with 100 ml of spent culture medium. Mixture was incubated for 10 minutes at 4°C followed by centrifugation for 5 minutes at 14,000 rpm. Supernatant was carefully removed and white fluffy protein pellet was washed with 200 μ l acetone and centrifuged again for 5 minutes at 14,000 rpm. This acetone washing step was repeated twice and pellet was dried at 95°C for 15 minutes to remove residual acetone. For SDS-PAGE pellet was mixed with 4X sample buffer, boiled at 95°C for 10 minutes and loaded in the 12% SDS gel.

3.13 Optimization of physical conditions for IFN- γ expression

As the protein was not visible on SDS-PAGE gels, ELISA assay was used to optimize protein expression conditions at shake flask level. YPLAC growth medium was used for the KL12:pVVDRP02 cell growth in all the experiments. All experiments were carried out in duplicate.

3.13.1 pH optimization

KL12:pVVDRP02 were grown at four different initial pH conditions 6.5, 7.0, 7.5, 8.0, at 30°C and 250 rpm. Samples were collected at regular interval, biomass was monitored by taking OD₆₀₀ nm. IFN- γ was quantified by ELISA.

3.13.2 Incubation temperature optimization

KL12:pVVDRP02 were grown at four different initial temperature conditions 25°C, 28°C, 30°C, 32°C at 7 pH and 250 rpm. Samples were collected at regular interval, biomass was monitored by taking OD₆₀₀ nm. IFN- γ was quantified by ELISA.

3.13.3 Agitation rate optimization

KL12:pVVDRP02 were grown at four different initial RPM conditions 200, 250, 275, 300 at 7 pH and 28°C. Samples were collected at regular interval, biomass was monitored by taking OD₆₀₀ nm. IFN- γ was quantified by ELISA.

3.13.4 Incubation time optimization

KL12:pVVDRP02 were grown at optimized conditions 7.0 pH, 28°C incubation temperature & 275 RPM at shake flask level for 84 hours. Samples were collected at 48th, 60th, 72nd and 84th hour, biomass was monitored by taking OD₆₀₀ nm. IFN- γ was quantified by ELISA.

3.13.5 Inoculum age optimization

KL12:pVVDRP02 were grown at optimized conditions 7.0 pH, 28°C incubation temperature & 250 RPM in three different shake flasks for 60 hours. All the three flasks were inoculated with inoculums of 12 hrs (early log phase), 18 hrs (log phase) and 32 hrs (stationary phase). Samples were collected at regular interval, biomass was monitored by taking OD₆₀₀ nm. IFN- γ was quantified by ELISA.

3.14 Production medium optimization

In order to optimize the medium for production of IFN- γ , Lactose was analyzed as a carbon source for the growth of K. lactis cells and further analyzed for substrate inhibition studies. Finally production medium was optimized using response surface methodology and artificial neural network algorithms.

3.14.1 Carbon source comparison

KL12:pVVDRP02 cells were grown in the presence of two different carbon sources. Since Galactose is a routinely used and very expensive carbon source,

similar yet cheaper alternative is required for the production of IFN- γ . Lactose (YPLAC) and Galactose (YPGAL) were compared for the IFN- γ production from KL12:pVVDRP02. Samples were collected at regular interval, biomass was measured by taking OD_{600nm}. IFN- γ was quantified by ELISA.

3.14.2 Substrate inhibition studies

Shaking flask level experiments were carried out to study the effect of concentration of lactose on the growth of *K. lactis* and IFN- γ production. 9 different levels of Lactose concentration were considered for the study (20, 40, 80, 100, 120, 140, 160, 200, 250 gm/litre). 9 flasks containing YPLAC medium were inoculated with KL12:pVVDRP02 seed culture and incubated at 28°C and 250 RPM for 96 hours. Samples were collected at regular intervals, biomass was monitored by taking OD_{600 nm}. IFN- γ was quantified by ELISA. Samples were analyzed by HPLC for substrate consumption rate & metabolic by-products like ethanol and acetate.

3.14.3 Response surface methodology

Production medium optimization is very important when it comes to recombinant biotherapeutic production where even minor improvements can be decisive for commercial success (Dasu & Panda, 2000; Sanjay et al., 2011). In this study, the improvement in production level of IFN- γ was achieved by increasing the biomass which in turn was achieved by optimizing nutritional parameters. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for experiment design, model building,

evaluating the effects of factors, and searching optimum levels of parameters for desirable responses (Myers, R. H., & Montgomery, D. C., 2002). This method has been successfully applied in many areas of biotechnology such as bioconversion of cheese whey to mycelia of *Ganoderma lucidum* (Lee et al., 2003), enzyme production (Bocchini et al., 2002), enzyme kinetics (Beg et al., 2003). Physical parameters for Human IFN- γ production from *E. coli* were optimized using Response surface methodology (Hernández et al., 2008). In this study the optimization of nutritional parameters for enhanced production of biomass was performed by Box-Behnken design (George Box & Donald Behnken, 1960) (Table 3.20). The optimal levels of nutritional parameters were verified at shake flask level. The three components of the complex medium viz. Lactose, yeast extract and trace elements were considered for the optimization process. The Box-Behnken design (Table 3.23) evaluated three independent variables, each one at three levels with three replicates (Table 3.22) to fit a second order polynomial model (Eq. 1)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \dots (1)$$

Where Y is the production of biomass, X_1 , X_2 , X_3 are independent variables for Lactose concentration, Yeast extract concentration and Trace elements concentration (Table 3.21) respectively. β_0 is the intercept term. β_1 , β_2 , β_3 are linear coefficients, β_{12} , β_{13} , β_{23} are interactive coefficients and β_{11} , β_{22} , β_{33} are quadratic coefficients. The model was evaluated with significance, good fit

and R^2 values. The analysis of RSM, analysis of variance (ANOVA) and the optimal conditions were performed using the statistical software package MINITAB® (Version 16.1 Minitab Inc., USA). The significant effects of dependent variables were determined by t -test with a probability value (P -value) smaller than 0.05.

Table 3.21: Trace elements composition

Component	Concentration
MgSO ₄ .7H ₂ O	1.69 g/l
Na ₂ SO ₄	0.18 g/l
ZnSO ₄ .6H ₂ O	0.24 g/l
MnSO ₄ .6H ₂ O	1 g/l
KH ₂ PO ₄	7.66 g/l

Table 3.22: Values of independent variables and the levels used in experimental design.

Symbol	Independent variables	Level			Units
		-1	0	+1	
X_1	Lactose	20	50	80	gm/l
X_2	Yeast extract	5	10	15	gm/l
X_3	Trace elements	5	10	20	gm/l

Table 3.23: Experimental design of Box-Behnken experiment. Levels of independent variables are given in coded form

Experiment No.	(X_1) Lactose (g/l)	(X_2) Yeast extract (g/l)	(X_3) Trace elements (g/l)
1	-1	-1	0
2	+1	-1	0
3	-1	+1	0
4	+1	+1	0
5	-1	0	-1
6	+1	0	-1
7	-1	0	+1
8	+1	0	+1
9	0	-1	-1
10	0	+1	-1
11	0	-1	+1
12	0	+1	+1
13	0	0	0
14	0	0	0
15	0	0	0

3.14.4 Artificial neural network algorithms

Statistical approaches are one of the best available methods for the optimization of factors affecting growth medium (Sen, 1997). There are a wide variety of such approaches available and equally good amount of literature is also available on medium optimization using these methods. These include experimental designs such as Plackett–Burman design (R.L. Plackett and J.P. Burman, 1946), Box-Behnken design (George Box & Donald Behnken, 1960), central composite design (Myers, Raymond H., 1971) and Taguchi design (Genichi Taguchi, 1962). But in recent times other mathematical approaches such as Artificial Neural Network (ANN) in conjunction with Genetic Algorithm (GA) and Simulated Annealing algorithm (SAA), are gaining popularity for optimization of process parameters and moreover they are proven to be superior to statistical approaches such as response surface methodology (Pal et al., 2009). Experimental multi-objective medium optimization was performed using a genetic algorithm which is supported by ANN. Typical artificial neural network architecture is depicted in figure 3.6. The ANN is used to model objective functions with the medium components as variables each time a new data set has been produced. ANNs are used commonly as “black box” models of key variables whose relationship to other process entities are neither formally described nor mathematically established, but are assumed to occur. Training is referred as the minimization of an error norm, which is usually the least squared criterion with respect to the output of the network and the desired output. Through this training procedure the parallel

processing units of the ANN (parameters) are adapted iteratively enabling thus function approximation. Validation is done while presenting the network a dataset not used for training and evaluating the system performance under this situation. Once the neural network was created, it was trained to accurately model the given phenomenon by using the experimental data (Table 2) in MATLAB version 8.2 (Mathworks Inc., Natick, US).

In this study the optimization of nutritional parameters for enhanced production of biomass (Nine layer ANN architecture for the prediction of biomass production is explained in figure 3.7) was performed by general full factorial design by analyzing three critical medium components. In the present study three neurons were used in the input layer, nine neurons in hidden layer and one neuron was used in the output layer for biomass optimization (figure 3.7). The full factorial central composite design (CCD) consisting of 27 experiments is given in Table 3.25. The range and levels of the three independent variables are listed in table 3.24. Optimization of *K. lactis* biomass production was performed by using ANN-GA and ANN-SAA based nonlinear modeling and optimization. The model was linked with GA and SAA to find the maximum production level and the optimum concentrations of the critical medium components that affect significantly the production process.

Table 3.24: Values of independent variables and the levels used in experimental design.

Symbol	Independent variables	Level			Units
		-1	0	+1	
X_1	Lactose	20	50	80	gm/l
X_2	Yeast extract	5	10	15	gm/l
X_3	Trace elements	5	10	20	gm/l

Table 3.25: The full factorial central composite design (CCD) consisting of 27 experiments. Levels of independent variables are given in coded form

Experiment No.	(X_1) Lactose (g/l)	(X_2) Yeast extract (g/l)	(X_3) Trace elements (g/l)
1	-1 (20)	-1 (5)	-1 (5)
2	-1 (20)	-1 (5)	0 (10)
3	-1 (20)	-1 (5)	+1 (20)
4	-1 (20)	0 (10)	-1 (5)
5	-1 (20)	0 (10)	0 (10)
6	-1 (20)	0 (10)	+1 (20)
7	-1 (20)	+1 (15)	-1 (5)
8	-1 (20)	+1 (15)	0 (10)
9	-1 (20)	+1 (15)	+1 (20)
10	0 (50)	-1 (5)	-1 (5)
11	0 (50)	-1 (5)	0 (10)
12	0 (50)	-1 (5)	+1 (20)
13	0 (50)	0 (10)	-1 (5)
14	0 (50)	0 (10)	0 (10)
15	0 (50)	0 (10)	+1 (20)
16	0 (50)	+1 (15)	-1 (5)
17	0 (50)	+1 (15)	0 (10)
18	0 (50)	+1 (15)	+1 (20)
19	+1 (80)	-1 (5)	-1 (5)
20	+1 (80)	-1 (5)	0 (10)
21	+1 (80)	-1 (5)	+1 (20)
22	+1 (80)	0 (10)	-1 (5)
23	+1 (80)	0 (10)	0 (10)
24	+1 (80)	0 (10)	+1 (20)
25	+1 (80)	+1 (15)	-1 (5)
26	+1 (80)	+1 (15)	0 (10)
27	+1 (80)	+1 (15)	+1 (20)

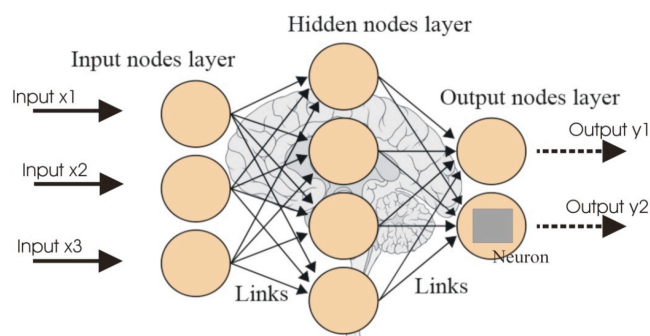


Figure 3.6: General artificial neural network architecture

Optimization of IFN- γ production was performed by using ANN-GA based nonlinear modeling and optimization (Five layer ANN architecture for the prediction of IFN- γ production is presented in figure 3.8). The model was linked with GA to find the maximum production level and the optimum concentrations of the critical medium components that affect significantly the production process. For IFN- γ production optimization study three neurons were used in the input layer, five neurons in hidden layer and one neuron was used in the output layer (Figure 3.8)

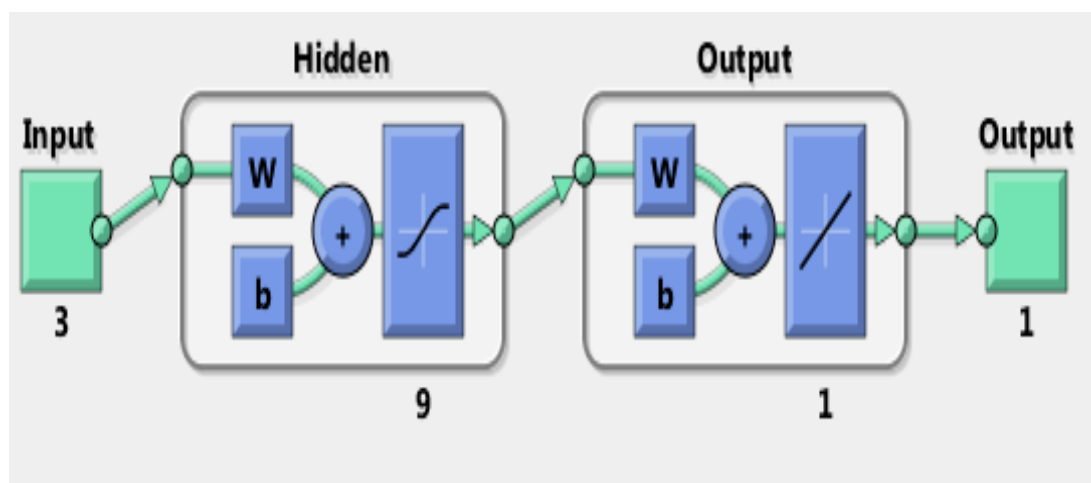


Figure 3.7: Nine layer ANN architecture for the prediction of biomass production

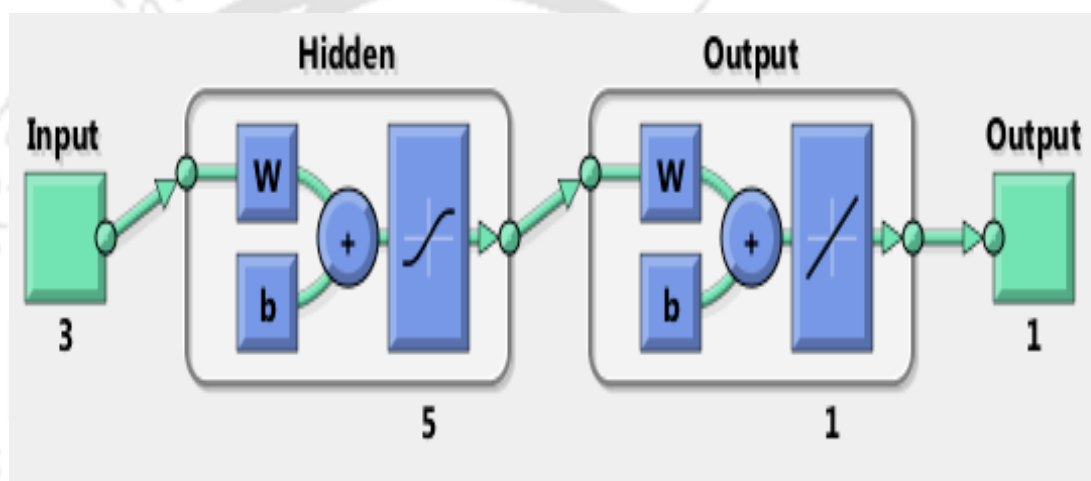


Figure 3.8: Five layer ANN architecture for the prediction of IFN- γ production

According to full factorial design, twenty-seven experiments were carried out in duplicate. Samples were collected at regular intervals. Biomass was analyzed by spectrophotometry at 600 nm. IFN- γ was quantified by ELISA. Maximum values of Biomass and IFN- γ for each experiment were fed to Genetic algorithm for model development.

3.15 Purification of IFN- γ

3.15.1 Iso-electric focusing

Iso-electric point of IFN- γ expressed in *K. lactis* fermentation broth was analyzed using immobilized pH gradient gel. *K. lactis* fermentation broth containing the IFN- γ was directly loaded onto the gel. All the proteins present in the broth were separated based on their pI.

3.15.2 Reverse micellar extraction

IFN- γ purification was performed using reverse micellar extraction (Figure 3.9). Process was optimized to maximize the yield of IFN- γ . Forward extraction was optimized using one variable at a time. Three parameters pH, CTAB & NaCl concentration were considered. Taguchi's method was employed to optimize Reverse extraction where three parameters: stripping phase pH, Iso propyl alcohol and KCl concentration were considered for optimization (Table 3.26).

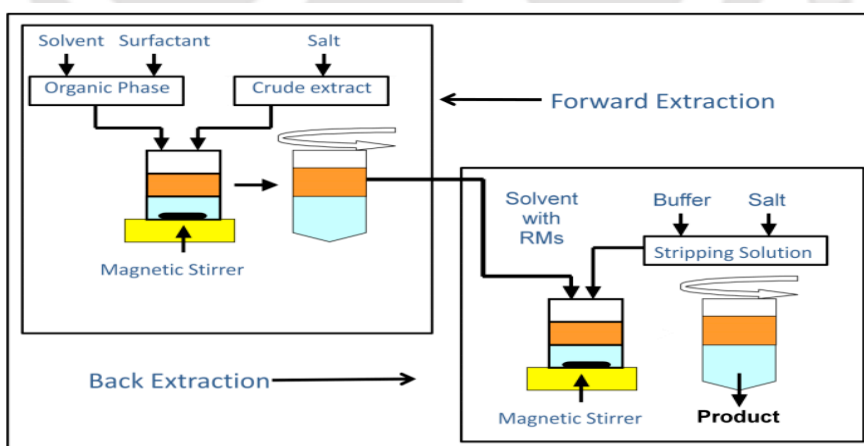


Figure 3.9: Schematic of the process of Reverse micellar extraction

Forward extraction was carried out with (CTAB/iso-octane with 10 % hexanol and 15 % n-butanol as co-solvents) with equal volume of aqueous phase (*K. lactis* fermentation broth) and known concentration of NaCl. Two solutions were allowed to mix for 45 min and were then separated by centrifugation at 5000 RPM for 15 min at 20°C. Back extraction experiment was carried out by mixing the organic phase obtained from forward extraction with a fresh aqueous phase, also termed as stripping phase (0.01 M phosphate buffer of known pH containing KCl). The phases were mixed thoroughly for 45 min and then centrifuged at 5000 RPM for 15 min at 20°C to obtain a complete phase separation.

IFN- γ concentration was determined using ELISA assay. Total Protein content in the aqueous phase was determined by Bradford method. Forward extraction optimization was carried out using OVAT (one variable at a time) with pH (9-12), CTAB concentration (50 mM-200mM) and NaCl concentration (0.05M-0.3M). The Back extraction optimization was carried out using Taguchi method.

Table 3.26: Parameters considered for back extraction optimization using Taguchi's method

Factors	Level 1	Level 2	Level 3	Units	Description
A	7	9	11	-	pH
B	0.3	0.5	0.8	Molar	Potassium chloride (KCl)
C	5	10	15	%	Iso-propyl alcohol (IPA)

3.16 IFN- γ production and fermentation in Bioreactor

Based upon the optimization data, production of IFN- γ was carried out in a four litre bench top bioreactor (Applikon Biotechnology) with a working volume of two litres. The batch culture was started by the addition of 200 mL of 18 hour aged incubated seed (KL12: pVVDRP02) culture ($OD_{600} = 3.0 \pm 0.5$) into bioreactor containing 1800 mL of optimized YPLAC medium. Total substrate concentration of lactose in the initial medium was 80 g/l. The pH was maintained at 7 by the addition of 1N HCl and 1N NaOH solution. Dissolved oxygen was controlled at 30-40% of air saturation by the control of both agitation rate and the inlet air. Foam was controlled by the addition of silicon-antifoaming reagent. Fermentation was carried out for 96 hours. Samples were collected at regular intervals, biomass was analyzed by spectrophotometry at 600 nm. IFN- γ was quantified by ELISA. Samples were analyzed by HPLC for substrate consumption rate, metabolites & metabolic by-products.



CHAPTER 4

RESULTS and DISCUSSION

4.1 Cloning and expression of IFN- γ in *E. coli* GalG20DE3 strain

Human *ifng* gene (501 bp) was successfully amplified from pUC19 vector using gene specific primers (Table 3.1). Temperature gradient PCR experiment amplified the human *ifng* gene at 5 different temperatures and 66°C was found to be the most suitable temperature for *ifng* amplification. An optimized PCR for *ifng* gene amplification was set up at 66°C (Fig. 4.1a and 4.1b).

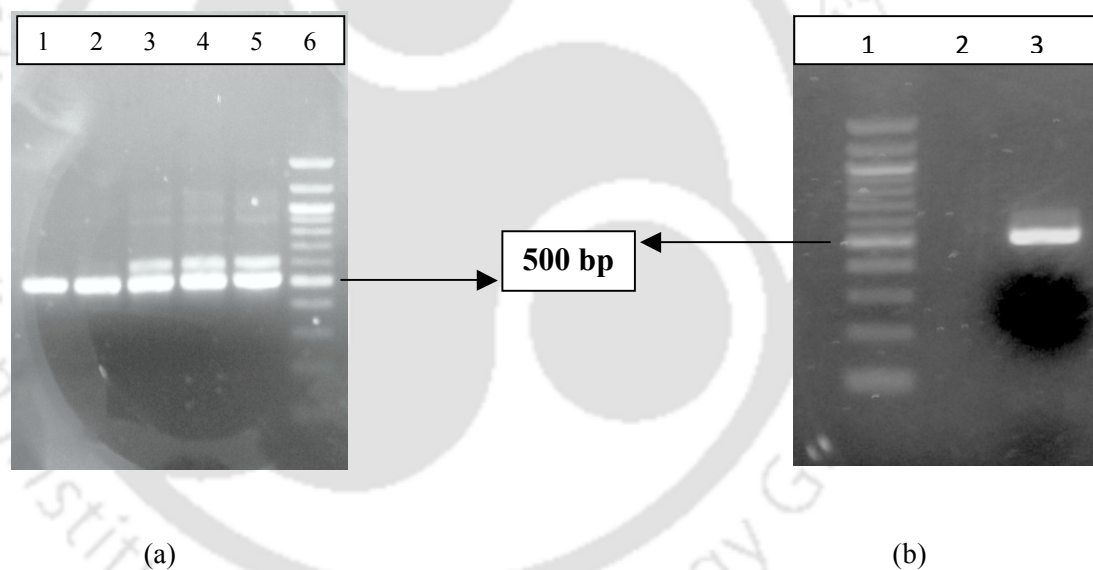


Figure 4.1: 501bp *ifng* amplification from *ifng* cDNA. (1a: Gradient PCR setup at different temperatures; Lane 1: 68°C, Lane 2: 66°C, Lane 3: 62°C, Lane 4: 58°C, Lane 5: 54°C, Lane 6: 100 bp NEB DNA ladder) (1b: Optimized PCR setup at 66°C; Lane 1: 100 bp NEB DNA ladder, Lane 2: Negative control, Lane 3: amplified *ifng* at 66°C).

ifng gene thus amplified was successfully digested (insert) with Nhe1 and Xho1 restriction enzymes to generate *ifng* insert with sticky ends. pET28a

vector was also successfully digested (vector) with the same enzymes. Insert and vector were successfully ligated and recombinant plasmid was named pVVDRP01. Recombinant plasmid was used to transform *E. coli* DH5 α . Single colonies were observed after an incubation period of 16 hours. In order to validate the cloning seven single colonies were randomly picked from the LB plate and subjected to colony PCR experiment. Colony PCR experiment detected all colonies to be positive for the presence of pVVDRP01 (Fig. 4.2).

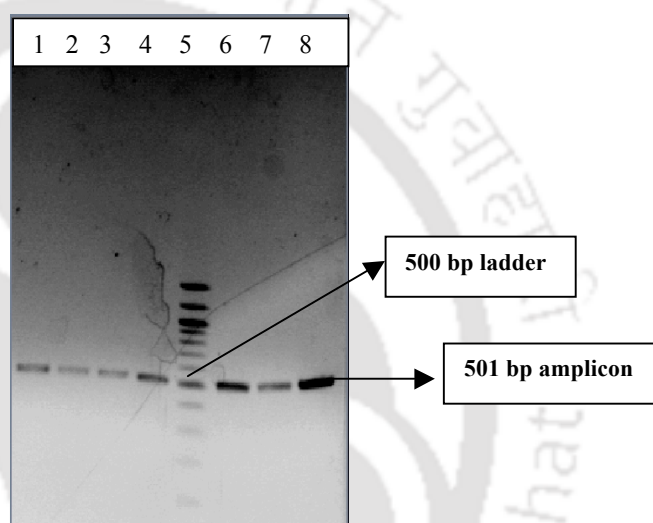


Figure 4.2: Colony PCR screening for positive clones of pVVDRP01 (Lane1: clone 1; Lane 2: clone 2; Lane 3: clone 3; Lane 4: clone 4; Lane 5: 100 bp NEB DNA ladder; Lane 6: clone 5; Lane 7: clone 6; Lane 8: clone 7).

To further validate the clones, four positive clones of pVVDRP01 were subjected to insert release check experiment. These four clones were enzymatically digested with NheI and XhoI restriction enzymes. All the clones successfully released the 501bp *ifng* insert (figure 4.3).

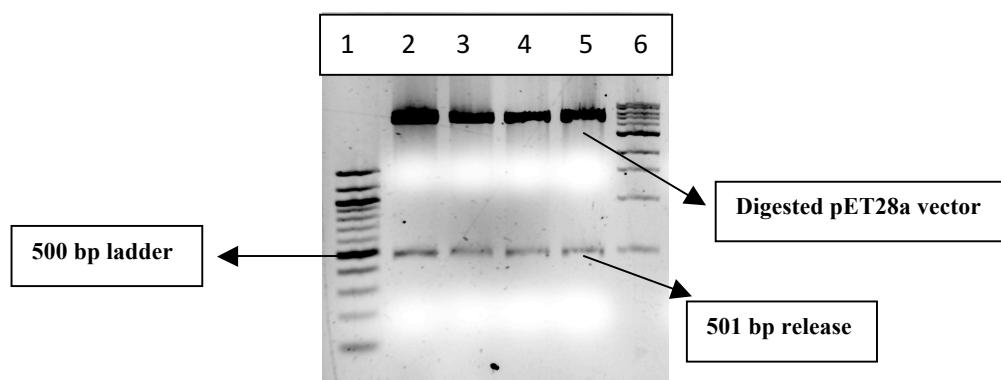


Figure 4.3: Clone confirmation by *Nhe*I and *Xho*I double digestion to release 501bp insert (Lane1: NEB low molecular wt. ladder; Lane 2: clone 2; Lane 3: clone 4; Lane 4: clone 5; Lane 5: clone 7; Lane 6: NEB high molecular wt. ladder).

Out of the four clones, two clones (no. 2 and 5) were selected for further IFN- γ expression studies. Clone no. 2 and 5 were also successfully confirmed for correct nucleotide sequence and open reading frame of *ifng* gene.

4.2 Development of a novel recombinant protein production host from *Δpgi* mutant *E. coli* GalG20, for production of IFN- γ

Although GalG20 was primarily metabolically engineered as a strain specialized for higher amounts of plasmid production, we hypothesized that it could be a hyper protein producer strain by the virtue of its enhanced plasmid producing capabilities. Lysogenization experiment was carried out to convert GalG20 cloning strain into a basic protein expression strain. *E. coli* strain GalG20 (MG1655 Δ endA Δ recA Δ pgi) strain was successfully lysogenized and verified by plaque formation technique. When λ DE3 phage, Helper Phage and Selection Phage were mixed with GalG20 strain and spread on LB plate, most of the surviving cells were GalG20DE3 lysogens.

4.3 IFN- γ expression from *E. coli* GalG20DE3, MG1655DE3 and BL21DE3 production hosts

IFN- γ was successfully expressed in GalG20DE3 (Figure 4.5 and 4.6), MG1655DE3 (Figure 4.7) and BL21DE3 (Figure 4.8) strain. Majority of the recombinant protein was getting expressed as insoluble inclusion bodies in all the three strains. IFN- γ was expressed as a 19 kDa protein. GALG20DE3 cells without pVVDRP01 plasmid which served as the negative control was also tested for IFN- γ expression and at the same location no protein band was observed (figure 4.5). GalG20DE3 (colony 1) produced 1.5 fold more IFN- γ as compared to BL21DE3 (colony 1) and 3.3 fold more as compared to MG1655DE3 (colony 2). In GalG20DE3 only 5%, in BL21DE3 only 3% and in MG1655DE3 28% of total IFN- γ protein was present in soluble form (Figure 4.4).

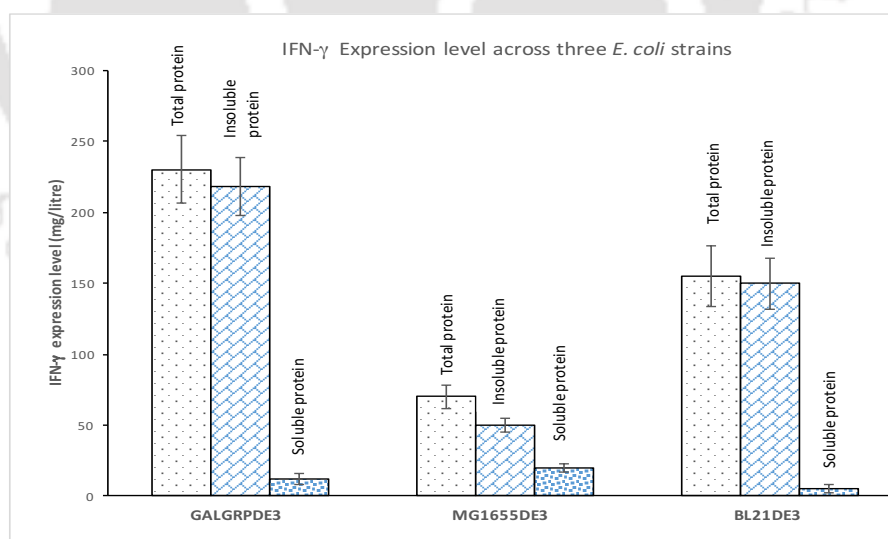


Figure 4.4: Production level of IFN- γ in three different *E. coli* strains.

In GalG20DE3 strain majority of IFN- γ was getting expressed in insoluble form i.e. forming inclusion bodies, soluble IFN- γ was almost negligible. The same case was observed with BL21DE3 strain. But in MG1655DE3 the amount of soluble IFN- γ was much higher as compared to other two strains. These findings suggest that due to high level of protein expression in GalG20DE3, the metabolic burden is very high and due to this IFN- γ is getting aggregated and forming inclusion body, similar pattern of lower fraction of soluble IFN- γ was observed in BL21DE3. Whereas in MG1655DE3 where metabolic burden is comparatively lower, amount of soluble IFN- γ is higher. These results clearly show that if expression conditions are properly optimized, GalG20DE3 can be developed into an excellent protein expression platform.

In order to validate the *ifng* cloning in pET28a followed by correct transformation of GalG20DE3 (Figure 4.5), BL21DE3 (data not shown) and MG1655DE3 (data not shown), colonies from the transformed population were screened for IFN- γ expression by inoculating single colony in 10 ml LB medium and expression was induced by 1 mM IPTG. In GalG20DE3 strain without pVVDRP01 expression cassette IFN- γ band was not observed, whereas in GalG20DE3 strain with pVVDRP01 expression cassette (GGVVDRP01), IFN- γ band of 19 kDa was clearly observed in insoluble fraction. Soluble fraction apparently did not contain the IFN- γ band.

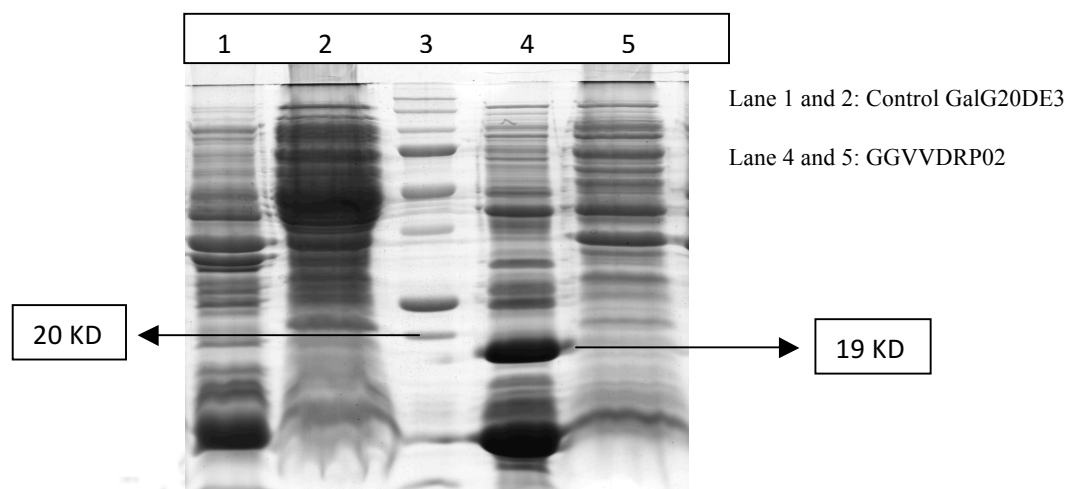


Figure 4.5: Expression profile of IFN- γ . Lane 1: Insoluble fraction, Lane 2: soluble fraction, Lane3: NZYBlue protein marker, Lane4: Insoluble fraction, Lane5: soluble fraction.

Two colonies from the transformed population of GalG20DE3 strain picked for expression analysis were grown in 10 ml LB medium, expression was induced by 1 mM IPTG. In GalG20DE3 strain successful IFN- γ expression was observed (figure 4.6). Although majority of the protein was getting aggregated and forming inclusion body, very minute amount of protein was visible in soluble fraction as well. Both the tested clones were found to be positive producer of IFN- γ .

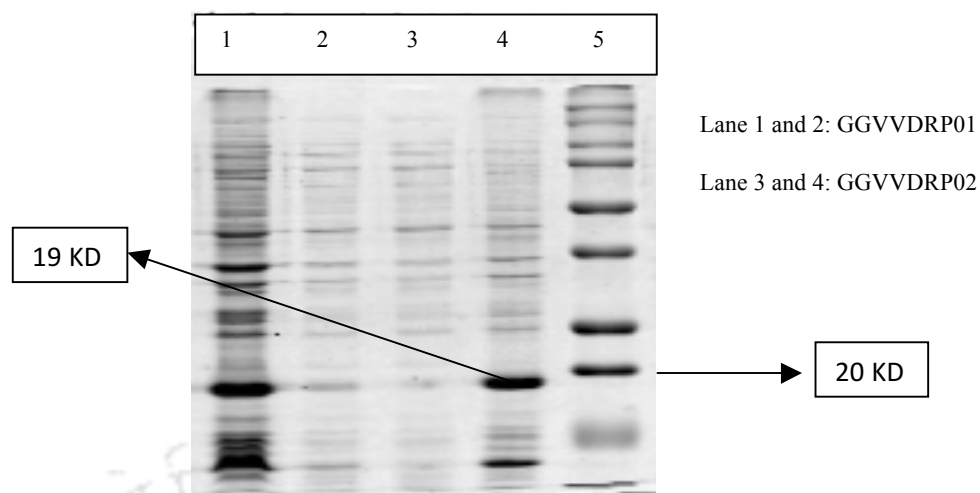


Figure 4.6: Expression profile of IFN- γ in GalG20DE3. Lane 1: Insoluble fraction of colony 1, Lane 2: soluble fraction of colony 1, Lane3: Insoluble fraction of colony 2, Lane4: soluble fraction of colony 2, Lane5: NZYBlue protein marker.++

Two colonies from the transformed population of MG1655DE3 strain picked for expression analysis were grown in 10 ml LB medium, expression was induced by 1 mM IPTG. Successful IFN- γ expression was observed in both the colonies (figure 4.7). IFN- γ expression profile was very similar to that of GalG20DE3 but with less amount of expressed protein as compared to GalG20DE3. Both the tested clones were found to be positive producer of IFN- γ . In MG1655DE3 strain amount of IFN- γ expressed as soluble protein was apparently much higher as compared to GalG20DE3 and BL21DE3 strains.

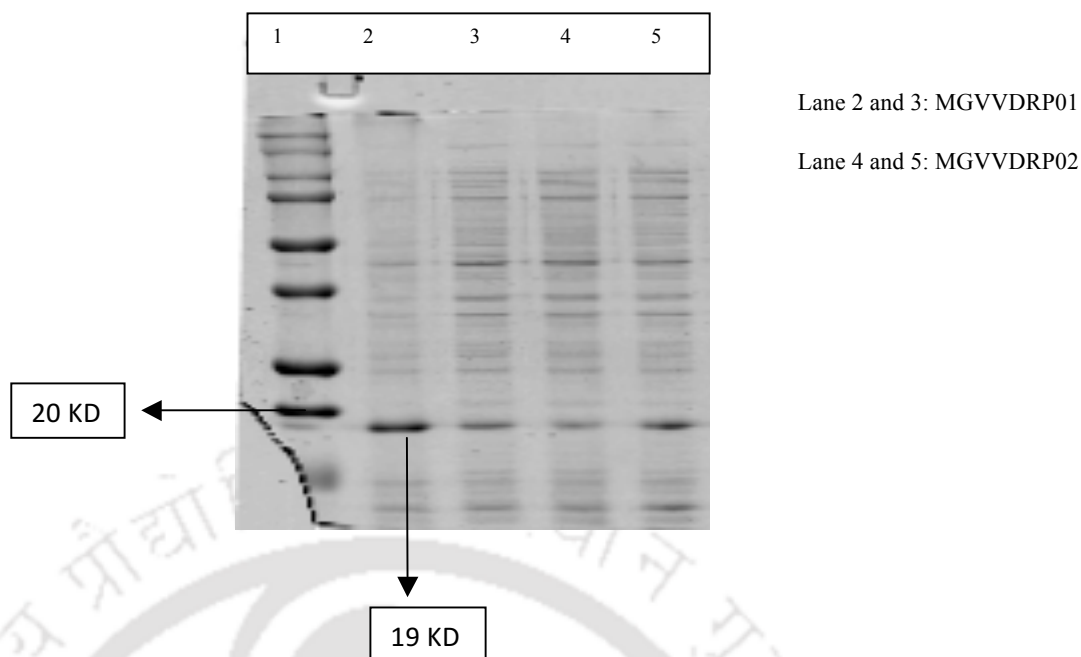


Figure 4.7: Expression profile of IFN- γ in MG1655DE3. Lane 1: NZYBlue protein marker, Lane 2: Insoluble fraction of colony 1, Lane3: soluble fraction of colony 1, Lane4: soluble fraction of colony 2, Lane5: insoluble fraction of colony 2.

Two colonies from the transformed population of BL21DE3 strain picked for expression analysis were grown in 10 ml LB medium, expression was induced by 1 mM IPTG. Successful IFN- γ expression was observed in both the colonies (figure 4.8). IFN- γ expression profile was very similar to that of other two strains. Both the tested clones were found to be positive producer of IFN- γ . In BL21DE3 strain almost all the IFN- γ was forming aggregates and apparently no protein was visible in the soluble fraction.

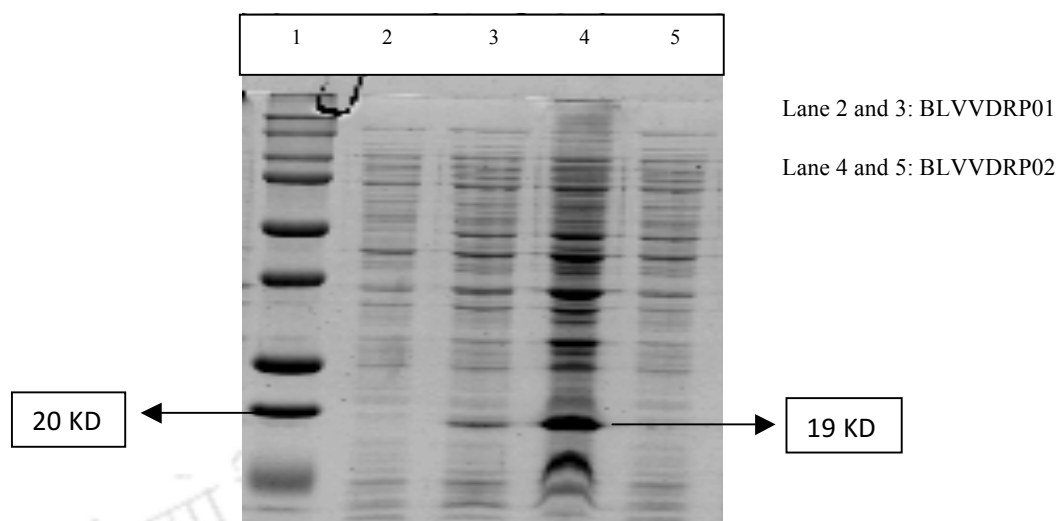


Figure 4.8: Expression profile of IFN- γ in BL21DE3. Lane 1: NZYBlue protein marker, Lane 2: soluble fraction of colony 1, Lane3: Insoluble fraction of colony 1, Lane4: insoluble fraction of colony 2, Lane5: soluble fraction of colony 2.

4.3.1 Western blot analysis of IFN- γ expressed in GGVVDRP, MGVVDRP and BLVVDRP cells

IFN- γ expressed in GGVVDRP, MGVVDRP and BLVVDRP in the form of inclusion body was denatured by 8M urea. Denaturation caused all the IFN- γ protein to reduce to its primary confirmation and because of the polyclonal antibody probing the protein became possible. Western blot confirmed the expression of IFN- γ from all three strains where highest amount of IFN- γ protein can be seen in GalG20DE3 followed by BL21DE3 and MG1655DE3.

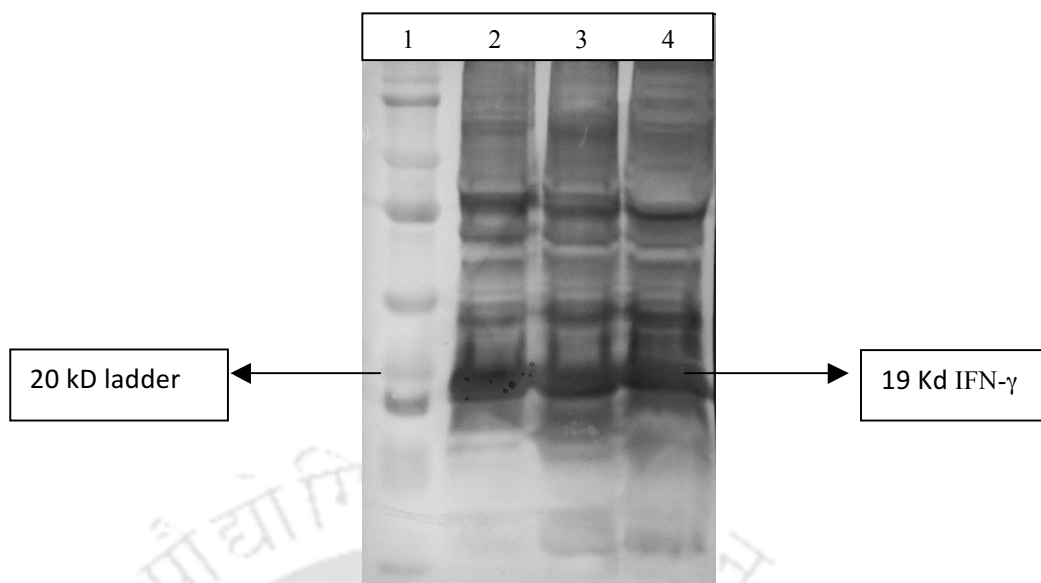


Figure 4.9: Western blot analysis of IFN- γ expressed in GGVVDRP, BLVVDRP and MGVVDRP strains. (lane 1: protein molecular ladder, lane 2: IFN- γ expressed in GGVVDRP, lane 3: IFN- γ expressed BLVVDRP, lane 4: IFN- γ expressed MGVVDRP)

4.3.2 Preparation of IFN- γ inclusion bodies

In order to assess the IFN- γ quantity expressed from GALG20DE3, MG1655DE3 and BL21DE3 cells, inclusion bodies were successfully solubilized and refolded to its native form using 8M Urea, 6M Guanidine HCL and 0.5 M Arginine. L-arginine mediated renaturation has been shown to be an effective method for renaturation of the recombinant proteins of human origin expressed in heterologous systems (Arora and Khanna, 1996). These arginine effects are apparently due to suppression of protein aggregation (Giardina et al. 2003; Engel and Wang 2009). Tsumoto et al. (2004) reviewed and showed that interactions between the guanidinium group of arginine and tryptophan side chains may be responsible for suppression of protein aggregation by arginine.

4.3.3 IFN- γ estimation

ELISA assay was used to estimate the total amount of IFN- γ expressed in GalG20DE3, MG1655DE3 and BL21DE3. GalG20DE3 expressed total of 230 mg/litre of IFN- γ , MG1655DE3 expressed 70 mg/litre of IFN- γ , whereas BL21DE3 expressed 155 mg/litre of IFN- γ (Figure 4.4). As mentioned previously in this report, IFN- γ was getting expressed as Inclusion body. For protein estimation both soluble and insoluble fractions were considered. Inclusion bodies were denatured, renatured and refolded back to its native form and protein estimation was carried out by ELISA assay. Similar ELISA assay was performed for the estimation of protein present in soluble fraction. Both the fractions together constituted the total amount of IFN- γ being produced by GalG20DE3, MG1655DE3 and BL21DE3.

It is clearly evident from the results obtained with *E. coli* expression system, IFN- γ expression level was very good. Although IFN- γ was getting produced in large quantities, 95% of IFN- γ was forming inclusion bodies in GalG20DE3. In case of MG1655DE3 also IFN- γ was getting expressed as Inclusion body but the amount of soluble form of IFN- γ was 6 fold higher as compared to GalG20DE3. Converting the insoluble inclusion body to soluble protein fraction required lots of efforts and time. Although at lab scale this issue does not seem significant but later on when the process will reach Pilot or higher scales, overall cost of producing IFN- γ will increase. The major objective of the present study is to produce IFN- γ in a cost effective manner, means the cost of end product should not be high and well within reach of

majority of Indian population. Producing IFN- γ in *E. coli* expression system does not seem to justify this objective. Moreover non GRAS status of *E. coli* strains can cause regulatory issues at the time of product approval from competent authorities. *E. coli* strains are well known for producing endotoxins, which is an exogenous pyrogen (external fever-inducing substance). For recombinant products produced from bacterial sources, LAL assay must be conducted in order to prove the product safe for human use. Conducting LAL assay would further increase the cost of production of IFN- γ . Keeping in view all the factors stated above, an expression system was explored which fits all the parameters. *Kluyveromyces lactis* is such a system; it is an eukaryotic expression system, means IFN- γ can safely be produced in soluble form. GRAS status of this organism paves the easy way for approval from regulatory authorities. It does not produce endotoxins (Walker, 1998). Moreover *Kluyveromyces lactis* has the ability to secrete the heterologous protein in the outside spent culture medium, thereby reducing the downstream processing cost of product drastically. Hence we decided to use *Kluyveromyces lactis* as the expression system for IFN- γ production.

4.4 Cloning and expression of IFN- γ in *Kluyveromyces lactis*

4.4.1 Cloning of human *ifng* gene in *Kluyveromyces lactis*

Human *ifng* gene (435 bp) was successfully amplified from pUC19 vector using gene specific primers (Table 3.11). Temperature gradient PCR experiment amplified the human *ifng* gene at five different temperatures, although *ifng* amplification was observed at all the temperatures but due to high visual intensity of DNA band (Figure 4.9), 61°C was found to be the most suitable temperature for *ifng* gene amplification. A final PCR for *ifng* amplification was set up at 61°C and *ifng* thus amplified was used for all subsequent cloning experiments.

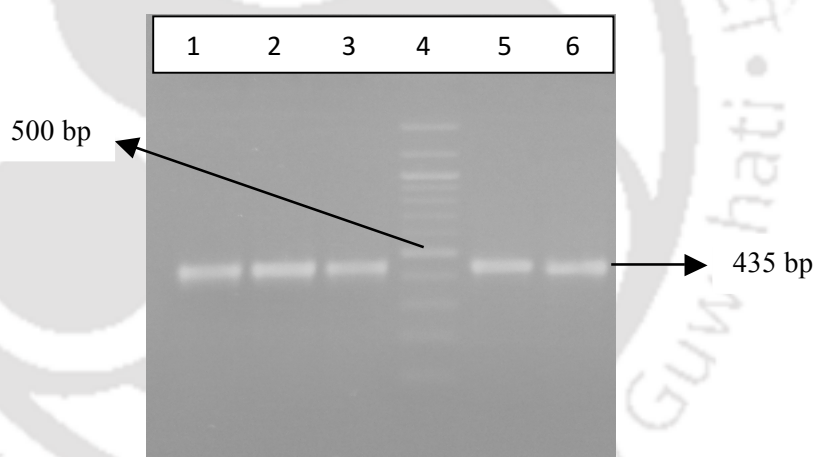


Figure 4.10: Gradient PCR setup for *ifng* gene amplification from pUC19 vector (Lane 1: 58°C, Lane 2: 61°C, Lane 3: 63°C, Lane 4: NEB 100 bp DNA ladder, Lane 5: 65°C, Lane 6: 68°C).

ifng thus amplified was enzymatically digested with restriction enzymes XhoI and BamHI. pKLAC2 vector was also digested successfully with XhoI and BamHI restriction enzymes. Digested *ifng* and pKLAC2 were successfully

ligated with each other to generate pVVDRP02 recombinant plasmid (figure 4.10 and 4.11).

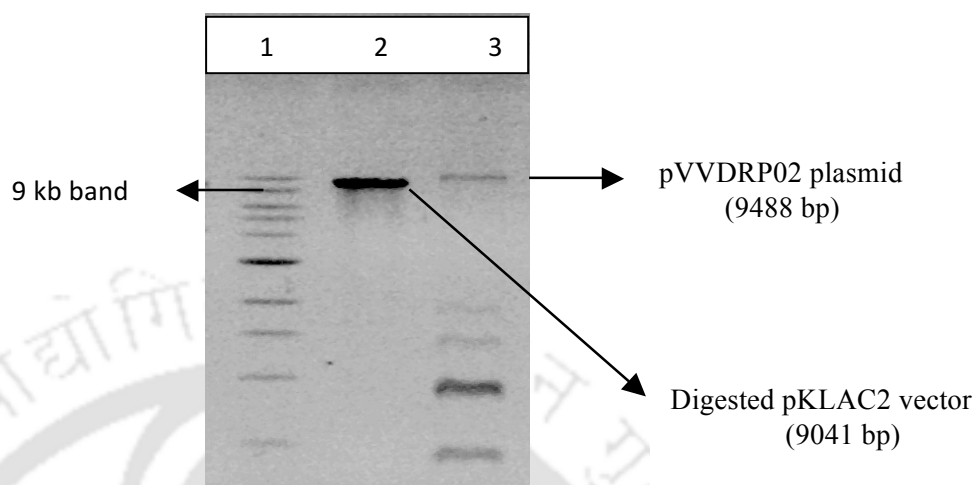


Figure 4.11: pVVDRP02 plasmid and digested pKLAC2 vector (Lane 1: 1 kb DNA ladder; Lane 2: Digested pKLAC2 vector; Lane 3: pVVDRP02 plasmid).

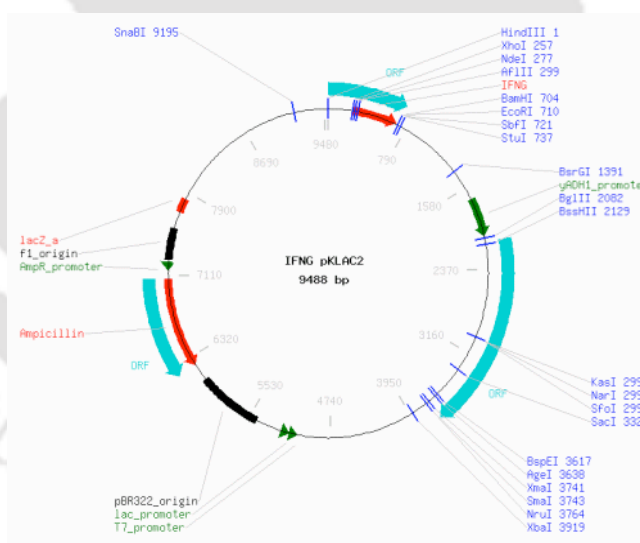


Figure 4.12: pVVDRP02 plasmid showing all the open reading frames and restriction sites.

E. coli DH5 α competent cells were chemically transformed with this resulting recombinant plasmid and plated on LB plates. LB plates were incubated at 37°C for 16 hours. Several single colonies were observed at the end of the incubation period.

In order to validate the cloning procedure four single colonies were randomly picked from the LB plate and subjected to colony PCR experiment with *ifng* gene specific primers. Colony PCR experiment detected all colonies to be positive clones of pVVDRP02 (Figure 4.12).

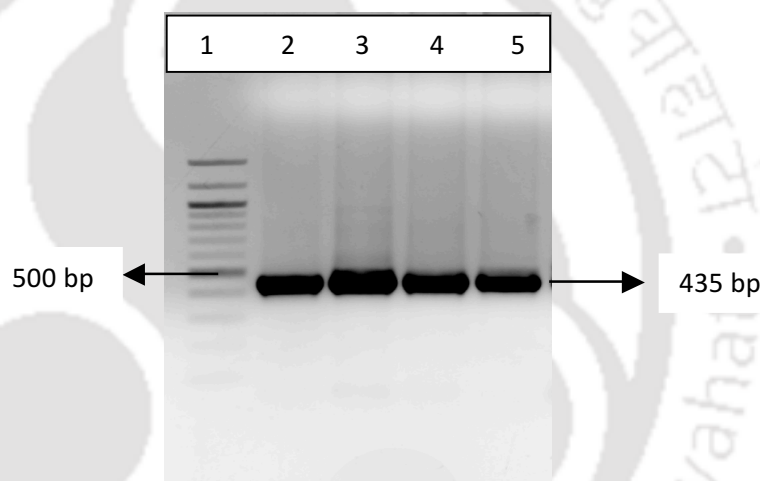


Figure 4.13: Colony PCR screening for positive clones of pVVDRP02 (Lane1: NEB high range DNA ladder; Lane 2: clone 1; Lane 3: clone 2; Lane 4: clone 3; Lane 5: clone 4).

To further validate the clones, four positive clones of pVVDRP02 were subjected to insert release check experiment. These four clones were enzymatically digested with XhoI and BamHI restriction enzymes. All the clones successfully released the 435bp *ifng* insert (figure 4.13).

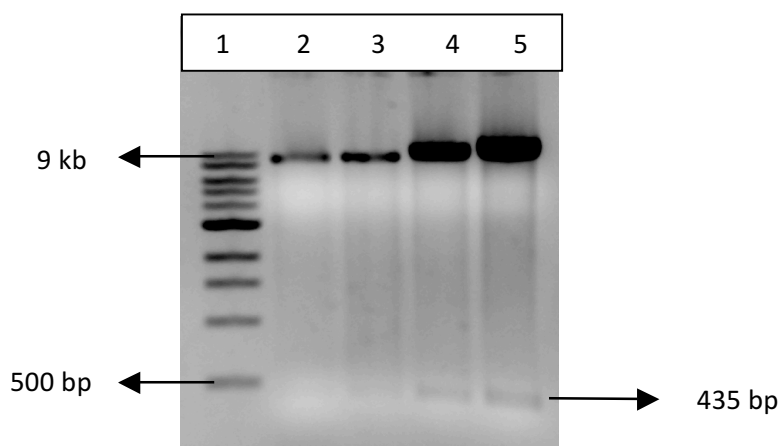


Figure 4.14: Clone confirmation by XhoI and BamHI double digestion to release 435 bp insert (Lane1: NEB high range DNA ladder; Lane 2: clone 1; Lane 3: clone 2; Lane 4: clone 3; Lane 5: clone 4).

Based on the results of double digestion experiment with XhoI and BamHI, all four pVVDRP02 clones were selected for nucleotide sequencing and open reading frame confirmation. All four clones were found to be correctly cloned in pKLAC2 with correct open reading frame.

In order to transform and integrate the recombinant pVVDRP02 expression cassette in *Kluyveromyces lactis* GG799 genome, all four clones were linearized with SacII restriction enzyme (Figure 4.14) which generated two DNA strands of 6661 bp and 2827 bp.

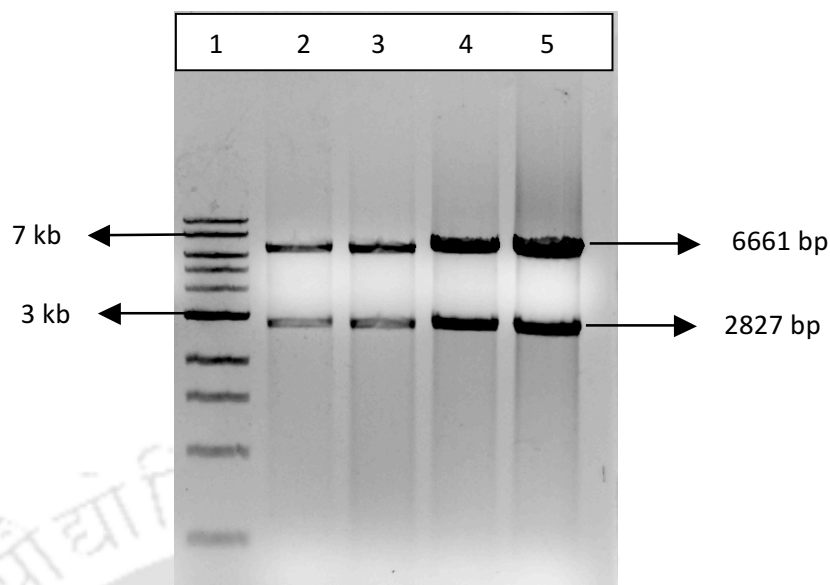


Figure 4.15: Linearization of pVVDRP02 plasmids with SacII restriction enzyme (Lane1: NEB high range DNA ladder; Lane 2: clone 1; Lane 3: clone 2; Lane 4: clone 3; Lane 5: clone 4).

4.4.2 Confirmation of proper integration of the pVVDRP02 in the genome of the transformed *K. lactis* clones

Transformants in which the pVVDRP02 expression cassette has correctly integrated into the *K. lactis* genome were identified by PCR using integration primers (Table 3.20) to yield a 2.4 kb PCR product. Total of 12 transformants (clones) were analyzed. Figure 4.16 clearly explains that clone no. 2, 4, 6, 8, 10 and 12 were found to be positive transformants of pVVDRP02 expression cassette.

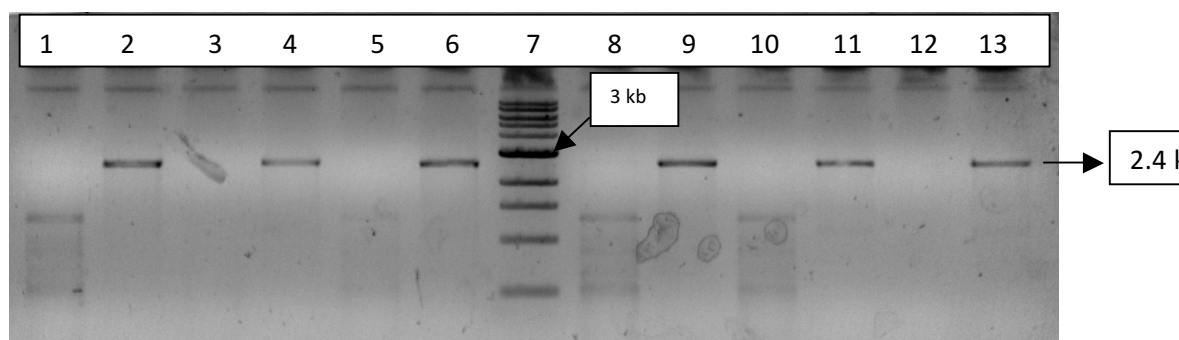


Figure 4.16: PCR amplification of *K. lactis* clones for identification of integrated pVVDRP02 expression cassette(Lane 1: clone 1; Lane 2: clone 2; Lane 3: clone 3; Lane 4: clone 4; Lane 5: clone 5; Lane 6: clone 6; Lane 7: NEB high range DNA ladder; Lane 8: clone 7; Lane 9: clone 8; Lane 10: clone 9; Lane 11: clone 10; Lane 12: clone 11; Lane 13: clone 12).

4.4.3 Identification and confirmation of multi-copy transformants

Multiply integrated cells were identified using whole-cell PCR with integration primers (Table 3.20) to yield a 2.3 kb PCR product (figure 4.17). Based on the results of this experiment clone no. 2, 6, 8, 10 and 12 were selected for IFN- γ expression analysis. Underlying mechanism behind identification of single and multi copies of transformants is explained in figure 3.5, page 70.

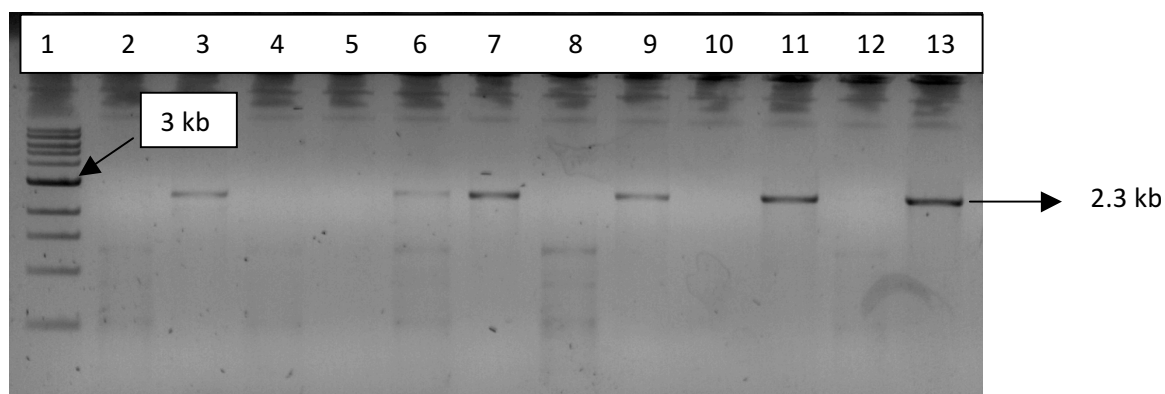


Figure 4.17: PCR amplification of *K. lactis* clones for identification of multiple integrated pVVDRP02 expression cassette. (Lane 1: NEB high range DNA ladder; Lane 2: clone 1; Lane 3: clone 2; Lane 4: clone 3; Lane 5: clone 4; Lane 6: clone 5; Lane 7: clone 6; Lane 8: clone 7; Lane 9: clone 8; Lane 10: clone 9; Lane 11: clone 10; Lane 12: clone 11; Lane 13: clone 12)

Interestingly the transformants in the first PCR experiment found to contain pVVDRP02 expression cassette, were also found positive in the second PCR experiment as they contain multiple copies of pVVDRP02 expression cassette. Only exception was transformant 4 which was positive in first but failed in second PCR experiment. This may be due to PCR error or absence of multiple copies of pVVDRP02 expression cassette. Transformant 5 also showed 2.3 kb band confirming the presence of multiple copies of pVVDRP02 expression cassette but rejected for further analysis due to multiple bands present in the PCR profile which was consistent with other negative transformants.

4.5 Expression of recombinant human Interferon gamma (IFN- γ)

4.5.1 Expression analysis of IFN- γ on SDS-PAGE

5 colonies (2, 6, 8, 10, 12) selected for IFN- γ expression, were analyzed by SDS-PAGE did not show any protein band (Figure 4.17). *K. lactis* cells not having the *ifng* gene serving as negative control did not show the protein band as well. As a matter of fact, there was no significant and visible difference between the expression profiles of *K. lactis* cells (with and without *ifng* gene). BSA served its purpose very well and a protein band of 66 kd consistent with the size of BSA was visible on the SDS-PAGE gel.

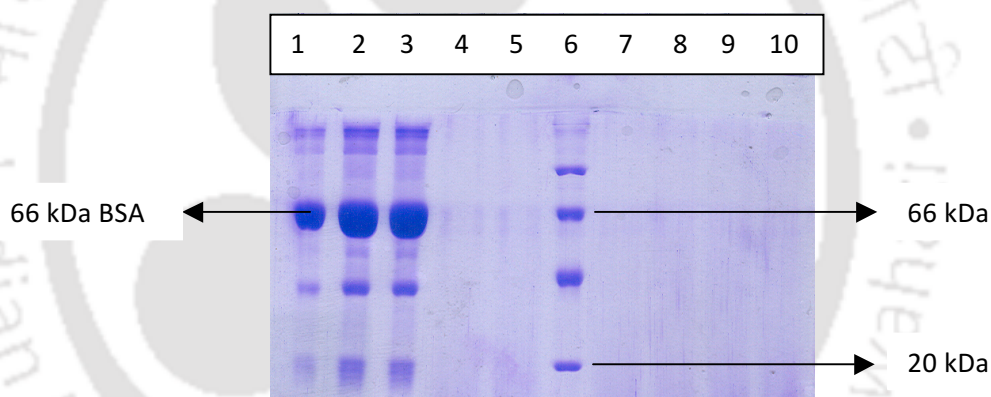


Figure 4.18: SDS PAGE gel of crude supernatant. Lane 1: BSA (0.1 mg/ml), Lane 2: BSA (0.5 mg/ml), Lane3: BSA (1.0 mg.ml), Lane4: clone 2, Lane5: clone 6, Lane 6: Bangalore genei protein ladder, Lane7: clone 8, Lane 8: clone 10, Lane 9: Clone 12, Lane 10: *K. lactis* cells without pVVDRP02.

4.5.2 Silver staining of SDS-PAGE gel

All the 5 clones previously examined with SDS-PAGE gel were used for this experiment. IFN- γ expression analyzed by silver staining also did not produce any conclusive results, as no IFN- γ bands were visible on the SDS-PAGE gel (Figure 4.18).

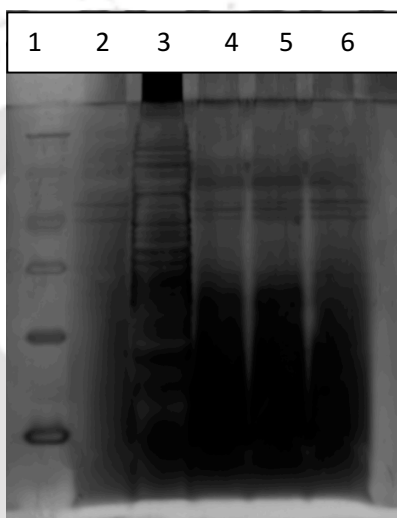


Figure 4.19: Silver staining of SDS PAGE gel. Lane 1: Bangalore genei protein ladder, Lane 2: clone 2, Lane 3: clone 6, Lane 4: clone 8, Lane 5: clone 10, Lane 6: clone 12.

4.5.3 Enzyme linked immunosorbent assay (ELISA) of IFN- γ

Human IFN- γ specific ELISA assay established the IFN- γ expression from *K. lactis* cells. All the five clones were found to secrete the IFN- γ . Manufacturer supplied human IFN- γ protein was used to plot standard curve (Figure 4.19) and R^2 value of 0.998 established the correctness of the test. Unknown quantities of IFN- γ from 5 clones was calculated (Table 4.1) by plotting the absorbance values of unknown on the standard curve.

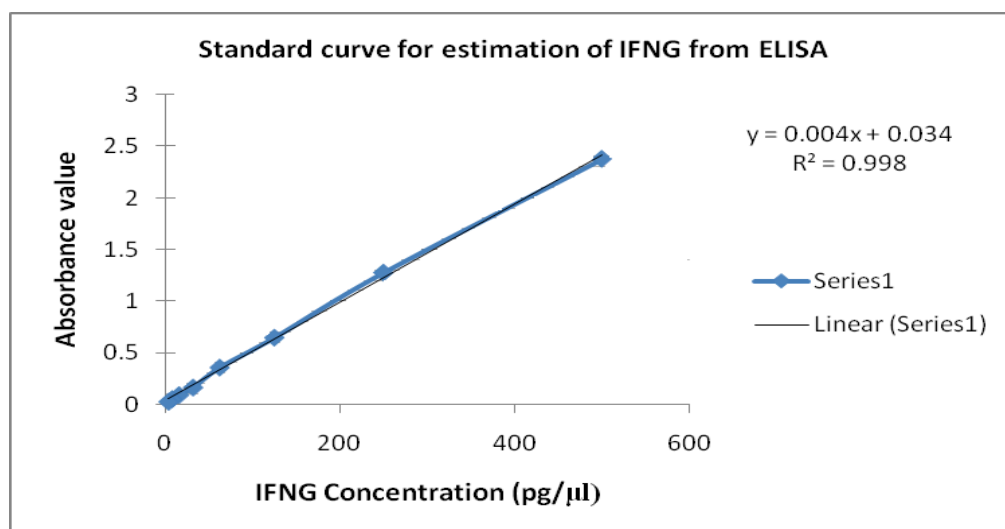


Figure 4.20: Standard curve of IFN- γ

Table 4.1: Comparative analysis of 5 clones of KL:pVVDRP02 for IFN- γ expression.

S. No.	Clone no.	Mean Absorbance value	Corresponding concentration ($\mu\text{g/litre}$)
1	2	0.4712 ± 0.023	109.3 ± 5.33
2	6	0.1279 ± 0.03	23.475 ± 5.50
3	8	0.5676 ± 0.034	133.4 ± 7.96
4	10	0.6070 ± 0.03	143.25 ± 7.06
5	12	0.7342 ± 0.044	175.05 ± 10.5

Clone 12 was selected for all future experiments due to highest production level among 5 transformants. Clone 12 was called as KL12:pVVDRP02.

4.5.4 Western blot analysis of IFN- γ expressed in *K. lactis*

100 ml TCA precipitated *K. lactis* fermentation broth was used to analyze presence of IFN- γ protein band on a nitrocellulose membrane. A band of 22 kD can be visualized on nitrocellulose membrane probed by human IFN- γ specific primary antibody. This experiment confirmed the presence of IFN- γ protein in *K. lactis* fermentation broth.

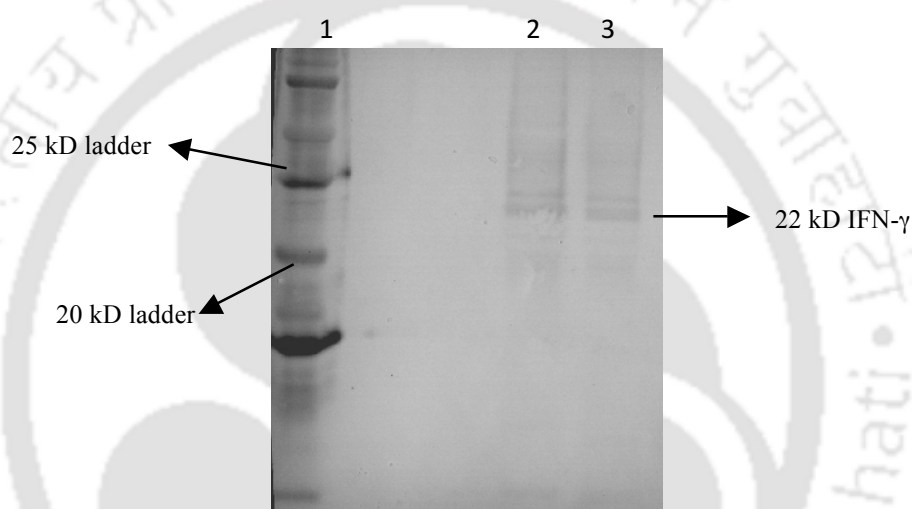


Figure 4.21: Western blot analysis of IFN- γ secreted in *K. lactis* fermentation broth. Lane 1: biobasic low range protein ladder, Lane 2: 30 μ l sample, Lane 3: 20 μ l sample.

4.5.5 Trichloro acetic acid (TCA) precipitation of *K. lactis* culture broth

KL12:pVVDRP02 was analyzed with TCA precipitation method to check the IFN- γ protein band on SDS-PAGE. 100 ml of fermentation broth of clone 12 was used for the experiment. Samples were loaded onto 1mm SDS-PAGE gel

in three different quantities of 30, 20 and 10 μ l. Analysis revealed the presence of a 22 kDa band consistent with the IFN- γ molecular weight of 22 kDa (figure 4.20) (glycosylated version expressed in a eukaryotic system).

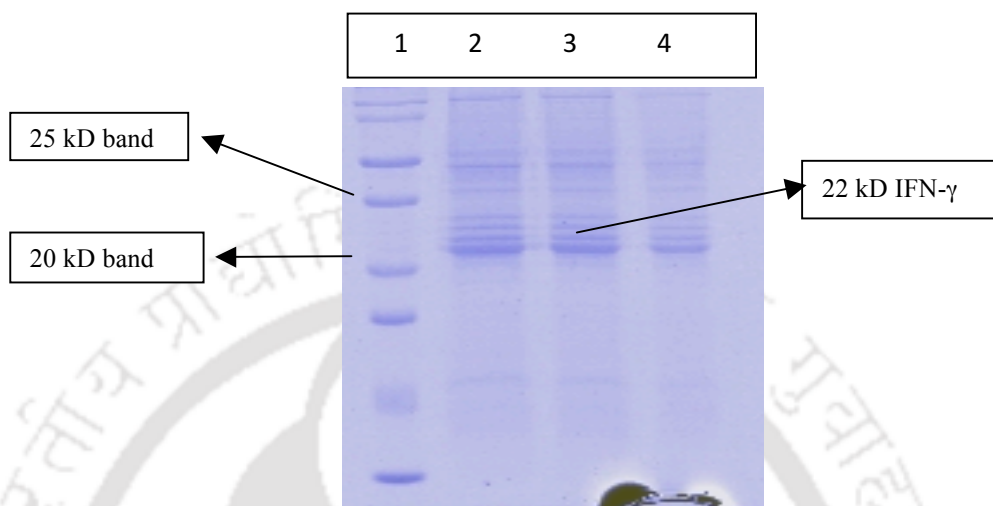


Figure 4.22: SDS PAGE gel of KL12:pVVDRP02 fermentation broth precipitated with TCA.

Lane 1: biobasic low range protein ladder, Lane 2: 30 μ l sample, Lane 3: 20 μ l sample, lane 4: 10 μ l sample.

4.6 Optimization of physical conditions for IFN- γ expression

KL12:pVVDRP02 was grown in YPGAL medium to optimize pH, incubation temperature, rpm, incubation time and age of inoculum. All experiments were carried out in triplicate. Results of optimization of all parameters are presented in figure 4.21.

4.6.1 pH optimization

KL12:pVVDRP02 was grown at four different initial pH conditions 6.5, 7.0, 7.5, 8.0, at 30 °C and 250 rpm. pH 7 was found to be the most suitable for IFN- γ production. pH 6.5 was found to produce 130 ± 6 μ g/litre of IFN- γ ,

whereas in the medium of pH 7, IFN- γ was found to be produced at a level of 200 ± 8.9 $\mu\text{g/litre}$. pH 7.5 produced IFN- γ at a slightly lower concentration as compared to pH 7 with 175 ± 9 $\mu\text{g/litre}$. Till now all expression experiments were carried out at pH 7.5 and 175 ± 10.5 was the expression level obtained for KL12:pVVDRP02 in the initial ELISA experiments. At pH 8, again expression level of IFN- γ reduced drastically and dropped to 140 ± 6 $\mu\text{g/litre}$. These results depicted in figure 4.21, clearly indicate that pH plays a very important role in the expression of IFN- γ .

4.6.2 Incubation temperature optimization

KL12:pVVDRP02 was grown at four different initial temperature conditions 25°C , 28°C , 30°C , 32°C at 7 pH and 250 rpm. 28°C was found to be the optimum temperature for IFN- γ production. At 25°C IFN- γ was produced at a level of 160 ± 7 $\mu\text{g/litre}$ and at 28°C expression level was highest at 220 ± 9 $\mu\text{g/litre}$ for IFN- γ . At 30°C expression reduced to 180 ± 6 $\mu\text{g/litre}$ of IFN- γ and at 32°C it further reduced to 170 ± 5 $\mu\text{g/litre}$. But it was clearly evident that with optimized pH, expression level in all the four different temperatures increased.

4.6.3 RPM optimization

KL12:pVVDRP02 was grown at four different initial rpm conditions 200, 250, 275, 300 at 7 pH and 28°C . At 200 rpm IFN- γ expression level was found to be 140 ± 5.5 $\mu\text{g/litre}$ and at 250 rpm it was found to be highest at 300 ± 8.5 $\mu\text{g/litre}$. At 275 rpm expression reduced to 260 ± 7.5 $\mu\text{g/litre}$ and at 300 rpm it

even more reduced to 230 ± 7 $\mu\text{g/litre}$ of IFN- γ . These results clearly indicate that 250 rpm is best for IFN- γ expression from KL12:pVVDRP02.

4.6.4 Incubation time optimization

KL12:pVVDRP02 was grown at optimized conditions 7.0 pH, 28°C incubation temperature and 250 RPM in the shaking flask level for four different durations of 48, 60, 72 and 84 hours. At 48 hours IFN- γ expression level was found to be 280 ± 9 $\mu\text{g/litre}$ and at 60 hours it was found to be highest at 370 ± 13.5 $\mu\text{g/litre}$, which was highest. At 72 hours expression level was reduced to 300 ± 13 $\mu\text{g/litre}$ and at 84 hours expression level was found to be lowest at 180 ± 9 $\mu\text{g/litre}$ of IFN- γ . Although biomass continued to increase till 72 hours, for production of IFN- γ , 60 hours was found to be the optimum time for fermentation.

4.6.5 Inoculum age optimization

KL12:pVVDRP02 was grown at optimized conditions 7.0 pH, 28°C incubation temperature and 250 RPM in three different shake flasks for 60 hours. All the 3 flasks were inoculated with inoculums of 12 hrs (early log phase), 18 hrs (log phase), 24 hours (mid log phase) and 30 hrs (stationary phase). Among the three different stages of inoculums used for the study, the 18 hour old inoculum was found to give the best results with 380 ± 12 $\mu\text{g/litre}$ of IFN- γ secreted in the culture medium. 12 hours old inoculum when used, KL12:pVVDRP02 was found to secrete 250 ± 9 $\mu\text{g/litre}$ of IFN- γ , whereas with

24 and 30 hour old inoculum, 300 ± 10 and 190 ± 8.5 $\mu\text{g/litre}$ of IFN- γ was found to be secreted in the culture medium respectively.

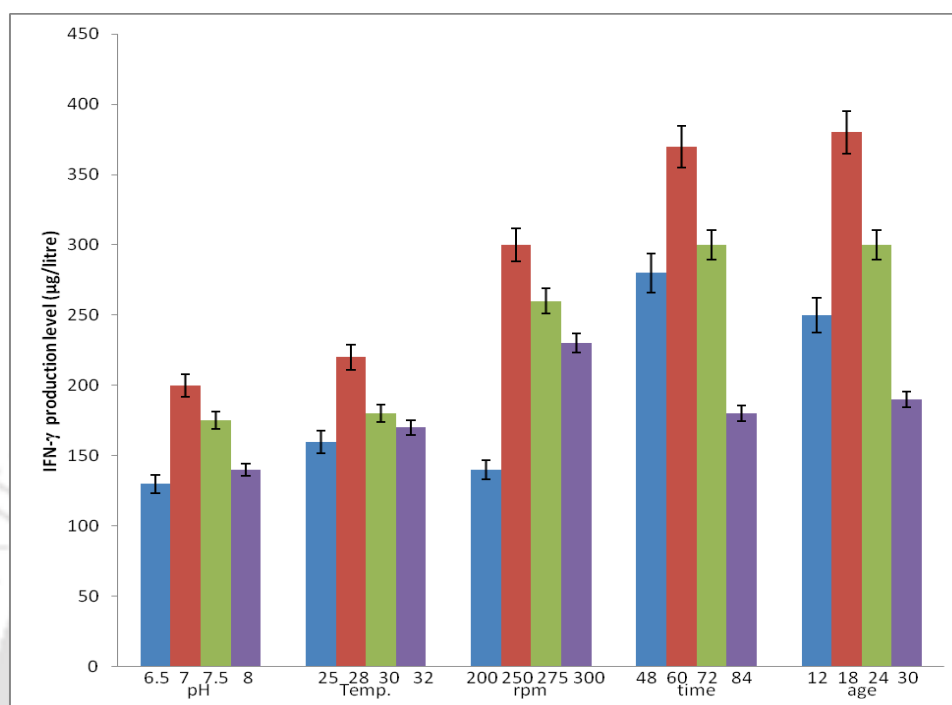


Figure 4.23: Bar-chart of protein expression conditions optimization results.

In the initial IFN- γ expression analysis, KL12:pVVDRP02 was found to produce 175 ± 10.5 $\mu\text{g/litre}$ of IFN- γ , which was highest among five analyzed clones. In the first experiment of pH optimization, pH 7 was found to produce highest IFN- γ . In the incubation temperature optimization experiment, it was found that 28°C gives the highest IFN- γ production level. Till now all the expression analysis experiments were carried out at 30°C . The next experiment of rpm optimization revealed 250 rpm to be the best suited for IFN- γ production as opposed to 275 rpm, which was being employed till now for IFN- γ expression and this experiment increased the IFN- γ expression level

by more than 1.5 folds. Incubation time optimization established 60 hours to be optimum IFN- γ production and an increase of two folds (approx.) was obtained in IFN- γ expression level. Finally age of inoculum optimization revealed 18 hours to be optimum age of inoculum for IFN- γ production. This is the phase where *K. lactis* cells grow rapidly and exponentially and cells are metabolically most active. After the optimization of physical conditions for IFN- γ expression a total increase of 2.2 fold in the expression level was observed.

4.7 Production medium optimization

4.7.1 Carbon source comparison

K. lactis cells were grown in the presence of two different carbon sources. galactose (Figure.4.22) and lactose (Figure 4.23) were utilized for the study. Lactose when used a carbon source for the growth of *K. lactis* cells gave results at par with those obtained with galactose as the carbon source. Because lactose is a much cheaper carbon source as compared to galactose, it was used instead of galactose as the carbon source for all the further experiments for the growth of *K. lactis* cells.

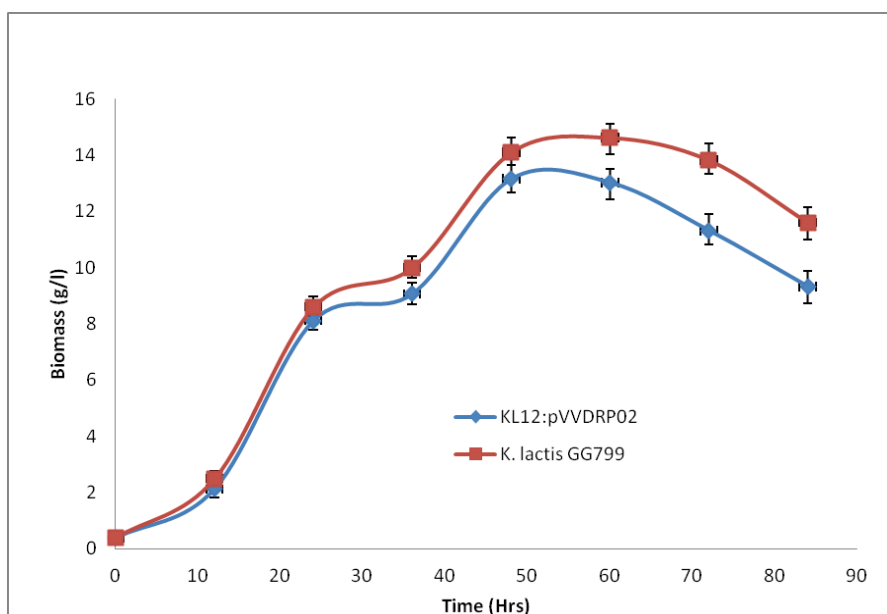


Figure 4.24: Growth profile of *K. lactis* on galactose

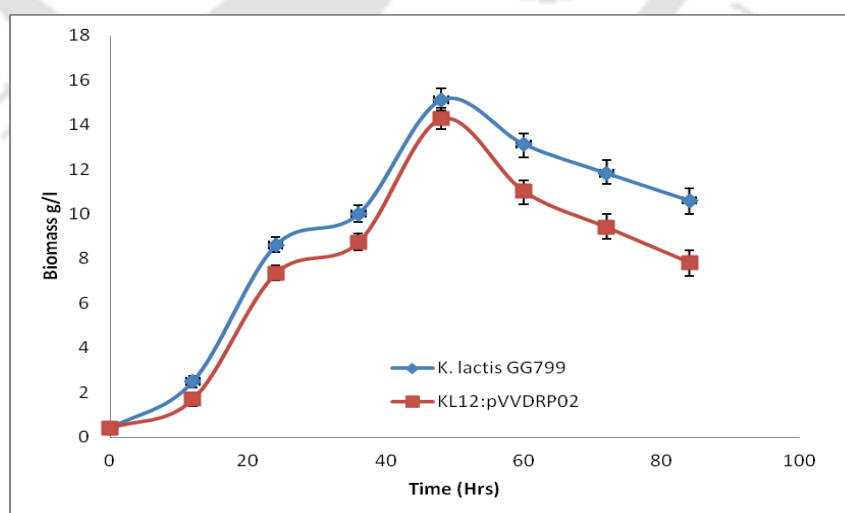


Figure 4.25: Growth profile of *K. lactis* on lactose

In this experiment *K. lactis* cells devoid of pVVDRP02 expression cassette were also used as a control. As the results clearly demonstrate that growth of KL12:pVVDRP02 and *K. lactis* control cells is almost comparable in the lag and exponential growth phases. Only after 36 hours growth of KL12:pVVDRP02 is hastened probably due to IFN- γ expression which poses a metabolic burden on *K. lactis* cells and that's why the growth of KL12:pVVDRP02 is slow as compared to *K. lactis* control cells. When grown on galactose maximum biomass of KL12:pVVDRP02 produced was 13.16 g/l, whereas control *K. lactis* cells produced a maximum of 14.6 g/l of biomass. When grown on lactose maximum biomass of KL12:pVVDRP02 produced was 14.26 g/l, whereas control *K. lactis* cells produced a maximum of 15.1 g/l of biomass. In case of lactose, *K. lactis* biomass level is better as compared to galactose.

4.7.2 Substrate inhibition studies

Substrate inhibition study revealed that up to 80 g/l of lactose is well tolerated by *K. lactis* cells for its growth but more than 80 g/l of lactose causes significant reduction in biomass production. HPLC analysis of spent culture medium revealed that *K. lactis* cells produce ethanol and acetate as metabolic by-product. Most of the lactose is consumed by the cells during the initial 20 hours of incubation. Ethanol is produced only after initial 20 hours of growth and we assumed ethanol is getting utilized by the cells as carbon source. Growth pattern of *K. lactis* shows a diauxic growth, after first lag phase there

can be observed a transient pause in the growth of organism which can be attributed to metabolic readjustments inside the cells, where organism prepares itself to consume ethanol for its growth. In growth profiles with 20, 40 and 80 g/l of substrate, it's clearly shown that ethanol production reaches its peak at 24 hours of growth and after that ethanol concentration starts to reduce (Figure 4.24, 4.25, 4.26). Acetate shows a similar profile as ethanol (Figure 4.24, 4.25, 4.26). Although the concentration of acetate is very low as compared to ethanol, it also appears to support the *K. lactis* cell's growth. Figure 4.33 clearly explains the mechanism of ethanol and acetate uptake in *K. lactis*. At very high concentrations of substrate cell do not utilize ethanol or acetate at all, moreover the biomass production is also highly reduced. All experiments were carried out in duplicate and the values plotted on the curves are the average of those two experiments.

KL12:pVVDRP02 when grown on 20 g/l of lactose shows a growth and metabolic profile (Figure 4.24) where maximum biomass observed is 15 g/l. Complete substrate utilization was observed at 20th hour. Ethanol production varied from 0.8 to 8.6 g/l, whereas acetate was in the range of 0.15 to 1.6 g/l. The event of complete substrate utilization coincided with the point from where the concentration of ethanol and acetate starts to decrease, supporting the theory of growth of KL12:pVVDRP02 dependent upon ethanol and acetate after 20 hours.

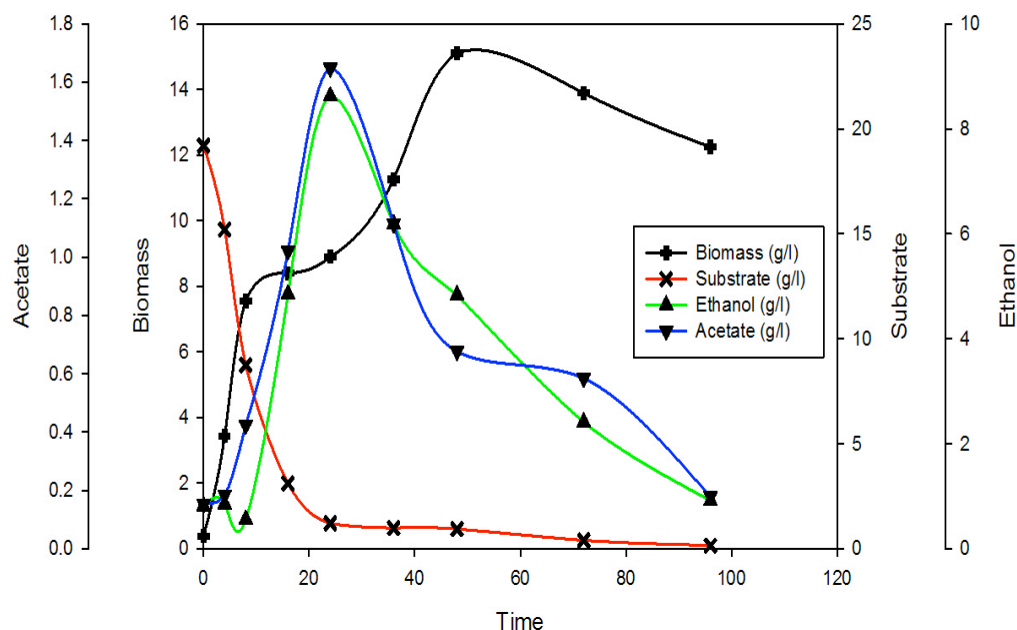


Figure 4.26: Performance of *K. lactis* on 20 g/l of lactose

KL12:pVVDRP02 when grown on 40 g/l of lactose, shows a growth and metabolic profile (Figure 4.25) where maximum biomass observed is 13.8 g/l. Complete substrate utilization was observed around 22nd hour. Ethanol production varied from 0.5 to 18 g/l, whereas acetate production level was in the range of 0.14 to 1.2 g/l. Highest ethanol production level (18 g/l) reached at 24th hour and after that ethanol level started to decrease and finally reached at 10 g/l at 96th hour.

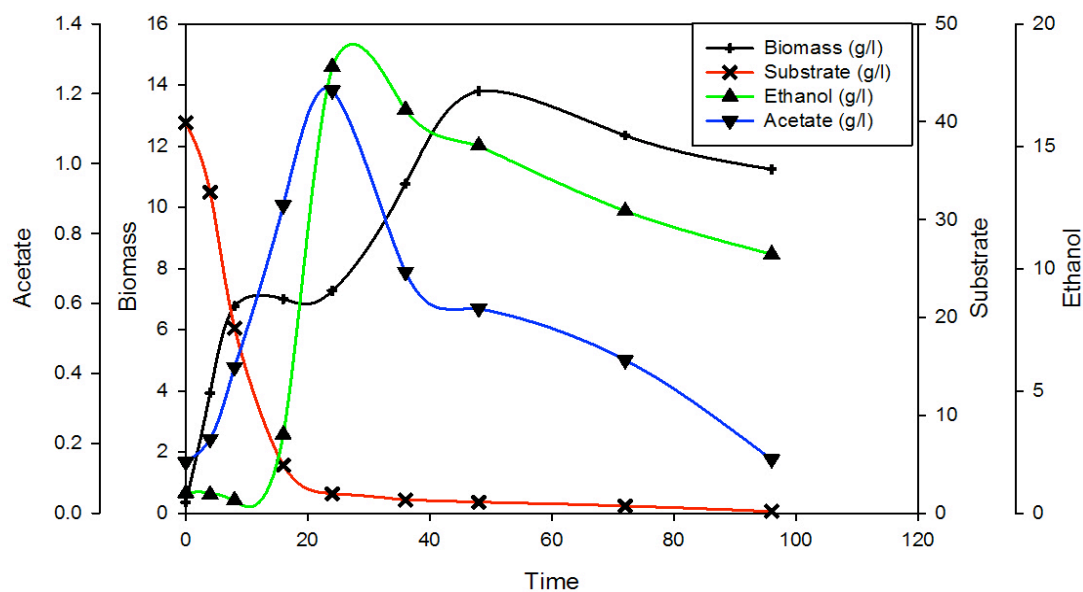


Figure 4.27: Performance of *K. lactis* on 40 g/l of lactose

Growth of KL12:pVVDRP02 on 80 g/l of lactose (figure 4.26) showed that complete substrate consumption did not take place and 11 g/l of lactose was left unutilized at 96th hour. Maximum biomass observed is 13.97 g/l. Ethanol production varied from 0.76 to 33 g/l, where as acetate production level was in the range of 0.18 to 1.6 g/l.

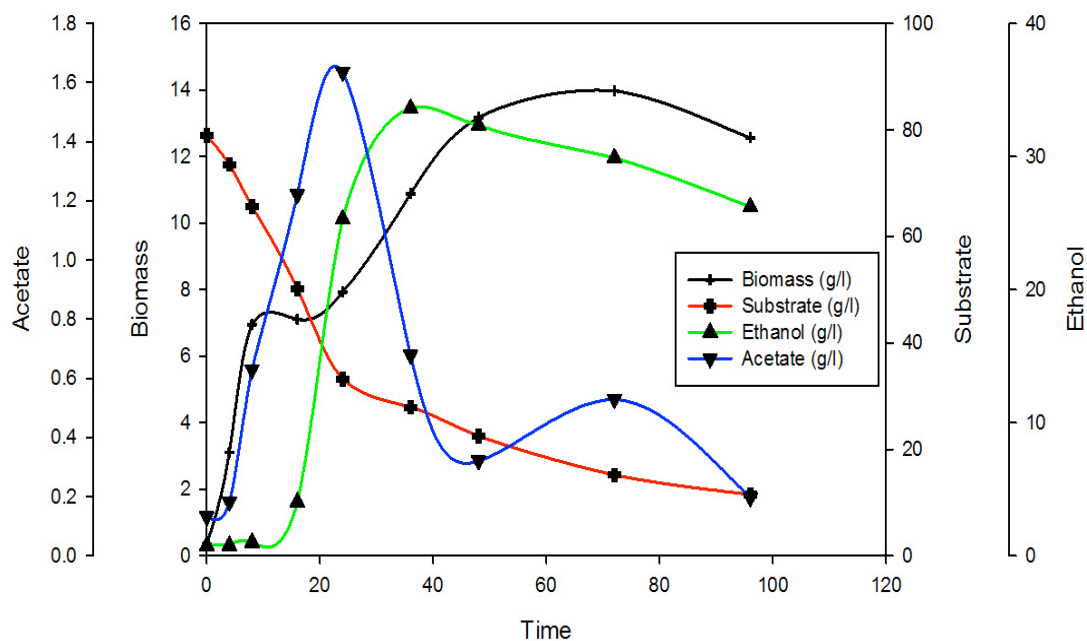


Figure 4.28: Performance of *K. lactis* on 80 g/l of lactose

Growth of KL12:pVVDRP02 on 100 g/l of lactose (figure 4.27) shows maximum biomass production level as 12 g/l while ethanol reaches its maxima 39 g/l at 72nd hour. Acetate production level varies between 0.15 to 1.3 g/l. Total substrate consumption did not take place and 16 g/l of lactose was left unutilized at 96th hour. At this stage cells do not seem to utilize ethanol for their growth, apparently due to substrate inhibition phenomenon.

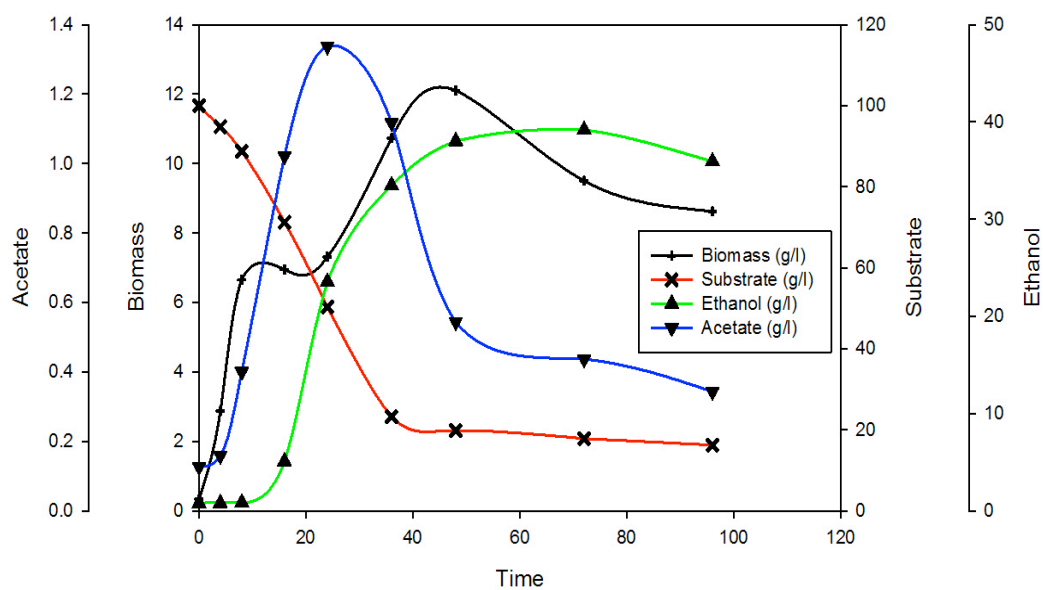


Figure 4.29: Performance of *K. lactis* on 100 g/l of lactose

More or less similar kind of profile was observed for 120, 140, 160 g/l of substrate where ethanol does not seem to be utilized for the growth of KL12:pVVDRP02 cells. Biomass falls in the range of 10-11 g/l.

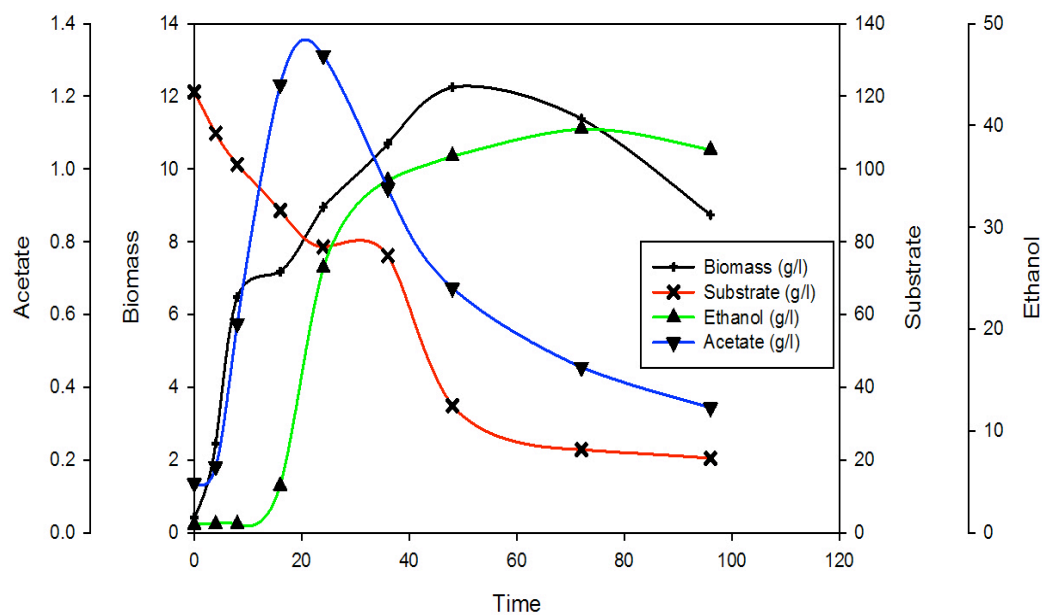


Figure 4.30: Performance of *K. lactis* on 120 g/l of lactose

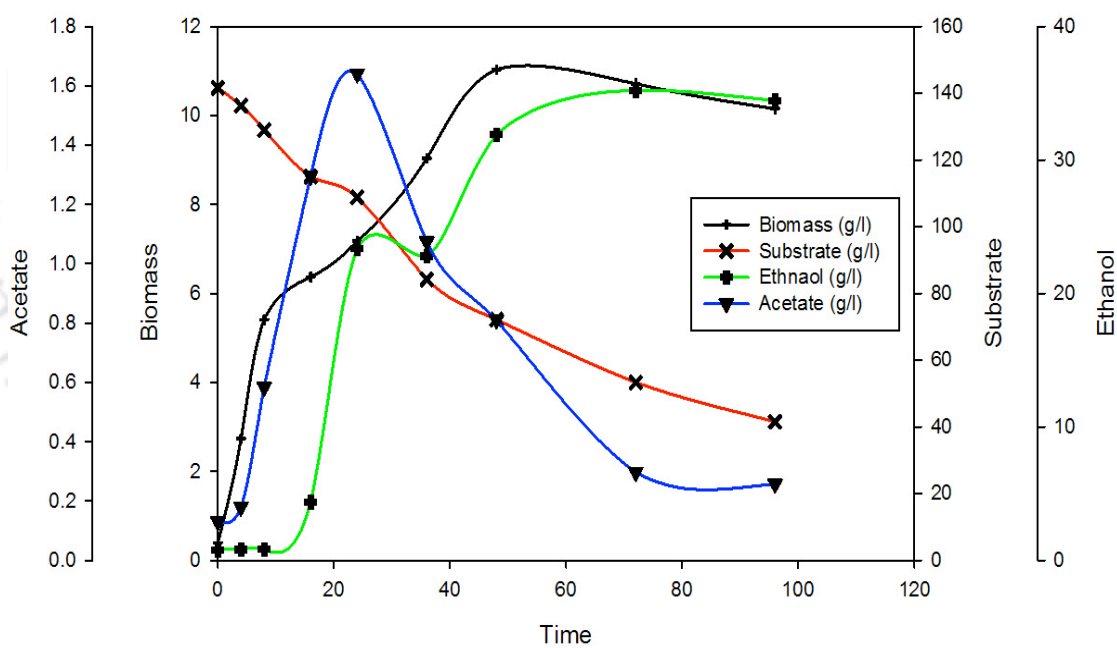


Figure 4.31: Performance of *K. lactis* on 140 g/l of lactose

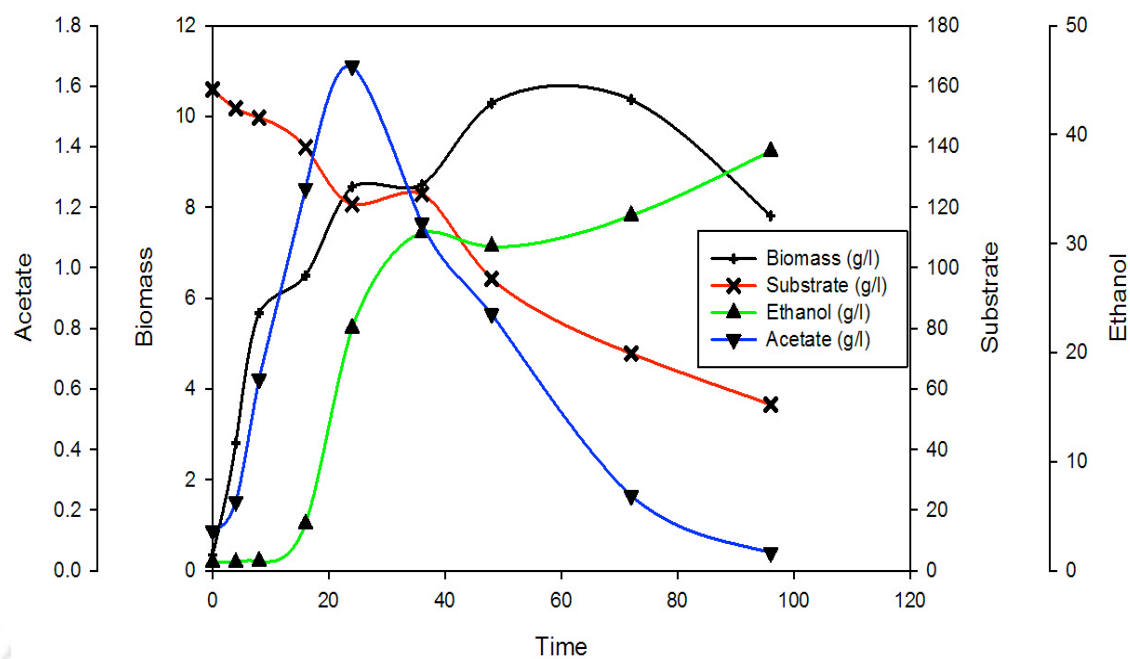


Figure 4.32: Performance of *K. lactis* on 160 g/l of lactose

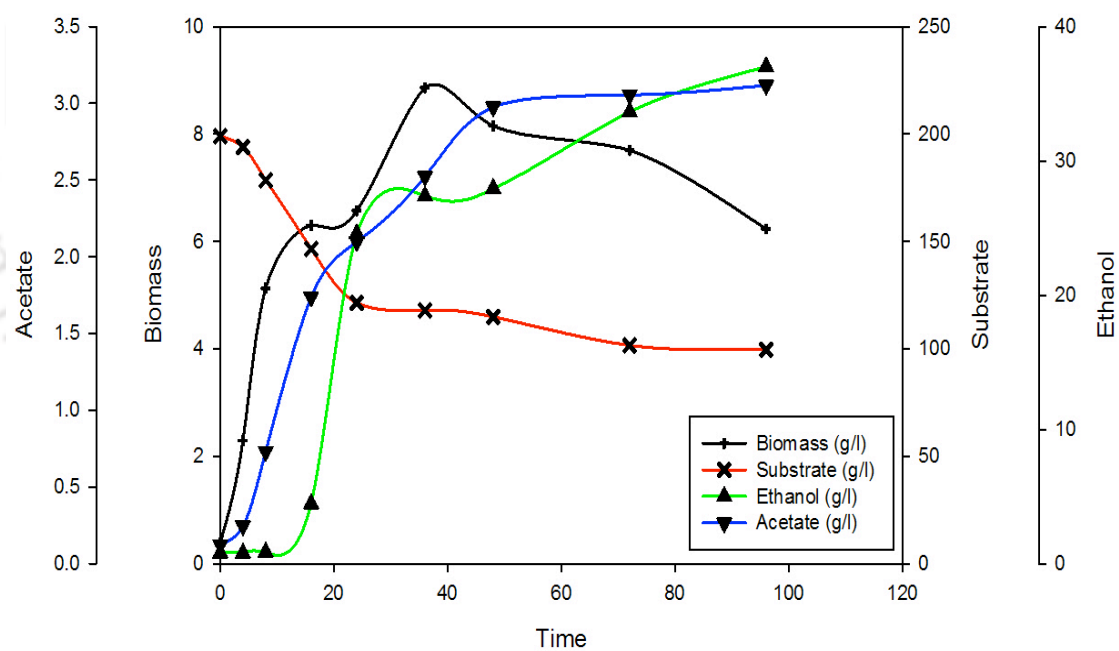


Figure 4.33: Performance of *K. lactis* on 200 g/l of lactose

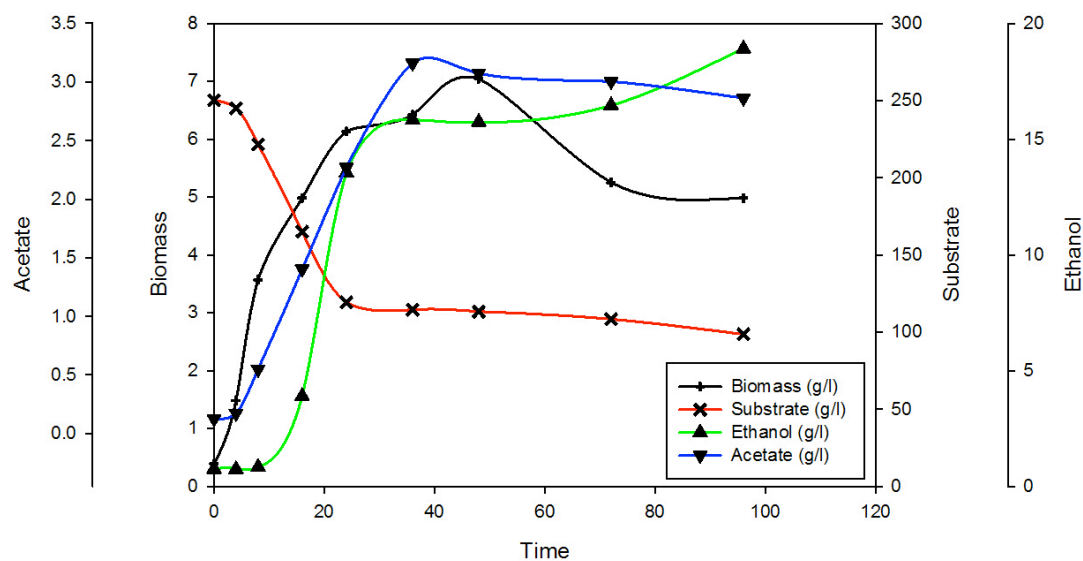


Figure 4.34: Performance of *K. lactis* on 250 g/l of lactose

KL12:pVVDRP02 cells grown on 200 and 250 g/l of substrate demonstrated complete substrate inhibition. Maximum biomass produced was in the range of 7-8 g/l. Despite the intake of 150 g/l lactose cells only produced 18 g/l of ethanol.

4.7.3 Overflow metabolism

At very high concentrations of substrate, *K. lactis* cells reorganize their metabolic pathways and starts utilizing ethanol and acetate as carbon source to support its growth.

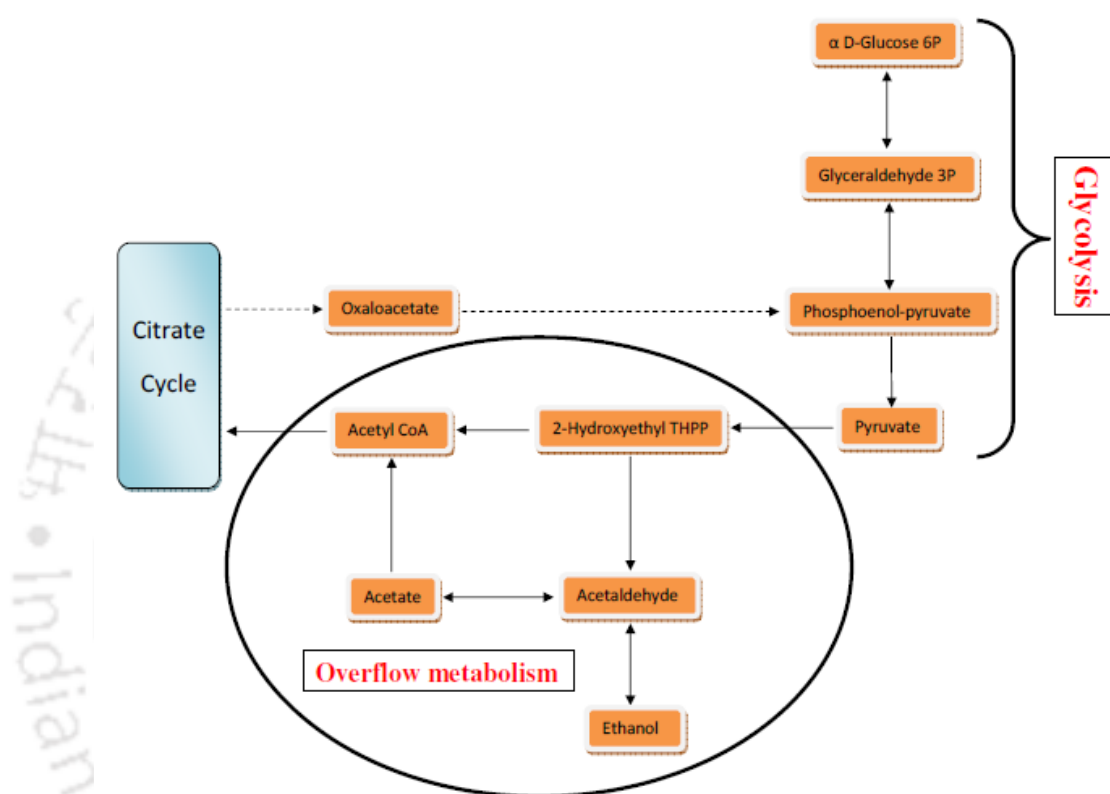


Figure 4.35: Schematic of Glycolysis and overflow metabolism in *K. lactis*.

lactose and galactose transport systems in *Kluyveromyces lactis* has been studied in depth and it has been shown that lactose uptake is by active transport (Boze H, 1987). The transport system is under monogenic control and is inducible. Lactose uptake is either by active transport or by simple diffusion process (Figure 4.34). As soon as lactose is taken up by

Kluyveromyces lactis cells, β -galactosidase (EC: 3.2.1.23) degrades it into D-galactose and α D-glucose. Hexokinase (EC: 2.7.1.1) converts α D-glucose to α D-glucose 6P and it enters into glycolysis. D-galactose also follows the same fate of entering into glycolysis pathway but after a multi step process involving at least 4 enzymes; namely a) Aldose 1-epimerase (EC: 5.1.3.3), b) Galactokinase (EC: 2.7.1.6), c) UDPglucose-hexose-1-phosphate uridylyltransferase (EC: 2.7.7.12), d) Phosphoglucomutase (EC: 5.4.2.2).

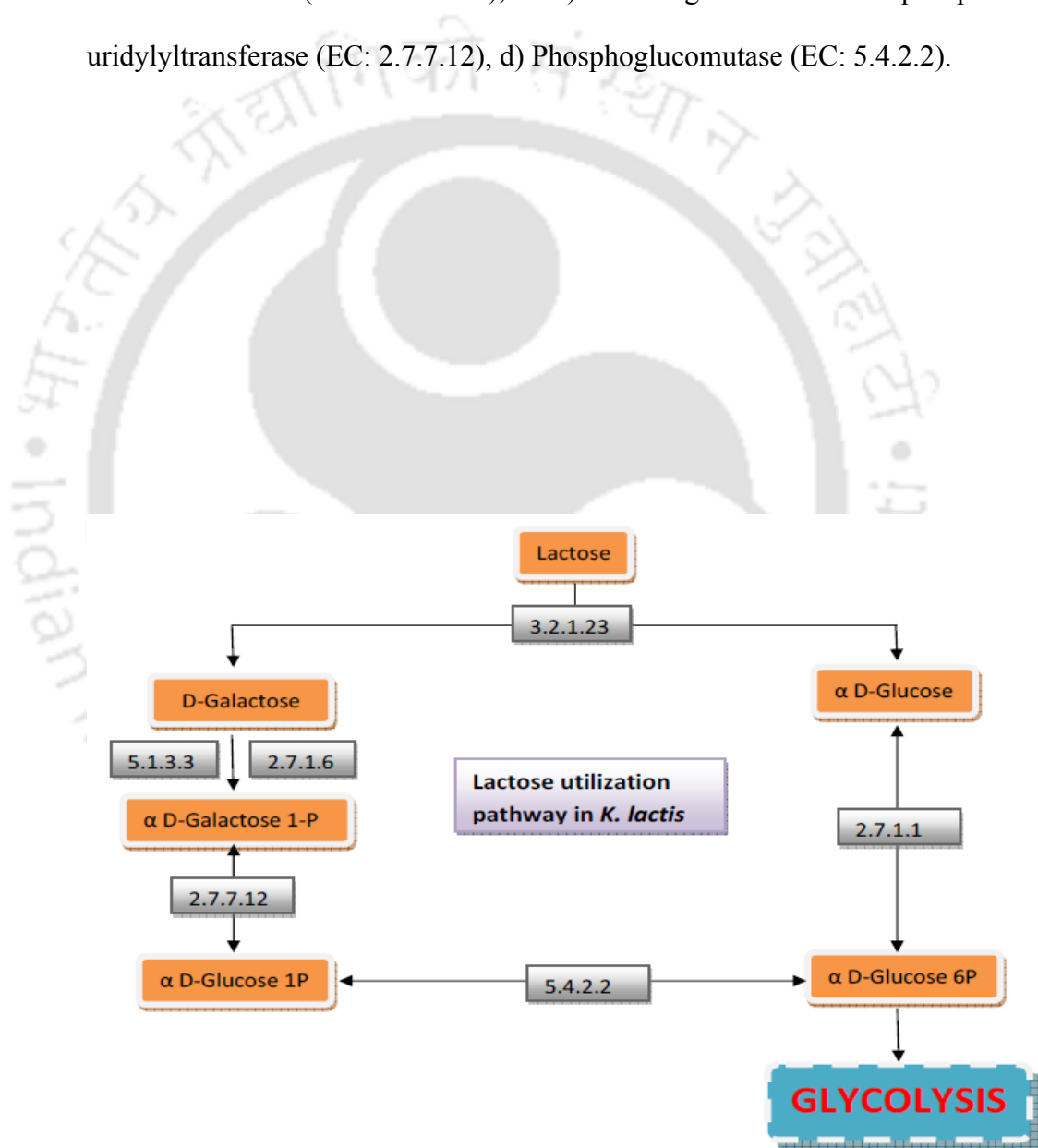


Figure 4.36: Lactose utilization pathway in *K. lactis*

4.7.4 Box-Behnken design for optimization of growth medium

Box-Behnken design predicted the optimum values of lactose, yeast extract and trace elements for producing highest possible biomass of *K. lactis* cells.

Table 4.2: Box-Behnken experimental design and result for biomass production in complex medium.

Experiment no.	X_1 Lactose (g/l)	X_2 Yeast extract (g/l)	X_3 Trace elements (g/l)	Biomass (g/l)	
				Experimental Data	Predicted Data
1	20	5	10	13.33 ± 0.21	13.30
2	80	5	10	16.57 ± 0.42	16.62
3	20	15	10	19.81 ± 0.36	20.25
4	80	15	10	16.20 ± 0.69	16.28
5	20	10	5	18.36 ± 0.30	16.63
6	80	10	5	15.09 ± 0.27	16.30
7	20	10	20	17.28 ± 0.39	19.55
8	80	10	20	21.02 ± 0.42	19.23
9	50	5	5	13.65 ± 0.28	14.19
10	50	15	5	15.98 ± 0.57	15.73
11	50	5	20	15.56 ± 0.43	15.37
12	50	15	20	20.63 ± 0.64	20.42
13	50	10	10	17.56 ± 0.39	17.79
14	50	10	10	18.23 ± 0.44	17.79
15	50	10	10	17.88 ± 0.39	17.79

The production of biomass varied in the range of 13.33 to 21.02 g/l for the treatments 1 and 8 respectively (Table 4.2). The analysis of variance (ANOVA) showed (Table 4.3) that production of biomass was significantly affected by linear and quadratic coefficients and their interactions. The mathematical model representing the production of biomass as a function of the evaluated variables in the experimental design studied is represented by the following equation:

$$Y = 50.71 - 0.4644X_1 + 4.7056X_2 + 4.1725X_3 - 5.1937X_1X_2 + 4.73X_1X_3 + 2.5275X_2X_3 + 0.4644X_1^2 - 3.8306X_2^2 - 0.0844X_3^2$$

Where Y is the production of biomass, X_1 , X_2 , X_3 are independent variables for lactose concentration, Yeast extract concentration and Trace elements concentration respectively. β_0 is the intercept term. The R^2 value was 97.80% indicating a good fit between the model and the experimental data. These values also suggest that the treatment was highly significant.

Table 4.3: Analysis of variance for the adjusted model for the predicted biomass in complex medium.

Source	Sum of squares	Degree of freedom	Mean square	F- ratio	P-value
Regression	1195.00	9	132.778	98.78	0.000
X_1	3.45	1	3.45	2.57	0.012
X_2	354.29	1	354.287	263.57	0.000
X_3	278.56	1	278.556	207.23	0.000
X_1^2	1.59	1	1.592	1.18	0.028
X_2^2	108.36	1	108.360	80.61	0.000
X_3^2	0.05	1	0.053	0.04	0.045
$X_1 * X_2$	215.80	1	215.800	160.54	0.000
$X_1 * X_3$	178.98	1	178.983	133.15	0.000
$X_2 * X_3$	51.11	1	51.106	38.02	0.000
Residual Error	26.88	20	1.344		
Lack-of-Fit	9.58	3	3.195	3.14	0.053
Pure Error	17.30	17	1.018		
Total	1221.89	29			

Table 4.4: Estimated Regression Coefficients for biomass

Source	Coefficients	Standard error of Coefficients	T-value
Constant	50.7100	0.4733	107.137
X_1	-0.4644	0.2898	-1.602
X_2	4.7056	0.2898	16.235
X_3	4.1725	0.2898	14.395
X_1^2	0.4644	0.4266	1.088
X_2^2	-3.8306	0.4266	-8.978
X_3^2	-0.0844	0.4266	-0.198
$X_1 * X_2$	-5.1937	0.4099	-12.671
$X_1 * X_3$	4.7300	0.4099	11.539
$X_2 * X_3$	2.5275	0.4099	6.166

According to Box-Behnken design analysis, increasing lactose concentration causes increase in biomass production. Trace elements also play an important role in biomass production and its increase causes biomass increase. But at high yeast extract concentrations biomass begins to decrease. This analysis is very clearly supported by artificial neural network algorithm prediction.

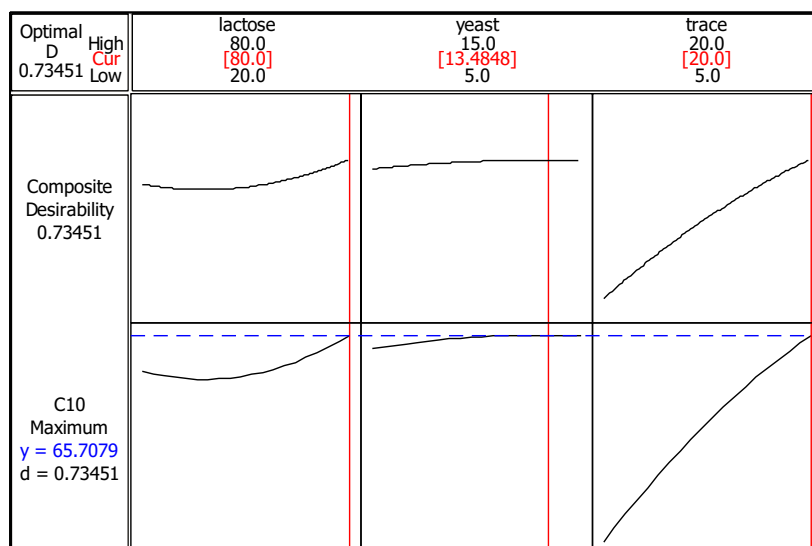


Figure 4.37: Response optimizer plot

4.7.5 Validation of Box-Behnken optimization

Based upon the optimization data obtained by Box-Behnken design of experiment and subsequent analysis, a validation experiment was performed. Results of validation experiment corroborated the predicted results. IFN- γ producing *K. lactis* cells were incubated in a medium composed of 80 g/l lactose, 13.5 g/l of yeast extract and 20 g/l of trace elements. Predicted biomass was 23.64 g/l and 24 g/l was achieved in the actual experiment.

Till now the best obtained *K. lactis* biomass level was 15.1 g/l (section 4.7.1), but after production medium optimization using Box-Behnken design *K. lactis* biomass level jumped to 24 g/l. An increase of 1.6 fold was observed in the biomass.

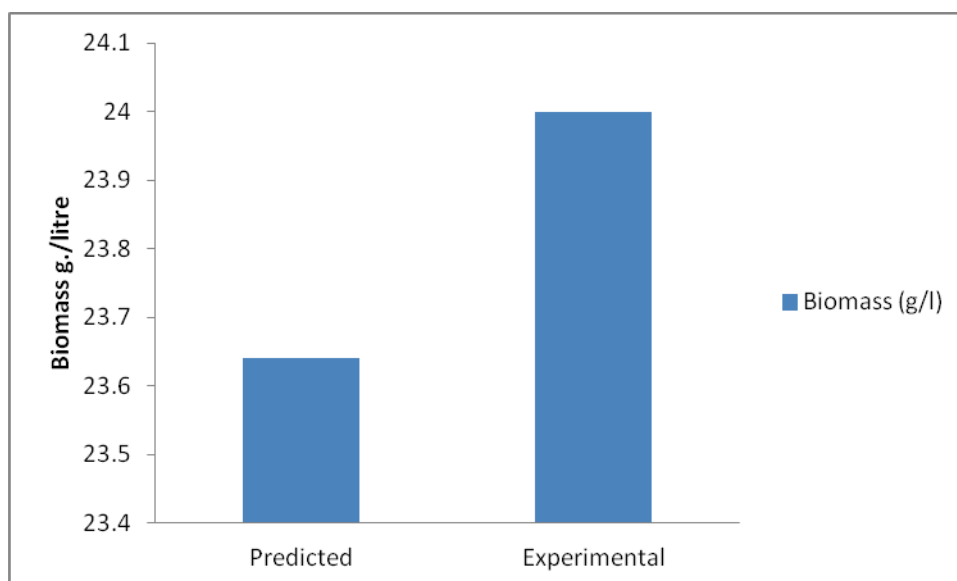


Figure 4.38: Validation of optimized parameters (Box-Behnken design) for biomass production.

4.7.6 Artificial neural network algorithms

Artificial neural network is a biologically inspired computational tool that was used in this experiment for modeling and optimization of growth medium for recombinant IFN- γ . For artificial neural network based optimization, two different algorithms were used to analyze the optimum value to each of three individual parameters for producing best possible biomass of *K. lactis* cells.

4.7.7 Genetic algorithm prediction

Genetic algorithm is a commonly used globalized optimization technique that optimizes a given function over a particular range, and is based on the evolutionary methods of natural selection of the best individuals in a population (Goldberg, 1989 and Imandi et al., 2008). Genetic algorithm was implemented to optimize the fermentation medium to maximize the biomass of *K. lactis* cells by using the ANN as the fitness function. A population of individuals was randomly generated, and a fitness value was assigned to each individual by the ANN fitness function. After several rounds of optimization calculation through Genetic algorithm, the maximum theoretical production of *K. lactis* biomass was $22.5 \text{ g}\cdot\text{L}^{-1}$. The optimal medium components were lactose, $78 \text{ g}\cdot\text{L}^{-1}$; yeast extract, $9.54 \text{ g}\cdot\text{L}^{-1}$; trace elements, $19.99 \text{ g}\cdot\text{L}^{-1}$.

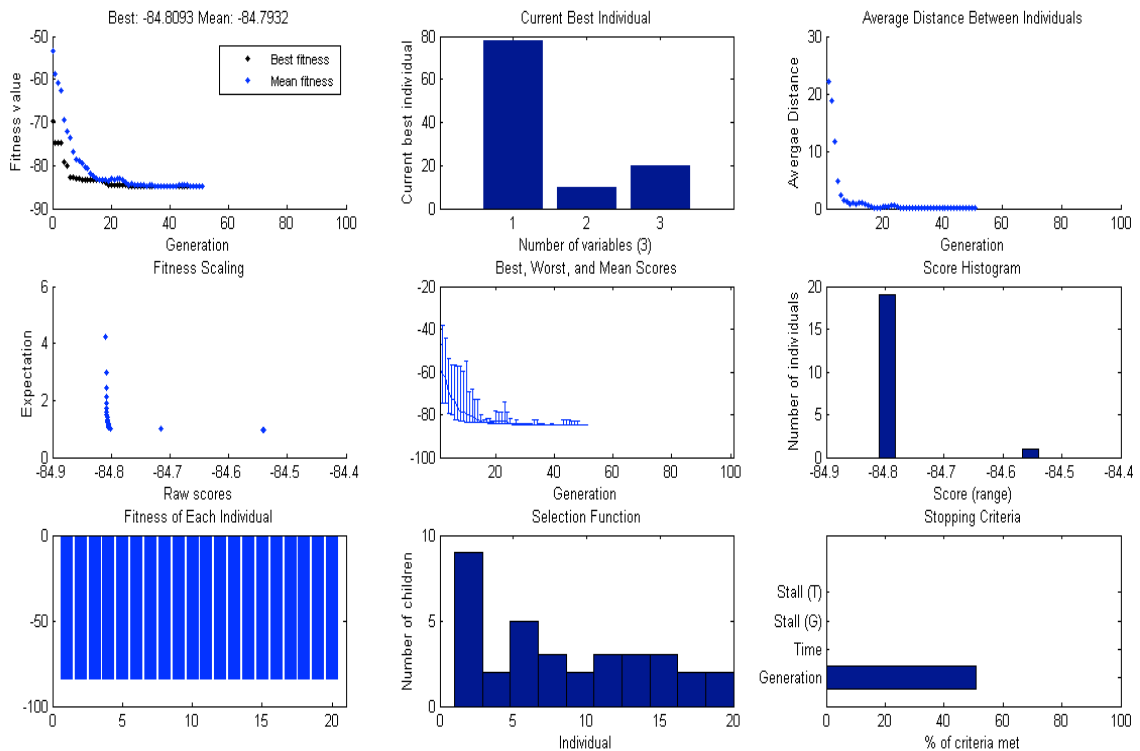


Figure 4.39: Analysis plots of genetic algorithm prediction for Biomass production.

A dynamic type of neural network used in this study was the feed forward backpropagation neural network (BPNN). This network consists of backpropagation algorithm which was developed by Rumerlhart in 1986 (Jain et al., 1996). The BPNN is a generalization of the delta rule used for training feed forward neural networks with non-linear units. This simple gradient descent method was designed to minimize the total error or mean error of the output computed by the network (Lee and Park, 2008). Since the initial configuration of ANN is arbitrary, the result of presenting a pattern to the ANN is likely to produce incorrect output. The errors for all input patterns were propagated backwards, from the output layer towards the input layer. The corrections to the weights were selected to minimize the residual error

between actual and desired outputs. Thus, the system ensured that the output is correctly within the specified range (Sencan and Kalogirou, 2005).

4.7.8 Simulated annealing algorithm prediction

Simulated annealing algorithm was implemented to optimize the fermentation medium to maximize the biomass of *K. lactis* cells by using the ANN as the fitness function. A population of individuals was randomly generated, and a fitness value was assigned to each individual by the ANN fitness function. After several rounds of optimization calculation through Simulated annealing algorithm, the maximum theoretical production of *K. lactis* biomass was 21.4 g·L⁻¹. The optimal medium components were lactose, 76.7 g·L⁻¹; yeast extract, 9.57 g·L⁻¹; trace elements, 20 g·L⁻¹.

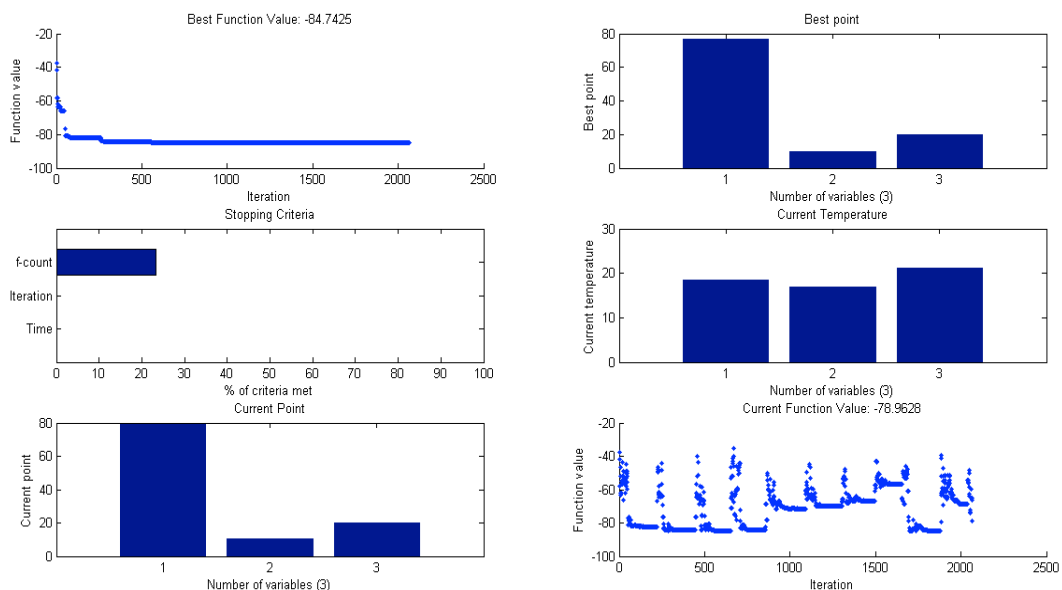


Figure 4.40: Analysis plots of simulated annealing algorithm prediction for Biomass production.

The results obtained out of simulated annealing algorithm corroborated the results of genetic algorithm. Both the algorithms predicted almost equal level of biomass and optimum concentration levels of medium components.

4.7.9 IFN- γ production prediction by genetic algorithm

Genetic algorithm was implemented to optimize the fermentation medium to maximize the IFN- γ production level by using the ANN as the fitness function. A population of individuals was randomly generated, and a fitness value was assigned to each individual by the ANN fitness function. After several rounds of optimization calculation through Genetic algorithm, the maximum theoretical IFN- γ production level was $900 \mu\text{g} \cdot \text{L}^{-1}$. The optimal medium

components were lactose, $58 \text{ g}\cdot\text{L}^{-1}$; yeast extract, $9.54 \text{ g}\cdot\text{L}^{-1}$; trace elements, $19.99 \text{ g}\cdot\text{L}^{-1}$.

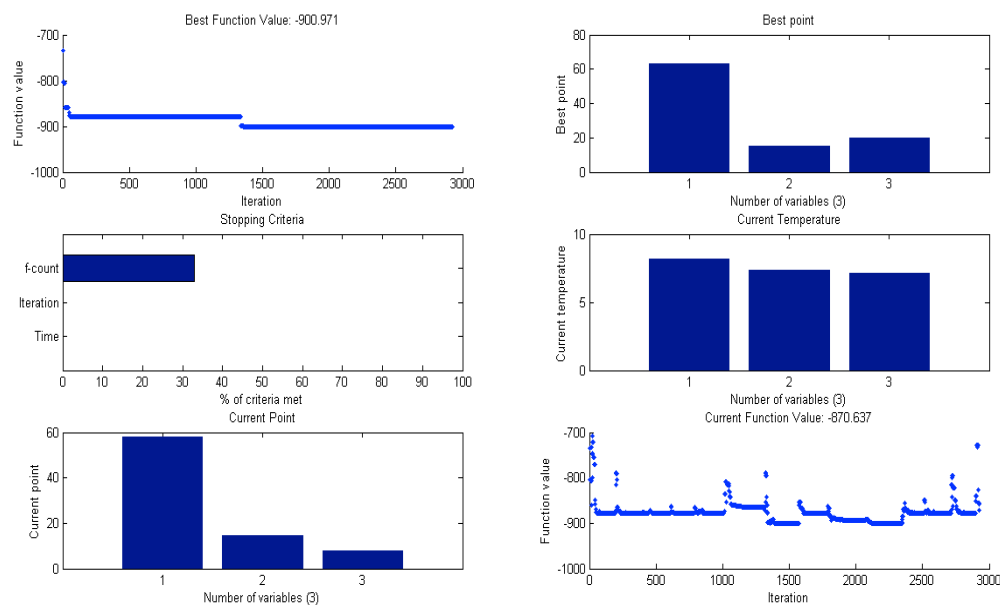


Figure 4.41: Analysis plots of genetic algorithm prediction for IFN- γ production

4.7.10 Validation study

The predicted results of *K. lactis* biomass based on genetic algorithm were validated by running an experiment at shake flask level. IFN- γ producing *K. lactis* cells were incubated in a medium composed of 78 g/l lactose, 9.54 g/l of yeast extract and 19.99 g/l of trace elements. Predicted biomass was 22.5 g/l and 23.1 g/l was achieved in an actual experiment.

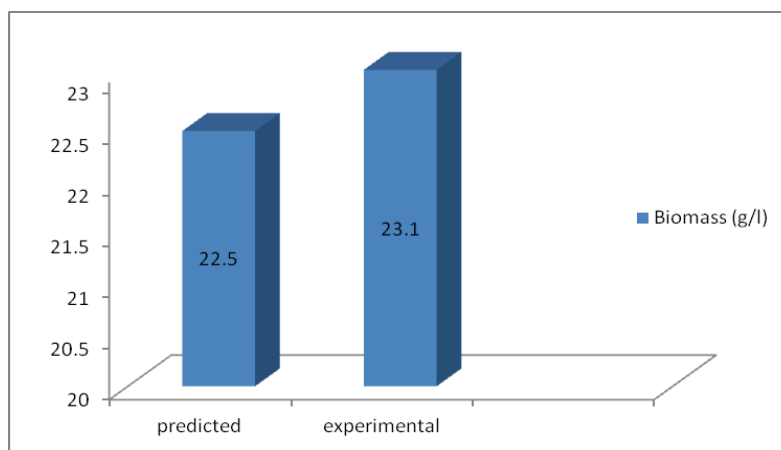


Figure 4.42: Validation of optimized parameters (artificial neural network genetic algorithm) for biomass production.

The predicted results of IFN- γ production based on genetic algorithm were validated by running an experiment at shake flask level. IFN- γ producing *K. lactis* cells were incubated in a medium composed of 58 g/l lactose, 9.54 g/l of yeast extract and 19.99 g/l of trace elements. Predicted IFN- γ production level was $900 \mu\text{g} \cdot \text{L}^{-1}$ and $882 \mu\text{g} \cdot \text{L}^{-1}$ was achieved in an actual experiment.

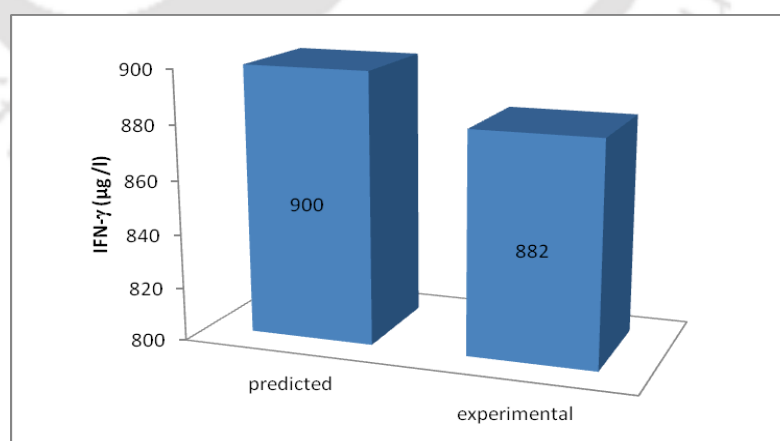


Figure 4.43: Validation of optimized parameters (artificial neural network algorithm) for IFN- γ production.

RSM is most widely used method in fermentation media optimization. It is one of the efficient methods for non-linear optimization. But its main limitation of RSM is that it assumes only quadratic non-linear correlation. So if we want to use RSM effectively, we need to narrow down search window appropriately (if we shrink the search window narrow enough, linear correlation may also suffice). This makes the search process highly dependent upon search space. It will require either extra experiments or good prior knowledge of the system to fix search window. Since ANN can inherently capture almost any form of non-linearity, it can easily overcome above discussed limitation of RSM. Thus, in case of ANN, more liberal search space can be chosen; even if, the correlation in that search space is more complex than quadratic.

4.8 Iso-electric focusing

Iso-electric focusing experiment revealed the pI of IFN- γ to be in the range of 9.3. Interestingly *K. lactis* broth doesn't contain any protein other than IFN- γ in the pI range of 5-10.

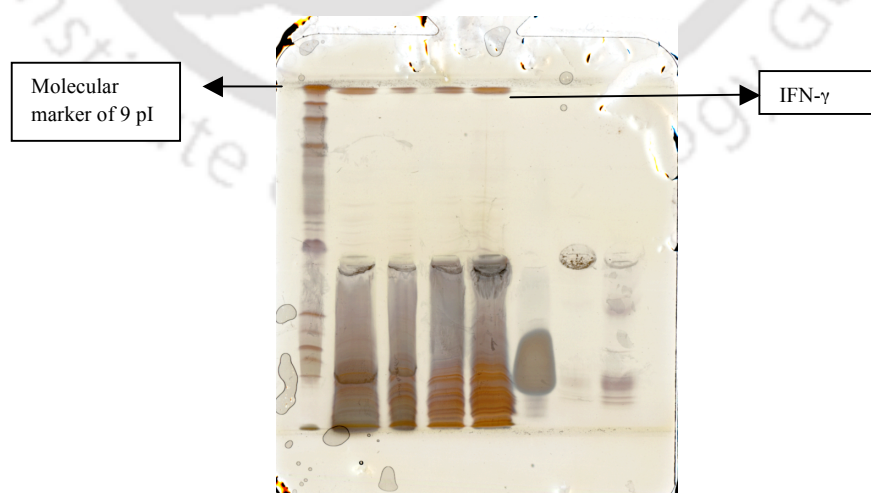


Figure 4.44: Iso-electric focusing gel of *K. lactis* broth.

4.9 Purification of IFN- γ by reverse micellar extraction

4.9.1 Forward extraction optimization with OVAT

Purification of IFN- γ was optimized in two steps. In the 1st step forward extraction was optimized with one variable at a time technique. pH optimization revealed that at high pH efficiency of forward extraction and % recovery of IFN- γ was highest. It may be due to the higher electrostatic interaction between positively charged CTAB (surfactant head group) and negatively charged IFN- γ ($\text{pH} > \text{pI}$). With the increase in pH extraction efficiency and protein recovery increased and at pH 12 highest values (88% forward extraction efficiency and 90% protein recovery) were obtained. Effect of surfactant (CATB) concentration also plays an important role in the forward extraction efficiency and protein recovery.

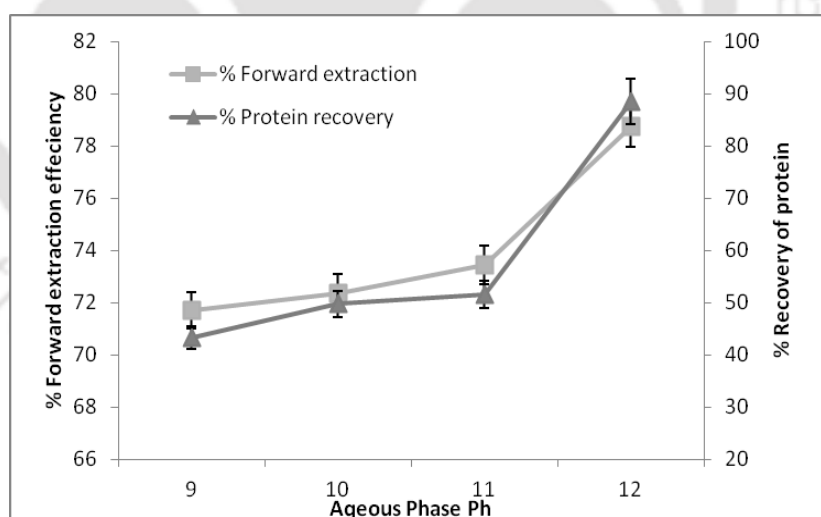


Figure 4.45: Effect of aqueous phase pH on reverse micellar extraction of IFN- γ from *K. lactis* broth.

Earlier studies have suggested that increasing the surfactant concentration, increases the forward extraction efficiency and protein recovery. CTAB concentration of 150 mM was found to be optimum for forward extraction efficiency. NaCl concentration of 0.2 M was found to be best suited for forward extraction and protein recovery.

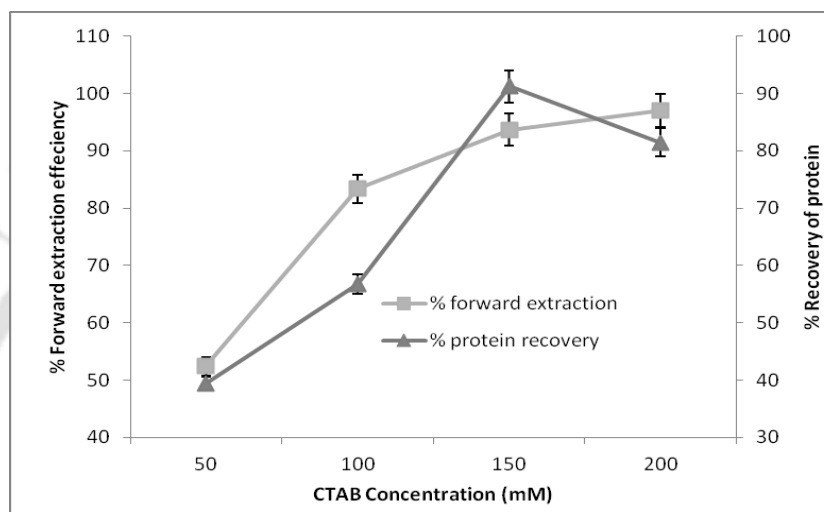


Figure 4.46: Effect of CTAB concentration on reverse micellar extraction of IFN- γ from *K. lactis* broth.

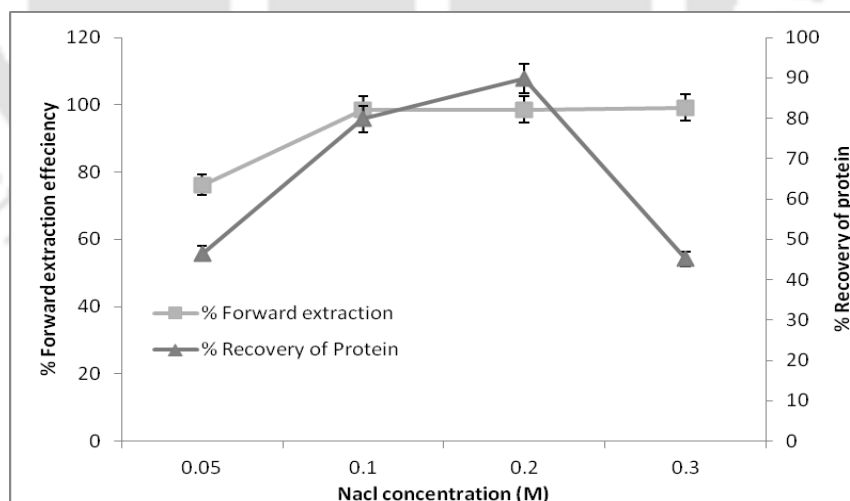


Figure 4.47: Effect of NaCl on reverse micellar extraction of IFN- γ from *K. lactis* broth.

4.9.2 Back extraction optimization with Taguchi method

Taguchi's method was employed to optimize back extraction efficiency for IFN- γ extraction from *K. lactis* broth. Orthogonal design of Taguchi's experimental model is shown in table 4.5. Nine experiments were conducted according to the experimental plan and response of each experiment was recorded. Minitab 16 software was used to analyze the data. The S/N ratio of each individual factor affecting the back extraction efficiency is shown in figure 4.49. The highest back extraction efficiency is given by highest number of S/N ratio. The maximum level in each factor was as following: pH 7, IPA 15% and KCl 0.8 M.

Table 4.5: $L^9(3^4)$ orthogonal array of Taguchi experimental design for the Reverse micellar extraction of IFN- γ . (BEE: back extraction efficiency; S/N: Signal to noise ratio; KCl: Potassium chloride; IPA: Iso propyl alcohol)

Run	pH	IPA (%)	KCl (M)	Actual BEE (%)	Predicted BEE (%)	S/N ratio actual
1	7	0.3	5	71.12 $\pm$ 2.39	72.187	37.03
2	7	0.5	10	77.66 $\pm$ 3.45	77.312	37.80
3	7	0.8	15	83.54 $\pm$ 5.90	83.594	38.43
4	9	0.3	10	71.55 $\pm$ 4.44	70.543	37.09
5	9	0.5	15	75.11 $\pm$ 5.15	75.039	37.54
6	9	0.8	5	80.28 $\pm$ 8.48	78.551	38.09
7	11	0.3	15	68.55 $\pm$ 5.33	68.27	36.72
8	11	0.5	5	70.26 $\pm$ 04.56	69.996	36.93
9	11	0.8	10	74.33 $\pm$ 3.19	76.278	37.42

Table 4.6: ANOVA of Reverse micellar extraction of IFN- γ .

		Regression coefficient			ANOVA Table					
Term	% Contribution	Coefficient	T	P	Source	D F	SS	MS	F	P
Constant		77.723	23.72	0	Regression	3	187.67	62.55	28.56	0.001
pH		-1.5983	-5.29	0.003	Residual error	5	10.952	2.19		
KCl (Mm)		17.862	7.44	0.001	Total	8	198.62			
IPA (%)		0.1847	0.120	0.187						
R square	94.5%									
R square (predicted)	91.2%									

Analysis of variance for the back extraction optimization of reverse micellar extraction revealed that all three parameters namely pH, KCl and IPA are highly significant for the process. Rsquare value was found to be 94.5% which indicates that only 5.6% of the anomalies can not be explained by this model. Predicted Rsquare value was 91.2%.

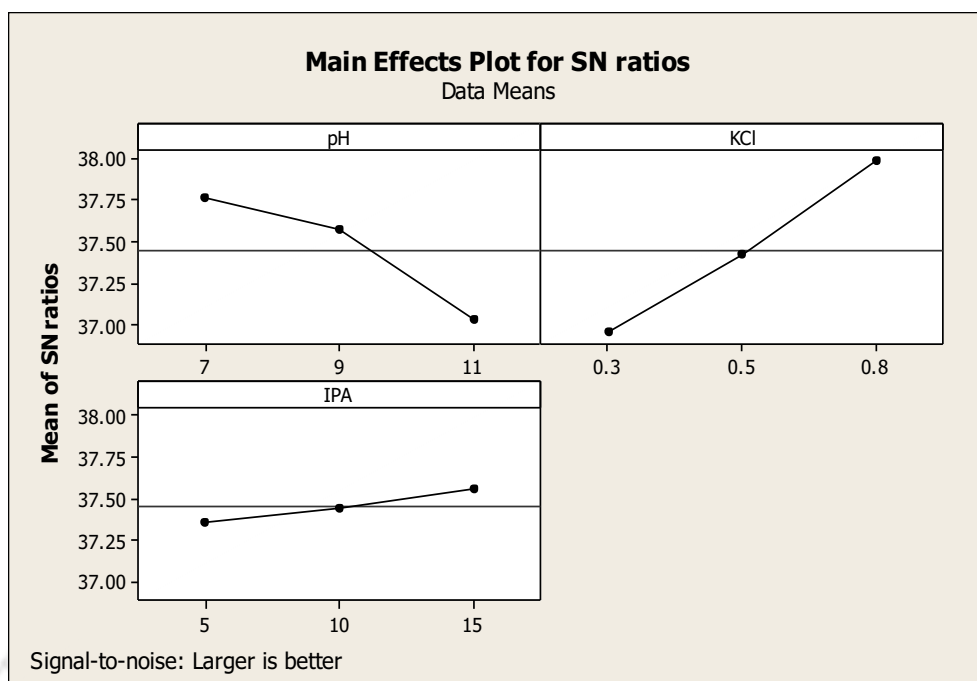


Fig 4.48: Variation of S/N ratio according to different level of pH, IPA (%) and KCl (M)

As already stated higher signal to noise ratio indicates the best value, pH 7 was found to be optimum for the back extraction as its S/N ratio was highest among the three tested pH of 7, 9 and 11. Optimum KCl concentration for back extraction was found to be 0.8 M as its S/N ratio was highest among the three tested concentrations of 0.3, 0.5 and 0.8 M. 15% iso propyl alcohol was found to be best suited for back extraction of IFN- γ among the three tested levels of 5, 10 and 15%.

4.10 Production studies

Based on the medium optimization data by Box-behnken design and artificial neural network algorithms, batch fermentation was carried out. Based on the data obtained by substrate inhibition studies, 80 g/l of substrate was used in the growth medium. *K. lactis* cells produced the best biomass level of 25 g/l. IFN- γ production level was also highest with 1100 μ g/l. Initial substrate of 80 g/l was consumed in the 98th hour. Maximum ethanol production levels reached 32 g/l in the bioreactor.

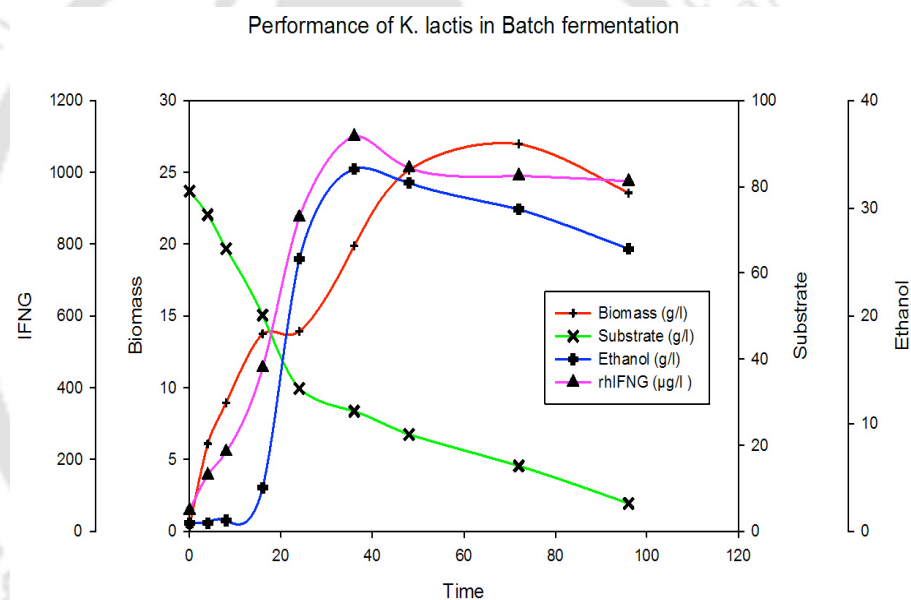


Figure 4.49: Growth profile of *K. lactis* cells in 2 litre batch fermentation.

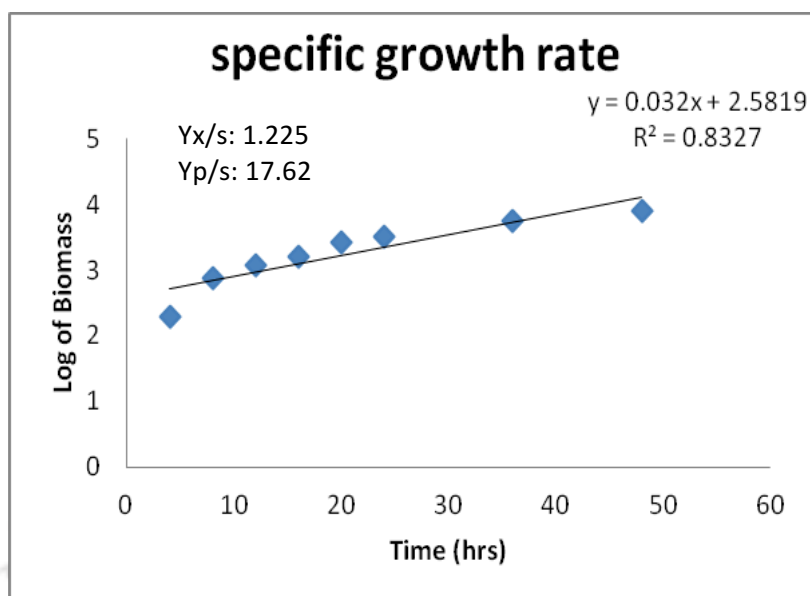


Figure 4.50: Specific growth rate of *K. lactis* cells in batch fermentation

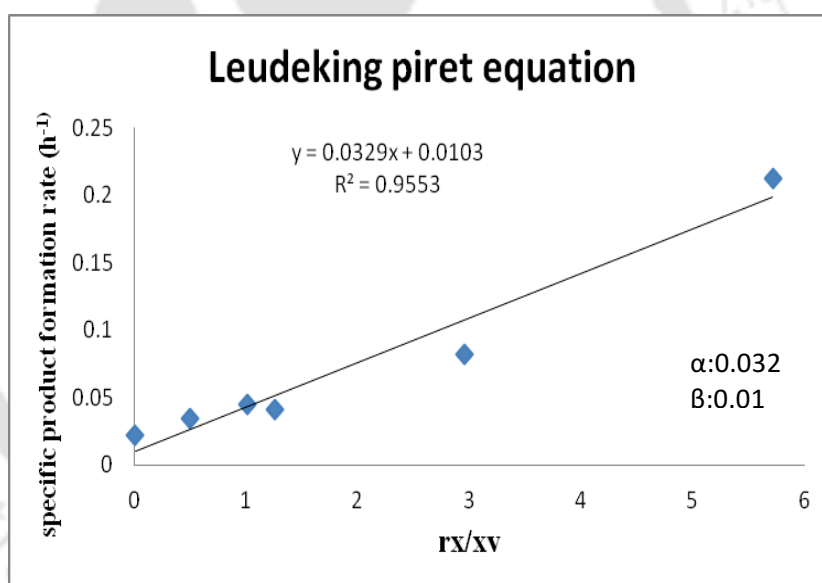


Figure 4.51: Leudeking piret equation for growth of *K. lactis* cells in batch fermentation

Specific biomass yield was found to be 1.225, whereas specific product yield was found to be 17.62. The Luedeking-Piret model was used to describe the relationship between *K. lactis* growth rate and IFN- γ formation rate.

$$dp/dt = Y_{p/x} dx/dt + \beta X$$

where dp/dt is the product formation rate (h^{-1}), dx/dt is the specific growth rate (h^{-1}), P is the product (IFN- γ), X is the cell concentration ($g\ L^{-1}$), $Y_{p/x}$ is the growth associated product yield coefficient, and β is the non-growth associated product yield coefficient. leudeking pirt equation suggested the IFN- γ production to be mixed growth associated since β was greater than zero.



4.11 Conclusion

In the present study IFN- γ was expressed in two different expression systems namely *E. coli* and *Kluyveromyces lactis*. Although IFN- γ has been previously expressed in *E. coli* several times, to the best of our knowledge, it was the first attempt to express IFN- γ in *Kluyveromyces lactis* expression system. As far as *E. coli* expression system was concerned, a novel protein expression strain, GalG20DE3 was developed for enhanced expression of IFN- γ . The newly engineered strain was developed from MG1655DE3 by λ DE3 lysogenization technique. Expression of IFN- γ was compared across three strains, GalG20DE3, MG1655DE3 and a commonly used lab strain BL21DE3. GalG20DE3 produced 1.5 fold more IFN- γ as compared to BL21DE3 and 3.3 fold more as compared to MG1655DE3. It was successfully demonstrated that GalG20DE3 is a hyper protein producing strain and if further developed, it can prove to be an excellent *E. coli* strain which will be equally useful for academic as well as industrial research. Although IFN- γ was getting produced in large quantities, 95% of IFN- γ was forming inclusion bodies in GalG20DE3. In case of MG1655DE3 also IFN- γ was getting expressed as Inclusion body but the amount of soluble form of IFN- γ was 6 fold higher as compared to GalG20DE3. *Kluyveromyces lactis* is an eukaryotic expression system, means IFN- γ can safely be produced in soluble form. GRAS status of this organism paves the easy way for approval from regulatory authorities. It does not produce endotoxins (Walker, 1998). Moreover *Kluyveromyces lactis* has the ability to secrete the heterologous protein in the outside spent culture

medium, thereby reducing the downstream processing cost of product drastically. Hence *Kluyveromyces lactis* was chosen as the expression system for IFN- γ production. ifng gene was successfully cloned in *K. lactis* GG799 cells. When expressed in suitable growth medium and analyzed on SDS-PAGE gel, IFN- γ protein could not be detected, later ELISA assay established the presence of IFN- γ protein the spent culture medium but the amount of the expressed IFN- γ protein was very low. In order to enhance the IFN- γ protein expression level, physical conditions were optimized after which a total increase of 2.2 fold in the expression level was observed. Substrate inhibition study revealed that up to 80 g/l of lactose is well tolerated by *K. lactis* cells for its growth but more than 80 g/l of lactose causes significant reduction in biomass production. HPLC analysis of spent culture medium revealed that *K. lactis* cells produce ethanol and acetate as metabolic by-product. Most of the substrate (carbon source) is consumed by the cells during the initial 20 hours of incubation. Ethanol is produced only after initial 20 hours of growth and we assumed ethanol is getting utilized by the cells as carbon source. Production medium for the growth of *K. lactis* cells was optimized using Box-behnken design which predicted the optimum values of lactose, yeast extract and trace elements for producing highest possible biomass of *K. lactis* cells. After production medium optimization using Box-Behnken design *K. lactis* biomass level jumped to 24 g/l. An increase of 1.6 fold was observed in the biomass. Further optimization of production medium was carried out using artificial neural network technique. Genetic and simulated annealing algorithms were implemented to optimize the fermentation medium to maximize the biomass

of *K. lactis* cells by using the ANN as the fitness function. After several rounds of optimization calculation through Genetic algorithm, the maximum theoretical production of *K. lactis* biomass was $22.5 \text{ g} \cdot \text{L}^{-1}$, similar results were obtained by simulated annealing algorithm which predicted the maximum theoretical production of *K. lactis* biomass as $21.4 \text{ g} \cdot \text{L}^{-1}$. Genetic algorithm was implemented to optimize the fermentation medium to maximize the IFN- γ production level by using the ANN as the fitness function which predicted the maximum theoretical IFN- γ production level as $900 \text{ } \mu\text{g} \cdot \text{L}^{-1}$. Iso-electric focusing experiment revealed the pI of IFN- γ to be in the range of 9.3. Purification of IFN- γ was performed by reverse micellar extraction. Forward extraction was optimized with one variable at a time technique. pH optimization revealed that at high pH efficiency of forward extraction and % recovery of IFN- γ was highest. CTAB concentration of 150 mM was found to be optimum for forward extraction efficiency. NaCl concentration of 0.2 M was found to be best suited for forward extraction and protein recovery. Taguchi's method was employed to optimize back extraction efficiency for IFN- γ extraction from *K. lactis* broth. The maximum level in each factor was as following: pH 7, IPA 15% and KCl 0.8 M. Analysis of variance for the back extraction optimization of reverse micellar extraction revealed that all three parameters namely pH, KCl and IPA are highly significant for the process. Based on the medium optimization data by Box-behnken design and artificial neural network algorithms, batch fermentation was carried out. *K. lactis* cells produced the best biomass level of 25 g/l. IFN- γ production level was also highest with 1100 $\mu\text{g/l}$.

4.12 Future scope of the work

- Production optimization of IFN- γ in fed batch mode. Interferon gamma was expressed in two different expression systems, *E. coli* and *K. lactis*. While expressing the protein in *E. coli* system we obtained very high amount of interferon gamma expressed but majority of the protein was getting formed as inclusion bodies, whereas with *K. lactis* system although the protein was in soluble form but was in very low amount. Producing Interferon gamma in these two expression systems has its own pros and cons. Economically speaking at lab scale both systems appear to be not so efficient and the cost of producing protein may be higher. But when the process is optimized at fed and continuous fed-batch mode of operation, cost may be reduced. Further medium/carbon source optimization may reduce the production cost more.
- Further modifications of GalG20DE3 strain by **1)** Incorporating gene of T7 Lysozyme in GalG20 DE3 genome for tighter regulation of T7 RNA Polymerase (pLysS engineering), **2)** Introducing molecular chaperon into the *GalG20* DE3 cell to overcome the biggest challenge associated with heterologous protein expression in *E. coli* i.e. formation of Inclusion bodies, **3)** overcoming the problem of codon biasing (while expressing heterologous proteins in *E. coli*) by rare codon supplementation & **4)** Growth medium and expression condition optimization.



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3. Ashish A Prabhu, S Chityala, N Kumar, B Mandal, **R Pandey**, V V Dasu. Bioprocess development of therapeutic proteins and Industrial enzymes. Research conclave 2014, IIT Guwahati, Guwahati, Assam, India.

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Rajat Pandey, Nitin kumar, Gabriel A. Monteiro, Veeranki Venkata Dasu, D. M. F. Prazeres. Development of a novel *E. coli* K-12 expression host for efficient production of recombinant therapeutic protein. Communicated to Metabolic engineering.

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1. **Pandey R**, Dasu V V. Optimizing secretory expression of recombinant human Interferon gamma from *Kluyveromyces Lactis*. (Manuscript in writing)
2. **Pandey R**, Ashish A Prabhu, Dasu V V. Optimization of process parameters for Reverse micellar extraction of Interferon gamma. (Manuscript in writing)
3. **Pandey R**, Dasu V V. Augmenting recombinant human Interferon gamma expression from *Kluyveromyces Lactis* by medium optimization tools. (Manuscript in writing)