OPTIMIZATION OF MEDIUM COMPONENTS AND PROCESS PARAMETERS FOR ENHANCED PRODUCTION OF LACTASE BY A BACTERIUM ISOLATED FROM DAIRY EFFLUENT

A THESIS

submitted by

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for the award of the degree

of

DOCTOR OF PHILOSOPHY

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This is to certify that the thesis entitled "**OPTIMIZATION OF MEDIUM COMPONENTS AND PROCESS PARAMETERS FOR ENHANCED PRODUCTION OF LACTASE BY A BACTERIUM ISOLATED FROM DAIRY EFFLUENT"** submitted by **T.C.VENKATESWARULU** to the Vignan's Foundation for Science, Technology and Research University, Vadlamudi, Guntur for the award of the degree of **Doctor of Philosophy** is a bonafide record of the research work done by him under our supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

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ABSTRACT

OPTIMIZATION OF MEDIUM COMPONENTS AND PROCESS PARAMETERS FOR ENHANCED PRODUCTION OF LACTASE BY A BACTERIUM ISOLATED FROM DAIRY EFFLUENT

Lactose intolerance is characterized by indigestion of milk sugar called as lactose. Indigestion of milk and milk based products in lactose intolerants can be treated with supplementing lactase enzyme. Bacteria that produce lactase have been used to treat the lactose intolerants. Commercially, this enzyme production from bacterial sources was not reported for higher production. In the present study, dairy effluent was screened for lactase producing bacteria by biochemical methods and found 46 lactase positive isolates. The highest lactase yielding isolate was named as VUVD001 and this isolate was characterized by morphological, biochemical and molecular methods. The VUVD001 isolate was stable at extreme pH and temperature conditions. The o-nitrophenyl-β-D-galactopyranoside (ONPG) disc analysis was performed for the confirmation of lactase producing activity. The produced lactase was partially purified followed by zymogram analysis for confirmation of extracellular activity. The isolate VUVD001 was identified as *Bacillus subtilis* by 16S rRNA gene sequencing and this strain was able to survive in a temperature range of 20-55 ^{o}C, pH range of 5-8 and a salt concentration upto 8% of NaCl. In addition, the production of lactase was improved by designing new medium through optimization of nutrient components by one-factor-at-a-time (OFAT) and statistical methods. Lactose and yeast extract were selected as preferable carbon and nitrogen sources for lactase production. Further, addition of tryptophan and $MgSO_4$ showed enhanced lactase production. Statistical analysis proved to be a powerful tool in exploring optimum fermentation conditions. The individual and interactive role of lactose, yeast extract, magnesium sulfate and tryptophan concentration on lactase production was examined by central composite design. The submerged fermentation with *B. subtilis* VUVD001 strain produced an enhanced lactase activity of 63.54 U/ml in optimized medium. The activity was improved 2.5 folds compared to classical optimization of medium components. This result of the study confirmed that the designed medium was

useful to produce higher yield of lactase. Similarly, the optimal physical conditions were determined in batch fermentation process using OFAT approach for the parsimonious yield of lactase. The influence of physical conditions pH, temperature, incubation time and inoculum size on enzyme production was also studied. The maximum activity of lactase in shake flask culture was found to be 15.27 U/ml at optimized conditions of incubation period 36 h, Temperature 37 ^{o}C, pH 7.0 and inoculums size 5%. Further, the modeling and optimization were performed to enhance the production of lactase through submerged fermentation by *B. subtilis* VUVD001 using artificial neural networks (ANN) and response surface methodology (RSM). The effect of process parameters namely temperature (^{o}C), pH and incubation time (h) and their combinational interactions on production was studied in shake flask culture by Box-Behnken design. The model was validated by conducting an experiment at optimized process variables which gave the maximum lactase activity of 91.32 U/ml. The production of lactase 6 folds improved after RSM optimization. This study clearly showed that both RSM and ANN models provided desired predictions. However, the ANN model (R^2=0.99456) gave a better prediction when compared with RSM (R^2=0.9496) for production of lactase. The findings of the present study revealed that the *B. subtilis* VUVD001 strain could be used as a potential strain for commercial production of lactase.

KEYWORDS: Response surface methodology, Artificial neural networks, Lactase, *B. subtilis* VUVD001 strain

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
µg/ml	microgram per milliliter
ANN	Artificial neural networks
ANOVA	Analysis of Variance
APS	Ammonium persulfate
g/l	gram per liter
h	hour
kDa	Kilo Dalton
LB	Luria Bertani
mg/ml	milligram per milliliter
min	minute
ml	milliliter
nm	nanometer
OD600	Optical Density at 600 nm
OFAT	One Factor at a Time
ONP	o- nitrophenol
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
R1	Response (lactase activity, U/ml)
RPM	Revolutions per minute
sp.	Species
TEMED	Tetramethylethylenediamine
v/v	volume by volume
VUVD	Vignan University Vadlamudi
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER-I

INTRODUCTION

1.1 Lactose and Lactose intolerance

The milk sugar lactose is a disaccharide which found in mammal's milk and important for the sustenance of newborn infants. The lactose sweetness is low in comparison with sucrose and it cannot be directly absorbed by the intestines. Lactose is hydrolysed into glucose and galactose by the action of lactase during digestion process. Lactose intolerance is malabsorption of dietary lactose due to lactase deficiency, which is normally produced by small-intestinal mucosa. The lactase is disappears after the weaning period of young mammals. Intestinal lactase activity was high in most of infants during the prenatal period. But, after two to twelve years of age, two separate groups come into view such as "lactase non-persistence" group with low lactase activity (hypolactasia) and "lactase-persistence" group of people who maintain their neonatal level of lactase activity into adulthood (Jurgen Schrezenmeir and Michael de Vrese, 2001; Mattar et al, 2010).

Lactose intolerance is a symptomatic malabsorption of lactose which slowly reduces the consumption of milk and other dairy products by humans. The lactose intolerance causes various disorders namely gas, bloating, nausea, or diarrhea and non-allergic (Houts, 1988; Vasiljevic and Jelen 2001). Lactose intolerance is not limited to gut symptoms. Systemic complaints, namely headache, vertigo, memory impairment, lethargy, muscle and joint pains, allergy, cardiac arrhythmia, mouth ulcers, and sore throat (Matthews et al, 2005; Harrington and Mayberry, 2008). Approximately, two-third of world population suffers from lactose intolerance and it significantly reduces their quality of life (Alazzeh et al, 2009). The lactase deficiency can be resolved either by using supplementary probiotic microorganisms or using lactase in dairy products that hydrolyze lactose present in food. This enzyme has commercial value in pharmaceutical and food industries. Particularly, in dairy industry it is used for hydrolysis of lactose in milk or other products like cheese and whey (Gasteiger et al. 2003). The prevalence of lactase deficiency in the population groups in the world is shown in Table 1.1.

Table 1.1 Lactose intolerance prevalence in different populations of the world

Ethnic group	Prevalence (%)
Germany	14.8
Austria	20.1
Brazil (white)	57
Brazil (Terenas Indians)	89.3
Brazil (Japanese)	100
Brazil (mulattos)	57
Brazil (Negroes)	80
China	87.3
Estonia (Finnish-background)	24.8
France	23.4
Hungary	37
India (North)	67.5
India (South)	86.8
Italy	51
Japan (adults)	89
Jordan (Bedouin)	24
Jordan (West) and Palestine	75
Russia (Northeast)	35.6
Siberia (Khants)	94
Somalis	76
Sudan (Béja tribe. farmers)	16.8
Sudan (Nilotis tribe. farmers)	74.5
Sweden (Caucasian children)	10
Sweden (non-Caucasian children)	66
Sweden (Caucasian elderly)	5
Tuareg	12.7
Turkey	71.3

(Source: Jellema et al, 2010; Rangel et al, 2016)

1.2 **Types of intolerance**

The deficiency is mainly three types such as congenital, primary and secondary. Out of these the congenital lactase deficiency (CLD) is very severe problem and is known as lactasia. In CLD individuals, the lactase levels are severely goes down at birth and which is remains abnormal throughout the life. They need to take the lactose free diet for entire life and they cannot tolerate even little amounts of lactose (Miller et al, 2000).

The primary lactase deficiency (PLD) is due to the decrease in lactase activity between two and twenty years of age after weaning and is common in most of the individuals. The reduction of lactase activity is probably of genetically predisposed reasons. The symptoms of PLD includes dehydration, deprived calcium absorption, diarrhea induced bloating etc. The watery diarrhea is one of the most dreadful consequences of PLD depending on the amount of lactose consumed by the individual and at the level of his tolerance (McBean and Miller, 1998; Rasinpera et al, 2006). Secondary lactase deficiency results from the damage of small and large intestinal epithelial linings which leads to gastrectomy (Vesa et al, 2000).

1.3 Milk allergy

Milk is a basic food for infants and an important supplement in the diet of adults and has elevated biological value consists of high nutrients. However, in few cases the consumption of milk is associated with reactions like cow's milk protein allergy (CMPA) (Bahna, 2002). The CMPA is the most common food allergy in infants and affecting 2% to 5% of world child population with less than 3 years of age (Huang & Kim, 2012). Cow's milk contains many allergens such as whey and casein components. The whey components such as α & β lactoglobulins & bovine immunoglobulin are allergic to children's. Similarly the adults are allergic to casein (α & β) components (Eigenmann et al, 1998). The milk allergy affects the immune system and causing symptoms like skin allergy, Food Protein-Induced Enterocolitis Syndrome (FPIES), gastrointestinal and anaphylaxis after food ingestion (Gasparin et al, 2010). Sometimes lactose intolerance is mystified with milk allergy in which one or more milk proteins stimulate the immune system of the body. Table 1.2 indicates the differences between these two diseases.

Table 1.2 Characteristic features of milk allergy and lactose intolerance

Characteristics	Lactose intolerance	Milk allergy
Definition	Difficulty in digestion of lactose	Allergic reaction to cow's milk
Cause	Low intestinal levels of lactase	Negative immune response for ingestion of milk protein
Dairy Food Use/ Avoidance	No need to eliminate dairy foods, only adjust the dose of lactose consumed	Eliminate cow's milk from the diet
Symptoms	Abdominal gas, bloating, cramps, diarrhea	Abdominal pain, vomiting, diarrhea, nasal congestion and skin rash
Age	Most common in adults	Common in children's especially infants

Source: (Miller et al, 2000; Tumas and Cardoso, 2008)

1.4 Diagnosis

The lactose is not completely digested in the intestine of intolerance individuals and the leftover lactose is fermented by the colonic bacteria and forms hydrogen gas. The released gas is analyzed for diagnosis of lactose intolerance by Breath hydrogen test and this test is appropriate for primary screening of the intolerance. Lactose intolerance can also be diagnosed through quantification of lactase activity for biopsy intestinal samples. This measured lactase activity is compared with the activity of another small intestine enzyme. There are also genetic studies to find a reliable and fast method for diagnosis of the disease (Troelsen, 2005). Urinary galactose measurement using an enzyme strip is another method for diagnosis (Vesa et al, 2000).

1.5 Solution to lactose intolerance with probiotics

Doing away with lactose containing dietary products is not a permanent and wise solution, considering the fact that those food supplements are quite necessary for our health. Also, lactase enzyme has been tried in the form of drugs or encapsulations. The people suffering from lactose mal-digestion for some unknown reason were able to utilize yoghurt lactose but not the milk lactose. It also resulted in lower hydrogen breath. Gradually it was found that the probiotic bacteria present in the yoghurt could exert such beneficial trait. Milk containing *L. acidophilus* has shown similar results. The non-pathogenic bacterial strains like *S. thermophilus* and *L. bulgaricus* could produce lactase (Gallagher et al, 2004; Tuohy et al, 2003). The beneficial effects associated with the probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* on

human health were shown in the Fig. 1. These bacteria are used as functional ingredients, especially in dairy products like yoghurts and other milk products.

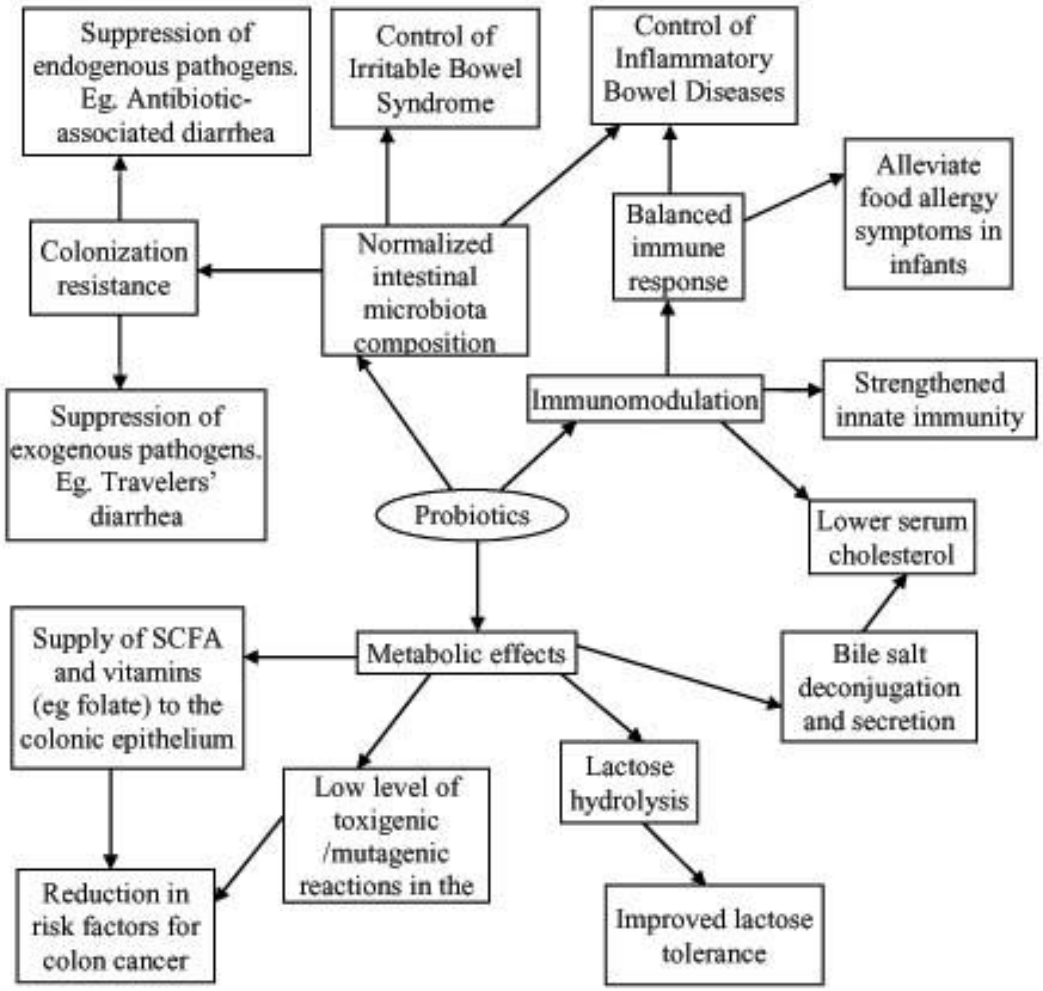

Fig. 1.1 Health benefits of probiotics (Source: Saarela et al, 2002)

1.6 Microbial enzymes

Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in various industries and applications. They are produced by living organisms to increase the rate of an immense and diverse set of chemical reactions required for life. They are involved in all processes essential for life such as DNA replication and transcription, protein synthesis, metabolism and signal transduction, etc. and their ability to perform very specific chemical transformations has made them increasingly useful in industrial processes. The end use market for industrial enzymes is extremely wide-spread with numerous commercial applications. The demand for industrial enzymes is on a continuous rise driven by a growing need for sustainable solutions (Adrio and Demain, 2005; Johannes and Zhao 2006; Kumar and Singh 2006).

1.7 Global market for enzymes

Enzymes are used in various sectors which include food manufacturing, cosmetics and pharmaceutical industries. At present, almost 4000 enzymes are known, among these, approximately 200 enzymes were originated from microbial sources. However, only about 20 enzymes are produced on truly industrial scale. The world enzyme demand is satisfied by about 12 major producers and 400 minor suppliers. Nearly 75% of the total enzymes are produced by three top enzyme companies, i.e., Denmark-based Novozymes, US-based DuPont (through the May 2011 acquisition of Denmark-based Danisco) and Switzerland-based Roche. The market is highly competitive, has small profit margins and is technologically intensive. According to a research report from Austrian Federal Environment Agency, about 158 enzymes were used in food industry, 64 enzymes in technical application and 57 enzymes in feedstuff, of which 24 enzymes are used in three industrial sectors. Almost 75% of all industrial enzymes are hydrolytic enzymes. Carbohydrases, proteases and lipases dominate the enzyme market, accounting for more than 70% of all enzyme sales. Many industrial processes are already adapted the use of microbial sources for large scale production because the chemical synthesis method have some disadvantages such as low catalytic efficiency and stability (Demain and Adrio 2010). Internationally the industrial enzymes market marginal value was around $105 million in 2012 but expected to grow significantly with an average of ≥10 % per year through 2017 to reach nearly $173 (Singh et al, 2016).

1.8 Lactase

Lactase catalyzes the breakdown of a disaccharide sugar found in milk known as lactose, into two monosaccharide sugars, galactose and glucose by the addition of a water molecule. The oxygen bridge connecting the two sides of the lactose molecule is cleaved and this phenomenon called as hydrolysis (Fig. 1.2). Yin et al, (2017) reported that the microbial lactase was used as biocatalyst in industry to produce prebiotic galactooligosaccharides(GOS) from lactose. Lactase (E.C. 3. 2. 1. 23) (β-D-galactohydrolase, β-D-galactoside galactohydrolase, galactosyltransferase and β-galactosidase), can catalyze both hydrolytic and transfer reactions. This enzyme also cleaves *o*-glycosidic bond of other β-D galactopyranosides. The resulting sugars, glucose & galactose are sweeter, more soluble and easily digested. Thus, lactase increases sweetness, solubility and digestibility of the final product.

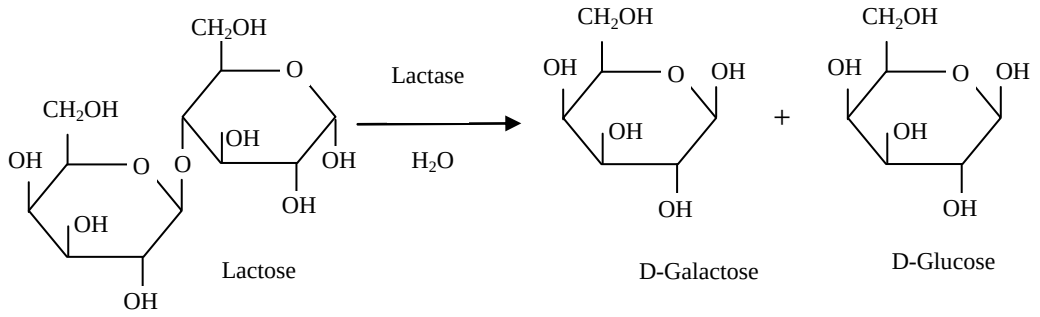

Fig. 1.2 Schematic presentation of lactose hydrolysis by lactase enzyme (Source: Prescott et al, 1996)

1.9 Structural properties of lactase

Lactases produced mainly by microorganisms and are released from the cytosol as oligomeric proteins. Lactase is composed of four polypeptide chains which is a tetramer and each monomer contains 1023 amino acids which form the distinct regions of structural domains with a compact three dimensional structure. The overall structure has horizontal two-fold axis of symmetry which forms long interface, one vertical which is activate interface and third is perpendicular. The crystal structure was initially proposed with four asymmetrical units of tetramers. Later the structure was elucidated with confined resolution as a single unit of tetramer, but asymmetrically. Deletion of amino acid residue from the lactase at the vertical position leads to weakening of active site and further dissociates into dimmers (Juers et al, 2012).

The molecular weight of lactase is a 464 kDa as a homotetramer. Each subunit of lactase consists of five domains and domain 1 is a jelly-roll type barrel, domain 2 and 4 are fibronectin type III-like barrels, domain 5 is a β-sandwich and domain 3 is a TIM-type barrel. The active site is present in the third domain of lactase and made up of elements from two subunits of the tetramer. The amino-terminal sequences of lactase and the α-peptide involved in α-complementation. The residue in active site region helps in stabilization of a four-helix bundle. The domains of structure were shown with different colors in Fig. 1.3. The complementation peptide, orange; domain 1, blue; domain 2, green; domain 3, yellow; domain 4, cyan; domain 5, red. The lighter and darker shades were used to differentiate the same domain in different subunits. The metal ions in each of the four active sites are shown as spheres: Na^{+}, green and Mg^{2+}, blue (Matthews, 2005).

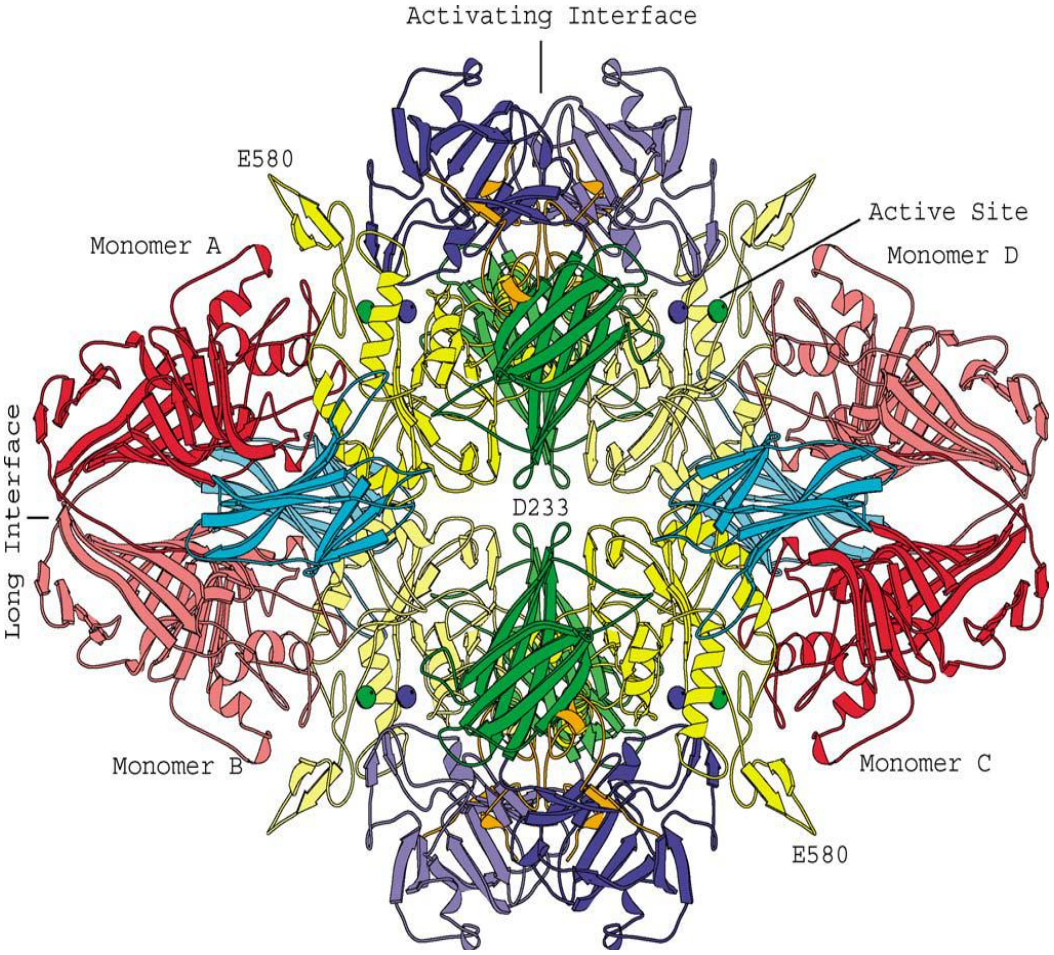

Fig. 1.3 3D-Structural view of lactase (source: Matthews, 2005)

1.10 Applications of lactase

Lactase main application area is dairy industry for production of low lactose containing milk to meet the need of populations who are suffering from lactose intolerance, sensitivity to lactose due to their lack of intestinal lactase (Albayrak and Yang, 2002). Lactose can also be hydrolyzed using acid, but this cause's color formation and fouling of the ion-exchange resins used in processing. Thus, an enzyme has more demand in industries because enzymes are able to hydrolyze lactose without side reactions. Low-lactose milk is produced in processing plant by supplying the lactase to milk and also the products like lactose reduced cottage cheese, processed cheese, ice-creams were commercially produced (McBean et al, 1998). Another use of lactase in dairy industry is to increase sweetness and digestibility of the final product. Since low solubility of lactose leads to its crystallization causing sandy, gritty texture which creates trouble for some dairy product such as ice-cream, frozen milk, dry milk, condensed and evaporated milk. Lactase also improves the utilization of high protein supplements containing milk. Lactose conversion is also used in sweetener production

from whey, a biological by-product of cheese processing, since most of the lactose pass to whey during cheese production (Qureshi et al, 2015). Lactose is utilized in bread making because of its physiological properties such as providing suitable texture and color. However, lactose is not fermented by ordinary baker's yeast. Thus, lactase can be used since its hydrolysis products can be readily fermented and improve the toasting of bread (Singh et al, 2016). Lactase was also used in the design of amperometric lactose biosensors which are used for estimation of lactose in milk and other milk based products to prevent lactose intolerance (Goktug et al, 2005). Pharmaceutical industries use the lactase enzyme for capsulation of chewable lactase tablets, which helps in the digestion of milk and dairy foods without gas, cramps, bloating, or diarrhea (Nath et al, 2014).

1.11 THESIS OBJECTIVES

The objectives of the present work are

1. To isolate and screen the lactase producing bacterium from dairy industrial effluents.
2. To characterize the lactase producing bacterium for strain identification.
3. To optimize the components of medium for the production of lactase using One Factor at a Time (OFAT) and Response Surface Methodology (RSM).
4. To optimize process parameters using OFAT and RSM for the parsimonious yield of lactase at laboratory scale and further modeling of physical parameters using Artificial Neural Networks (ANN).

1.12 THESIS ORGANIZATIONS

Thesis comprised with five chapters; Chapter 1 provides the information about lactose intolerance and lactase applications in dairy and other fields. Chapter 2 furnish the detailed review of literature related to different sources availability for production of lactase and methods and tools adapted in previous research works for enhanced production of higher lactase. The chapters 3, 4 and 5 gives the information about the methodologies and reagents used in experimental work and also provides an insight idea about the findings of the work and discussion part. The conclusions and scope for future work were summarized in chapter 6 and thesis is end up with references and appendices.

CHAPTER-II

REVIEW OF LITERATURE

2.1 Sources of lactases

In the current scenario, microbial strains have employed for commercial production of various essential enzymes. In previous studies, lactase was isolated from different sources which include bacteria, fungi and yeast (Haider and Husain, 2007).

2.1.1 Bacteria

Microbial species was found to be efficient for large scale production of lactase as compared to plant and animal sources (Nam and Ahn, 2011). The benefits associated with bacterial fermentation are secretion abilities, simple fermentation, high enzyme activity and stability, rapid growth and metabolic diversity and simple purification of the enzyme and hence still the *Bacillus* strains were ideal for commercial production of lactase (Picard et al, 2005; Prakaham et al, 2008). Lactase has been found in various bacterial strains belonging to the *Enterobacteriaceae* and *Pseudomonadaceae* (Tryland and Fiksdal, 1998). The dairy industry uses bacterial genera *Lactobacillus* and *Bifidobacterium* that are capable of producing lactase enzyme and enhances the digestion capacity in lactose intolerance patients. These bacilli are generally regarded as safe (GRAS) and thus, the lactase secreted by them can be consumed without excessive purification by lactose intolerance patients (Somkuti et al, 1998; Vinderola et al, 2003; He et al, 2008). *Bifidobacterium* was selected as a model bacterium for hydrolysis of lactose by the colonic microbiota (Arunachalam, 2004). Lactase production from *Bifidobacterium longum* CCRC15708, *B.circulans*, *Bifidobacterium adolescentis*, *L.reteri*, *L.plantarum*, *Bifidobacterium longum* B6 and *Bifidobacterium infantis* CCRC14633, *Lactobacillus delbrueckii*, *bulgaricus* 11842 were also investigated for the production of lactase (Burya and Jelen, 2000; Batra et al, 2002; Hsu et al, 2005). The bacterial group namely *Lactococci*, *Streptococci*, *Lactobacill*, *B. licheniformis*, *B.amyloliquefaciens* are well studied for relatively high quantity of lactase (Sani et al, 1999; Husain et al, 2010). The lactase producing bacterial species were represented in Table 2.1.

Table 2.1 Bacterial species reported for lactase production

S.No	Bacterial species	Location	Reference
1.	*Arthrobacter oxydans*	I	Banerjee et al, 2016
2.	*L. plantarum* MTCC2156	I	Gobinath et al, 2015
3.	*L. acidophilus* ATCC4356	I	Milika carevic et al, 2015
4.	*B. animalis* ssp. *lactis* Bb12	I	Prasad et al, 2013
5.	*Streptococcus thermophilus*	I	Princely et al, 2013
6.	*Bacillus safensi*	I	Nath et al, 2012
7.	*Bacillus* sp. MPTK121	-	Mukesh Kumar et al, 2012
8.	*Citrobacter freundii*	E	Lokuge & Mathew, 2010
9.	*Bacillus licheniformis* ATCC 12759	E	Akcan, 2011
10.	*Rahnella aquatilis* KNOUC601	E	Nam and Ahn, 2011
11.	*Bacillus* sp. NV1	-	Batra et al, 2011
12.	*Lactobacillus acidophilus*	I	Choonia and Lele, 2011
13.	*Bacillus stearothermophilus*	I	Chen et al, 2009
14.	*L. acidophilus* ATCC0291	-	Fung et al, 2008
15.	*B. longum* CCRC15708	I	Hsu et al, 2006
16.	*Saccharopolyspora erythraea*	E	Post and Luebke, 2005
17.	*Lactobacillus plantarum*	Cytoplasm	Silvestroni et al, 2002
18.	*Bacillus coagulans RCS3*	I	Batra et al, 2002
19.	*Bacillus circulans*	I	Boon et al, 2000
20.	*Escherichia coli*	I	Giacomini et al, 2001
21.	*Lactobacillus thermophilus*	I	Greenberg et al, 1981
22.	*Bacillus stearothermophilus*	--	Pederson, 1980
23.	*Leuconostoc citrovorum*	I	Singh et al, 1979

I- intra cellular; E-extra cellular

2.1.2 Fungi

Fungal lactase was very sensitive to inhibition especially with high concentration of galactose. Lactase production from fungal sources has acidic pH optima in the range of 2.5–5.4 (Boon et al, 2000). Traditionally, lactase was obtained from *Aspergillus niger* and *Aspergillus oryzae* because of acceptable yields from the fermentation of these cultures (Torsvik et al. 1998; Picard et al. 2005; Kosseva et al. 2009; Bibi et al. 2014). Otieno (2010) was used thermophilic fungus namely, *Talaromyces thermophilus* CBS23658 for production of intracellular lactase. The purified lactase from *Aspergillus oryzae* showed optimum pH 4.5 with o-nitrophenyl β-D galactopyranoside (ONPG) and 4.8 with lactose.

2.1.3 Yeasts

The yeast species, *Kluyveromyces lactis* and *Kluyveromyces marxianus* and *Saccharomyces fragilis* used as sources for production of lactase (Shaikh et al, 1997; Santos et al. 1998). The *Kluyveromyces lactis* is still the major commercial source for production of lactase and its major drawback is lower thermo stability (Chen et al, 2009). Yeast lactases are most active in the buffers of p^H 6.0–7.0 (Genari et al, 2003). The recent reports on the lactase production from different species of fungi and yeast were listed in the Table 2.2.

Table 2.2 Fungal and yeast sources for production of lactase

Yeast species	Location	Reference
Kluyveromyces marxianus CCT7082	E	Machado et al, 2015
Kluyveromyces sp. CK8	E	Am-aiam et al, 2015
Kluyveromyces marxianus CCT7082	E	Manera et al, 2008
Kluyveromyces lactis NRRL Y-8279	I	Dagbagli, 2008
Kluyveromyces fragilis	Cell bound	Matioli et al, 2003
A.niger ATCC9142	E	Kazemi et al, 2016
Aspergillus tubengensis GR-1	E	Raol et al, 2014
Aspergillus oryzae	-	Shankar et al, 2007
Absidia sp.WL511	I	Li et al, 2006

I- intracellular; E- extracellular

2.1.4 Plant and Animal sources

Many plants like Rosaceae, almonds, wild roses, soy bean seeds and coffee and peanuts were reported for lactases, which are essential plant growth and fruit ripening. The lactase from papaya source was used in hydrolysis of its cell wall and softening of the fruit (Lopez et al, 2001; Bryant et al, 2003; Lazan et al, 2004). Lactase activity levels at different developmental stages in the cell wall of strawberry and in peaches, apricots, apples (*Fragaria ananassa*) fruits were also reported (Hadfield et al, 2000; Tateishi et al, 2001; Nagy et al, 2001; Flood and Kondo, 2004; Haider and Husain, 2007).

The lactase enzyme has also been found in intestine of various animals like dogs, rabbits, calves, sheep, goats, rats, rams, bulls, boars (Wallenfels et al, 1960). Moreover, numerous studies have shown the presence of enzyme in human saliva,

distribution of lactase in fetuses of primates and farm animals and in tissues of rats and mice and in plasma serum (Heilskov et al, 1951).

2.2 Optimization of nutritional components and physical factors for production of lactase

The cost of industrial enzymes primarily depends on method of fermentation and medium used for production. Hence, the production was enhanced through the design of medium consist of special inducers. Usually, defined medium was not established for the maximum production of any enzyme due to genetic diversity of various microbial sources. Each strain has its own favorable conditions for higher yields. Therefore, study of preferable conditions and suitable for a newly isolated strain is an important phenomenon in bioprocess industries to achieve the desirable production (Prakasham et al, 2005). The various chemical and physical variables namely, carbon, nitrogen and other supplementary sources like amino acids and metal ions and pH, temperature, rpm , dissolved oxygen (DO) may influence the production. The component of medium has a critical role in multiplication of cell number and to attain higher production. The activity of enzymes was affected by different parameters such as type of strain, cultivation conditions and the ratio of carbon and nitrogen sources in medium (Jurado et al, 2004). Therefore, the notable fermentation variables were studied for optimization of lactase production through submerged fermentation (Schneider et al, 2001; Jurado et al, 2004). Most of industrial lactases are produced by liquid fermentation in comparison with other fermentation methods.

2.2.1 Medium components effect on the lactase production

Alazzeh et al (2009) described that the carbon source in fermentation media is primary energy source and essential for growth and production of lactase and it also regulates the biosynthesis of lactase. Rezessy et al (2002) has studied the effect of various sugars such as galactose, melibiose, stachyose and raffinose on production of lactase. Duffaud et al (1997) was examined the effect of complex carbon sources namely, locust bean gum and guar gum on the production of lactase. Marisa et al (1996) was found that the stachyose was the favorable inducer for lactase production through cultivation of *Lactobacillus fermentum.* Similarly, Guar gum as carbon source was also reported to raise the lactase production through *Bacillus megaterium* VHM1 (Patil et al, 2010). The probiotic strain *Lactobacillus fermentum* CM33 in submerged process yielded high production when the medium was supplemented with lactose (Sriphannam et al, 2012).

Shaikh et al (1997) reported that the organic and inorganic nitrogen sources provide the favorable condition for production of lactase. Lokuge and Mathew (2010) have reported the nitrogen source soya bean meal highly enhanced the production of lactase by *Geobacillus* sp. The organic nitrogen sources such as peptone, tryptone and yeast extract have been studied for their effect on production by *Bacillus stearothermophilus* (Delente, 1974). In other hand, the combinational effect of yeast extract with ammonium sulphate was also studied for enhanced production of lactase by *Bacillus megaterium* VHM1 (Patil et al, 2010). Apart from carbon and nitrogen sources, the ionic components are also highly essential for enhanced production of lactase. The molar concentration of ions like Ca^{2+}, Mg^{2+}, Na^{+}, NH^{4+} and K^{+} may influence the production and stability of lactase (Garman et al, 1996). Similarly, Liu et al, (2007) was found that the minerals like $CaCl_2$, K_2HPO_4 and $MgSO_4$ and vitamins such as vitamin C and B shown the high effect on production.

2.2.2 Physical parameters effect on production of lactase

The fermentation process parameters such as inoculums size, temperature, pH, incubation time and agitation etc., may affect the yield. Therefore, optimization of factors is the primary step in designing the process for improving production. Growth of cells and production of lactase was strictly depends on the pH of the medium. The optimum pH range 6.0 to 7.5 highly preferred for lactase production from bacterial fermentation (Kamaran et al, 2015). The neutral pH for optimum production of lactase as reported through submerged fermentation by *E.coli* (Nagao et al, 1988) and *L.curvatus* R08 (Yoon and Hwang, 2008). Lactase from *Kluyveromyces marxianus* was produced at pH 3 through submerged fermentation (Al-Jazairi et al, 2015). The favorable temperature for production of enzymes varied based on type of organisms. Most of the probiotic organisms like *Lactobacillus* and *Bfidobacterium* species were grown at 30-37 °C for lactase production (Xiao et al, 2000). Rezessy-Szabo et al (2003) examined the lactase production from *Thermomyces lanuginosus* and it can grow easily on yeast phosphate soluble starch (YpSs) medium at 45 ºC. Optimum growth occurs between 45-50 ºC. No growth is observed at temperatures either below 30 ºC or above 60 ºC. The optimum temperature 30 °C for lactase production by the cultivation of by *lactobacilli* strain was reported (Murad et al, 2011). Mixing is required for uniform distribution of nutritional components of medium to the growing cells. The mixing conditions were varied based on type of the organism and design of the vessels used. Maximum production of lactase was attained at 180 rpm in shake

fermentation from *Bacillus megaterium* VHM1 (Patil et al, 2010). Different process parameters for production of lactase from various sources were listed in Table 2.3.

Table 2.3 Fermentation variables for production of lactase from various microbial sources

Microorganism	**Carbon**	**Nitrogen**	**pH**	**Temp. °C**	RPM	**Incubation time, h**	**Activity, U/ml**	**Reference**
Lactobacillus delbruekii subsp. *bulgaricus*	Lactose	peptone	7.2	37	-	48	7.69	Mazhar Ali et al, 2016
Lactobacillus acidophilus ATCC 4356	Lactose	Yeast extract and Meat extract	6.5–7.5.	37	150	48	1.05	MilicaCarevic et al, 2015
Streptococcus thermophilus	Lactose	Whey	7.2	40	-	24	7.76	Princely et al, 2013
B. animalis ssp. *Lactis* Bb12	Lactose	Yeast extract and peptone	6.8	37	180	18	6.84	Prasad et al, 2013
Bacillus sp.	Lactose	peptone	7.0	35	200	48	0.294	Jayasree natarajan et al, 2012
Bacillus megaterium VHM1	Guar gum	Peptone	7.5	50	180	28	1.6	Patil et al, 2010
Lactobacillus curvatus R08	Raffinose	Peptone, Yeast extract and Meat extract	7.0	35	-	12	40	Yoon and Hwang, 2008
Leuconostoc mesenteriodes JK55	Raffinose	Peptone, Yeast extract and Meat extract	7.0	35	-	12	40	Yoon and Hwang, 2008
Bacillus stearothermophilus	soybean Meal	Yeast extract	6.5–7.0	60	-	16	1.08	Gote et al, 2004
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842	Lactose	Yeast extract	5.6	43	150	48	3.09	Vasiljevic and Jelen, 2001
Citrobacter freundii	Raffinose	Peptone	8.0	30	115	12	14	Lokuge et al, 2000
Escherichia coli	Raffinose	Peptone	7.0	30	115	6	1.2	Lokuge et al, 2000

2.3 Statistical tools for improved lactase production from microbial sources

Many researchers were tried to enhance the yield by adapting a statistical approaches like Taguchi and RSM. The statistical studies for design of experiments using the tools like RSM, orthogonal array, artificial neural network and genetic algorithms are well recognized for providing the economically feasible solutions for optimization in bioprocessing sector. The classical optimization method was time taking and it cannot give a complete picture of independent factors affecting the production and also the combinational interaction of variables on process could not be understood. But in Response Surface Methodology (RSM), the effect of independent variables and their interactions on yield were predicted (Sreenivas Rao et al, 2004; Prakasham et al, 2005; Ravichandra et al, 2007).

2.3.1 Response Surface Methodology (RSM)

RSM is an acquisition of statistical and mathematical techniques which are used to describe the relative effects between process output and variables in process. RSM is a more convenient tool for designing experiments, plotting models, evaluating the effects of factors and exploring optimum conditions of factors for significant responses. RSM is also used for optimization of prominent varieties of fermentation media and studying interactions among various bioprocess parameters with the minimum number experiments (Amid et al, 2011; Sanjivani et al, 2016). RSM involves assumptions to describe the relative influence of variables for the objective function and the frequent model employed for optimization was second order polynomial. RSM model also generates mathematical equation based on the experimental data (Basri et al, 2007; Bas and Boyaci, 2007). Banerjee et al (2016) studied the lactase production through the cultivation of bacterium *Arthrobacter oxydans* using Box-Behnken design. The optimum production of lactase was found at pH, 6.76; temperature, 36.1 °C and rpm, 121.37. Am-aiam et al (2015) reported that the highest lactase activity was produced from the cultivation of thermotolerant yeast achieved through optimization nutritional variables of the medium consists of yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, lactose and $MgSO_4.7H_2O$ by a central composite design (CCD). The statistical tools employed for the production of lactase from different microbial sources were represented in Table 2.4.

Table 2.4 Statistical tools for enhanced lactase production from microbial species

S.No	Organism	I/E	Fermentation Type	Tool used	Variables in model design	Activity	Reference
1	*Bacillus* sp. LX	E	Submerged	RSM	galactose, peptone , $MnSO_4$	1.06	Jaekoo Lee et al, 2013
2	*S.griseoloalbus*	E	Submerged	RSM	carbon source, yeast extract, $MgSO_4$, $FeSO_4$ and salinity	50	Anisha et al, 2008
3	*Lactobacillus fermentum* CM33	-	Submerged	RSM	lactose, tryptone and Tween8	4.98	Sriphannam et al, 2012
4	*Aspergillus foetidus* ZU-G1	E	Submerged	RSM	soybean meal, wheat bran, KH_2PO_4, $FeSO_4.7H_2O$ and the medium initial	64.75	Liu et al, 2007
5	*Kluyveromyces marxianus* CCT 7082	E	Submerged	RSM	pH, Lactose, Glycerol	31.8	Machado et al, 2015
6	*Kluyveromyces* sp. CK8	E	Submerged	RSM	yeast extract, lactose, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4.7H_2O$	8.82	Am-aiam et al, 2015
7	*Kluyveromyces marxianus* CCT7082	E	Submerged	RSM	Lactose, Yeast t extract) And $((NH_4)_2SO_4)$	10.6	Manera et al, 2008

I- intracellular; E- extracellular

2.3.2 Artificial neural networks (ANN)

In practice, both in RSM and orthogonal array tools have limitation towards the variable level in plotting of variables on response (Fang et al, 2003). To overcome these problems artificial Feed-Forward Neural Networks (FFNN) and genetic algorithms (GA) were used. ANN model was the well established and fashionable tool in statistical analysis of experimental data and also used to understand the biotechnology applications like expression function, functional analysis of genomics and proteomics. The ANN approach has the wide applications in solving nonlinear

problems. An artificial neural network was extremely connected network structure consisting of many processing elements which are capable of performing parallel computation for data processing (Manohar et al, 2005). In the literature survey, it was observed that many researchers were carried out their studies on comparison of ANN and RSM to predict accuracy of the models (Arulsudar et al, 2005). Some researchers used hybrid GA-FNN for optimization studies. Hanai et al (1999), Hongwen et al (2005), Nagata and chu (2003) and Kazemi et al (2016) were conducted the research on optimization study by using Orthogonal arrays design for production of lactase through *A.niger* ATCC9142 by solid state fermentation. They reported the high significant range of variables, which includes the solid substrates (wheat straw, rice straw and peanut pod), carbon/nitrogen (C/N) ratios, incubation time and inducer. The yeast species, *Kluyveromyces marxianus* capable of producing homologous enzymes, such as β galactosidase, as well as heterologous proteins and also can grow on different substrates including lactose as the sole carbon and energy source (Furlan et al, 2000; Martins et al, 2002; Ribeiro et al, 2007). The lactase production from *Aspergillus niger* MRSS234 through the screening of various nitrogen sources, minerals and enzyme inducers by statistical method was reported (Srinivas et al, 1994). In solid state fermentation, the lactase production was investigated by statistical approach through screening of process variables and their optimization from *Aspergillus foetidus* ZU-G1 (Liu et al, 2007) and *Streptomyces griseoloalbus* (Anisha et al, 2008). To the best of our knowledge no reports were available for optimization of different variables for extracellular lactase production from *Bacillus subtilis.* Many studies reported on production of lactase from fungal sources across the world, but till date not many studies was reported on lactase enzyme produced by *Bacillus* sp. for higher yields. Hence, the effort has been made for improvement of the lactase production. In present study, screening and isolation followed by identification has resulted in a new strain of *Bacillus* sp. that produces higher lactase which is discussed in the later part of this thesis.

2.4 MOTIVATION

Reducing the lactose intolerance has thrown a challenge for researchers, because 65% of human adults (and most adult mammals) were surveyed as lactose intolerants due to the lack of the secretion of intestinal lactase after weaning. To overcome from lactose intolerance the dairy and other milk products were supplemented with lactase producing strain or through the large scale production. There are few reports and

literature available on the production of lactase from bacteria. Lactase has excellent application in dairy industry for hydrolysis of lactose and can reduce the intolerance. Hence, the importance of the study is to explore microbial sources to make supplements available for genetically predisposed lactose intolerant subjects.

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CHAPTER-III

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF LACTASE PRODUCING BACTERIUM

3.1 INTRODUCTION

The dairy effluents are fully occupied with nutrients, lactose and other sanitizing agents which facilitate a suitable environment for rapid population of microbial species. The bacterial species present in dairy effluent has proved for probiotics and these species were highly preferred for production of lactase (Sreekumar and Krishnan, 2010). So far, most of the probiotic and prebiotic bacterial oligosaccharides have been used in combination with dairy products, since these products often contain large amounts of lactose. Much attention has been focused on the lactase, which involved in the bacterial metabolism of lactose. In addition to normal hydrolysis of the β-D-galactoside linkage in lactose and lactase also catalyze the formation of galactooligosaccharides through transfer of one or more D-galactosyl units onto the D-galactose moiety of lactose. This transgalactosylation reaction has been shown to be a characteristic of lactase enzyme from a variety of bacterial and fungal species (Moller et al, 2001). Picard et al (2005) reported that the bacterial lactase has highly preferred for hydrolysis of lactose due to their superior activity and stability. From the past three decades many researchers proved that lactase has found use in numerous applications such as dairy, confectionary, baking and soft drinks industries (Grosova et al, 2008; Panesar et al, 2010).

Ladero et al (2001) studied the biochemical parameters like stability and activity of lactase from *E.coli* using ONPG as substrate. Sharma et al (2014) was isolated and screened the lactase producing bacterial species by X-gal plate method and activity of the lactase was measured by ONPG assay. In previous studies many researchers were used the 16S rRNA technique for identification of bacterial strains due to its reliability (Macrae et al, 2000; Cardinale et al, 2004). The strain identification of bacteria that exist in the environment has been based on the determination of 16S rRNA sequences of amplified genes (Mota et al, 2005).

3.2 MATERIALS AND METHODS

3.2.1 Morphological characterization of lactase producing isolate

The cell morphology and motility of isolate were examined by light microscopy. Spore-staining was done on the basis of Schaeffer and Fulton's method. Smears were made with 56 h old cultures and fixed with gentle heat. The smear was immersed in malachite green stain for 3 to 6 min at 37 °C in dark followed by washing with tap water and counter stained with Schaeffer and Fulton spore stain-B for 30 s. The smear was air dried and viewed under a microscope with 100x resolution (Miller, 1972).

3.2.2 Growth curve

The flask contains 100 ml of lactose broth medium and was inoculated with 5 ml of the inoculum for growth analysis. The flask was kept for incubation in orbital shaker at 37 ^{o}C and speed of 160 rpm. For every 3 h, 5 ml of the culture medium was taken and the absorbance was measured at 600 nm using calorimeter against an uninoculated blank and the same step was repeated till the cell growth had reached to stationary phase (Kumar et al, 2014)

3.2.3 Antimicrobial activity by agar diffusion method

The antimicrobial activity of isolate was screened on agar plate and was overlaid with 25 ml of overnight active culture. Different wells were made in agar plate and were crammed with 100 micro liters cell free supernatant of overnight culture obtained by centrifuging broth at 15000 rpm, 4 °C for 20 min. The plates were sealed with parafilm and incubated at 37 ^{o}C for 24 h in incubator. The diameter of inhibition zones extending across the well was measured and clear zone of more than 1 mm was considered as positive inhibition (Asimi et al, 2013).

3.3 Biochemical characterization

3.3.1 X-gal plate method

Dairy effluent sample was collected from Sangam Dairy industry, Vadlamudi village, Guntur (district), Andhra Pradesh, India. The sample was serially diluted and plated on lactose agar medium containing contains 50 μg/ml of 5-bromo-4-chloro-3-idolyl-β-D-galactopyranoside (X-gal) for isolation of lactase producing bacterial isolates. The plates were incubated for 24 h at room temperature. The blue colonies on the plate were subcultured on nutrient agar medium by streak plate method (Maity et al, 2013).

3.3.2 Lactase enzyme assay

The lactase activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The ONPG solution was prepared with phosphate buffer and used for assay. 0.5 ml of enzyme source was added with 2.0 ml of substrate and incubated for 30 min. The reaction was stopped with addition of 0.5 ml of 1 M Na_2CO_3 and absorbance was recorded at 420 nm. Activity of lactase was determined from ONP standard graph. One unit of activity defined as amount of enzyme that liberates 1 micromole of ONP from the substrate per min under assay conditions (Ghosh et al, 2012).

3.3.3 ONPG disc assay

The lactase producing ability of VUVD001 isolate was tested by using ONPG discs (Kalogridou-Vassiliadou, 1992).

3.3.4 Zymogram Analysis for Lactase Activity

Zymogram assay for cell free extract of isolated strain was performed using native-PAGE method with 10% (w/v) polyacrylamide gel. After electrophoresis the gel was incubated in 0.25 mM X-gal solution at room temperature for 10 min and the hydrolysis of X-Gal was confirmed by the formation of blue band on the gel (Trimbur et al, 1994).

3.3.5 IMViC, Sugar fermentation and enzyme tests

The IMViC test was done for determination of biochemical properties of the VUVD001 isolate. The sugar fermentation test for different sugars like lactose, glucose, sucrose, maltose and starch was performed to determine the metabolization ability of VUVD001 isolate. The VUDV 001 was also tested for enzyme activity of catalase and amylase (Princely et al, 2013).

3.3.6 Salt tolerance test

Salinity test was carried out to determine the ability of isolate in a salt-rich environment. The sterile nutrient broth medium was prepared with different NaCl concentrations like 2%, 4%, 6%, 8% and 10%. Then, the tubes were inoculated with one loop of VUVD001 culture and incubated for 24 h at 37 °C in shaking incubator. The biomass absorbance was measured at 600 nm using calorimeter (Patil, 2014).

3.3.7 Thermo stability test

Thermo stability test was done to determine the survival efficiency of the bacterium at high temperature. A loop full culture of VUVD001 isolate was streaked onto the agar medium. This plate was incubated in inverted position for 24 h at different temperatures range from 20 to 55 ^{o}C in an incubator (Panda et al, 2013).

3.4 Molecular characterization of the lactase producing isolate

3.4.1 Bacterial DNA isolation

The standard protocol of Hoffman and Winston was used for bacterial DNA extraction (Hoffman and Winston, 198) Lactase producing colony was inoculated in nutrient broth and was grown for 36 h at 37 ºC. The cells were collected from 5 ml of the broth culture. Lysozyme (100 µl) was added and incubated at room temperature for 30 min, followed by the addition of 800 µl cell lysis buffer (1X TE buffer (pH-8.4); 1 % SDS and 100 µg proteinase K). Nucleic acids was separated with two rounds of extraction with phenol: chloroform: isoamylalcohol (25:24:1) mixture. The aqueous layer containing genomic DNA was separated and then 800 µl of isopropanol was added on top of the solution. The two layers were mixed gently to precipitate the genomic DNA. Genomic DNA was then harvested by centrifugation at 12,000 rpm for 10 min at 4 ^{o}C. Pellet was washed with 70% ethyl alcohol. The DNA pellet was air dried and dissolved in 50 µl of 1X TE buffer. The quality of DNA was determined by running on 0.8% agarose gel stained with ethidium bromide (Sen et al, 2010).

3.4.2 PCR amplification

PCR reaction was done in a gradient thermal cycler (Eppendorf, Germany). The universal primers 27F and 1429R were used for 16S rDNA fragment amplification. The reaction mixture of 100 ng genomic DNA, 2.5 Units *Taq* DNA polymerase, 5 µl of 10X PCR amplification buffer, 200 µM of dNTPs, 10 pico moles each of the two universal primers and 1.5mM $MgCl_2$. Amplification was done by initial denaturation for 3 min at 94 ºC and then subjected to 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 ºC for 30 s and elongation at 72 °C for 2 min and a final extension conducted at 72 °C period of 10 min. The amplicon of 16S rDNA was confirmed by agarose gel electrophoresis and visualized on UV transilluminator by staining with ethidium bromide (Sen et al, 2010).

3.4.3 DNA sequencing

DNA sequencing was performed at Helini Biomolecules Pvt Ltd at Chennai, India. Obtained DNA sequences were subjected to BLAST analysis through Pub Med database (http://www.ncbi.nlm.nih.gov/blast) using the algorithm BLASTN and compared with other sequences to analyze bacterial strain and its phylogeny.

3.5 RESULTS AND DISCUSSION

3.5.1 Morphological characterization

3.5.1.1 Gram staining

The VUVD001 isolate was sub-cultured on nutrient agar plates by streak plate method to acquire pure culture (Fig.3.1a) and the same culture was examined for microscopic observations. The morphology of the VUVD001 was observed as Gram-positive and rod shaped (Fig. 3.1b).

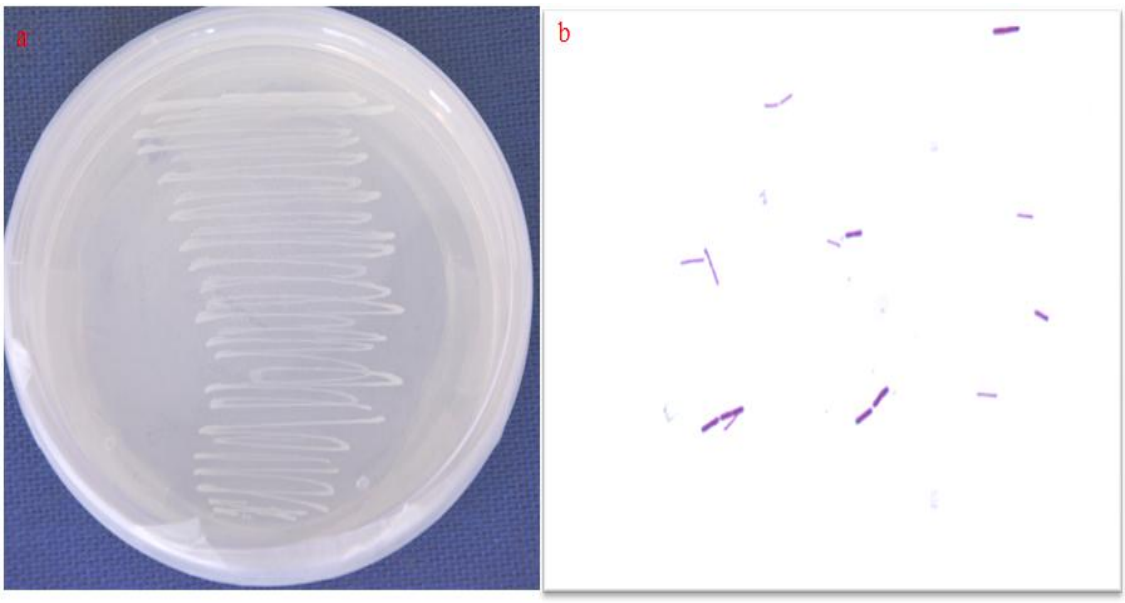

Fig. 3.1 Morphological study of VUVD001 isolate; a) pure culture b) Gram staining

3.5.1.2 Spore staining

Spore production was the vital characteristic feature of a few microorganisms. Bacterial spores are microbial cysts which can exist in the unfavorable conditions like heat, dehydrated and radiation state. The spores of *B. subtilis* species were being used as probiotics and competitive exclusion agents for human consumption (Casula and Cutting, 2002). Schaeffer and Fulton's spore stain was showed the positive results for isolate VUVD001 which produced the light green spores (Fig.3.2).

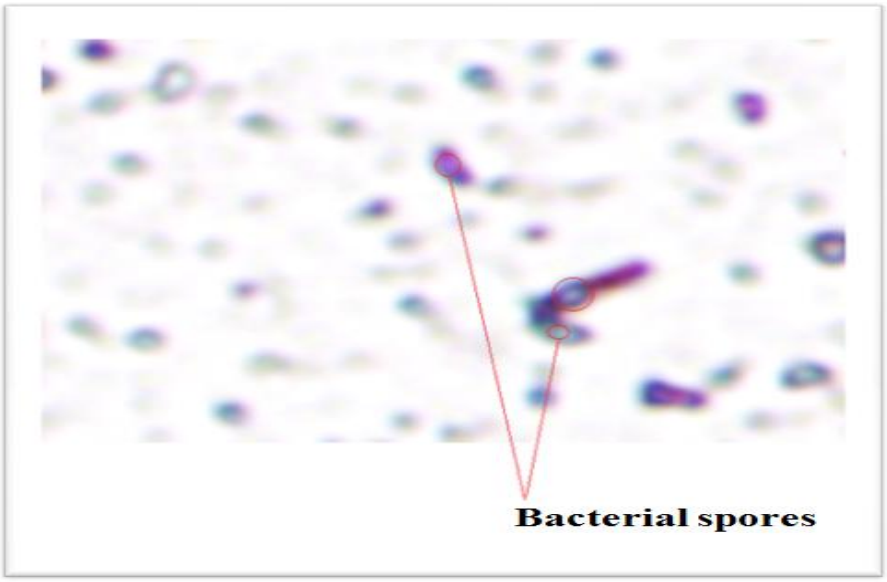

Fig.3.2 Spore staining of VUVD001 isolate

3.5.1.3 Growth curve

The growth profile of *B.subtilis* VUVD001 isolate was studied at different time intervals up to 48 h. During incubation period, the concentration cell mass was measured by taking an absorbance for every 3 h. The production of lactase enzyme from cultivation of *B.subtilis* VUVD001 strain was started after 9 h and then lactase activity was improved as the growth of cell increased up to 36 h. The maximum lactase activity was found to be 15.13 U/ml after 36 h of incubation and beyond this point, the growth and activity was slowly reduced upto 48 h (Fig.3.3).

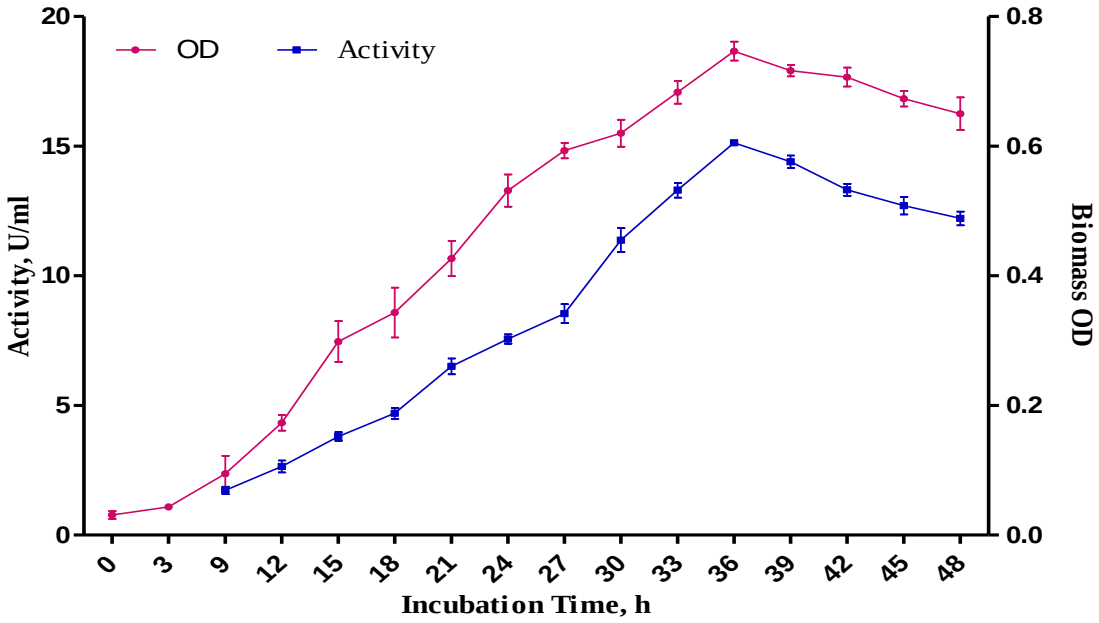

Fig. 3.3 Growth curve analysis for VUVD001

3.5.1.4 Antimicrobial activity

The cell-free supernatant of VUVD001 isolate was tested for antimicrobial effect against the pathogenic test organisms. The maximum activity was observed on the *Enterobacter aerogenes* MTCC111 and *Klebsiella pneumonia* MTCC109 (Fig.3.4). The minimal activity was found against *Staphylococcus aureus* MTCC96. The zones of inhibition on the growth of selected pathogenic strains were shown in the Table 3.1.

Table 3.1 Antimicrobial activity of cell-free supernatant of VUVD001

Test organism	**Zone diameter in mm**		
	20 µl	**50 µl**	**100 µl**
E. aerogenes MTCC111	15	21	26
K. pneumonia MTCC109	14	16	22
S. aureus MTCC96	-	10	10

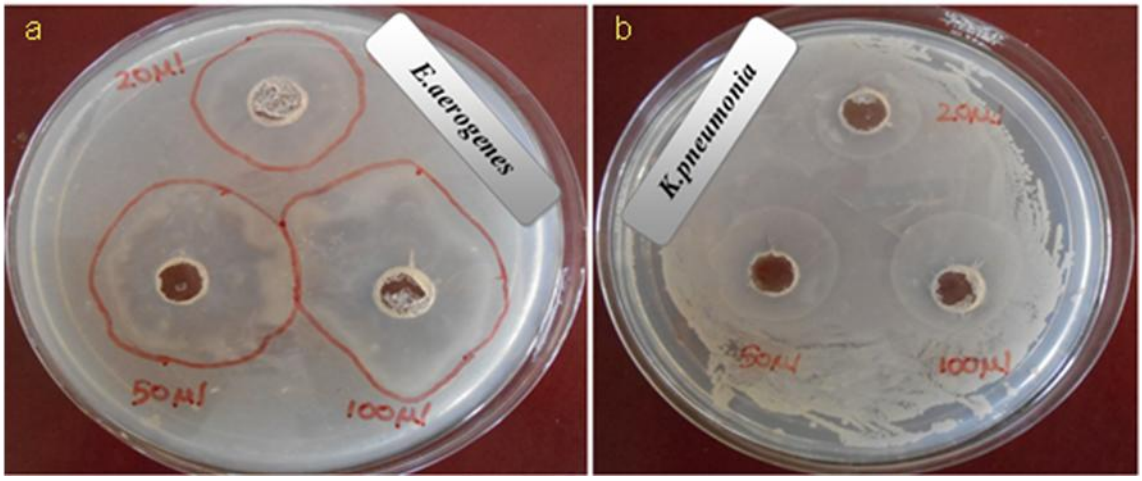

Fig. 3.4 Antimicrobial activity of VUVD001 isolate against pathogenic strains; a) *E.aerogenes* and b) *K.pneumonia*

3.6 Biochemical characterization

3.6.1 X-gal plate method

The lactase producing bacterial colonies were isolated from dairy effluent by X-gal plating method. A total of 46 isolates obtained from dairy effluent, a highest lactase producing bacterium, named as VUVD001 (working sample id) was selected for the study (Fig.3.5). Sreekumar and Krishnan (2010) were also reported that the X-gal method for screening of lactase producing bacterial isolates. Maity et al (2013) were also isolated the lactase producing bacterial strains from soil sample.

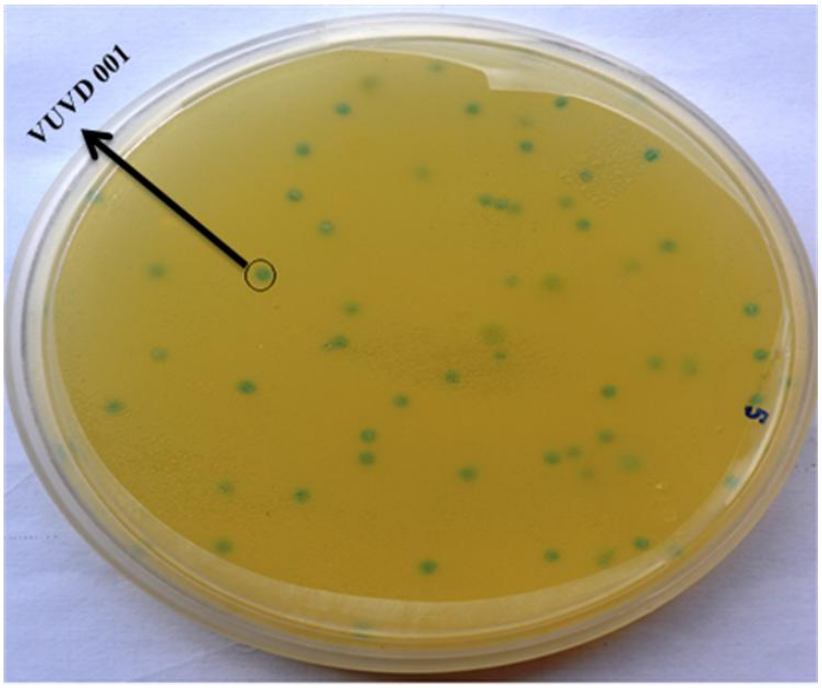

Fig. 3.5 Isolation of lactase producing bacterial isolates from dairy effluent by X-gal plating method

3.6.2 Lactase enzyme assay for blue bacterial isolates

Among all blue isolates the VUVD001 produced highest activity and was found to be 15.10 U/ml after 36 h incubation. The activity of lactase positive isolates was showed in Table 3.2. Our experimental results showed the improved lactase activity compared with other previous results. Arekal et al (2014) reported that highest lactase activity of 10.6 U/ml was obtained with *Lactobacillus plantarum* MTCC 5422 in the soy whey based medium at optimized conditions of incubation time 33 h, pH 6.6 and temperature 37 °C. Hsu et al (2005) reported the maximum lactase activity of 18.6 U/ml was obtained in submerged fermentation with *Bifidobacterium longum* CCRC 15708. Based on biochemical characterization, it was interpreted as the isolate was positive for lactase activity.

3.2 Lactase activity for the isolated bacterial colonies

S.No	Strain name	Activity, U/ml
1.	VUVD001	15.10
2.	VUVD 002	12.23
3.	VUVD 003	11.35
4.	VUVD 004	10.26
5.	VUVD 005	7.64
6.	VUVD 006	7.41
7.	VUVD 007	6.45
8.	VUVD 008	9.54
9.	VUVD 009	2.68
10.	VUVD 010	3.67
11.	VUVD 011	4.42
12.	VUVD 012	5.24
13.	VUVD 013	3.47
14.	VUVD 014	4.75
15.	VUVD 015	5.67
16.	VUVD 016	6.47
17.	VUVD 017	6.45
18.	VUVD 018	4.27
19.	VUVD 019	7.84
20.	VUVD 020	1.86
21.	VUVD 021	5.67
22.	VUVD 022	5.74
23.	VUVD 023	4.26
24.	VUVD 024	3.12
25.	VUVD 025	4.26
26.	VUVD 026	5.27
27.	VUVD 027	4.26
28.	VUVD 028	3.54
29.	VUVD 029	4.26
30.	VUVD 030	4.26
31.	VUVD 031	3.47
32.	VUVD 032	5.24
33.	VUVD 033	6.10
34.	VUVD 034	5.22
35.	VUVD 035	3.84
36.	VUVD 036	7.45
37.	VUVD 037	6.41
38.	VUVD 038	5.31
39.	VUVD 039	4.28
40.	VUVD 040	3.64
41.	VUVD 041	5.24
42.	VUVD 042	12.20
43.	VUVD 043	6.23
44.	VUVD 044	4.26
45.	VUVD 045	2.67
46.	VUVD046	4.65

3.6.3 Qualitative assay for VUVD001 by ONPG disc method

In our study lactase producing bacterial strain VUVD001 was screened by ONPG disc method and observed that the reaction mixture turned to deep yellow color after incubation indicating that our bacterial strain has ability to hydrolyze ONPG into ONP (Fig.3.6). Favier et al (2011) reported similar method in the biochemical screening of *Bifidobacteria* for lactase activity.

Fig. 3.6 Primary screening of lactase producing activity of VUVD001 by ONPG disc method

3.6.4 Zymogram Analysis

Lactose hydrolyzing enzyme activity in crude cell free extracts of *B.subtilis* VUVD001 strain was confirmed through zymogram assay. The native PAGE gel was stained with chromogenic X-gal. The enzyme performed hydrolysis of X-gal which was observed in gel with distinct blue color band (Fig. 3.7). Previous reports have been revealed that the X-gal hydrolysis through zymogram assay was used for confirmation of lactase producing nature of a cold-adapted bacterium named as *Rahnella aquatilis* KNOUC601 (Nam et al, 2011)**.**

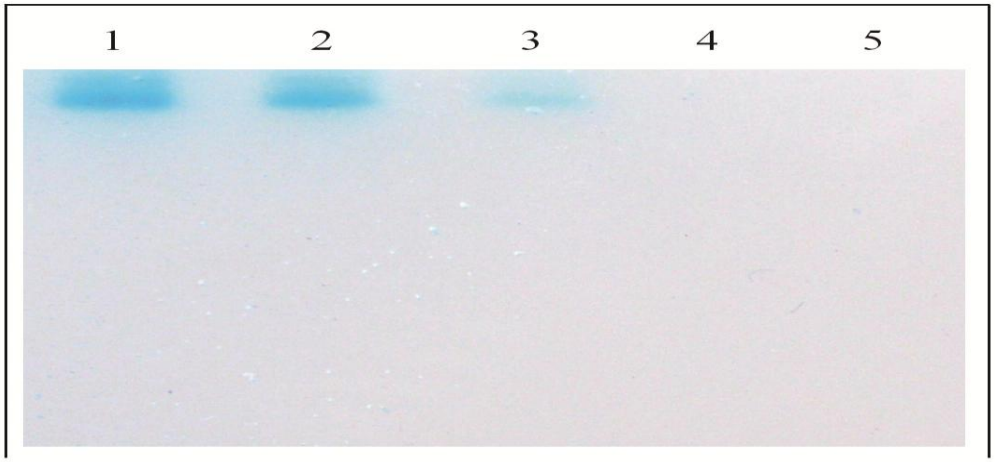

Fig.3.7 Zymogram analysis of concentrated cell free extracts of *B. subtilis* VUVD001 isolate. Lane 1 & 2 represents X-gal hydrolyzing enzyme from VUVD001 grown at different temperatures (37 ^{o}C & 35 ^{o}C); Lane 3 – positive control (*Lactobacillus* sp. ATCC-8008); Lane 4 – Negative control (*B. cereus* ATCC-10876); Lane 5- blank.

3.6.5 IMViC test

The yellow color in the tube is an indicative that the VUVD001 strain is negative for indole test (Fig. 3.8a). Upon the addition of two drops of methyl red reagent there is no color change the culture tube that indicates that the isolate is negative for methyl red test (Fig. 3.8b). Voges-Proskauer test was performed to differentiate organism based on the ability of isolate to produce acetylmethylcarbinol product from glucose fermentation. The overnight culture medium of isolate was turned into reddish color on addition of Voges-proskauer reagents. It indicates that the isolate was positive and no color change was observed in negative control, *E.coli* and blank. Hence, the results support that isolate was positive for acetylmethylcarbinol (Fig. 3.8c**)**. Citrate utilization test was performed to determine the ability of isolate to utilize sodium citrate as carbon source and inorganic ammonium di hydrogen phosphate ($NH_4H_2PO_4$) as nitrogen source. After four days of incubation the isolate color on agar medium was changed from green to blue. Hence, the isolate successfully utilizes sodium citrate and ammonium di hydrogen phosphate for growth (Fig. 3.8d). Similar studies were conducted by Abiola and Oyetayo (2016) for the biochemical characterization of bacterial species.

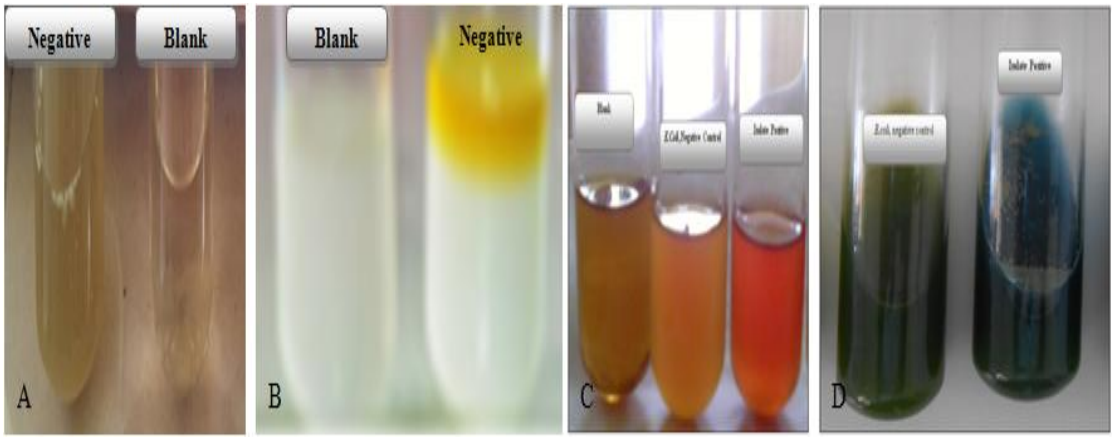

Fig.3.8 (a)-(d) IMViC test for biochemical characterization of VUVD001

3.6.6 Carbohydrate utilization test

The VUVD001 isolate shown positive result for utilizing various carbon sources namely glucose, lactose, fructose, sucrose and starch by changing the color of phenol red broth medium from red to yellow due to change in pH of medium after 24 h of incubation. Based on this observation it was found that the VUVD001 strain was successfully utilized these sugars as carbon source for growth and also no gas was trapped into Durham tube (Fig.3.9).

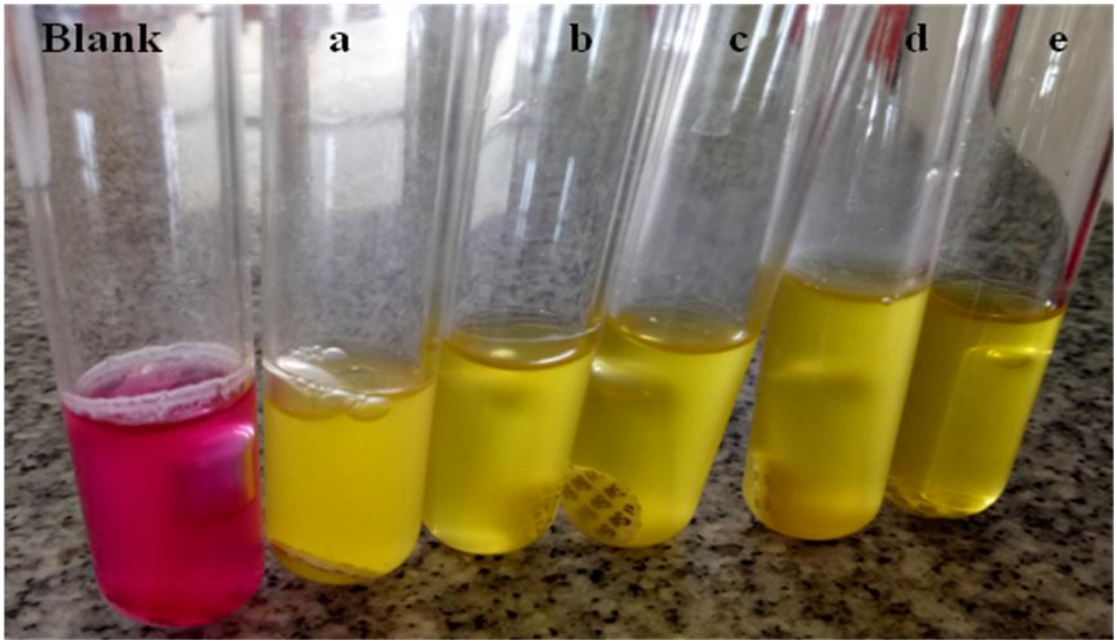

Fig. 3.9 Carbohydrate fermentation tests for VUVD001 strain; a) glucose, b) lactose, c) fructose, d) sucrose and e) starch

3.6.7 Starch hydrolysis and catalase test

The isolate was also screened for amylase by starch hydrolysis. The yellow colored plate shown that the isolate was positive for amylase and the bacterium *E.coli* was negative control for amylase (Fig. 3.10a&b). The formation of bubbles was observed by placing loop full culture of isolate on a glass slide containing few drops of hydrogen peroxide. It means that isolate produces catalase enzyme and it splits hydrogen peroxide into water and oxygen, hence the isolate was catalase positive (Fig. 3.10c)**.**

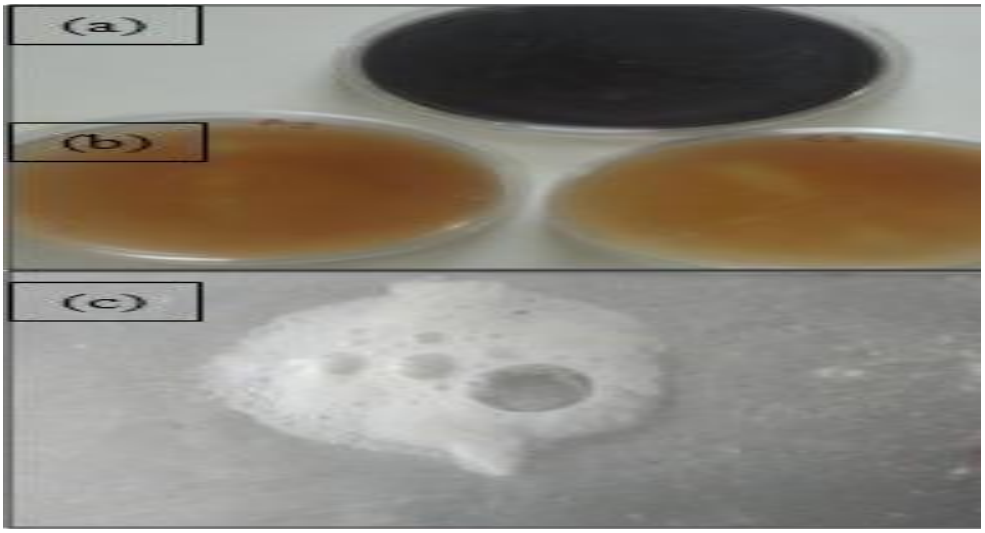

Fig. 3.10 (a)-(c) Starch hydrolysis and catalase test for the VUVD001 strain

3.6.8 Salt tolerance test

The growth efficiency of isolate in salt rich environment was studied by salt tolerance test and isolate was able to survive at high salt concentration (8% NaCl). The maximum growth was observed with 4% (w/v) sodium chloride concentration (Fig.3.11). The mineral sources may influence the proliferation rate of microorganisms. Hudaaneetoo et al (2015) reported that the lactic acid bacterium was able to tolerate up to 4% NaCl concentrations. Here, our isolated strain has shown significant growth up to the concentration of 6% NaCl. Similarly, in the earlier studies the salts like NaCl and $MgCl_2$ were proved as growth influencing factors (Pulicherla et al, 2013).

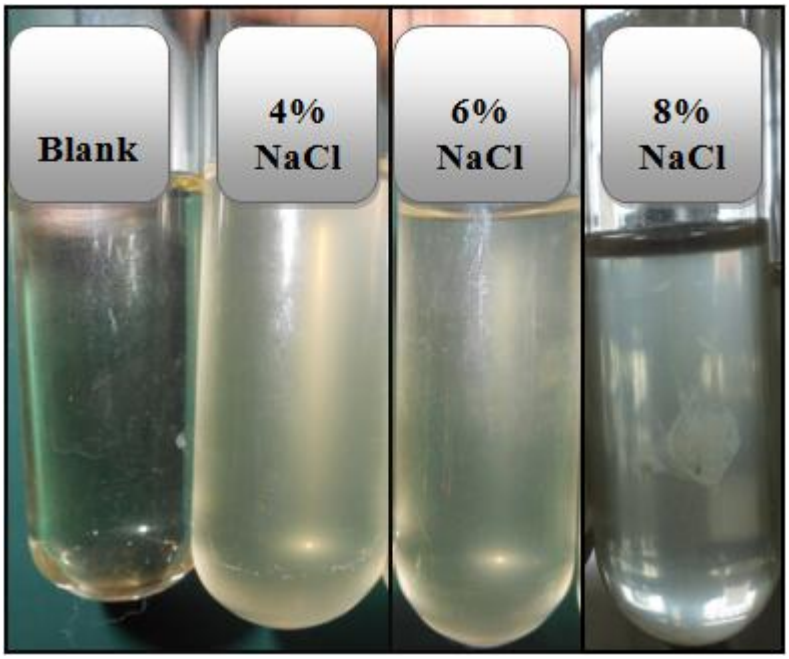

Fig. 3.11 Salt tolerance test for VUVD001 isolate

3.6.9 Thermo stability test

From the results of thermo stability test it was observed that the VUVD001 isolate was able to survive at 55 ^{o}C. The plate has shown growth of VUVD001 after 24 h of incubation in an incubator (Fig.3.12). The physiological and biochemical characteristics of VUVD001 isolate was represented in Table 3.3.

Fig.3.12 Thermostability test for VUVD001

Table 3.3 The physiological and biochemical characteristics of isolate

Tests	**VUVD001 isolate**
Gram staining	+
Spore staining	+
Growth at different pH	
5	+
6	+
7	+
8	+
9	-
Growth on NaCl %	
2	+
4	+
6	+
8	+
10	-
Growth at temperatures (^{o}C)	
20	+
25	+
30	+
35	+
40	+
55	+
Starch hydrolysis	+
Indole test	-
Methyl red test	-
Voges-proskauer	+
Citrate utilization	+
Oxidation–fermentation	Fermentative
Aerobic growth	+
Lactase	+
Catalase	+

3.7 Molecular Characterization and identification of VUVD001

3.7.1 Amplification

The PCR amplification of ribosomal DNA was amplified with universal primers of rDNA and produced amplicon of 1400 bp was obtained (Fig.3.13). Since DNA sequencing was performed directly on PCR amplicon, a 1000 bp DNA was obtained after merging forward and reverse sequencing reads. This highly conserved region of 16S rDNA was identified by blast search and multiple sequence alignment was done with sequences of other *Bacillus* species obtained from GenBank.

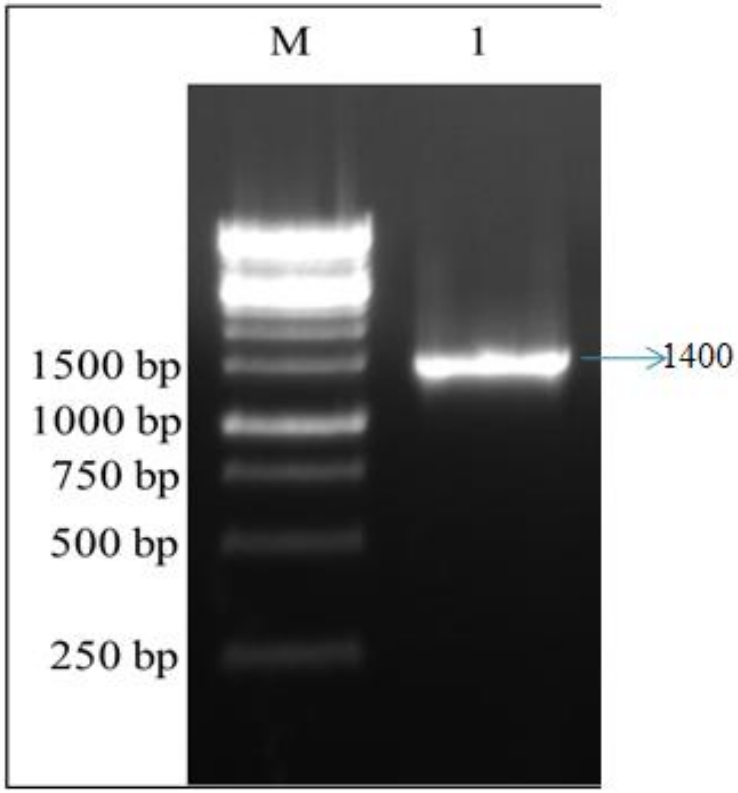

Fig. 3.13 Agarose gel analysis of PCR amplified 16S rDNA; Lane M- 1 KB ladder; Lane 1- 16S rDNA of VUVD001

3.7.2 Multiple sequence alignment (MSA)

The 16S rDNA sequence of VUVD001 was blasted through PubMed database using the algorithm BLASTN to figure out the highly conserved and similar 16S rDNA sequences (Fig. 3.14).

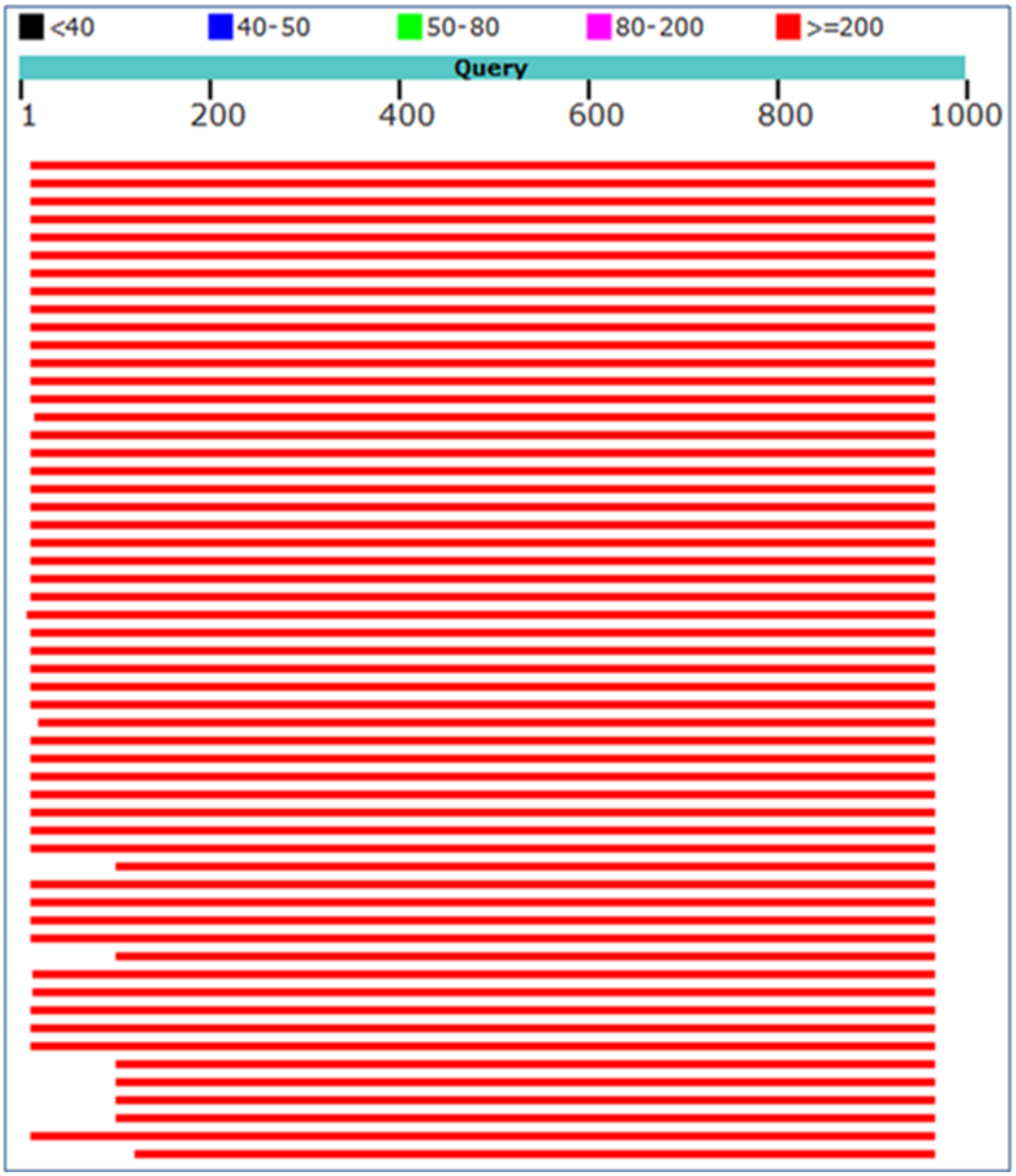

Fig. 3.14 Color key for alignment scores by BLASTN

The MSA was performed for the selected set of bacterial 16S partial sequences by Multalin software version 5.4.1 and the similarity among the partial sequences of selected bacterial species were showed in the alignment (Fig. 3.15). The species obtained from GenBank database are as follows: Bss (*Bacillus subtilis* strain DBT13), Bma (*Bacillus malacitensis* strain LMG22477), Bvs (*Bacillus vallismortis* strain NBRC101236), Bas (*Bacillus vallismortis* strain NBRC101236), Bns (*Bacillus nematocida* strainB-16), Bsi (*Bacillus siamensis* strain PD-A10), Bls *(Bacillus licheniformis* strain BCRC11702), Bme (*Bacillus methylotrophicus* strain CBMB205) and VUVD was the identified strain and the gaps are shown by dot (.)

```
                1                                                   50
VUVD            .......... .......... ..GACGGACG CTGTC..... .........A
Bss             .......... .......... .........C ATGGCGGCA. GCTATA...A
Bma             .......... .......... ....CGAACG CTGGCGGCGT GCCTAATACA
Bvs             .......... .......... ..GACGAACG CTGGCGGCGT GCCTAATACA
Bas             .......... .......... ..GACGAACG CTGGCGGCGT GCCTAATACA
Bns             .......... .TCCTGGCTC AGGACGAACG CTGGCGGCGT GCCTAATACA
Bsi             TTTGAGTTTG ATCCTGGCTC AGGACGAACG CTGGCGGCGT GCCTAATACA
Bls             .......... .......... ....CGAACG CTGGCGGCGT GCCTAATACA
Bme             .......... .......... .......... .GGGNGGCNN GCTATA...A
Consensus       .......... .......... ..gacg.acg cTGgCggc.. gc...a...A

               51                                                  100
VUVD            TGCA.GTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bss             TGCA.GTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bma             TGCAAGTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bvs             TGCAAGTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bas             TGCAAGTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bns             TGCAAGTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bsi             TGCAAGTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Bls             TGCAAGTCGA GCGGACCGAC GGGAGCTTGC TCCCTTAGGT CAGCGGCGGA
Bme             TGCAAGTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA
Consensus       TGCA.GTCGA GCGGACAGAT GGGAGCTTGC TCCCTGATGT TAGCGGCGGA

               101                                                 150
VUVD            CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bss             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bma             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bvs             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bas             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bns             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bsi             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bls             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Bme             CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG
Consensus       CGGGTGAGTA ACACGTGGGT AACCTGCCTG TAAGACTGGG ATAACTCCGG

               151                                                 200
VUVD            GAAACCGGGG CTAATACCGG ATGGTTGTTT GAACCGCATG GTTCAAACAT
Bss             GAAACCGGGG CTAATACCGG ATGGTTGTTT GAACCGCATG GTTCAAACAT
Bma             GAAACCGGGG CTAATACCGG ATGCTTGTTT GAACCGCATG GTTCAAACAT
Bvs             GAAACCGGGG CTAATACCGG ATGCTTGTTT GAACCGCATG GTTCAAACAT
Bas             GAAACCGGGG CTAATACCGG ATGCTTGTTT GAACCGCATG GTTCAAACAT
Bns             GAAACCGGGG CTAATACCGG ATGCTTGTTT GAACCGCATG GTTCAGACAT
Bsi             GAAACCGGGG CTAATACCGG ATGGTTGTTT GAACCGCATG GTTCAGACAT
Bls             GAAACCGGGG CTAATACCGG ATGCTTGATT GAACCGCATG GTTCAATCAT
Bme             GAAACCGGGG CTAATACCGG ATGGTTGTTT GAACCGCATG GTTCAGACAT
Consensus       GAAACCGGGG CTAATACCGG ATGgTTGTTT GAACCGCATG GTTCAaACAT

               201                                                 250
VUVD            AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bss             AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bma             AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bvs             AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bas             AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bns             AAAAAGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bsi             AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bls             AAAAGGTGGC TTTTAGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Bme             AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
Consensus       AAAAGGTGGC TTC.GGCTAC CACTTACAGA TGGACCCGCG GCGCATTAGC
```

```
            251                                                 300
 VUVD       TAGTTGGTGA GGTAACGGCT CACCAAGGCA ACGATGCGTA GCCGACCTGA
 Bss        TAGTTGGTGA GGTAACGGCT CACCAAGGCA ACGATGCGTA GCCGACCTGA
 Bma        TAGTTGGTGA GGTAATGGCT CACCAAGGCA ACGATGCGTA GCCGACCTGA
 Bvs        TAGTTGGTGA GGTAATGGCT CACCAAGGCA ACGATGCGTA GCCGACCTGA
 Bas        TAGTTGGTGA GGTAACGGCT CACCAAGGCA ACGATGCGTA GCCGACCTGA
 Bns        TAGTTGGTGA GGTAACGGCT CACCAAGGCA ACGATGCGTA GCCGACCTGA
 Bsi        TAGTTGGTGA GGTAACGGCT CACCAAGGCG ACGATGCGTA GCCGACCTGA
 Bls        TAGTTGGTGA GGTAACGGCT CACCAAGGCG ACGATGCGTA GCCGACCTGA
 Bme        TAGTTGGTGA GGTAACGGCT CACCAAGGCG ACGATGCGTA GCCGACCTGA
 Consensus  TAGTTGGTGA GGTAAcGGCT CACCAAGGCa ACGATGCGTA GCCGACCTGA

            301                                                 350
VUVD        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bss         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bma         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bvs         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bas         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bns         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bsi         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bls         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Bme         GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
Consensus   GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG

            351                                                 400
VUVD        AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bss         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bma         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bvs         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bas         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bns         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bsi         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bls         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Bme         AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC
Consensus   AGGCAGCAGT AGGGAATCTT CCGCAATGGA CGAAAGTCTG ACGGAGCAAC

            401                                                 450
VUVD        GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bss         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bma         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bvs         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bas         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bns         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bsi         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Bls         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAACTCTG TTGTTAGGGA
Bme         GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA
Consensus   GCCGCGTGAG TGATGAAGGT TTTCGGATCG TAAAGCTCTG TTGTTAGGGA

            451                                                 500
VUVD        AGAACAAGTA CCGTTCGAAT AGGGCGGTAC CTTGACGGTA CCTAACCAGA
Bss         AGAACAAGTA CCGTTCGAAT AGGGCGGTAC CTTGACGGTA CCTAACCAGA
Bma         AGAACAAGTA CCGTTCGAAT AGGGCGGTAC CTTGACGGTA CCTAACCAGA
Bvs         AGAACAAGTG CCGTTCNAAT AGGGCGGCAC CTTGACGGTA CCTAACCAGA
Bas         AGAACAAGTG CCGTTCAAAT AGGGCGGCAC CTTGACGGTA CCTAACCAGA
Bns         AGAACAAGTG CCGTTCAAAT AGGGCGGCAC CTTGACGGTA CCTAACCAGA
Bsi         AGAACAAGTG CCGTTCAAAT AGGGCGGCAC CTTGACGGTA CCTAACCAGA
Bls         AGAACAAGTA CCGTTCGAAT AGGGCGGTAC CTTGACGGTA CCTAACCAGA
Bme         AGAACAAGTG CCGTTCAAAT AGGGCGGCAC CTTGACGGTA CCTAACCAGA
Consensus   AGAACAAGTa CCGTTCgAAT AGGGCGGtAC CTTGACGGTA CCTAACCAGA
```

```
              501                                                   550
VUVD          AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bss           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bma           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bvs           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bas           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bns           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bsi           AAGCCACGGC TAATTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bls           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Bme           AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA
Consensus     AAGCCACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGTGGCAA

              551                                                   600
VUVD          GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Bss           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Bma           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTCCTTAAG
Bvs           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Bas           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Bns           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Bsi           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Bls           GCGTTGTCCG GAATTATTGG GCGTAAAGCG CGCGCAGGCG GTTTCTTAAG
Bme           GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG
Consensus     GCGTTGTCCG GAATTATTGG GCGTAAAGGG CTCGCAGGCG GTTTCTTAAG

              601                                                   650
VUVD          TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bss           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bma           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bvs           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bas           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bns           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bsi           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bls           TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG
Bme           TCTGATGTGA AAGCCCCCGG CTCAACCCGG GGAGGGTCAT TGGAAACTGG
Consensus     TCTGATGTGA AAGCCCCCGG CTCAACC.GG GGAGGGTCAT TGGAAACTGG

              651                                                   700
VUVD          GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bss           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bma           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bvs           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bas           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bns           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TAC.ACGTGT AGCGGTGAAA
Bsi           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bls           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Bme           GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA
Consensus     GGAACTTGAG TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA

              701                                                   750
VUVD          TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bss           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bma           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bvs           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bas           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bns           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bsi           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bls           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Bme           TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
Consensus     TGCGTAGAGA TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG
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```
                751                                                   800
VUVD            TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bss             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bma             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bvs             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bas             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bns             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bsi             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bls             TAACTGACGC TGAGGCGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Bme             TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC
Consensus       TAACTGACGC TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC

               801                                                   850
VUVD            CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bss             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bma             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bvs             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bas             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bns             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bsi             CTGGTATTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Bls             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG AGGGTTTCCG
Bme             CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG
Consensus       CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG

                851                                                   900
VUVD            CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bss             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bma             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bvs             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bas             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bns             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bsi             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGT
Bls             CCCTTTAGTG CTGCAGCAAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Bme             CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG
Consensus       CCCCTTAGTG CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG

                901                                                   950
VUVD            TCGCAAGACT GAAACTCAAA GGAATTGACG GGGGGCCCGC ACAAGCGGTG
Bss             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bma             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bvs             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bas             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bns             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bsi             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bls             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Bme             TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG
Consensus       TCGCAAGACT GAAACTCAAA GGAATTGACG GGGG.CCCGC ACAAGCGGTG

                951                                                  1000
VUVD            GAGCATGTGG TTTAATTCGA AGCAACGCGA AGGAACCCTT ACCAGGGTCT
Bss             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bma             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bvs             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bas             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bns             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bsi             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bls             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Bme             GAGCATGTGG TTTAATTCGA AGCAACGCGA AGAA..CCTT ACCAGG....
Consensus       GAGCATGTGG TTTAATTCGA AGCAACGCGA AGaA..CCTT ACCAGG....
```

```
            1001                                                1050
VUVD        TTGACAGGTC TTGACATCCT CTGACAATCC TAGAGATAG. ..........
Bss         ........TC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
Bma         ........TC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
Bvs         ........TC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
Bas         ........TC TTGACATCCT CTGACANCCC TAGAGATAGG GCTTCCCCTT
Bns         ........TC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
Bsi         ........TC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
Bls         ........TC TTGACATCCT CTGACAACCC TAGAGATAGG GCTTCCCCTT
Bme         ........TC TTGACATCCT CTGACAATCC TAGAGATAGG ACGTCCCCTT
Consensus   ........TC TTGACATCCT CTGACAAtCC TAGAGATAGg acgtcccctt

            1051                                                1100
VUVD        .......... .......... .......... .......... ..........
Bss         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bma         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bvs         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bas         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bns         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bsi         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bls         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Bme         CGGGGGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA
Consensus   cgggggcaga gtgacaggtg gtgcatggtt gtcgtcagct cgtgtcgtga

            1101                                                1150
VUVD        .......... .......... .......... .......... ..........
Bss         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bma         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bvs         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bas         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bns         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bsi         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bls         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Bme         GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA
Consensus   gatgttgggt taagtcccgc aacgagcgca acccttgatc ttagttgcca

            1151                                                1200
VUVD        .......... .......... .......... .......... ..........
Bss         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bma         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bvs         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bas         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bns         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bsi         GCATTCAGTT GGGCCACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bls         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Bme         GCATTCAGTT GGGC.ACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA
Consensus   gcattcagtt gggc.actct aaggtgactg ccggtgacaa accggaggaa

            1201                                                1250
VUVD        .......... .......... .......... .......... ..........
Bss         GGT....... .......... .......... .......... ..........
Bma         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Bvs         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Bas         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Bns         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Bsi         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Bls         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Bme         GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC
Consensus   ggt....... .......... .......... .......... ..........
```

```
            1251                                                1300
VUVD        .......... .......... .......... .......... ..........
Bss         .......... .......... .......... .......... ..........
Bma         GTGCTACAAT GGACAGAACA AAGGGCAGCG AAACCGCGAG GTTAAGCCAA
Bvs         GTGCTACAAT GGACAGAACA AAGGGCAGCG AAACCGCGAG GTTAAGCCAA
Bas         GTGCTACAAT GGACAGAACA AAGGGCAGCG AGACCGCGAG GTTAAGCCAA
Bns         GTGCTACAAT GGNCAGAACA AAGGGCAGCG AAACCGCGAG GTTAAGCCAA
Bsi         GTGCTACAAT GGGCAGAACA AAGGGCAGCG AAACCGCGAG GTTAAGCCAA
Bls         GTGCTACAAT GGGCAGAACA AAGGGCAGCG AAGCCGCGAG GCTAAGCCAA
Bme         GTGCTACAAT GGACAGAACA AAGGGCAGCG AAACCGCGAG GTTAAGCCAA
Consensus   .......... .......... .......... .......... ..........

            1301                                                1350
VUVD        .......... .......... .......... .......... ..........
Bss         .......... .......... .......... .......... ..........
Bma         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Bvs         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Bas         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Bns         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Bsi         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Bls         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Bme         TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC TCGACTGCGT
Consensus   .......... .......... .......... .......... ..........

            1351                                                1400
VUVD        .......... .......... .......... .......... ..........
Bss         .......... .......... .......... .......... ..........
Bma         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Bvs         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Bas         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Bns         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Bsi         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Bls         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Bme         GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT
Consensus   .......... .......... .......... .......... ..........
            1401                                                1450
VUVD        .......... .......... .......... .......... ..........
Bss         .......... .......... .......... .......... ..........
Bma         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Bvs         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Bas         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Bns         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Bsi         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Bls         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Bme         TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC
Consensus   .......... .......... .......... .......... ..........

            1451                                                1500
VUVD        .......... .......... .......... .......... ..........
Bss         .......... .......... .......... .......... ..........
Bma         CGAAGTCGGT GAGGTAACCT TTATGGAGCC AGCCGCCGAA GGTGGGAC.A
Bvs         CGAAGTCGGT GAGGTAACCT TTTAGGAGCC AGCCGCCGAA GGTGGGAC.A
Bas         CGAAGTCGGT GAGGTAACCT TTATGGAGCC AGCCGCCGAA GGTGGGAC.A
Bns         CGAAGTCGGT GAGGTAACCT TTTAGGAGCC AGCCGCCGAA GGTGGGAC.A
Bsi         CGAAGTCGGT GAGGTAACCT TTATGGAGCC AGCCGCCGAA GGTGGGACGA
Bls         CGAAGTCGGT GAGGTAACCT TT.TGGAGCC AGCCGCCGAA GGTGGGAC.A
Bme         CGAAGTCGGT GAGGTAACCT TTTAGGAGCC AGCCGCCGAA GGTGAC....
Consensus   .......... .......... .......... .......... ..........
```

```
                1501                                                1543
VUVD            .......... .......... .......... .......... ...
Bss             .......... .......... .......... .......... ...
Bma              GATGATTGGG G......... .......... .......... ..
Bvs             GATGATTGGG GTGAAG.... .......... .......... ...
Bas             GATGATTGGG GTGAAG.... .......... .......... ...
Bns             GATGATTGGG GTGAAGTC.G TAACAAGGTA GCCGTATCGG AAG
Bsi             GATGATTGGG GTGAATGCAG TAACAAGGTA GCCG.ATCGA TGC
Bls             GATGATTGGG G......... .......... .......... ...
Bme             .......... .......... .......... .......... ...
Consensus       .......... .......... .......... .......... ...
```

Fig. 3.15 Multiple sequence alignment of 16S rDNA nucleotides from species related to *Bacillus* using Multalin software

3.7.3 Cluster analysis

The bacterial species could be recognized based on similarity in the clusters of genotypes. The researchers used MSA approach, to find the relationships among the closely related species within a single genus (Christensen et al. 2004; Hanage et al, 2006). The sequence clustering of species was done by Multalin software version 5.4.1 and this study revealed that the VUVD (VUVD001 isolate) was highly similar to the Bss (*B.subtilis* starin DBT13) (Fig. 3.16)

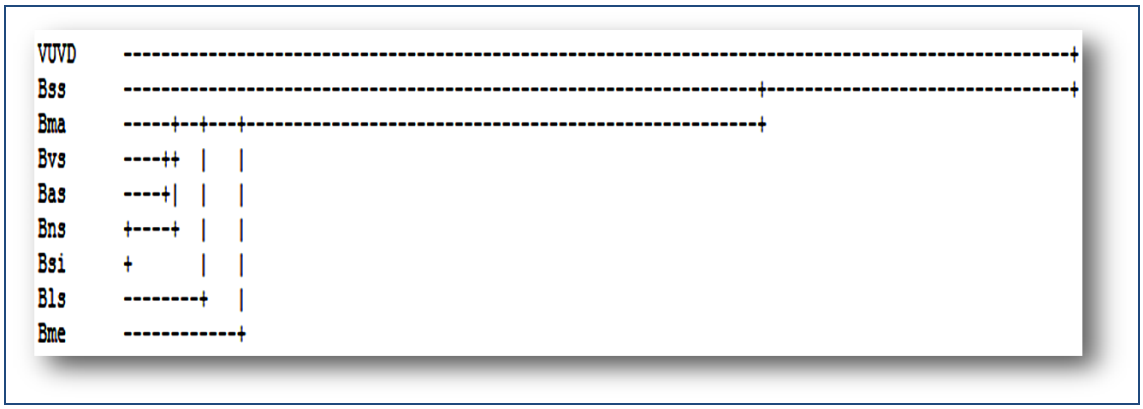


Fig. 3.16 Cluster sequencing for VUVD001 by Multalin software

3.7.4 Phylogenetic tree construction

The evolutionary analysis was conducted using MEGA6. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of taxa analyzed. The results of phylogenetic tree showed that the strain VUVD001 was 77% similar to *Bacillus subtilis* strain DBT13 from GenBank database (Fig.3.17).

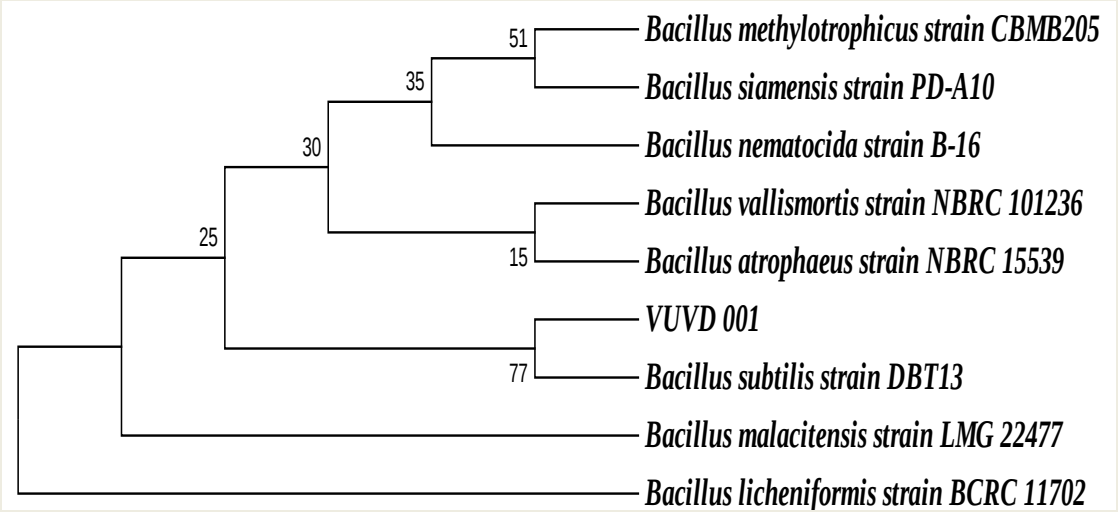


Fig. 3.17 Dendrogram constructed by maximum-likelihood method using MEGA 5v

The 16S partial sequence of VUVD001 isolate was submitted to the National Center for Biotechnology Information (NCBI) and accepted as new strain of *Bacillus subtilis* VUVD0001 strain with the accession number of KT894158 (Fig. 3.18).

Bacillus subtilis strain VUVD001 16S ribosomal RNA gene, partial seque

GenBank: KT894158.1

FASTA Graphics

Go to:

```
LOCUS       KT894158                1000 bp    DNA     linear   BCT 18-OCT-2015
DEFINITION  Bacillus subtilis strain VUVD001 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KT894158
VERSION     KT894158.1
KEYWORDS    .
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1000)
  AUTHORS   Venkateswarulu,T.C. and Prabhakar,K.V.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-OCT-2015) Department of Biotechnology, Vignan's
            Foundation for Science, Technology & Research, Vadlamudi, Andhra
            Pradesh 522 213, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..1000
                     /organism="Bacillus subtilis"
                     /mol_type="genomic DNA"
                     /strain="VUVD001"
                     /isolation_source="dairy effluent"
                     /db_xref="taxon:1423"
                     /country="India"
                     /collection_date="23-Mar-2014"
                     /collected_by="TC Venkateswarulu"
     rRNA            <1..>1000
                     /product="16S ribosomal RNA"
ORIGIN
        1 gacggacgct gtcatgcagt cgagcggaca gatgggagct tgctccctga tgttagcggc
       61 ggacggggtga gtaacacgtg ggtaacctgc ctgtaagact gggataactc cgggaaaccg
      121 gggctaatac cggatggttg tttgaaccgc atggttcaaa cataaaaggt ggcttcggct
      181 accacttaca gatggacccg cggcgcatta gctagttggt gaggtaacgg ctcaccaagg
      241 caacgatgcg tagccgacct gagagggtga tcggccacac tgggactgag acacggccca
      301 gactcctacg ggaggcagca gtagggaatc ttccgcaatg gacgaaagtc tgacggagca
      361 acgccgcgtg agtgatgaag gttttcggat cgtaaagctc tgttgttagg gaagaacaag
      421 taccgttcga atagggcggt accttgacgg tacctaacca gaaagccacg gctaactacg
      481 tgccagcagc cgcggtaata cgtaggtggc aagcgttgtc cggaattatt gggcgtaaag
      541 ggctcgcagg cggtttctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca
      601 ttggaaactg gggaacttga gtgcagaaga ggagagtgga attccacgtg tagcggtgaa
      661 atgcgtagag atgtggagga acaccagtgg cgaaggcgac tctctggtct gtaactgacg
      721 ctgaggagcg aaagcgtggg gagcgaacag gattagatac cctggtagtc cacgccgtaa
      781 acgatgagtg ctaagtgtta gggggtttcc gccccttagt gctgcagcta acgcattaag
      841 cactccgcct ggggagtacg gtcgcaagac tgaaactcaa aggaattgac gggggcccg
      901 cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg aaggaaccct taccagggtc
      961 tttgacaggt ctttgacatcc tctgacaatc ctagagatag
```

Fig. 3.18 The 16S partial sequence of *B.subtilis* VUVD001 strain to NCBI

3.8 SUMMARY

The lactase producing potential bacterial strain was isolated from dairy effluent and named as VUVD001. Based on morphological and biochemical studies the VUVD001 confirmed that it belongs to genus of *Bacillus.* To further confirm the particular strain the VUVD001 isolate was subjected to 16S rRNA analysis. From this study the strain was identified as *Bacillus subtilis* VUVD001 strain which is 77% closer to the *B.subtilis* strain DBT13. The antimicrobial activity of VUVD001 isolate has shown the highest inhibition activity on *Enterobacter aerogenes* and *Klebsiella pneumonia.* Prior to the production the growth proliferation ability of *B.subtilis* VUVD001 strain was studied by growth curve experiment.

CHAPTER-IV

OPTIMIZATION OF NUTRITIONAL COMPONENTS OF THE MEDIUM FOR THE ENHANCED LACTASE PRODUCTION

4.1 INTRODUCTION

The production medium cost and low productivity of the enzymes were the main problem in commercial scale production. The production level was highly influenced by nutritional composition of medium particularly, carbon and nitrogen sources. Hence, the design of suitable medium with lower cost and higher lactase activity was highly essential (Tari et al, 2007). The traditional optimization method (OFAT) involves varying one variable at a time and other factors were maintained at a fixed level. However, OFAT is time consuming and effect of interaction of multiple factors on production was unable study in a single experiment. To overcome this, Response Surface Methodology (RSM) has been employed in the optimization for production of lactase. The RSM is a collection of statistical tools used for experimental design and evaluating the effects of variables and identifying the most desirable conditions for enhanced production of lactase (Siham and Hashem, 2009; Charyulu and Gnanamani). Hsu et al, (2005) was investigated the effect of medium components on lactase production by RSM and they found the maximum lactase production by the cultivation of *Bifidobacteria*. Liu et al (2013) was applied the RSM to investigate the impact of process factors such as wheat bran, soybean meal, KH_2PO_4, $MnSO_4 \cdot H_2O$ and $CuSO_4 \cdot 5H_2O$ on lactase production from *Aspergillus foetidus.* Abdul Khalil et al, (2014) was used the RSM tool for the optimization of milk based medium. They were reported that the yeast extract is a potential nitrogen source for enhanced production of lactase through the cultivation of *Bifidobacterium pseudocatenulatum* G4. Liu et al (2007) was applied Response Surface Methodology for improved production of lactase by assess the impact of factors such as concentrations of wheat bran, soybean meal, KH_2PO_4, $MnSO_4.H_2O$ and $CuSO_4.5H_2O$ on production from the microbial species. Adikane et al (2015) examined the *Pantoea* sp. for enhanced production of lactase through the optimization of milk whey medium by RSM.

4.2 MATERIALS AND METHODS

4.2.1 Microorganism and Shake flask fermentation

The organism used in this study was *B.subtilis* strain VUVD001. The culture was maintained in our laboratory at room temperature and preserved at 4 ^{o}C on nutrient agar medium. The original fermentation medium consisted of 4 g/l of Lactose, 4 g/l of Yeast extract, 1 g/l of $MgSO_4 \cdot 7H_2O$ and 0.1g/l of Tryptophan. The shake-flask fermentation was carried out by inoculating 5 ml seed culture in a conical flask containing 100 ml of production medium. Then, the flasks were incubated at 36 °C on a rotary shaker (180 rpm) for 36 h.

4.2.2 Effect of various nutrients sources

The suitable carbon and nitrogen sources were identified for the production of enzyme by allowing bacterial strain proliferation in production medium. The medium was supplemented with different carbon sources such as lactose, glucose, sucrose, fructose and starch in concentration range of 0.4% and 0.4% of nitrogen sources like yeast extract, urea, sodium nitrate, ammonium nitrate were added to the culture medium to investigate their effect on enzyme production. Similarly, the metal ions (0.1%), $MgSO_4$, $MnSO_4$ and $ZnSO_4$ and 0.01% of different amino acids namely glycine, tryptophan and cysteine were added to the medium to investigate its significant effect on production (Sharma and Singh, 2014).

4.2.3 RSM for optimization of Medium

The Design Expert software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA), was used to optimize the production medium variables namely Lactose (A), Yeast extract (B), Tryptophan (C) and $MgSO_4$ (D) are shown in Table 4.1. The range of variables from low (-1) to high (+1) were used to study the effect of independent variables on production. Regression analysis of experimental data and response surface plots were performed. The model was validated by conducting experiment at given optimal variables of the medium.

Table 4.1 Variable ranges used in experimental design

Symbol	Name of the Variable	Range
A	Lactose, g/l	5 – 20
B	Yeast extract, g/l	5 - 15
C	Tryptophan, g/l	0.2 – 0.6
D	MgSO4, g/l	2 – 6

4.3 RESULTS AND DISCUSSION

4.3.1 Optimization of nutritional components of medium by one-factor-at-a-time method

4.3.2 Effect of carbon sources on enzyme production

The amount of carbon source in fermentation media is primary energy source which is essential for bacterial growth and production of lactase in submerged fermentation. Carbon source may regulate the biosynthesis of lactase in different microorganisms (Alazzeh et al, 2009). The fermentation medium was supplemented with 0.4% of different carbon sources to study their individual influence on production by traditional optimization. The molecules like glucose and fructose were also showed the increase of biomass and yield but they were less efficient as compared with lactose (15.14 U/ml) (Fig.4.1a). Further the lactose quantity was optimized by varying the concentration from 0.5 to 2% and found that significant improvement in lactase activity (24.84 U/ml) and biomass with the addition of 1.5% of lactose (Fig.4.1b). Sriphannam et al (2012) has reported lactose may enhance the production of lactase by probiotic strain *Lactobacillus fermentum* CM33 in submerged process. In previous study, the strain was reported for improved activity of lactase by using lactose as sole carbon source from a *Thalassospira frigidphilosprofundus* through submerged fermentation and hence our results were supported by the previous reports (Pulicherla et al, 2013).

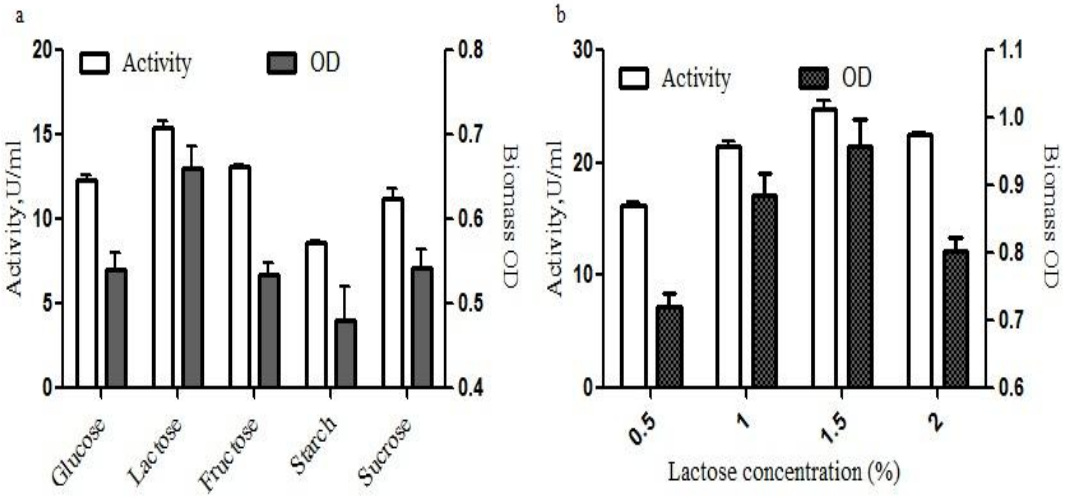


Fig.4.1 Effect of various carbon sources on Lactase production and Biomass. a) Different carbon sources of glucose, lactose, fructose, starch and sucrose and b) different concentrations of lactose in %

4.3.3 Effect of nitrogen source

The nitrogen was an important factor which may affect microbial biosynthesis of lactase (Shaikh et al, 1997). The effect of different nitrogen sources namely sodium nitrate, ammonium nitrate, yeast extract and urea on cell growth and lactase production were investigated. In addition, yeast extract and ammonium nitrate were showed the enhancement in lactase activity but the effectiveness of ammonium nitrate was less as compared with yeast extract (Fig. 4.2a). Further, the concentration of yeast extract for production was evaluated simultaneously by varying the concentration and the maximum lactase activity of 22.19 U/ml was obtained at one % of yeast extract (Fig. 4.2b). The similar study was also conducted by Hsu et al (2007) using *Bifidobacterium longum* CCRC15708 in shake flask culture system. He reported highest lactase was produced with yeast extract as nitrogen source. Similarly Mukesh Kumar et al (2012) also reported the activity of lactase was improved with the supplementation of 1% of yeast extract in the submerged fermentation with *Bacillus* sp. MNJ23.

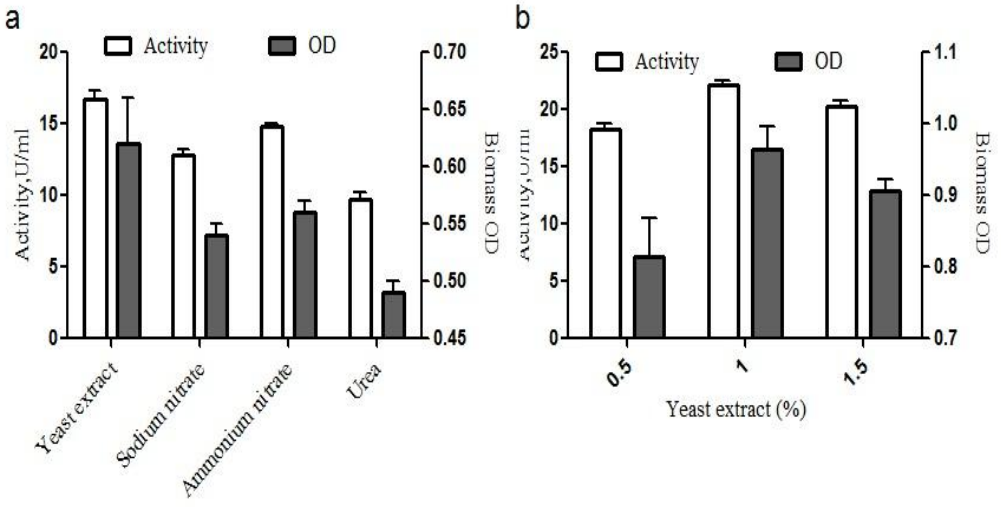


Fig.4.2 Effect of nitrogen source on Lactase production and Biomass. a) Different sources i.e., Yeast extract, sodium nitrate, ammonium nitrate and urea and b) Yeast extract concentrations in %.

4.3.4 Effect of tryptophan on lactase activity

The amino acid role in medium was described as stimulator for biosynthesis and excretion of enzymes (Gupta et al, 2003). Therefore, production medium with amino acid may consider as experimental tool for significant enhancement of enzyme production. Based on this observation the amino acids tryptophan, glycine and cysteine, were selected to study its impact on biomass and lactase production. The

results indicated that activity was improved with addition of tryptophan. Among the three amino acids tryptophan resulted the highest activity and the corresponding report were represented in Fig.4.3a. Therefore, the effect of tryptophan concentration on production was further evaluated for improving the production rate. However the medium feeding with 0.04% tryptophan was significantly enhanced the enzyme production. Under this condition, the production had reached 25.10 U/ml in the shake-flask fermentation (Fig.4.3b). Akcan (2011) reported the addition of L-tryptophan may enhace the biosynyhesis of lactase by *Bacillus licheniformis* ATCC12759 in submerged fermentation.

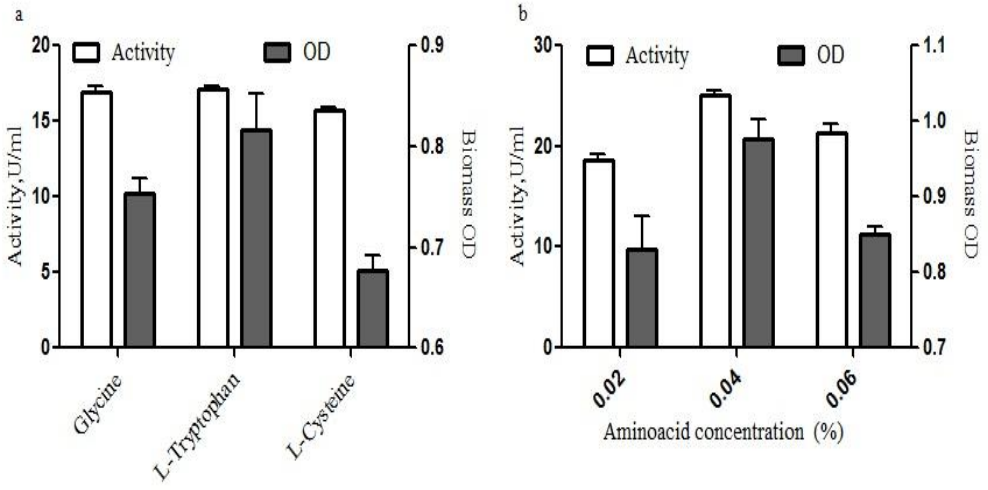


Fig.4.3 Effect of amino acids on Lactase production and Biomass. a) Different amino acids i.e., Glycine, L-Tryptophan and L-Cysteine and b) Tryptophan concentrations in %

4.3.5 Effect of metal sources on Lactase activity

The effect of metal ions on biosynthesis of enzyme production was screened by supplementing medium with 0.1% of metal sources like $MgSO_4$, $MnSO_4$ and $ZnSO_4$. The enzyme activity was improved with $MgSO_4$ compared to $MnSO_4$ and $ZnSO_4$ and the results were displayed in Fig.4.4a. In addition the impact of $MgSO_4$ was further investigated at different concentration ranges. Among three ionic compounds, $MgSO_4$ enhanced the production with a highest activity of 22.12 U/ml at 0.4%. (Fig.4.4b). The contribution of this metal ion as an ion channeling agent in membrane permeabilization has been reported (Karpen and Ruiz, 2002). Previous report revealed that the production of lactase was significantly improved with the supplementation of 10 mg of $MgSO_4$ per gram of substrate (Gopal et al, 2015). The $MnSO_4$ also showed the considerable effect on the production of lactase but it was less efficient than

$MgSO_4$. In previous report addition of 1 mM of Mn^{2+} to the medium was showed the significant improvement in lactase activity through *L.crispatus* fermentation (Kim and Rajagopal, 2000). Previous report showed a slight stimulatory influence on lactase activity by addition of $MnCl_2$ with concentration of 0.02 M in shake flask culture with *L.acidophilus* NRRL4495**.** Based on the experimental results it was found that the addition of divalent ion sources such as Mg^{2+} and Mn^{2+} to growth medium may enhance the yield.

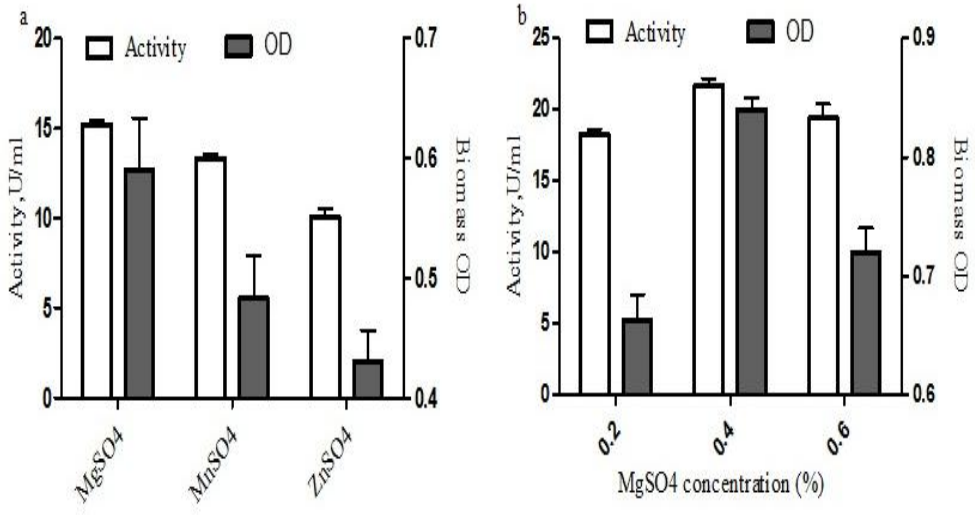


Fig.4.4 Effect of minerals on Lactase production and Biomass. a) Different minerals i.e., $MgSO_4$, $ZnSO_4$ and $MnSO_4$ and b) $MgSO_4$ concentrations in %

4.3.6 Optimization of nutritional components of medium by Central Composite Design (CCD)

4.3.6.1 CCD for medium optimization

After one factor at a time optimization study, activity of lactase was significantly improved as compared with basal medium. For further enhancing the lactase yield, the Central Composite Design (CCD) was adapted for design of optimum concentration of nutrient components such as lactose, yeast extract tryptophan and $MgSO_4$. The experimental results of CCD experimental designs for production were shown in Table 4.2.

Table 4.2 The design data of CCD and lactase activity values

Run No	Lactose (g/l)	Yeast extract (g/l)	Tryptophan (g/l)	$MgSO_4$ (g/l)	Activity, U/ml	
					Experimental	Predicted
1	5	5	0.6	6	28.31±0.007	26.17
2	20	5	0.6	6	27.46±0.011	29.10
3	5	5	0.2	2	25.18 ±0.009	24.11
4	5	5	0.6	2	28.75±0.005	30.48
5	12.5	10	0.4	4	63.04±0.013	60.70
6	5	15	0.2	6	34.25 ±0.006	34.73
7	5	15	0.6	6	41.09 ±0.004	41.76
8	12.5	10	0.4	2	61.74 ±0.010	61.87
9	5	5	0.2	6	27.41 ±0.008	27.06
10	20	10	0.4	4	51.62 ±0.007	52.46
11	12.5	5	0.4	4	38.75±0.012	42.04
12	12.5	10	0.4	4	62.84±0.002	60.70
13	12.5	10	0.4	4	61.02±0.014	60.70
14	5	15	0.2	2	23.14 ±0.005	22.39
15	12.5	10	0.4	4	60.54 ±0.013	60.70
16	20	5	0.2	6	37.91 ±0.009	36.57
17	20	5	0.6	2	35.75 ±0.005	33.77
18	5	15	0.6	2	36.85 ±0.011	36.69
19	20	15	0.2	6	57.12 ±0.006	56.28
20	12.5	15	0.4	4	55.85 ±0.010	54.99
21	20	15	0.6	6	57.16 ±0.003	56.73
22	12.5	10	0.6	4	62.47 ±0.016	61.89
23	20	15	0.6	2	50.76 ±0.013	52.00
24	20	15	0.2	2	43.65 ±0.009	44.29
25	20	5	0.2	2	33.75 ±0.023	33.97
26	12.5	10	0.4	6	63.42 ±0.012	65.71
27	5	10	0.4	4	38.45 ±0.009	40.04
28	12.5	10	0.2	4	55.48 ±0.003	58.48
29	12.5	10	0.4	4	63.01 ±0.014	60.70
30	12.5	10	0.4	4	61.05 ±0.008	60.70

The response, Y was fitted with the second-order polynomial equation.

Lactase activity (U/ml), R1 = +60.70 +6.21*A+6.48 * B+1.71* C+1.92 * D+3.01 *A* B-1.64*A*C-0.087*A*D+1.98 *B* C+2.35*B*D-1.82*C*D-14.46*A^2-12.19* B^2-0.52* C^2+3.09 * D^2

The model equation importance was statistically evaluated by the *F* test for the analysis of variance (ANOVA). The prob > *F* values (<0.0001) for the Lactase

production are lower than 0.05, indicating that goodness of designed quadratic model was significant. Based on observation from ANOVA table it was found that the variables A, B, C, D, AB, AC, BC, BD, CD, A^2, B^2 and D^2 are significant model terms. The coefficient (*R*2) value was found to be 0.98 and this value also supported a high correlation between experimental and predicted values. The lower consistency of the experiment is generally indicated by high value of coefficient of variation (CV). In this case, low % CV (4.55) represents that the experiment performed is reliable. Adequate precision ratio greater than 4.00 is desirable. In present study, the ratio is 29.117 which indicate an adequate signal (Table 4.3).

Table 4.3 ANOVA analysis of model and medium components

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value**
					Prob > F
Model	5707.57	14	407.684	92.0795	< 0.0001
A-Lactose	693.781	1	693.781	156.698	< 0.0001
B-Yeast extract	755.309	1	755.309	170.594	< 0.0001
C-Tryptophan	52.3947	1	52.3947	11.8339	0.0036
D-$MgSO_4$	66.3552	1	66.3552	14.987	0.0015
AB	144.841	1	144.841	32.7138	< 0.0001
AC	43.2964	1	43.2964	9.77893	0.0069
AD	0.1225	1	0.1225	0.02767	0.8701
BC	62.7264	1	62.7264	14.1674	0.0019
BD	88.1721	1	88.1721	19.9146	0.0005
CD	52.7802	1	52.7802	11.9209	0.0036
A^2	541.389	1	541.389	122.278	< 0.0001
B^2	385.021	1	385.021	86.9609	< 0.0001
C^2	0.68811	1	0.68811	0.15542	0.699
D^2	24.7326	1	24.7326	5.58612	0.032
Residual	66.4128	15	4.42752		
Lack of Fit	59.6527	10	5.96527	4.41209	0.0575
Pure Error	6.76013	5	1.35203		
Cor Total	5773.99	29			

R-Squared 0.98 Adeq Precision 29.117 C.V. % 4.55

The 3-D response surface curves were plotted to evaluate interactions of combinational medium variables effects on response and to find optimal concentrations of nutrient sources for lactase production (Fig. 4.5 to 4.10).

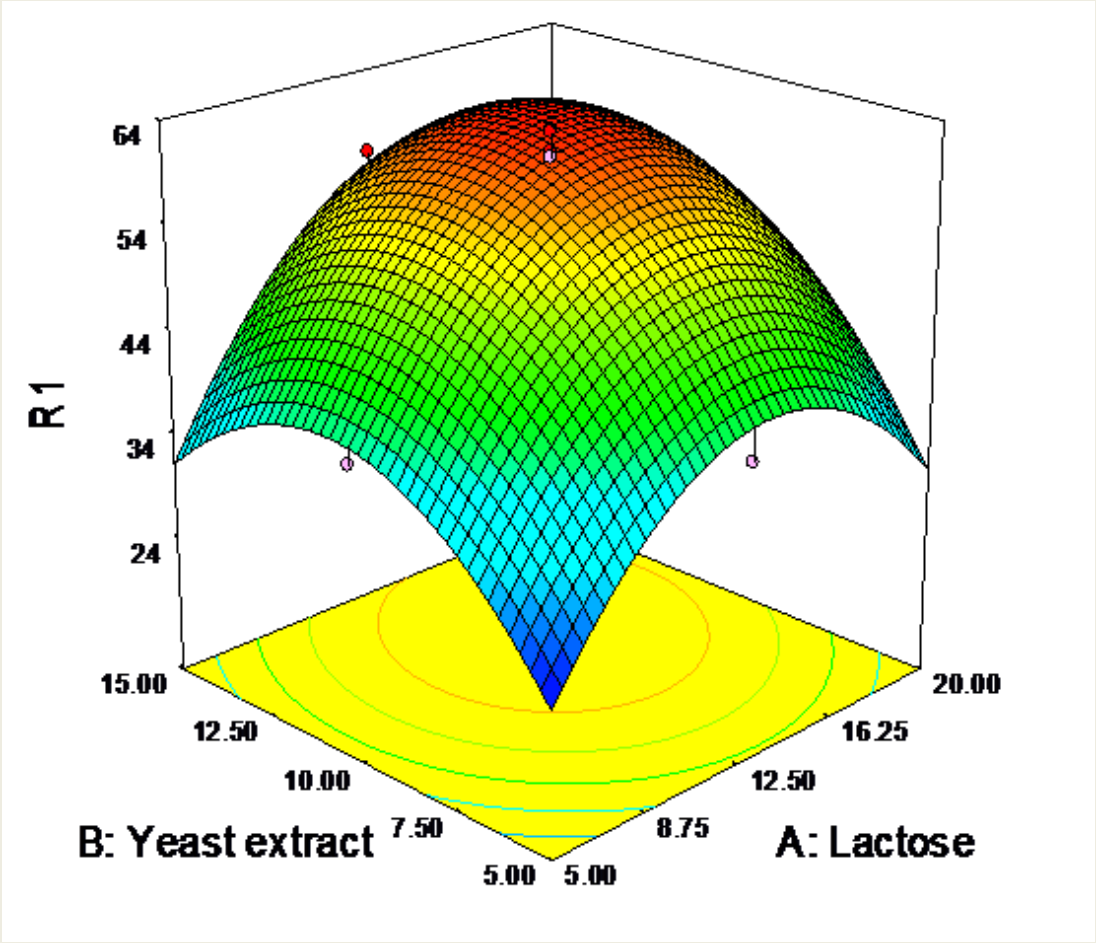


Fig.4.5 Effect of yeast extract and lactose on lactase activity

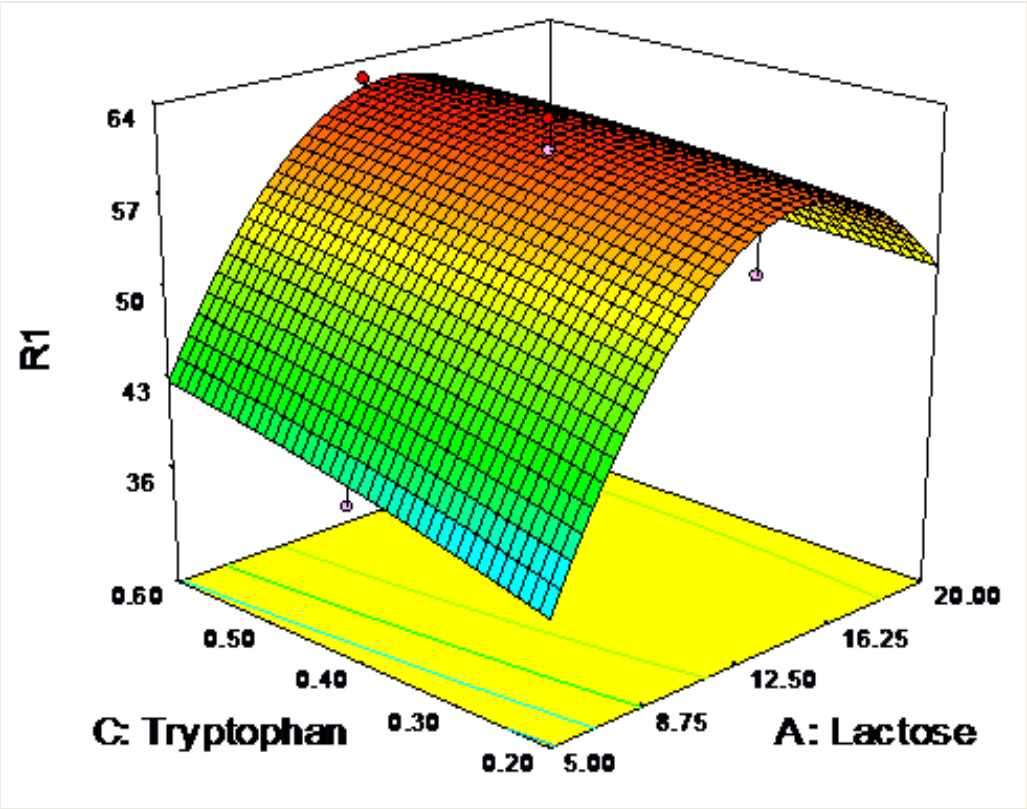


Fig. 4.6 Effect of tryptophan and lactose on lactase activity

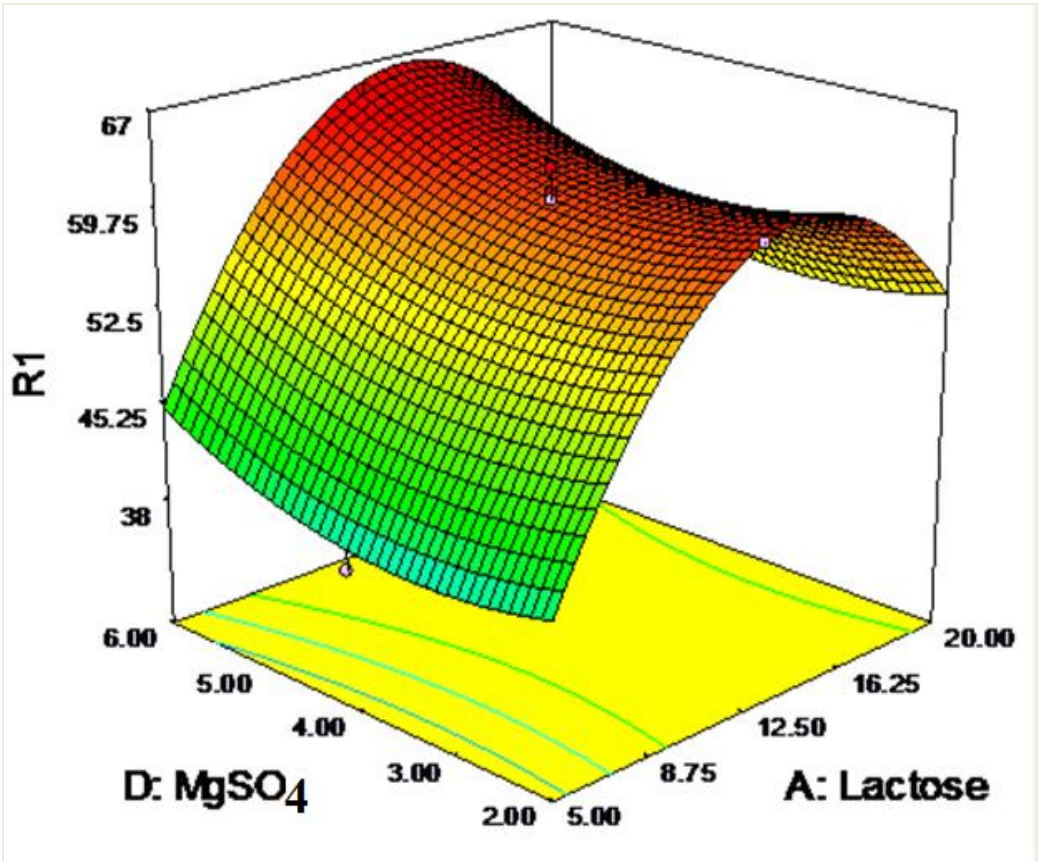


Fig. 4.7 Effect of $MgSO_4$ and lactose on lactase activity

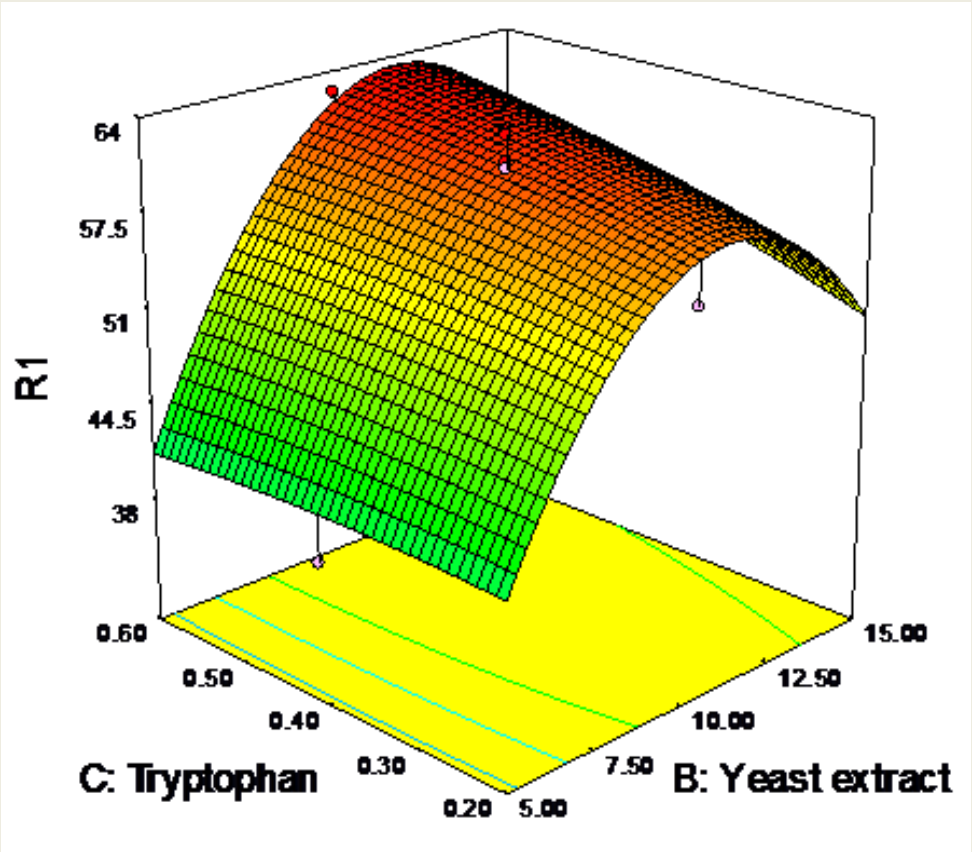


Fig. 4.8 Effect of tryptophan and yeast extract on lactase activity

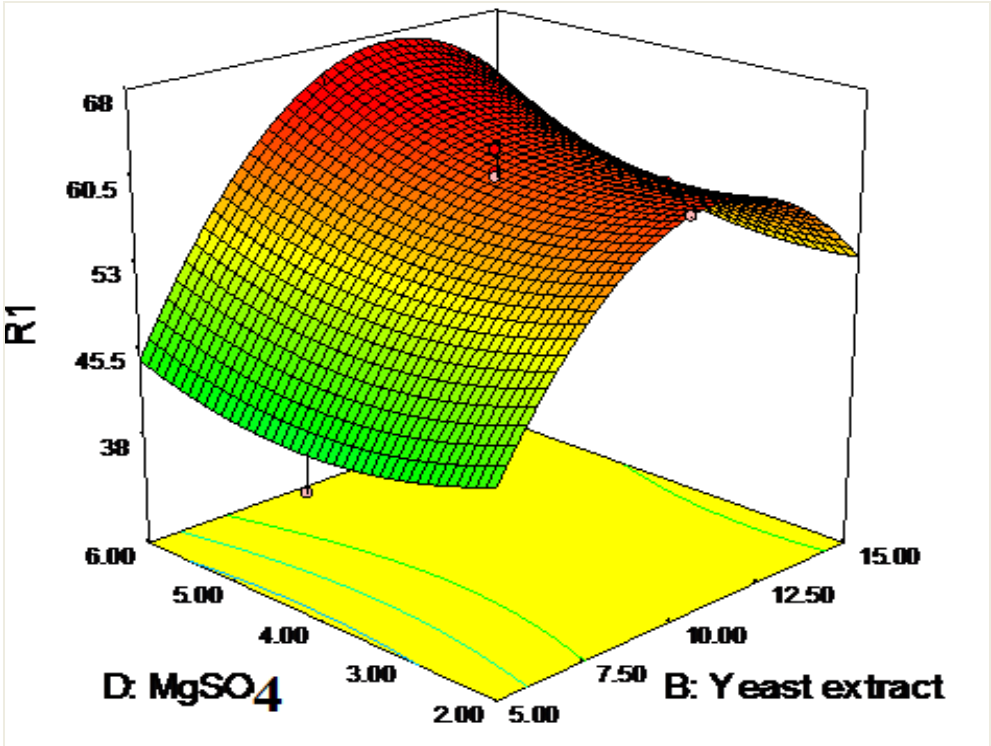


Fig. 4.9 Effect of $MgSO_4$ and yeast extract on lactase activity

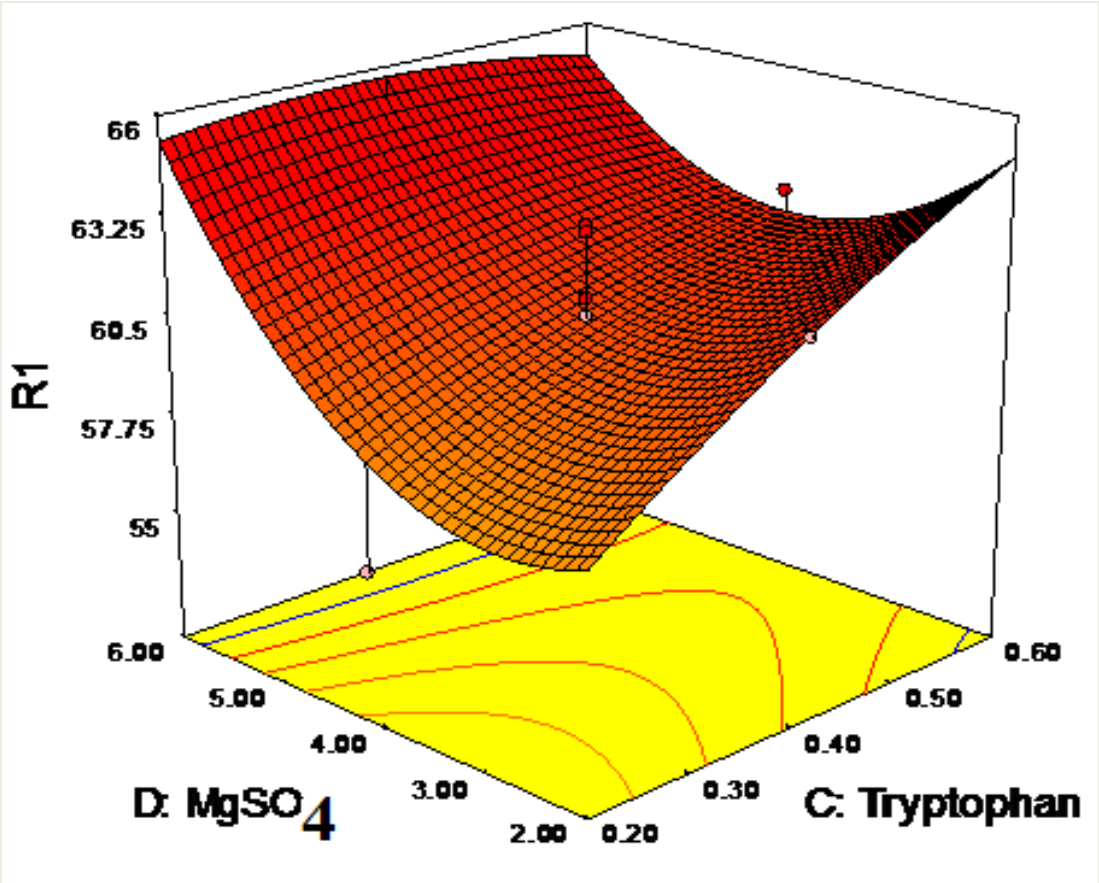


Fig.4.10 Effect of $MgSO_4$ and tryptophan on lactase activity

4.3.6.2 Validation of RSM Model

The RSM model is validated by conducting an experiment at best predicted solution for production of lactase. Under optimized conditions the enzyme activity was reached to 63.54 U/ml from *Bacillus subtilis* VUVD001 this value is almost near to the RSM predicted value (Table 4.4).

Table 4.4 RSM Optimized medium variables for lactase activity

A-Lactose (g/l)	B-Yeast extract (g/l)	C-Tryptophan (g/l)	D-$MgSO_4$ (g/l)	Activity, U/ml	
				Predicted	Experimental
14.01	10.30	0.43	5.32	64.49	63.54±0.021

Previously, reported the maximum lactase activity of 18.6 U/ml was obtained in submerged fermentation with *Bifidobacterium longum* CCRC 15708. The process was run at optimum conditions of pH 6.5, temperature 37 ^{o}C and incubation time 16 h in liquid medium contains 4% lactose, 3.5% yeast extract, 0.3% K_2HPO_4, 0.1%

KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.03% L-cysteine (Hsu et al, 2007). Similarly Prasad et al (2013) reported that the highest intracellular lactase activity of 6.80 U/ml and 7.7 U/ml were observed by cultivation of *Bifidobacterium animalis* spp. *lactis Bb12* and *Lactobacillus delbrueckii* spp. *bulgaricus* ATCC 11842 on whey. Both microbes were grown in protein free whey medium containing yeast extract (3.0 g/l), peptone (5.0 g/l) and glucose (10.0 g/l) for 18 h, at 37 ºC for *B. animalis* ssp. *lactis* Bb12 and at 45 ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC11842. Compared to the Prasad et al (2013) report our strain is an extracellular producer and the lactase activity is increased around 9.3 and 8.4 folds. In the fermentation process they used multiple nitrogen sources which increase the overall cost of the process but in our study we used the single nitrogen sources which may reduce cost of process.

4.4 SUMMARY

The production was improved through optimization methods like OFAT and RSM, in OFAT method first the mineral sources $MgSO_4$ was optimized and then followed by yeast extract, lactose and tryptophan. The activity was improved from 15.27 U/ml to 25.10 U/ml through the optimization of nutrient components by OFAT method. The production was further improved from 25.10 U/ml to 63.54 U/ml through the statistical optimization of the same nutritional components by Response Surface Methodology. The production was 2.5 folds superior when compared to traditional optimization.

CHAPTER-V

MODELING AND OPTIMIZATION OF PHYSICAL VARIABLES BY ARTIFICIAL NEURAL NETWORKS AND RESPONSE SURFACE METHODOLOGY

5.1 INTRODUCTION

The physical variables like pH, temperature and incubation time, aeration and agitation are the important factors and have vigilant effect on metabolic activities of microorganisms (Al-Jazairi et al, 2015). Modeling and optimization are main stages in biotechnology manufacturing sector to enhance the effectiveness of fermentation process and lowering the cost of process. The classical optimization method is not only time-consuming and it will not predict the complete effects of the variables in the process and also ignores combined interactions between the parameters (Baş and Boyaci, 2007). Meanwhile, the Response Surface Methodology (RSM) is an empirical modeling algorithm for maximizing and optimizing the various factors. RSM assesses -the relationships between independent variables and defines its effect on process (Chen et al, 2002). Many researchers were successfully applied the RSM for production of industrially important enzymes from various microorganisms (Basri et al, 2007). Tari et al (2009) was studied the production of lactase from the *Streptococcus thermophilus* 95/2 (St 95/2) and *Lactobacillus delbrueckii* ssp. *bulgaricus* 77 (Lb 77) through optimization of process variables by RSM. Dagbaglı et al (2009) applied the RSM for optimization of physical parameters such as aeration rate, agitation speed, incubation time for production of lactase from yeast species. The modeling techniques namely artificial intelligence and evolutionary computing were developed based on the phenomenon of applied biological systems. In the present scenario, Artificial Neural Networks (ANN) are the most popular learning technique in the field of biotechnology for optimization of various factors in the enzymes production from microbes (Dutta et al, 2004; Manohar et al, 2005). In previous studies Afshin et al (2008) was used the ANN for modeling of bioprocess parameters in the production of enzyme from *Geobacillus* sp. strain ARM. Rao et al (2008) carried out their research work on modeling and optimization of process parameters for improved production of enzyme from *B. circulans* by ANN and genetic algorithm (GA).

5.2 MATERIALS AND METHODS

5.2.1 Optimization of physical factors by OFAT

The medium consists of (in g/l); lactose, 4; yeast extract, 4; $MgSO_4 \cdot 7H_2O$, 1 and tryptophan, 0.1 was used for optimization study by OFAT. The physical factors of the process were optimized by conducting the experiments at different temperatures; 27 ^oC, 37 ^oC and 47 ^oC and different pH values ranges from 5 to 8.0. The isolate was also tested for inoculums size from 1% to 6% and incubation time at different intervals 12 h, 24 h, 36 h and 42 h.

5.3 Optimization of physical variables by RSM

5.3.1 Medium and shake flask experiment

One loop full bacterial culture of *B. subtilis* VUVD001 from agar slant of 36 h old was transferred to lactose broth medium for inoculum preparation and 5 ml of inoculums was transferred to the 100 ml of production medium. The compositions of medium were: (in g/l) lactose 14.01, yeast extract 10.30, tryptophan 0.43 and $MgSO_4$ 5.32l.

5.3.2 Culture conditions

The liquid fermentation was conducted batch wise in 250 ml flasks using the lactose broth medium and sterilized at 121 ^oC for 15 min. The flasks were inoculated with 5 % inoculum and incubated at 37 ^oC on rotary shaker at 150 rpm. The growth condition levels of Temperature, pH and incubation time used in the optimization study by an application of Response Surface Methodology are given in Table 5.1.

Table 5.1 Physical variable ranges used in experimental design

Symbol	Name of the Variable	Range
A	Temperature,^oC	35 – 40
B	pH	5 – 8
C	Incubation time, h	10 – 50

5.3.3 Prediction of variables effect on production

Box-Behnken method was applied to predict maximum ranges of the significant factors (temperature, pH and incubation time) and their combinational interaction on production. Design Expert version 7.0 software was utilized for design of experiment, data analysis and development of statistical model. Each experiment was studied in triplicate and average yield obtained was taken as the response (R), while the predicted values of response were obtained from quadratic model. The variables

impact on response was predicted through regression analysis of data. The three dimensional surface plots was obtained to understand the variable effect and to determine their optimum levels for production. Response surface model was fit to response i.e. activity of lactase (U/ml). The second order response function for three variables is given by the following equation.

$$R = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2$$

Where R is dependent variable (lactase production) and A, B and C are independent variables (temperature, pH and incubation time respectively), β_0 is an intercept term, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are the interaction coefficients, β_{11}, β_{22} and β_{33} are the quadratic coefficients.

5.4 Modeling using Artificial Neural Networks (ANN)

ANN modeling is an alternative tool to RSM for regression analysis of polynomial non-linear systems. ANN architecture is an interlinked complex with elements like neurons and the connections between the neurons were described by weights (w) and bias (b). The neurons were controlled by transfer and summing functions and general transfer functions includes *purelin, log sig* and *tan sig* (Das et al, 2015). In the present study, the predictive model was developed using temperature (^oC), pH and incubation time (h) as input variables and lactase activity (U/ml) as the output for the model. The input layer function is to pass the scaled input values to hidden layer through weights. A back-propagation algorithm is used with one hidden layer enhanced with Levenberg-Marquardt optimization method (Arcaklioglu et al, 2004).

5.5 RESULTS AND DISCUSSION

5.5.1 Effect of incubation time on production

The changes in enzyme activity were observed during incubation periods from 12 to 48 h. The maximum enzyme activity of 15.13 U/ml was found at 36 h of incubation beyond this the activity was declined due to depletion of nutrients (Fig. 5.1a). Similarly, Qian et al (2013) reported that highest lactase activity was found at 36 h of incubation time through fermentation in shake flasks with thermotolerant strain. Mukesh Kumar et al (2012) and Jayashree Natarajan et al (2012) were also found optimum lactase activity at 48 h of incubation period in submerged fermentation process by using *Bacillus* sp. MPTK121 and *Bacillus* sp. correspondingly.

5.5.2 Effect temperature on production

The submerged fermentation process from *B. subtilis* VUVD001 strain was shown the maximum enzyme activity at 37 °C and then enzyme activity slowed down beyond

this temperature. Thus, 37 °C was found to be optimum for lactase production by *B. subtilis* strain VUVD001 (Fig.5.1b). Tryland and Fiksdal (1998) were reported that 35 °C is the maximum temperature for lactase activity and beyond this temperature the enzyme activity slowly reduce up to 44 °C. Murad et al (2011) stated that lactase production was increased by *lactobacilli* strain when the cultivation temperature is maintained at 30 °C.

5.5.3 Effect of inoculum size on production

Inoculum size of bacterial culture has an important effect on production of maximum quantity of enzyme. The maximum lactase activity of 15.20 U/ml was observed with inoculum size of 5% and minimal enzyme activity was found with concentration of 1% inoculum respectively (Fig.5.1c). Gowdhami et al (2014) was observed highest lactase production by *L. bifermentans* at 2% v/v inoculums.

5.5.4 Effect of pH on enzyme production

The effect pH on enzyme activity revealed that activity gradually increased from pH 5.0 to 7.0 and beyond this the activity slowly decreased with increase in pH values. The highest enzyme activity was found to be 15.04 U/ml at pH 7.0 (Fig.5.1d). Cherabarti et al (2003) proved that relative activity of lactase was higher at pH 7 through submerged fermentation using *B.poymyxa*. Prasad et al (2013) was found the optimum intracellular lactase activity at pH 6.8 through cultivation of *L. delbrueckiispp.bulgaricus* ATCC11842 and *B. animalis* spp.*lactis* Bp12.

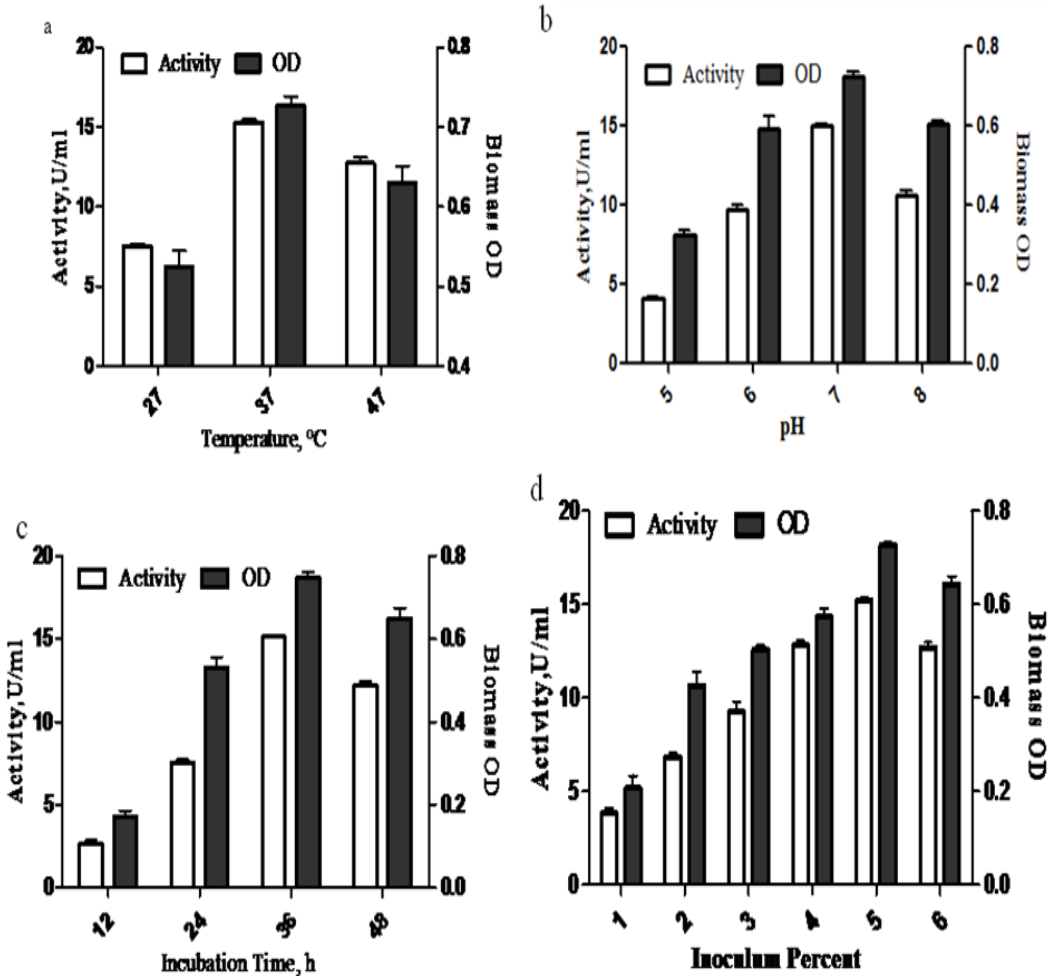


Fig.5.1 Optimization of physical parameters for production of lactase; a) Temperature, ^{o}C b) pH c) Incubation time, h and d) Inoculum size, %

5.6 Optimization of physical variables by Box-Behnken Design

Statistical model for experimental design is an essential tool in optimizing conditions that may bring several fold increase in production. The effect of parameters and their interaction on lactase synthesis was determined by conducting 17 experiments given by the model. Box-Behnken design provides necessary information about effects of variables on response. The actual data given by the model was showed in Table 5.2.

Table 5.2 Actual data for design of Experiments

Run	Temp.(^{o}C)	pH	Incubation Time, (h)	Activity, (U/ml)		
				Experimental	RSM Predicted	ANN Predicted
1	37.5	6.5	30	88.64±0.007	86.67	85.59
2	35	8	30	67.35±0.018	61.25	67.35
3	37.5	5	50	41.26±0.011	45.07	43.13
4	35	6.5	10	28.46 ±0.018	38.37	28.46
5	40	6.5	10	9.57 ±0.012	13.66	12.79
6	37.5	5	10	30.16±0.006	19.96	30.16
7	37.5	8	50	46.53 ±0.003	56.72	44.03
8	37.5	6.5	30	81.45±0.002	86.67	85.59
9	40	6.5	50	43.44±0.015	33.52	41.98
10	37.5	6.5	30	91.04±0.014	86.67	85.59
11	37.5	6.5	30	80.12±0.017	86.67	85.59
12	40	5	30	13.55±0.008	19.64	13.55
13	40	8	30	15.65 ±0.015	15.37	15.69
14	35	5	30	23.15 ±0.009	23.42	23.15
15	37.5	8	10	45.68±0.010	41.86	45.68
16	37.5	6.5	30	92.14±0.015	86.67	85.59
17	35	6.5	50	62.57±0.001	58.47	62.57

The quadratic model equation was obtained by Box-Behnken Design, which predicts the variables impact on response.

$$R1 = +86.68 - 12.42 * A + 8.39 * B + 9.99 * C - 10.52 * A * B - 0.060 * A * C - 2.56 * B * C - 30.83 * A^2 - 25.93 * B^2 - 19.84 * C^2$$

Where the response (R1) indicates lactase production and other variables A, B, C represents temperature, pH and incubation time respectively.

The coefficient R-squared value 0.9496 recommend the design was significant to predict effect of variables on production by *B.subtilis* VUVD001. The Model F-value of 14.64 implies the model is significant. The values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 5.87 implies the Lack of Fit is not significant relative to the pure error (Table 5.3).

Table 5.3 Analysis of variance of quadratic model for production of lactase

Source	Sum of squares	df	Mean squares	F-value	P-value
Quadratic Model	12508.84	9	1389.871	14.63993	0.0009
A-Temperature	1233.058	1	1233.058	12.98816	0.0087
B-pH	562.6335	1	562.6335	5.926386	0.0451
C-Incubation Time	798.6006	1	798.6006	8.411898	0.0230
AB	443.1025	1	443.1025	4.667331	0.0676
AC	0.0144	1	0.0144	0.000152	0.9905
BC	26.26563	1	26.26563	0.276664	0.6151
A^2	4000.825	1	4000.825	42.14189	0.0003
B^2	2830.519	1	2830.519	29.8147	0.0009
C^2	1657.83	1	1657.83	17.46242	0.0041
Residual	664.5592	7	94.93703		
Lack of Fit	541.5099	3	180.5033	5.87	0.0602
Pure Error	123.0493	4	30.76232		
Cor Total	13173.4	16			

R-Squared = 0.9496, Adj. R-Squared = 0.8847, C.V. = 19.24%, Adeq. Precision = 9.770

The three dimensional response plots give understandable information to predict the relationship between response and variable range. The graphs showed an oval shape curve that suggests optimum conditions and combinational effects on production (Fig.5.2 to 5.4).

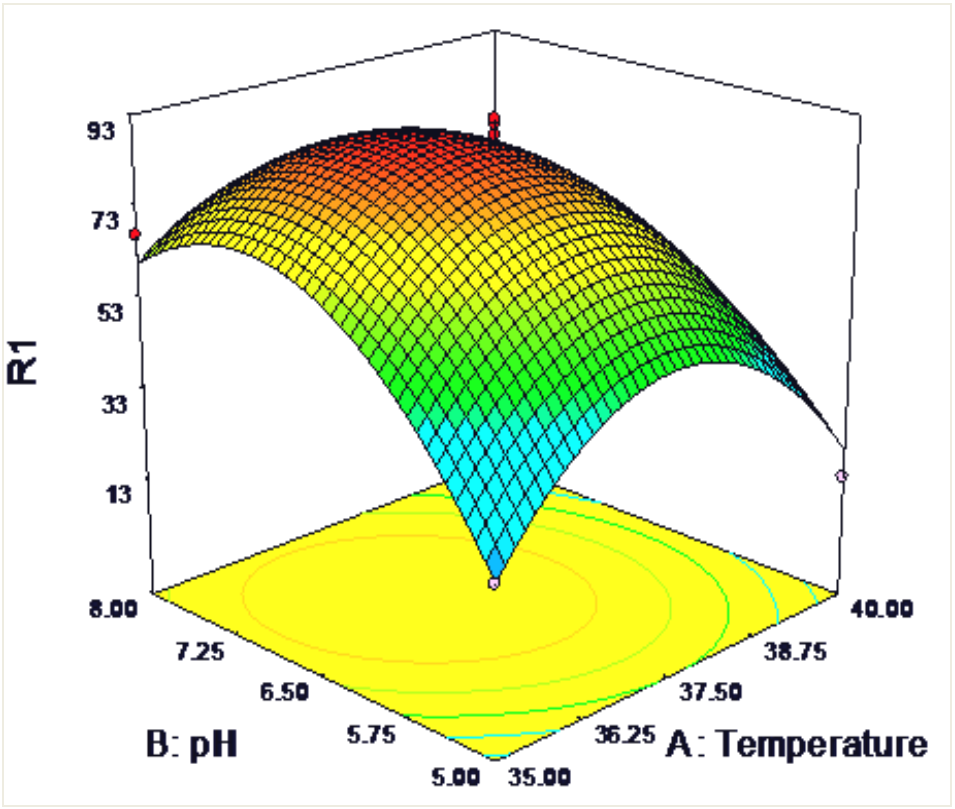


Fig. 5.2 Effect of temperature and pH on lactase production

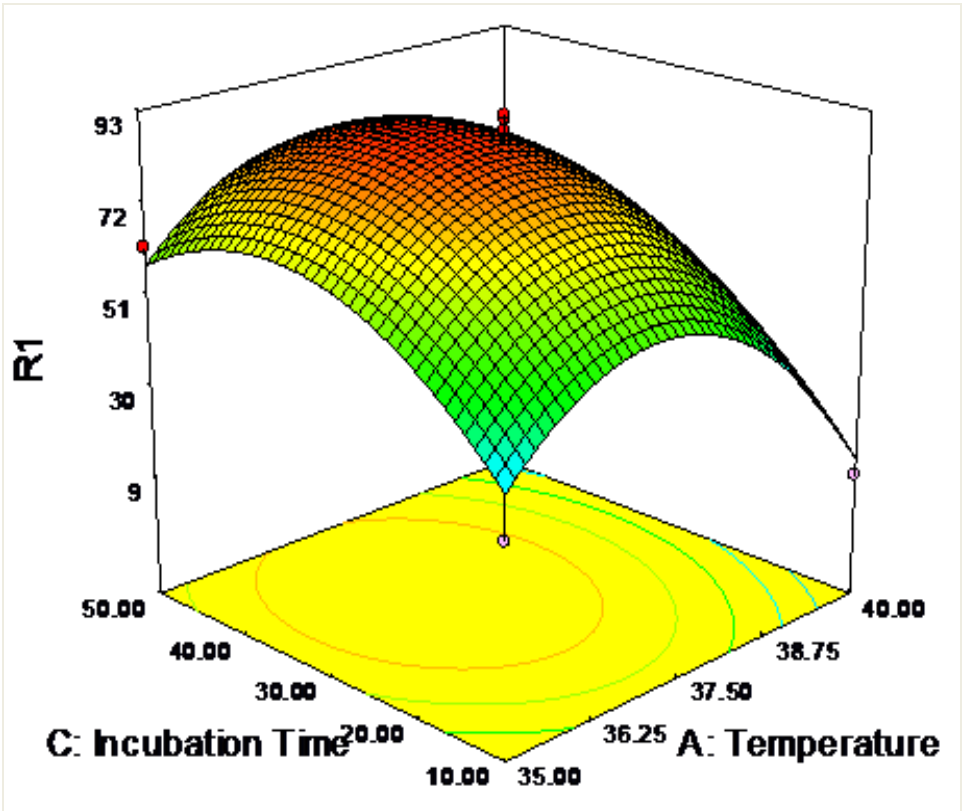


Fig. 5.3 Effect of temperature and incubation on the lactase production

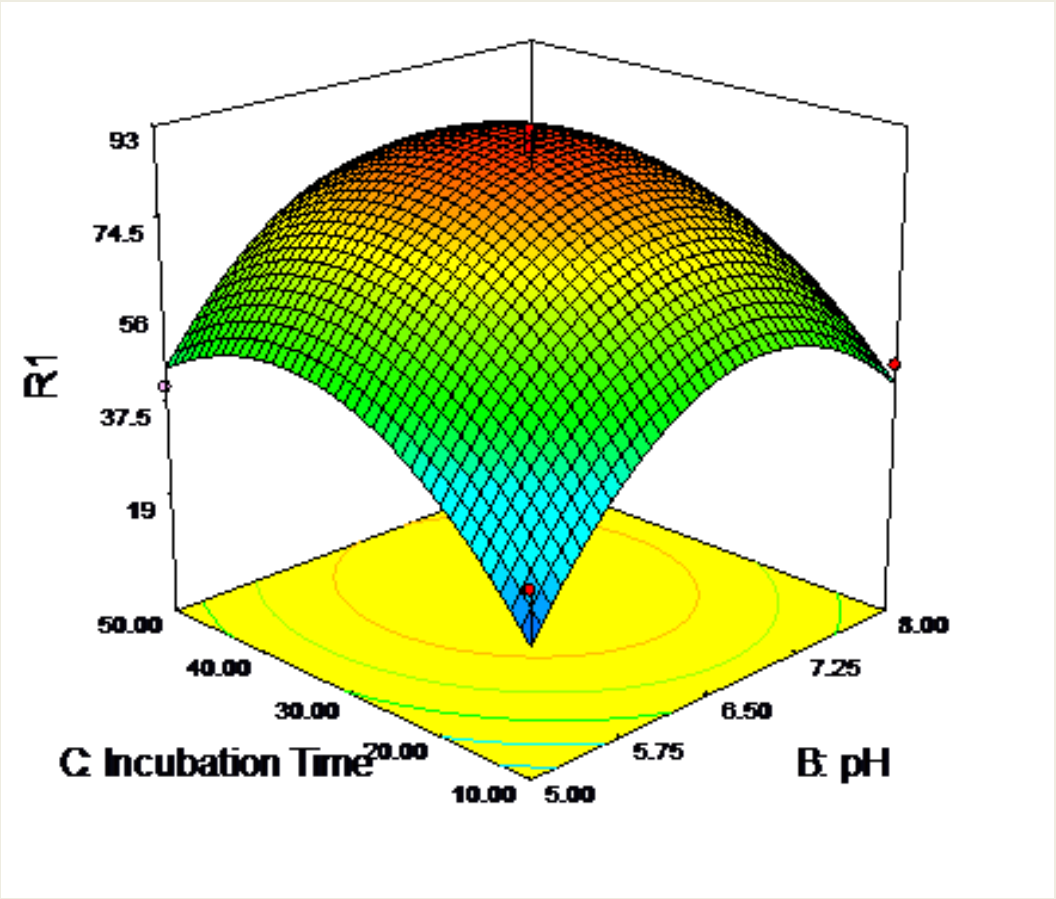


Fig. 5.4 Effects of incubation time and pH on the producttion of lactase

5.6.1 Validation of RSM Model

The RSM model is validated by conducting an experiment at best predicted solution given by RSM for production of lactase. The enzyme activity was reached to 91.32 U/ml from a *B. subtilis* VUVD001 after statistical optimization and this value is almost near to the RSM predicted value (Table 5.4).

Table 5.4 RSM optimized process variables for maximum lactase activity

Temp.(°C)	**pH**	**Incubation time (h)**	**Activity, U/ml**	
A	B	C	**Predicted**	**Experimental**
36.91	6.8	34.77	90.16	91.32 ±0.014

It has been observed that maximum production 91.32 U/ml was attained at 36.91 ^{o}C, pH 6.8 and incubation time of 34.77 h. In previous reports several workers achieved highest enzyme activity at 37 ^{o}C and 24 h incubation time through submerged fermentation with *Bacillus* strain B-2 (Mukesh Kumar et al, 2012), *Lactobacillus amylophilu*s GV6 at 48 h (Mozumder et al, 2012), *Lactobacillus acidophilus (*Milica Carevic et al, 2015), *Bacillus* Sp.MPTK 121 (Mukesh Kumar et al, 2012) and *Lactobacillus delbrueckii* (Mozumder NHMR et al, 2012). Similarly, Milica Carevic et al in 2015 has observed optimum activity of 1.01IU ml^{-1} at 37 ^{o}C, pH 6.5 – 7.5 and incubation time 48 h in shake flask culture fermentation using *Lactobacillus acidophilus* ATCC 4356 (Milica Carevic et al, 2015). Earlier reported the lactase of 0.31 U/ml was achieved by *Aspergillus terreus* even after the RSM model (Rashmi et al, 2011). Compared with this fungal strain our bacterium is potential for Lactase production. Hsu et al (2007) reported the maximum beta-galactosidase activity of 18.6 U/ml was obtained in submerged fermentation with *Bifidobacterium longum* CCRC15708. The process was run at optimum conditions of pH 6.5, Temperature 37 ^{o}C and Incubation time 16 h in liquid medium contain 4% lactose, 3.5% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.03% L-cysteine. Anisha et al (2008) reported the activity is reached from 17 to 50 U/ml under the conditions of temp 33 ^{o}C and pH 7 in submerged fermentation by *Streptomyces griseoloalbus* in Box-Behnken Design. Jaekoo Lee et al (2013) were gained maximum activity of lactase is 1.06 U/ml in submerged cultivation with *Bacillus* sp. LX-1. In the same way, Edupuganti et al (2014) reported that the lactase production in submerged fermentation by *Acinetobacter* sp.was achievd the activity of 10.2 U/ml after the modeling and genetic algorithm optimization. The process was done under the optimum conditions of temperature 37 °C, pH 7.2 and agitation speed 183 rpm. Similarly Arekal et al (2014) reported that highest lactase activity of 10.6 U/ml was obtained with *Lactobacillus plantarum* MTCC5422 in the soy whey based medium at RSM optimized conditions of incubation time 33 h, temperature 37 °C and pH 6.6. Based these observations it was proved that the predicted model found to be significant and the optimized process conditions were used in bioindistries for large scale production.

5.7 Development of Neural Network Model and Result Analysis

Training, Testing and validation of neural network were performed with three input variables and one output by using tools namely feed forward back propagation network and TRAINLM in MATLAB R2013a version. The elevated regression value of 0.9945 was attained from ANN model. The performance curve was developed using MATLAB R2013a for training, testing and validation of data. The regression plot of output versus target was developed with ten hidden nodes and R-squared value 0.99 was accomplishing the validation of model (Fig.5.5).

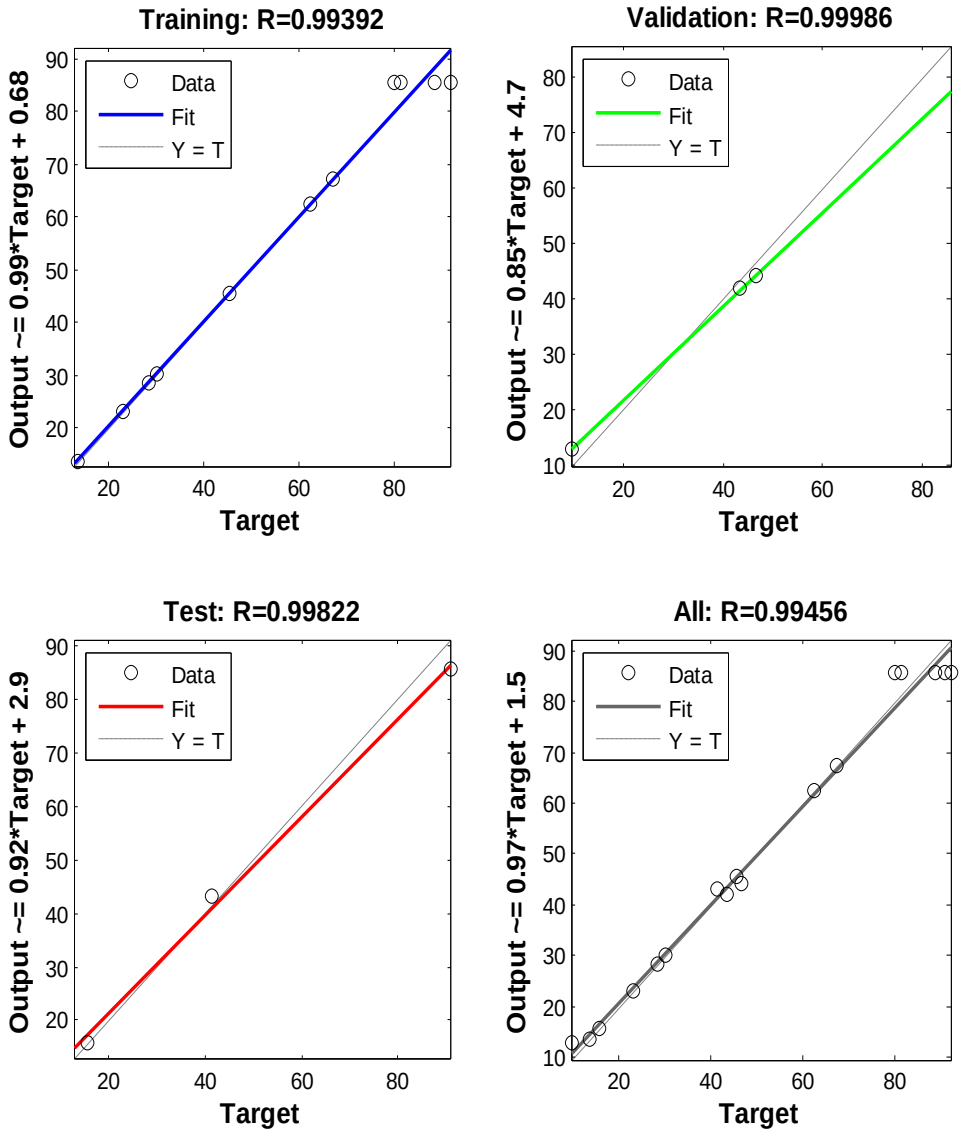


Fig. 5.5 Output vs. target regression plot

5.8 RSM and ANN predicted values Vs Experimental data

The RSM and ANN predicted data was compared with experimental values. It was observed that the ANN prediction is almost similar to experimental values (Fig. 5.6)

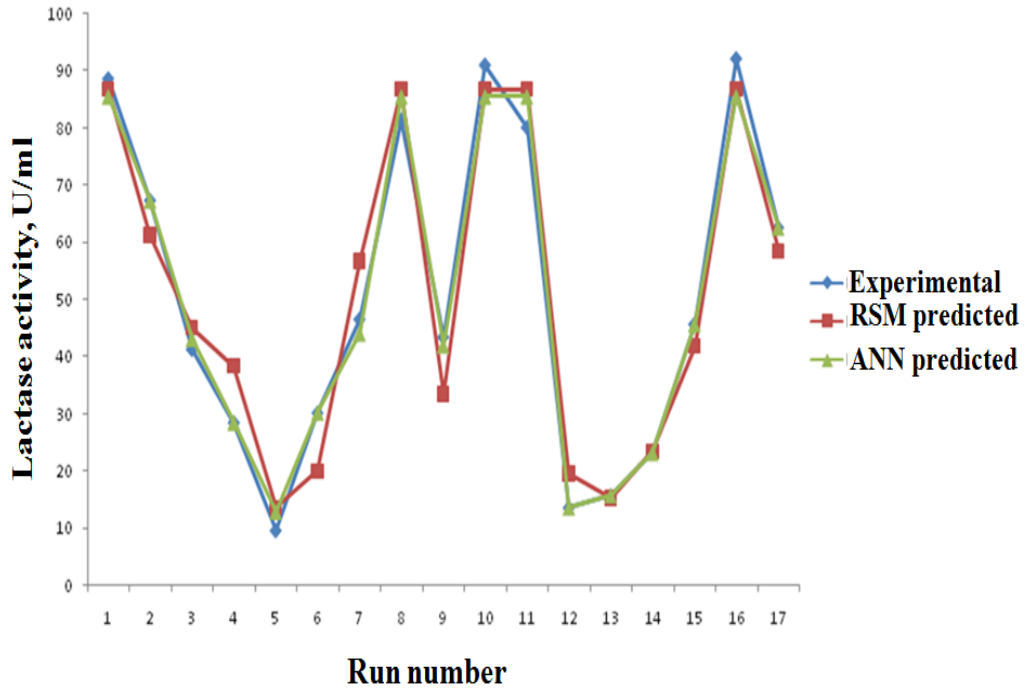


Fig. 5.6 Comparison between the experimental data Vs RSM and ANN predicted data

5.9 SUMMARY

The production level was more enhanced through the optimization of physical components such as, temperature, pH and incubation time by Response Surface Methodology (RSM). The optimized medium components by RSM were considered for the streamlining of these physical factors. Henceforth, the production was enhanced from 63.54 U/ml to 91.32 U/ml, which were 6 folds higher in comparison with initial activity.

CHAPTER-VI

CONCLUSIONS AND SCOPE FOR FUTURE WORK

6.1 Major findings from the thesis

- The dairy effluent was screened for lactase producing bacterial strains. It was observed that 46 bacterial isolates were able to produce lactase. The highest yielding potential bacterial strain was named as VUVD001 and this strain was identified as *Bacillus subtilis* through 16S rRNA sequence analysis.
- The *B. subtilis* VUVD001 strain was also tested for an extracellular lactase activity by native PAGE method with X-gal staining and found that the *B.subtilis* VUVD001 produced an extracellular lactase enzyme.
- The lactase activity was found to be 25.10 U/ml after optimization of fermentation factors by OFAT.
- The desired quantities of medium components were established by RSM for enhanced production of lactase was lactose, 14.01 g/l; yeast extract, 10.30 g/l; tryptophan, 0.43 g/l and $MgSO_4$, 5.32 g/l. The optimized medium has produced the lactase activity of 63.54 U/ml and it was almost three folds higher than the unoptimized medium.
- The effects of physical variables namely, temperature, pH and incubation period on production were also studied by Box-Behnken Design. Under the optimized conditions highest activity of lactase, 91.32 U/ml was obtained.
- The bioprocess modeling study was carried out to relate the experimental data with predicted observations by RSM. ANN model gave R^2 value of 0.9945 for RSM predicted data. Hence our findings reveal that *B. subtilis* VUVD001 was also an alternative promising strain for commercial production of lactase.

6.2 Scope for future work

Lactase enzyme has commercial applications in dairy and pharmaceutical industries. It has already been proved that many bacteria produced lactase enzyme to fulfill the needs of lactose intolerants. The improved productivity of lactase enzyme has achieved through the optimization of various process parameters by classical and statistical approaches from newly isolated *B.subtilis* VUVD001 strain. However, there is a lot of scope to enhance lactase production by strain improvement through genetic modifications and also to increase the yield of lactase by scale-up of the process from

shake flask to reactor level. In addition, there is a further scope in the area of structural characterization for complete elucidation of lactase from *B. subtilis* VUVD001.

REFERENCES

A. Kumar, N. Bhoot, I. Soni, P.J John, "Isolation and characterization of a *Bacillus subtilis* strain that degrades endosulfan and endosulfan sulphate," 3 Biotech, (2014), 4(5), 467-475.

A. Kumar, S. Singh, "Directed evolution: Tailoring biocatalysis for industrial application derivatives," International Dairy Journal, (2008), 18, 685–94.

A. Macrae "The use of 16S rDNA methods in soil microbiology ecology," Brazilian Journal of Microbiology, (2000), 31, 77-82.

A. Nath, S. Sarkar, M. Maitra, C. Bhattacharjee, R. Chowdhury, "An experimental study on production of intracellular β-galactosidase at different conditions by batch process using isolated *Bacillus safensis* (JUCHE1) and characterization of synthesized β-galactosidase," Journal of The Institution of Engineers (India): Series E, (2012), 93(2), 55-60.

A. Silverstone, A. Connes, C. Sesma, G.S. de Giori, J.C. Piard, "Characterization of the melA locus for α-galactosidase in *Lactobacillus plantarum*," Applied and environmental microbiology, (2002), 68(11), 5464-5471.

A. Tateishi , H. Inoue , S. Yamaki . "Fluctuations in activities of three β-galactosidase isoforms from ripening avocado (*Persea americana)* fruit and their different activities against its cell wall polysaccharides as substrates," Journal of the Japanese Society for Horticultural Science, (2001), 70, 586–592.

A.C. Lopez , A.C. Hernandez , A.C. Trejo , F.G. Lara , O.P. Lopez , "Hydrolytic activity and ultrastructure in fruit skins from two prickly pear (*Opuntia* sp.) varieties during storage," Journal of Agricultural and Food Chemistry, (2001),50, 1681–1685.

A.G. Patil, S.K. Praveen Kumar, V.H. Mulimani, Y. Veeranagouda, K. Lee, "α-galactosidase from *Bacillus megaterium* VHM1 and its application in removal of flatulence-causing factors from soymilk," Journal of Microbiology and Biotechnology, (2010), 20(20), 1546-1554.

A.H.D.N. Rangel, D.C. Sales, S.A. Urbano, J.G.B. Galvao Junior, J.C.D. Andrade Neto, C.D.S. Macedo, "Lactose intolerance and cow's milk protein allergy," Food Science and Technology (Campinas), (2016),179 – 187.

A.I. Siham, A.M. Hashem, "Nutrition requirement for the production of *Penicillium chrysogenum* α-galactosidase and its potential for hydrolysis of raffinose family oligosaccharides," Journal of Applied Sciences and Research, (2012), 8, 945–952.

A.L.S. Schneider, R. Merkle, M.F. Carvalho-jonas, R. Jonas, S. Furlan, "Oxygen transfer on β-D-galactosidase production by *Kluyveromyces marxianus* using sugar cane molasses as carbon source," Biotechnology Letters (2001), 23, 547-550.

A.P. Manera, J.C. Ores, V.A. Ribeiro, C.A.V. Burkert, S.J. Kalil, "Optimization of the culture medium for the production of galactosidase from *Kluyveromyces marxianus* CCT 7082," Food Technology and Biotechnology, (2008), 46(1), 66-72.

A.Y. Alazzeh, S.A. Ibrahım, D. Song, A. Shahbazi, A.A. AbuGhazaleh, "Carbohydrate and protein sources influence the induction of β-galactosidases in *Lactobacillus reuteri*," Food Chemistry, (2009), 4, 654-659.

B. Jost, I. Duluc, J.L. Vilotte, J.N. Freund, "Lactase is unchanged in suckling mice fed with lactose-free milk," Gastroenterologie Clinique Et Biologique, (1998), 22(1), 863-7.

B. Manohar , S. Divakar, "An artificial neural network analysis of porcine pancreas lipase catalysed esterification of anthranilic acid with methanol," Process Biochemistry, (2005), 40, 3372-3376.

B.A. Pribila, S.R. Hertzler, B.R. Martin B, C.M. Weaver, D.A. Savaiano, "Improved lactose digestion and intolerance among African-American adolescent girls fed a dairy-rich diet," Journal of the American Dietetic Association**,** (2000), 100(5), 524-8.

B.W. Matthews, "The structure of *E. coli* β-galactosidase," Comptes rendus biologies, (2005), 328(6), 549-556.

C. Abiola and V.O. Oyetayo, "Research Article Isolation and Biochemical Characterization of Microorganisms Associated with the Fermentation of Kersting's Groundnut (Macrotyloma geocarpum)," Research Journal of Microbiology, (2016), 11(2), 47-55.

C. Favier, C. Neut, C. Mizon, A. Cortot, J.F. Clombel, J. Mizon, "Fecal β-D-Galactosidase production and Bifidobacteria are decreased in crohn's Disease," Journal of Microbiological Methods, (2011), 279(1), 25-31.

C. Giacomini, G. Irazoqui, F. Batista-Viera, B.M. Brena, "Influence of immobilization chemistry on the properties of immobilized β-galactosidase," Journal of Molecular Catalysis B: Enzymatic, (2001), 11, 597-606.

C. Liu, H. Ruan, H. Shen, Q. Chen, B. Zhou, Y. Li, G. He, "Optimization of the fermentation medium for galactosidase production from *Aspergillus foetidus* ZU-G1 using response surface methodology," Journal of Food Science, (2007), 72, 120–125.

C. Picard, J. Fioramonti, A. Francois, T. Robinson, F. Neant, C. Matuchansky," *Bifidobacteria* as probiotic agents-physiological effects and clinical benefits," Alimentary Pharmacology & Therapeutics, (2005), 22(6), 495-512.

C. Tari, F.I. Ustok, S. Harsa, "Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface methodology," International dairy journal, (2009), 19(4), 236-243.

C. Tari, N. Gogus, F. Tokatli, "Optimization of biomass, pellet size and polygalacturonase production by *Aspergillus sojae* ATCC20235 using response surface methodology," Enzyme and microbial technology, (2007), 40(5), 1108-1116.

C.A. Hsu, R.C. Yu, C.C. Chou, "Production of beta-galactosidase by *Bifidobacteria* as influenced by various culture conditions," International Journal of Food Microbiology, (2005), 104(2), 197–206.

C.A. Hsu, R.C. Yu, C.C. Chou, "Purification and characterization of a sodium-stimulated β-galactosidase from *Bifidobacterium longum* CCRC15708". World Journal of Microbiology and Biotechnology, (2006), 22, 355–361.

C.A. Hsu, Y.R.C Lee, C.C. Chou "Cultural condition affecting the growth and production of β-galactosidase by *Bifidobacterium longum* CCRC 15708 in a jar fermenter," International Journal of Food Microbiology, (2007), 116, 186–189.

C.Q. Liu, Q.H. Chen, B. Tang, H. Ruan, G.Q. He, "Response Surface Methodology for optimizing the fermentation medium of alpha-galactosidase in solid-state fermentation," Letters in applied microbiology, (2007), 45(2), 206-212.

C.S. Chen, K.J. Liu, Y.H. Lou, C.J. Shieh, "Optimisation of kojic acid monolaurate synthesis with lipase PS from *Pseudomonas cepacia*," Journal of the Science of Food and Agriculture, (2002), 82(6), 601-605.

C.S. Rao, T. Sathish, M. Mahalaxmi, G.S. Laxmi, R.S. Rao, R. S. Prakasham, "Modelling and optimization of fermentation factors for enhancement of alkaline protease production by isolated *Bacillus circulans* using feed-forward neural network and genetic algorithm," Journal of Applied Microbiology, (2008), 104(3), 889-898.

D. A. Post, V.E Luebke, "Purification, cloning, and properties of α-galactosidase from *Saccharopolyspora erythraea* and its use as a reporter system". Applied microbiology and biotechnology, (2005), 67(1), 91-96.

D. Gobinath and S.G. Prapulla, "Transgalactosylating β-galactosidase from probiotic *Lactobacillus plantarum* MCC2156: production and permeabilization for use as whole cell biocatalyst," Journal of food science and technology, (2015), 52(9), 6003-6009.

D. Kalogridou-Vassiliadou, "Biochemical activities of *Bacillus* species isolated from flat sour evaporated milk," Journal of Dairy Science, (1992), 75(10), 2681–2686.

D.Baş and I.H. Boyaci, "Modeling and optimization I: Usability of response surface methodology," Journal of food engineering, (2007), 78(3), 836-845.

D.E. Trimbur, K.R. Gutshall K, P. Prema, J.E. Brenchley, "Characterization of a psychrotrophic *Arthrobacter* gene and its coldactive beta-galactosidase," Applied and Environmental Microbiology, (1994), 60(1), 4554-4552.

D.H. Juers, B.W. Matthews, R.E. Huber, "LacZ β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance," Protein Science, (2012), *21*(12), 1792-1807.

D.J. Mukesh Kumar, M. Sudha, S. Devika, M.D. Balakumaran, M. Ravi Kumar, P.T. Kalaichelvan, "Production and Optimization of β-galactosidase by *Bacillus* Sp. MPTK 121, Isolated from Dairy Plant Soil," Annals of Biological Research, (2012), 3 (4), 1712-1718.

D.M. Pederson and R.E. Goodman, "Isozymes of α-galactosidase from *Bacillu stearothermophilus*," Canadian journal of microbiology, (1980), 26(8), 978-984.

D.O. Otieno, "Synthesis of β-Galactooligosaccharides from lactose using microbial β-Galactosidases," Comprehensive Reviews in Food Science and Food Safety, (2010), 9(5), 471-482.

E. Afshin, R.N. Zaliha, R. A. Rahman, D.H.E. Chng, M. Basri, A.B. Salle, "A modelling study by Response Surface Methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic *Geobacillus* sp. strain ARM, BMC Biotechnology, (2008), 96(8), 1-15.

E. Arcaklioglu, A. Cavusoglu, A. Erisen, "Thermodynamic analyses of refrigerant mixtures using artificial neural networks," Applied Energy, (2004), 78(2), 219-230.

E. Gallagher, T.R. Gormley, E.K. Arendt, "Recent advances in the formulation of gluten-free cereal-based products," Trends in Food Science & Technology, (2004), 15(3), 143-152.

E. Gasteiger, A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel, A. Bairoch, ExPASy: "the proteomics server for in-depth protein knowledge and analysis." Nucleic Acids Research (2003), 31, 3784–8.

E. Jurado, F. Camacho, G. Luzón, J.M. Vicaria, "Kinetic models of activity for β-galactosidases influence of pH, ionic concentration and temperature," Enzyme and Microbial Technology, (2004), 34(2004), 33-40.

E.M. Charyulu and A. Gnanamani, "Condition stabilization for *Pseudomonas Aeruginosa* MTCC5210 to yield high titers of extra cellular antimicrobial secondary metabolite using response surface methodology," Current research in bacteriology, (2010), 3(4), 197-213.

E.S. Nam and J.K. Ahn, "Isolation and characterization of cold adapted bacteria producing lactose hydrolyzing enzyme isolated from soils of Nome area in Alaska,". International Research Journal of Microbiology, (2011), 2(9), 348-355.

F. Huang & J.S. Kim, "IgE-mediated cow's milk allergy in children," Pediatric Allergy and Immunology," (2012), 12(6), 630-640.

F.F. Mota, E.A. Gomes, E. Paiva, L. Seldin, "Assessment of the biodiversity of *Paenibacillus* species in environmental samples by a novel rpoB-based PCRDGGE Method," FEMS Microbiolology and Ecology, (2005), 53, 317-28.

F.S.R. Gasparin, J.M. Teles, S.C. Araújo, "Alergia à proteína do leite de vaca versus intolerância à lactose: as diferenças esemelhanças," Revista Saude and Pesquisa, (2010), 3(1), 107-114.

G. Banerjee, A. Ray, S. Das, R. Kumar, A.K. Ray, A. K, "Chemical extraction and optimization of intracellular β-galactosidase production from the bacterium *Arthrobacter oxydans* using Box-Behnken design of response surface methodology" Acta Alimentaria, (2016), 45(1), 93-103.

G. Casula and S. Cutting, "*Bacillus* Probiotics: Spore Germination in the Gastrointestinal Tract," Applied and Environmental Microbiology, (2002), 68(5), 2344–2352.

G. D. Miller, J. K. Jarvis, L.D. McBean, "Handbook of Dairy Foods and Nutrition," Second Edition, CRC Press LLC, Rosemont, Illinois, 2000.

G. Matioli, F.F. De Moraes, G.M. Zanin, "Operational stability and kinetics of lactose hydrolysis by β-galactosidase from *Kluyveromyces fragilis,"* Acta Scientiarum, (2003), *25*, 7-12.

G. Sreekumar and S. Krishnan, "Isolation and characterization of probiotic *Bacillus subtilis* SK09 from dairy effluent," Indian Journal of Science and Technology, (2010), 3(8), 863-866.

G.D. Duffaud, C.M. McCutchen, P. Leduc, K.N. Parker, R.M. Kelly, "Purification and characterization of extremely thermostable beta-mannanase, beta-mannosidase, and alpha-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068," Applied and environmental microbiology, (1997), *63*(1), 169-177.

G.G. Raol, V.S. Prajapati, B.V. Raol, "Formulation of low-cost, lactose-free production medium by Response Surface Methodology for the production of β-galactosidase using halotolerant *Aspergillus tubengensis* GR-1," Biocatalysis and Agricultural Biotechnology, (2014), 3(4), 181-187.

G.S. Anisha, R.K. Sukumaran, P. Prema, "Evaluation of *α*-galactosidase biosynthesis by *Streptomyces griseoloalbus*in solid-state fermentation using response surface methodology," Letters in Applied Microbiology, (2008), 4, 338–343.

H. Christensen, P. Kuhnert, J.E. Olsen, M. Bisgaard, "Comparative phylogenies of the housekeeping genes atpD, infB and rpoB and the 16S rRNA gene within the Pasteurellaceae," International journal of systematic and evolutionary microbiology, (2004), 54(5), 1601-1609.

H. Lazan, S.Y. Ng, L.Y. Goh, ZM Ali, "Papaya beta-galactosidase/galactanase isoforms in differential cell wall hydrolysis and fruit softening during ripening," Plant Physiology and Biochemistry, (2004), 42, 847–853.

H. Li, W.Q. Liang, Z.Y. Wang, N. Luo, X.N. Wu, J.M. Hu, Y.H. Liu, "Enhanced production and partial characterization of thermostable α-galactosidase by thermotolerant *Absidia* sp. WL511 in solid-state fermentation using response surface methodology," World Journal of Microbiology and Biotechnology, (2006), 22(1), 1-7.

H. Rasinpera, K. Saarinen, A. Pelkonen, L. Järvelä, E. Savilahti, K.L. Kolho, "Molecularly defined adult-type hypolactasia in school-aged children with a previous history of cow's milk allergy," World Journal of Gastroenterology, (2006), 12(14), 2264-2268.

H.A. Murad, R.I. Refaea, E.M. Aly, "Utilization of UF-permeate for production of beta-galactosidase by lactic acid bacteria," Polish Journal of Microbiology, 2011, 60(2), 139- 144.

H.P. Singh, M.V.R. Rao, S.M. Dutta, "Partial-purification and properties of *Leuconostoc citrovo-rum* beta-galactosidase," Milk Science International, (1979), 34, 475-478.

H.S. Choonia and S.S. Lele, "Release of β-galactosidase from indigenous *Lactobacillus acidophilus* by ultrasonication: process optimization," Chemical Engineering Communications, (2011), 198(5), 668-677.

H.V. Adikane, J.V. Shah, S.S. Biswas, "Studies of β-Galactosidase production and immobilization using novel bacteria isolated from the soil enriched with whey," Current Biotechnology, (2015), 4(3), 373-379.

H.Yin, T. Pijning, X. Meng, L. Dijkhuizen, S.S. van Leeuwen, "Engineering of the *Bacillus circulans* β-galactosidase product specificity," Biochemistry, (2017), 56(5), 704-711.

Hudaaneetoo, Nabiihahboubadoor, Mala ranghoosanmukhiya, "isolation, characterization and determination of probiotic Properties of lactic acid bacteria from effluents of a dairy Plant in Mauritius," International Journal of Biology, Pharmacy and Allied Sciences, (2015), 4(2), 320-333.

I. Tryland and L. Fiksdal, "Enzyme Characteristics of β-D-Galactosidase-and β-D-glucuronidase-positive bacteria and their Interference in rapid methods for detection of waterborne coliforms and *Escherichia coli,"* Applied and Environmental Microbiology, (1998), 64(3), 1018-1023.

J. Delente, J.H. Johnson, M.J. Kuo, R.J. O. Connor, L.E. Weeks, "Production of a new thermostable neutral α-galactosidase from a strain of *Bacillus stearothermophilus*." Biotechnology and bioengineering, (1974), 16(9), 1227-1243.

J. Garman, T. Coolbear, J. Smart, "The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β -galactosidase from six strains of lactic acid bacteria," Applied Microbiology and Biotechnology, (1996), 46, 22–7.

J. Miller, "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory press, New York, (1972).

J.C. Jayashree Natarajan, D.J. Christobell, M.D. Mukesh Kumar, M. Balakumaran, P.T. Kalaichelvan, "Isolation and characterization of β-galactosidase producing *Bacillus* sp. from dairy effluent," World Applied Sciences Journal, 2012, 17(11), 1466-1474.

J.E. Prenosil, E. Stuker, J.R. Bourne, "Formation of oligosaccharides during enzymatic hydrolysis of lactose," Biotechnology and Bioengineering, (1987), 30, 1019-1025.

J.L. Adrio, A.L. Demain, "Microbial Cells and Enzymes—A Century of Progress. In Methods in Biotechnology," Microbial Enzymes and Biotransformations, (2005), 52, 120–5.

J.M. Rezessy-Szabó, Q.D. Nguyen, E. Bujna, K. Takács, M. Kovács & A. Hoschk, "*Thermomyces lanuginosus* CBS 395.62/b strain as rich source of alpha-galactosidase enzyme," *Food* Technology and Biotechnology, (2003), 41(1), 55-60.

J.P. Troelsen, "Adult type hypolactasia and regulation of lactase expression," *Biochimica et Biophysica Acta*, (2005), 1723, 19-32.

J.R. Dutta, P.K. Dutta, R. Banerjee, "Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models," Process Biochemistry, (2004), 39, 2193–2198.

J.R. Machado, M.B. Behling, A.R.C. Braga, S.J. Kalil, "β-Galactosidase production using glycerol and byproducts: Whey and residual glycerin," Biocatalysis and Biotransformation, (2015), 33(4), 208-215.

J.V. Ganeva, B. Galutzov, N. Eynard, J. Teissie, "Electroinduced extraction of β-galactosidase from *Kluyveromyceslactis,"* Applied Microbiology and Biotechnology, (2001), 56(1), 411-3.

J.W. Bartholomew, M.D. Lechtman, H. Finkelstein, "Differential spore & lipid staining at room temperature by using fluorescent dye," Journal of Bacteriology, (1965), 90, 1146–1147.

J.W. Karpen, M.L. Ruiz, "Ion channels: does each subunit do something on its own?," Trends in Biochemical Sciences, (2002), 27, 402–409.

J.W. Kim, S.N. Rajagopal, "Isolation and characterization of b-galactosidase from *Lactobacillus crispatus*," Folia Microbiologica, (2000), 45, 29–34.

Jaekoo Lee, Inkyung Park, Sangbuem Cho, Sooki Kim, Yunjaie Choi, Jaiesoon Cho,"Optimization of medium composition for α- galactosidase production by Antarctic bacterial isolate, *Bacillus* sp. LX-1 using response surface methodology," African journal of microbiology, (2013), 7, 3494 – 3500.

Jurgen Schrezenmeir and Michael de Vrese, "Probiotics, prebiotics, and synbiotics-approaching a definition," American Journal of Clinical Nutrition, (2001), 73, 361-364.

K. Abdul Khalil, S. Mustafa, R. Mohammad, A. Bin Ariff, Y. Shaari, Y. Abdul Manap, F.A. Dahalan, "Optimization of milk-based medium for efficient cultivation of *Bifidobacterium pseudocatenulatum* G4 using face-centered central composite-response surface methodology," BioMed research international,(2014), 1-10.

K. Wallenfels and O.P. Malhotra, "β-galactosidase in The Enzymes," second edition, Academic Press, NewYork, (1960), 4, 409.

K.A. Hadfield , T. Dang , M. Guis , J.C. Pech , M. Bouzayen, A.B. Bennett, "Characterization of ripening-regulated cDNAs and their expression in ethylene-suppressed charentais melon fruit," Plant Physiology, (2000), 122, 977–983.

K.D. Arunachalam, "Role of *bifidobacteria* in nutrition, medicine and Technology," Nutritional Research, (2004), 19, 1559–1597.

K.K. Pulicherla, P.S. Kumar, K. Manideep, K, Rekha, M. Ghosh, K.R.S.Sambasiva Rao, "Statistical approach for the enhanced production of cold-active β-galactosidase from *Thalassospirafrigidphilosprofundus* a novel marine psychrophile from deep waters of Bay of Bengal," Preparative Biochemistry & Biotechnology, (2013), 43(8), 766-80.

K.M. Tuohy, H.M Probert, C.W. Smejkal, G.R. Gibson, "Using probiotics and prebiotics to improve gut health," Drug discovery today, (2003), 8(15), 692-700.

L. D. McBean, G.D. Miller, "Allaying fears and fallacies about lactose intolerance, Journal of the American Dietetic Association," 1998, 98(6), 671-676.

L. Prasad, N. Ghosh, B.C. Sherkat, "Extraction and characterisation of β-galactosidase produced by *Bifidobacterium animalis* spp. *Lactis* Bb12 and *Lactobacillus delbrueckii* spp. *Bulgaricus* ATCC11842 grown in whey," International Food Research Journal, (2013), 20(1), 487-494.

L.K. Harrington and J.F. Mayberry, "A re-appraisal of lactose intolerance" International Journal of Clinical Practice, (2008), 62, 1541–1546.

L.M. Prescott, J.P. Harley, D. Klein, "Microbiology," Wm. C. C. Brown Publisher, Dubuque, IA, (1996).

M. Al-Jazairi, S. Abou-Ghorra, Y. Bakri, M. Mustafa, "Optimization of β-galactosidase production by Response Surface Methodology using locally isolated Kluyveromyces marxianus". International Food Research Journal, (2015), 22(4), 1361-1367.

M. Basri, R.N.Z.R.A. Rahman, A. Ebrahimpour, A.B. Salleh, E.R. Gunawan, M.B.A Rahman, "Comparison of estimation capabilities of Response Surface Methodology

(RSM) with artificial neural network (ANN) in lipase-catalyzed synthesis of palm-based wax ester," BMC biotechnology, (2007), 7(1), 1-14.

M. Cardinale, L. Brusetti , P.Quatrini, S. Borin, A.Maria Puglia, A. Rizzi, E. Zanardini C. Sorlini, C. Corselli, DD. Daffonchio D, "Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities," Applied and Environmental Microbiology, (2004), 70, 6146–6156.

M. Ghosh, K.K.Pulicherla, V.P.B. Rekha, P. Kumarraja, K.R.S.S. Rao, "Cold Active b-Galactosidasefrom*Thalassospira sp.* 3SC-21 to Use in Milk Lactose Hydrolysis, A Novel Source from Deep Watersof Bay-of-Bengal," World Journal of Microbiology *and* Biotechnology, (2012), 28(1), 2859–2869.

M. Ladero, A. Santos, J.L Garcia, F. Garcia-Ochoa, "Activity over lactose and ONPG of a genetically engineered galactosidase from *Escherichia coli* in solution and immobilized kinetic modeling," Enzymes and Microbial Technology (2001), 29, 181-193.

M. Maity, S. Sanyal, J. Bhowal, D.K. Bhattacharyya, "Studies on isolation and characterization of lactase produced from soil bacteria," Research Journal of Recent Sciences, (2013), *2*(8), 92-94.

M. Saarela, L. Lähteenmaki, R. Crittenden, S. Salminen, T. Mattila Sandholm, "Gut bacteria and health foods—the European perspective," International journal of food microbiology, (2002), 78(1), 99-117.

M. Xiao, K. Tanaka, X.M. Qian, K.Y. amamoto, H. Kumagai, "High-yield production and characterization of α-galactosidase from *Bifidobacterium breve* grown on raffinose," Biotechnology letters, (2000), *22*(9), 747-751.

M.A. Boon, A.E.M. Janssen, K. van't Riet, "Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides," Enzyme and Microbial Technology, (2000), 26, 271-281.

M.A. Lokuge and C.D. Mathew, "Isolation from soil of bacteria producing extracellular alpha galactosidase," Journal of the National Science Foundation of Sri Lanka, (2010), *28*(4), 243 – 252.

M.A. Qureshi, A.K. Khare, A. Pervez, S. Uprit, "Enzymes used in dairy industries. International Journal of Applied Research, (2015), *1*(10), 523-527.

M.K. Panda, M. K. Sahu, K. Tayung, "Isolation and characterization of a thermophilic Bacillus sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India" Iranian Journal of Microbiology, (2013), *5*(2), 159-165.

M.Y. Yoon, H.J. Hwang, "Reduction of soybean oligosaccharides and properties of α-D-galactosidase from *Lactobacillus curvatus* R08 and *Leuconostoc mesenteriodes* JK55," Food microbiology, (2008), *25*(6), 815-823.

MilicaCarevic, Maja Vukasinovic-Sekulic, SanjaGrbavcic, MarijaStojanovic, MladenMihailovic, Aleksandra Dimitrijevic, DejanBezbradica, "Optimization of β-galactosidase production from lactic acid bacteria," Hemijska industrija, (2015), 69, 305-312.

N. A. Greenberg, R. R. Mahoney, "Immobilization of lactase (β-galactosidase) for use in dairy processing," A review. Process Biochemistry, (1981), 16, 2-8.

N. Akcan, "High level production of extracellular galactosidase from *Bacillus licheniformis* ATCC12759 in submerged fermentation," African Journal of Microbiology Research, (2011), 5, 4615–4621.

N. Albayrak and S.T. Yang, Immobilization of *Aspergillus oryzae* β galactosidase on tosylated cotton cloth. Enzymes and Microbial Technology, (2002), 31, 371–383.

N. Arekal,Roopashri, C. Mandyam,Varadaraj, "Soy whey based medium for optimized phytase activity in *Saccharomyces cerevisiae* MTCC5421 and α-D-galactosidase and antibacterial activities in *Lactobacillus plantarum* MTCC5422 by response surface methodology," Journal of Food Science and Technology, (2004), 51, 519–526.

N. Batra, J. Singh, S. Bhatia, A. Behal, A. Joshi, "Lactose hydrolysis by β-galactosidase from a newly isolated bacterial strain bacillus sp. *NV1*. Current Trends in Biotechnology and Chemical Research, (2011), *1*, 111-120.

N. Batra, J.Singh , U.C. Banerjee, P.R. Patnaik, R.C. Sobti, "Production and characterization of a thermostable β-galactosidase from *Bacillus coagulans* RCS3". Biotechnology and applied biochemistry, (2002), 36(1), 1-6.

N.C. Heilskov, "Studies on animal lactase," Acta Physiologica, (1951), *22*(2), 267-276.

N.H.M.R. Mozumder, M. Akhtaruzzaman, M.A. Baki, Fatema-Tuj-Zohra," Study on isolation and partial purification of Lactase enzyme from *Lactobacillus* bacteria isolated from yogurt," Journal of Scientific Research, (2012), 4, 239 – 249.

Nath, S. Mondal, S. Chakraborty, C. Bhattacharjee, R. Chowdhury, "Production, purification, characterization, immobilization, and application of β-galactosidase: a review," Asia-Pacific Journal of Chemical Engineering, (2014), 9(3), 330-348.

Nazish Mazhar Ali, Saiqa Andleeb, Bushra Mazhar and Agha Usman Ali Khan, "Optimization of β-galactosidase Production from Yogurt and Dairy Soil Associated Bacteria Using Different Fermentation Media," British Microbiology Research Journal, (2016), 11(2), 1-15

O.A. Asimi, N.P. Sahu, and A.K. Pal, "Antioxidant activity and antimicrobial property of some Indian spices," International Journal of Scientific and Research Publications Scientific, (2013), 3(3), 541-549

P. Jellema, F.G. Schellevis, D.A.W.M. Van Der Windt, C.M.F. Kneepkens, H.E. Van Der Horst, "Lactose malabsorption and intolerance: a systematic review on the diagnostic value of gastrointestinal symptoms and self-reported milk intolerance," QJM, (2010), 103(8), 555-572.

P. Sujatha, B. Naresh Kumar, V. Kalarani, "Isolation, characterization and molecular identification of bacteria from tannery effluent using 16S rRNA sequencing," Current Biotica, 2012, 6(2), 198-207.

P.A. Eigenmann, S.H. Sicherer, T.A. Borkowski, B.D. Cohen, H.A. Sampson, "Prevalence of IgE-mediated food allergy among children with atopic dermatitis. *Pediatrics*," (1998), 101(3), 1-8.

P.L. Moller, F. Jorgensen, O.C. Hansen, S.M. Madsen, P. Stougaard, "Intra-and Extracellular β-Galactosidases from *Bifidobacterium bifidum* and *B. infantis*: Molecular Cloning, Heterologous Expression, and Comparative Characterization," Applied and environmental microbiology, (2001), *67*(5), 2276-2283

P.S. Panesar, R. Panesar, R.S. Singh, J.F. Kennedy, H. Kumar, "Microbial production, immobilization and applications of beta-D-galactosidase," Journal of Chemical Technology and Biotechnology, (2006), 81, 530–43.

P.S. Panesar, S. Kumari, R. Panesar, "Potential applications of immobilized β-galactosidase in food processing industries," Enzyme Research, (2010), 2010, 1-16.

Q.Husain, "Beta galactosidases and their potential applications-a-review," Critical Reviews in Biotechnology, (2010), 30, 41–62.

R. Gupta, P.Gorgas, H. Mohapatra, V.K. Goswami VK, B. Chauhan, "Microbial α-amylases: a biotechnological perspective," Process Biochemistry, (2003), 38, 1599-1616.

R. Mattar, D.F. de Campos Mazo, F.J. Carrilho, "Lactose intolerance: diagnosis, genetic, and clinical factors," Clinical and Experimental Gastroenterology, (2012), *5*, 113-121.

R. Rashmi, K.R. Siddalingamurthy, “Optimization of β-galactosidase production by response surface methodology,” International Journal of Biosciences, (2011), 1, 119-127.

R. Singh, M. Kumar, A. Mittal, P.K. Mehta, “Microbial enzymes: industrial progress in 21st century,” 3 Biotech, (2016), 6(2), 174.

R. Tumas and A.L. Cardoso, “Como conceituar, diagnosticar e tratar a intolerância à lactose,” Revista Brasileira de Clínica e Terapêutica, (2008), 34(1), 13-20.

R.J. Bryant, D.R. Rao, S. Ogutu, “α and β-galactosidase activities and oligosaccharide content in peanuts,” Plant Foods for Human Nutrition (Formerly *Qualitas Plantarum),* (2003), 58(3), 213-223.

R.J. Bryant, D.R. Rao, S. Ogutu, “α and β-galactosidase activities and oligosaccharide content in peanuts,” Plant Foods for Human Nutrition (Formerly Qualitas Plantarum*),* (2008), *58*(3), 213-223.

R.S. Prakasham, C. Rao, R.S. Rao, P.N. Sarma, “Alkaline Protease Production by an Isolated *Bacillus circulans* under Solid-State Fermentation Using Agroindustrial Waste: Process Parameters Optimization,” Biotechnology progress, (2005). 21(5), 1380-1388.

R.Sen, D. Pal, V.P. Kodali, S. Das, S.K. Ghosh, “Molecular characterization and In Vitro Analyses of a Sporogenous Bacterium with Potential Probiotic Properties,” Probiotics and antimicrobial proteins, (2010), 2(1), 152–161.

R.Sen, K.S. Babu, “Modeling and optimization of the process conditions for biomass production and sporulation of a probiotic culture,” Process Biochemistry, 2005, 40(7), 2531–2538.

S. Am-aiam and C. Khanongnuch, “Medium optimization for βgalactosidase production by thermotolerant yeast.,” Chiang Mai Journal of Science , (2015), *42*, 480-489.

S. Am-aiam and C. Khanongnuch, “Medium optimization for βgalactosidase production by thermotolerant yeast,” Chiang Mai Journal of Science, (2015), *42*, 480-489.

S. Dagbagli and Y. Goksungur, “Optimization of b-galactosidase production using *Kluyveromyces lactis* NRRL Y-8279 by response surface methodology,” Electronic Journal of Biotechnology, (2008), 11(4), 11-12.

S. Dagbaglı and Y. Göksungur, “Optimization of β-Galactosidase production in stirred tank bioreactor using *Kluyveromyces lactis* NRRL Y-8279. Food Science and Biotechnology, (2009), *18*(6), 1342-1350.

S. Das, A. Bhattacharya, S. Haldar, A. Ganguly, Y.P. Sai Gu, P.K. Ting, “Optimization of enzymatic saccharification of water hyacinth biomass for bio-ethanol: comparison between artificial neural network and response surface methodology,” Sustainable Materials and Technologies, (2015), 3, 17–28.

S. Edupuganti, R. Potumarthi, T. Sathish, L.N. Mangamoori, “Role of feed forward neural networks coupled with genetic algorithm in capitalizing of intracellular alpha-Galactosidase production by *Acinetobacter* sp.,” BioMed Research International, (2014), 2014, 1- 8.

S. Kazemi, G. Khayati, M. Faezi-Ghasemi, β-galactosidase, “Production by *Aspergillus niger* ATCC 9142 using inexpensive substrates in solid-state fermentation: optimization by Orthogonal arrays design,” Iranian biomedical journal, (2016), 20(5), 287- 294.

S. Princely, N.S. Basha, J.J. Kirubakaran , M.D. Dhanaraju, “Biochemical characterization, partial purification, and production of an intracellular beta-galactosidase from *Streptococcus thermophilus* grown in whey,” European Journal of Experimental Biology, (2013), 3(2), 242-251.

S. Salminen, C. Bouley, M.C. Boutron-Ruault, J.H. Cummings, A. Franck, G.R. Gibson, E. Isolauri, M.C. Moreau, M. Roberfroid, I. Rowland, “Functional food science and gastrointestinal physiology and function,” British Journal of Nutrition, (1998), 80, 147– 171.

S. Sharma, P.Singh, “Isolation and characterization of galactosidase enzyme producing microbe and optimization of its enzyme activity under different culture condition,” International Journal of Current Microbiology and Applied Sciences, (2012), 3(7), 148 – 155.

S.A. Shaikh, J.M. Khire, M.I. Khan, “Production of a-galactosidase from theromphillic fungus *Rhizomucor* sp.,” Journal of Industrial Microbiology and Biotecnology, (1977), 17:239-245.

S.B. Matthews, J.P. Waud, A.G. Roberts, A.K. Campbell, “Systemic lactose intolerance: a new perspective on an old problem,” Postgraduate Medical Journal” (2005), 81, 167–173.

S.K. Akolkar, A.D. Sajgure, S.S. Lele, "β-Galactosidase from *Lactobacillus acidophilus* isolated from fermented ragi (*Eleusine coracan*)," Indian Journal of Biotechnology, (2006), 5(1), 184-188.

S.K. Shankar and V.H. Mulimani, "α-Galactosidase production by *Aspergillus oryzae* in solid-state fermentation," Bioresource technology, (2007), 98(4), 958-961.

S.L. Bahna, "Cow's milk allergy versus cow milk intolerance," Annals of Allergy Asthma and Immunology, (2002), 89(6), 56-60.

S.S. Houts, "Lactose Intolerance. Food Technol," (1988), 42(3), 110.

T. Goktug, M.K. Sezginturk, E. Dinckaya, "Glucose oxidase-β galactosidase hybrid biosensor based on glassy carbon electrode modified with mercury for lactose determination. Analytica Chimica **Acta**, (2005), 551, 51–56.

T. H. Vesa, P. Marteau, R. Korpela, "Lactose intolerance," Journal of the American College of Nutrition, (2000), 19(2), 165-175.

T. Haider and Q.Husain, "Preparation of lactose free milk by using ammonium sulphate fractionated proteins from almonds," Journal of the *Science* of *Food* and Agriculture, (2007), 87, 1278–1283.

T. He, M.G. Priebe, Y. Zhong, C. Huang, H.J. Harmsen, G.C. Raangs, J.M. Antoine, G.W. Welling, R.J. Vonk, "Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects," Journal of Applied Microbiology, (2008), 104(2), 595-604.

T. Vasiljevic, P. Jelen, "Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria," Innovative Food Science & Emerging Technologies, (2001), *2*(2), 75-85.

T. Vasiljevic, P. Jelen, "Lactose hydrolysis in milk as affected by neutralizers used for the preparation of crude β galactosidase extracts from *Lactobacillus bulgaricus,"* Innovative Food Science *and* Emerging Technologies, (2002), 3, 175–184.

T.H. Vesa, P. Marteau, R. Korpela, "Lactose intolerance," Journal of the American College of Nutrition, 2000, 19(sup2), 165S-175S.

T.W. Johannes & H. Zhao, "Directed evolution of enzymes and biosynthetic pathways," Current opinion in microbiology, (2006). 9(3), 261-267.

V.S. Patil, "*Bacillus subtilis*-A potential salt tolerant phosphate solubilizing bacterial agent," International Journal of Life Sciences Biotechnology and Pharma Research, (2014), 3(2), 141-145.

W. Chen, H. Chen, Y. Xia, J. Yang, J. Zhao, F. Tian, H. Zhang, "Immobilization of recombinant thermostable β-galactosidase from *Bacillus stearothermophilus* for lactose hydrolysis in milk," Journal of dairy science, (2009), *92*(2), 491-498.

W. Chen, H. Chen, Y. Xia, J. Zhao, F. Tian, H. Zhang, "Production, purification, and characterization of a potential thermostable galactosidase for milk lactose hydrolysis from *Bacillus stearothermophilus,"* Journal of Dairy Science, (2008), 91(5), 1751-8.

W. Sriphannam, K. Unban, H. Ashida, K. Yamamoto, C. Khanongnuch, "Medium component improvement for β-galactosidase production by a probiotic strain *Lactobacillus fermentum* CM33," African Journal of Biotechnology, (2012), 11, 11242-11251.

W.P. Hanage, C. Fraser, B.G. Spratt, "Sequences, sequence clusters and bacterial species," Philosophical Transactions of the Royal Society of London B: Biological Sciences, (2006), *361*(1475), 1917-1927

W.Y. Fung, Y.P. Woo, M.T. Lion, "Optimization of growth of Lactobacillus acidophilus FTCC 0291 and evaluation of growth characteristics in soy whey medium: a Response Surface Methodology approach," Journal of agricultural and food chemistry, (2008), *56*(17), 7910-7918.

Y. Nagao, T. Nakada, M. Imoto, T. Shimamoto, S. Sakai, M. Tsuda, T. Tsuchiya, "Purification and analysis of the structure of α-galactosidase from *Escherichiacoli*," Biochemical and biophysical research communications, (1988), 151(1), 236-241.

Z. Grosová, M. Rosenberg, M. Rebroš, M. Šipocz, B. Sedláčková "Entrapment of β-galactosidase in polyvinylalcohol hydrogel," *Biotechnology letters*, (2008), *30*(4), 763-767.

Z.M.M. Rosenberg, "Current trends of β-galactosidase application in food technology," Journal of Food and Nutrition Research, (2006), 45(2), 47-54.

APPENDIX-A

(Chemical composition for lactase assay)

- **2.5mM ONP:** To prepare 10 ml of 2.5mM ONP, 0.00347775 g of ONP was dissolved into 10 ml of phosphate buffer.
- **1M Na_2CO_3:** To prepare 1M Na_2CO_3, 10.6 grams of Na_2CO_3 was dissolved into 100ml of distilled water.
- **4mg/ml ONPG:** To prepare 4mg/ml of ONPG, 0.020 grams of ONPG was dissolved into 5 ml of phosphate buffer.
- One liter of 0.1 M sodium phosphate buffer, pH 7 can be made by mixing the 57.7 ml 1M Na_2HPO_4 and 42.3 ml of 1M NaH_2PO_4 and the final volume make up to one liter with distilled H_2O.

Formula for caluculation of lactase activity

Enzyme activity (U/ml) = a x DF/ ε x T x enzyme suspension

Where ‘a’ denotes the absorbance at 420 nm, ‘ε’ represents the molar extinction coefficient, ‘T’ represents the incubation time and ‘DF’ indicates dilution factor

APPENDIX-B

(Bacterial nutritional requirements)

Starch agar medium

Component	g/l
Beef Extract	3
Soluble starch	10
Agar-Agar	12

MR-VP broth medium

Component	g/l
Peptone	5
Dextrose or Glucose	5
di-potassium hydrogen phosphate (K_2HPO_4)	5

Simmons citrate agar medium

Component	g/l
Magnesium sulphate	0.2
Ammonium di hydrogen phosphate	1.0
Di potassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.080
Agar-Agar	15

Nitrate broth medium

Component	g/l
Peptone	5
Beef extract	3
Potassium nitrate	1

Phenol red broth medium

Component	g/l
Peptone	10
Beef extract	1
NaCl	5
Phenol red indicator	0.018

2 X Electrophoresis buffer or tank buffer

Chemical	Final Concentration	Amount
Tris base (MW 121.1)	250 Mm	30.4 g
Glycine	1.92 M	144.0 g
Milli - Q water	Dissolve & Make up To 1 lit	

10% Resolving gel (10 ml)

Chemical	Amount
Monomer	4 ml
1.5M tris pH 8.8	2.5 ml
Milli Qwater	3.3 ml
10% APS	0.1 ml
TEMED	0.004 ml

5% Stacking gel (5 ml)

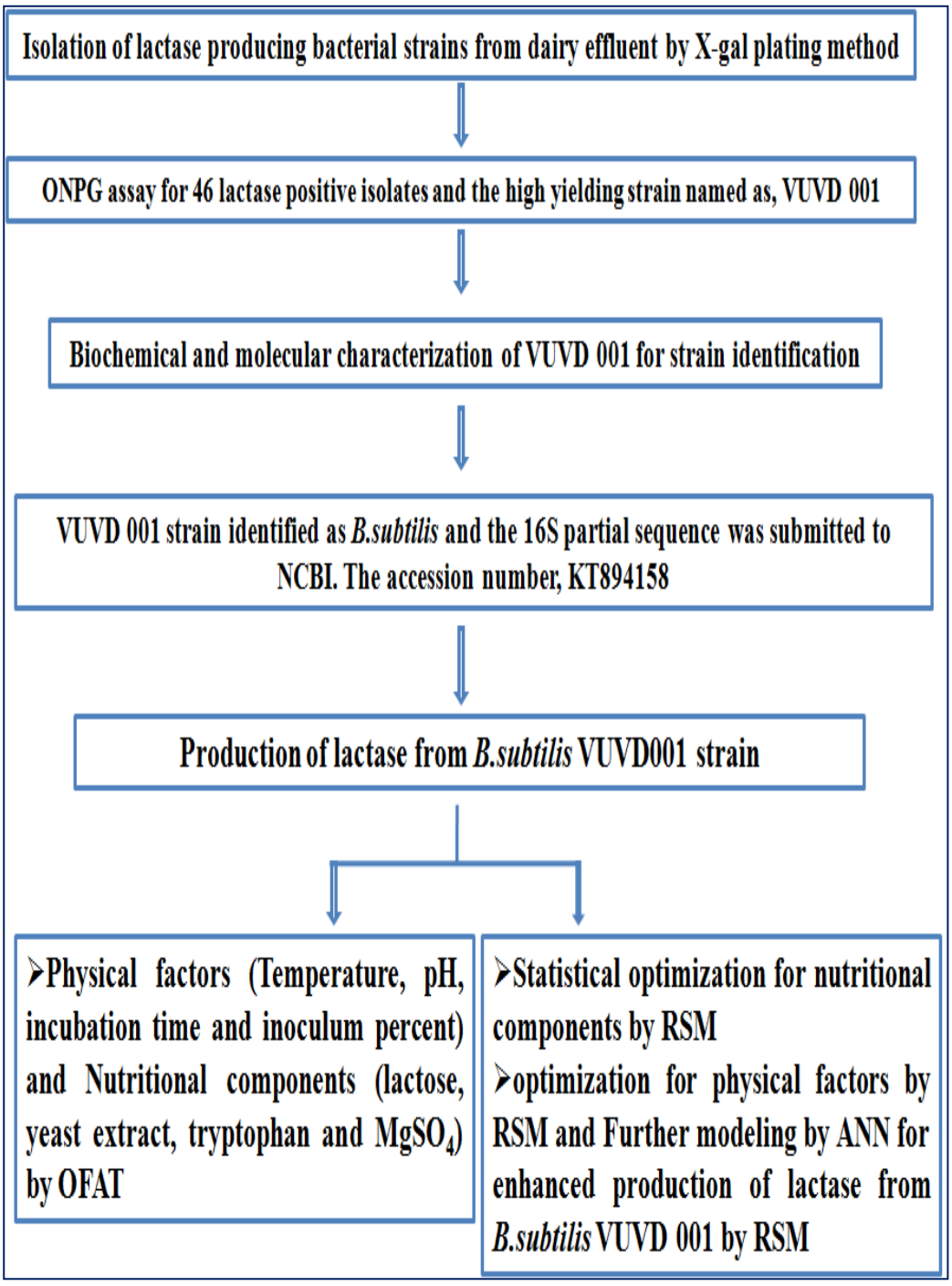
Chemical	Amount
Monomer	0.83 ml
1.5M tris pH 6.8	0.63 ml
Milli Qwater	3.4 ml
10% APS	0.005 ml
TEMED	0.005 ml

6X Sample loading buffer (100 ml)

Chemical	Amount
Tris HCl	5.91 g
100% glycerol	48 ml
Bromophenol blue	30 mg

APPENDIX-C

(Flow chart of the work)



LIST OF PUBLICATIONS FROM THESIS

- T.C.Venkateswarulu, K. Vidya Prabhakar, R.Bharath Kumar, S.Krupanidhi, Modeling and Optimization of Fermentation Variables for Enhanced Production of Lactase by Isolated *Bacillus subtilis strain*VUVD001 using Artificial Neural Networking and Response Surface Methodology, (2017), 3 BIOTECH (**Accepted**)
- T.C.Venkateswarulu, K. Vidya Prabhakar, R.Bharath Kumar Optimization of Nutritional Components of Medium by Response Surface Methodology for Enhanced Production of Lactase, (2017), 3 BIOTECH (**Accepted**)

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Publications

SCIE Indexed

1. **T.C.Venkateswarulu**, K. Vidya Prabhakar, R.Bharath Kumar, S.Krupanidhi, Modeling and Optimization of Fermentation Variables for Enhanced Production of Lactase by Isolated *Bacillus subtilis strain*VUVD001 using Artificial Neural Networking and Response Surface Methodology, (2017), 3 BIOTECH (**Accepted**)
2. **T.C.Venkateswarulu**, K. Vidya Prabhakar, R.Bharath Kumar Optimization of Nutritional Components of Medium by Response Surface Methodology for Enhanced Production of Lactase, (2017), 3 BIOTECH (**Accepted**)
3. R. R.Nair, N.T. Raveendran, V.R. Dirisala, M.B. Nandhini, T. Sethuraman, **T.C. Venkateswarulu**, G. Doss, Mutational pressure drives evolution of synonymous codon usage in genetically distinct Oenothera plastomes. Iranian Journal of Biotechnology, (20014), 12(4), 58-72.

Scopus Indexed

1. Karlapudi, Abraham Peele, Indira Mikkili, **T. C. Venkateswarulu**, D. John Babu, A. Ranganadha Reddy Bioconcrete Build Buildings with Quorum Sensing Molecules of Biofilm Bacteria. Journal of Pharmaceutical sciences and Research, (2016), 8, 10-12.
2. Y. Evangelin, **T. C. Venkateswarulu**, D. John Babu, K. Kasturi, Bacteriocins from Lactic Acid Bacteria and Its Potential Applications. Int. J. Pharm. Sci. Rev. Res, (2015), 32(1), 306-309.
3. M. Indira, **T.C. Venkateswarulu**, K. Chakravarthy, A. Ranganadha Reddy, K.Vidya Prabhakar, Isolation and Characterization of Bacteriocin Producing Lactic Acid Bacteria from Diary Effluent. Research J. Pharm. and Tech, (2015), 8(11), 1560-1565.
4. D. Sravani, K. Aarathi, N.S. Sampath Kumar, S. Krupanidhi, D. Vijaya Ramu, **T.C. Venkateswarlu**, In Vitro Anti- Inflammatory Activity of *Mangifera indica* and *Manilkara zapota* Leaf Extract. Research J. Pharm. and Tech, (2015), 10, 1-4.
5. A. Ranganadha Reddy, **T.C.Venkateswarulu,** M. Indira, A.V. Narayana, Identification of Membrane Drug Targets by Subtractive Genomic Approach In Mycoplasma Pneumonia. Research J. Pharm. and Tech, (2015), 8(9), 707 -714.

6. **T.C.Venkateswarulu**, B. Bodaiah, D. John Babu, A. Venkata Naraya, Y. Evangelin, Bioethanol Production by yeast fermentation Using Pomace Waste, Research J. Pharm. and Tech, (2015), 8(7), 841 – 844.
7. A.Ranganadha Reddy, **T.C. Venkateswarulu**, D. John Babu, M.Indira, Homology Modeling Studies of Human Genome Receptor Using Modeller, Swiss-Model Server and Esypred-3D Tools. Int. J. Pharm. Sci. Rev. Res, (2015), 30(1), 1-10.
8. **T.C.Venkateswarulu**, Kodali. V. Prabhakar, D. John Babu, R. Bharath Kumar, A.Ranganadha Reddy, M. Indira, A. Venkatanarayan, Screening Studies on Isozyme Pattern in all leaves of Dura Variety of Oil Palm (*Elaeis guineensis* Jacq.) for Selection of Leaf Index. Research J. Pharm. and Tech, (2015), 8(1), 69 – 73.
9. **TC Venkateswarulu**, Kodali V Prabhakar, D John Babu, Rahul R Nair, A Ranganadh Reddy, and A.Venkata Narayana. Studies on Electrophoretic Band Pattern of Isozymes in all Leaves of Pisifera Variety of Oil Palm (Elaeis guineensis Jacq). Research Journal of Pharmacy and Technology, (2014), 7(4), 415-418.
10. **T.C. Venkateswarulu**, C.V. Raviteja, Kodali.V. Prabhaker, D. JohnBabu, M. Indira, A.RanganadhaReddy, A.Venkatanarayana, A Review on Methods of Transesterification of Oils and Fats in Bio-diesel Formation. International Journal of Chemtech Research, (2014), 6(4), 2568-2576.
11. **T.C. Venkateswarulu**,A. RanganadhaReddy, D. JohnBabu.Rahul.R.Nair, M. Indira and A.Venkatanarayana, Comparative Studies of H5N1 Gene Segments with other Subtypes of Influenza- A Virus Research Journal of Pharmaceutical, Biological and Chemical Sciences, (2014), 5(3), 1417-1429.
12. K. Seetha Ram, **T. C. Venkateswarulu**, D. John Babu, Y. Sudheer, Seetharami Reddy, J. B. Peravali and K. K. Pulicherla," Cost Effective Media Optimization for the Enhanced Production of Hyaluronic Acid Using a Mutant Strain *Streptococcus zooepidemicus* 3523–7: A Statistical Approach" International Journal of Advanced Science and Technology, (2013), 60, 83-96.
13. J.B. Peravali, S.R. Kotra, S. K. Suleyman, **T.C. Venkateswarulu**, K.V. Rajesh, K. Sobha, K.K Pulicherla, Fermentative Production of Engineered Cationic Antimicrobial Peptide from Economically Feasible Bacterial Host *E. coli* GJ1158. International Journal of Bio-Science and Bio-Technology, (2013), 5(5), 211-222.
14. B. Sumalatha, A. Venkata Narayana, K. Kiran Kumar, D. John Babu and **T. C. Venkateswarulu**, Design and simulation of a plant producing dimethyl ether

(DME) from methanol by using simulation software ASPEN PLUS, Journal of Chemical and Pharmaceutical Research, (2015), 7(1), 897-901.

15. A. Ranganadha Reddy, **T.C. Venkateswarulu**, D. John Babu, N. Shyamala Devi Homology Modeling, Simulation and Docking Studies of Tau-Protein Kinase. Research Journal of Pharmacy and Technology, (2014), 7(3), 376-388.
16. Indira Mikkili, Abraham P Karlapudi, **T.C.Venkateswarulu,** D. John Babu, S.B. Nath, Vidya P. Kodali, Isolation, Screening and Extraction of Polyhydroxybutyrate (PHB) producing bacteria from Sewage sample. International Journal of PharmTech Research, (2014), 6(2), 850-857.
17. B Sumalatha, Y Prasanna Kumar, K Kiran Kumar, D John Babu, A Venkata Narayana, Maria Das, and **TC Venkateswarulu**, Removal of Indigo Carmine from Aqueous Solution by Using Activated Carbon. RJPBCS, (2014), 5(2), 12-22.
18. D. John Babu, B. Sumalatha, **T.C. Venkateswrulu**, K. Mariya Das and Vidya P. Kodali, Kinetic, Equilibrium and Thermodynamic Studies of Biosorption of Chromium (VI) from Aqueous Solutions using Azolla filiculoidus. JPAM, (2014), 1, 3107 – 3116.
19. K. Murali Krishna, **T.C. venkateswarulu**, K. Ravikanth reddy, Venkat R. konda, G. T. Rajesh T. srivalli, Bioprocess optimization and characterization ofrecombinant urate oxidase expressed in *Escherichia coli*. International Journal of Pharma and Bio Sciences, (2013), 4(4), 608 – 616.
20. Vidya P. Kodali., Karlapudi P. Abraham., J. Srinivas, **T.C.Venkateswarulu, M.** Indira, D. John Babu, T. Diwakar, K.L. Vineeth, Biosynthesis and potential applications of bacteriocins. Journal of Pure and Applied Microbiology, (2013), 4, 2933-2945.
21. J. B. Peravali, K. Seetha Ram, S. K. Suleyman, **T. C. Venkateswarlu**, KV Rajesh, K. Sobha and K. K. Pulicherla, Fermentative Production of Engineered Cationic Antimicrobial Peptide from Economically Feasible Bacterial Host *E. coli* GJ1158. International Journal of Bio-Science and Bio-Technology, (2013), 5(5), 211-222.
22. Karlapudi P. Abraham, J. Sriniva, **T.C. Venkateswarulu,** M**.** Indira, D. John Babu, T. Diwakar, Vidya P. Kodali, Investigation of the Potential Antibiofilm Activities of Plant Extracts. International Journal of Pharmacy and Pharmaceutical Sciences, (2012), 4, 282-285.

23. Devika.P, R.OletiSreedara, **T.C.Venkateswarulu**, N.D.Prasanna Computational understanding of selective MMP inhibitors in COPD. International journal of pharmaceutical science and research, (2012), 3(12), 4959-4975.

National Conferences

1. Presented a paper entitled Comparitive modeling, simulation and docking studies of TAU-Protien kinase in National Seminar entitled New Horizons In Biotechnology organized by KVR Womens Degree college Kurnool sponsored by UGC NewDelhi on 9th Feb to 10th Feb, 2015.
2. Presented a paper entitled Isolation, screening and Characterization of Lactase producing bacteria from Dairy Effluent in Indian Youth Science Congress organized by MSSRF, SRM & ANU from 19th Jan to 21st Jan, 2015.
3. Presented Molecular cloning, Expression, Purification and Characterization of recombinant urate oxidase at A.P. Science Congress-2012 from14th Nov to 16th Nov 2012, Jointly Organized by Andhra Pradesh Academi Sciences, Hyderabad and Dept. of Biotechnology, Achyarya Nagarjuna University, Guntur-522510 A.P. India.
4. Presented a paper on Isiolation, Screening and Purification of Laccases From a Novel Fungal Species in national conference on Technological Advancements In Biotechnology organized by Department of Biotechnology JNTU, Pulivendula on 7th Aug to 8th Aug, 2012.

International Conferences

1. Presented a paper on Cellulase Production through Submerged Fermentation from Newly Isolated Bacterium from Sewage Waste at global summit on Emerging Science and Technology: Impact on Envirnoment and Human Health Jointly organized by VS University, Nellore, India and UNT Health Science Center, Forthworth, Texas, USA.
2. Presented a paper on Studies on Electrophoresis pattern of Isozymes and Proteins in Different Leaves of Dura variety of oil palm (*Elaeis guineensis* jacq) presented at "International Conference on Chemical and Bioprocess Engineering " Organized by Schools of Chemical Engineering of NIT Warangal, Warangal on 16th &17th, November, 2013.

3. Presented a paper on Biosorption of Cromium from Industrial Waste Water Using Azolla Filicuoidus organized by Department of Biotechnology, VFSTR University, Vadlamudi on 23rd Aug to 24th Aug, 2013.
4. Presented a paper on Expression and Purification of recombinant therapeutic protein (PDGF-AA) international conference on Biotechnology held at Hyderabad on 02th May to 05th May, 2012 conducted by OMICS Publishing group.
5. Presented a paper on Extracellular Biosynthesis of Silver nano particles by using Bacterial Species in the International symposium on Role of Nanotechnology& Biomedical in Health Care at ANU Campus, Guntur on 19th Dec to 21th Dec, 2009.

Training Programs and Workshops attended

1. Attended the five days workshop held at Deparment of Biotechnology, NIT, Warangal on 2nd to 6th Nov, 2015 on Modeling, Simulation and Optimization of Bioprocesses.
2. Attented the three day work shop from 26th Nov to 28th Nov, 2015 on Biosignal Processing and Measurments and Computing Everywhere organized by Department of Electronics and Communication Engineering, VFSTR University, Vadlamudi.
3. Attended a three day Indo-US work shop from 26th Sep to 28th Sep, 2014 on Bacterial Antibiotic Resistance and Nanotechnologies organized by Department of Biotechnology, VS University, Nellore.
4. Attended a three day INDO-US Workshop on Bacterial Antibiotic Resistance and Nanotechnologies held during 26th Sep to 28th Sep, 2014, at Department of Biotechnology, Vikrama Simhapuri University, Nellore.
5. Attended the workshop held at Dept. of Biotechnology, NIT, Warangal on 8th Nov to 10th Nov, 2013 on Modeling, Simulation and Optimization of Bioprocesses.
6. Attended the workshop held at Department of Biotechnology, Indian Institute of Technology, Madras on 5th Jul to 9th Jul, 2011 on Summer Workshop on Bioreactors.
7. Attended the workshop held at Sri Venkateswara College of Engineering, Sriperumbudur on 27th Sep to 28th Sep, 2010 on Recent Trends in Instrumentation for Quality Control and Quality Assurance in Bioprocess Industries.
8. Attended 10 days training held at VFSTR University, Vadlamudi from 26th July to 5th Aug, 2010 on Faclty Induction Program.

9. Attended the Indo-US 5 days work shop from 5th July to 9th July, 2010 at VFSTR University, Vadlamudi on Chemical and Biochemical Process Control.

Membership in Professional bodies:

- Life member of the International Association of Engineers (IAENG) with membership number 136737
- Life member of Asia-Pacific Chemical, Biological& Environmental Engineering Society (APCBEES) membership number 201729
- Life member of Society for Biotechnologists(India) with membership number 770

Personal Profile

Name	**:** T.C.Venkateswarulu
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Address	Erraguduru (P) Pamulapadu (M) Kurnool (D) Andhra Pradesh, PIN-518442
Languages Known	**:** English and Telugu

Declaration

I hereby confirm that the above information given here are true to the best of my knowledge and belief.

T.C.Venkateswarulu