VALORIZATION OF INDUSTRIAL WASTE (WHEY) MICROBIAL MANUPULATION FOR INDUSTRILIZATION.

M. Sc. Thesis

2022

For Partial fulfillment of the requirement for the Master of Science in Biotechnology

Submitted to

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by

Devraj Maidali

Roll no: BT 504/074

T.U.Regd No: 5-3-28-296-2017

Supervisor

Dr. Pramod Aryal

Senior Scientist

Central Department of Biotechnology

Tribhuvan University, Kirtipur Kathmandu, Nepal

ACKNOWLEDGEMENT

I am indebted to my supervisor, Dr. Pramod Aryal, Senior scientist, Central Department of Biotechnology, for your patience, guidance and support. Your unassuming approach to research and science is a source of inspiration. I am extremely grateful that you took me on as a student and continued to have faith in me over the entire work in waste management and farmer's payback research work.

Thank you to Prof. Dr. Krishna Das Manandhar Sir, Head of Department, Central Department of Biotechnology, Tribhuwan University, for all of the kind words and assistance you have provided.

I am fortunate to have been a part of the Alpha Agro research team. I acknowledge this Pvt. Ltd. for the generous financial support and the space for my research works and the team members for their kind help.

I would like to express my gratitude to the entire Central Department of Biotechnology family. Also, I extend my thankfulness to all my seniors; friends and juniors, who directly and indirectly provided me inspirations and valuable suggestion during the course of this study.

Heartful thanks to my parents for providing me endless support throughout all these years and through the process of research works. I strongly acknowledge them for giving me the strength and unconditional faith on me. Thank you for all of your love and supports .

Finally, distinguished personalities, the locals and the dairy stakeholders contacted and the source of information revealed are also thankful acknowledged.

Devraj Maidali

ACRONYMS

BOD	Biological Oxygen Demands
DO	Dissolved Oxygen
CFU	Colony Forming Unit
RT	Real Time
RH	Relative Humidity
LAB	Lactic Acid Bacteria
UN	United Nations
FAS-USDA	Foreign Agriculture service- U.S. Department of Agriculture
OECD-FAO	Organization for Economic Co-operation Development- Food and Agricultural Organization
WHO	World Health Organization
GDP	Gross Domestic Products
DFTQC	Department of Food Technology and Quality Control
NDDB	National Dairy Development Board
DNA	De-oxy ribonucleic Acid
ATP	Adenosine 5-Triphosphate
RO	Reverse Osmosis
EDTA	Ethylene di-amine tetra-acetic acid
KMS	Potassium meta bi- sulfate
OD	Optical Density
LB	Luria burtani miller
SDA	Sabouraud Dextrose agar
BSA	Bovine serum albumin
HPLC	High performance liquid chromatography
PPM	Part per million

LIST OF TABLES

Appendix

LIST OF TABLES

Appendix

TABLE OF CONTENTS

ABSTRACT

The looming food insecurity and changing climate demand the complete utilization of nutrient-rich residues from food industries as value added products. Agro-industrial by-products and residues can be transformed into valuable compounds in bio-refineries. Whey, the liquid remaining after the separation of milk fat and casein from whole milk, is a major problem as organic pollutant for the environmental natural quality, due to its high BOD which demands simple and economical solutions for valorization. Abundant in milk and other dairy products, lactose sugar is considered to have an important role in oral microbial ecology and can contribute to caries development in both adults and young children. Further, the growth and survival of probiotic bacteria in whey media and the influence of yeast addition on it for complete sugar conversion, for possible production of a nutritive highly valuable whey drink should be a good achievement. With all these possibilities and concept of transforming agro-industrial by-products and residues into valuable compounds, here we present a new concept in which production of bio-ethanol by direct bio-conversion of whey with *Saccharomyces cerevisiae* and then whey based fermented probiotic beverage from the co-culture of *Lactobacillus, Bacillus megaterium* and *Bacillus subtilis* using single batch fermentation technology. Round 32±0.9Kcal total calorific value was found in whey samples, fermentable sugar was around 5%. About 75% lactose was utilized by organisms, thereby reducing BOD of whey by 80% at the end. 0.9% (v/v) ethanol was produced in whey wine fermentation by co-culture, was vacuum distilled as a result whey based fermented probiotic beverage was prepared with zero ethanol. After 30 d of RT and RH conditions the organisms count was more than 10^7 Cfu/ml in the whey wine with no internal spoilage & cross-contamination.

Keywords: Whey; Yeast; Fermentation; Probiotics; Vacuum distillation; BOD.

CHAPTER 1
INTRODUCTION

1.1 Background

Cash income is one of the motivational factors among the entrepreneurs and frequent turnover of the cash is motivating acceleration apparatus. Milk is one of the livestock products that generate cash twice a day and transfer that cash from richer to poor. Due to awareness on the importance of health among the people, milk consumption trend is increasing day by day. Unprocessed liquid milk, which is practically not traded as most part of it is being water makes costly in transportation and also due to high chance of contamination and spoil. Moreover, lactose intolerance, purpose of reducing fat content also encourages dairy industries to convert raw milk into different products which have concentrated nutrition and long shelf-life.

Cheese, paneer, cheena are the most common milk-based solid dairy product, which features a variety of flavors and are produced all over the world by coagulating milk with the help of calf rennet or suitable coagulating agents (microbial or vegetable rennet, vinegar and lemon) and draining the whey liquid all over (Hickey, 2017). These milk based products comparatively acquired more health aspects and more liked by lactose intolerant population. Cheese is rich in most of the nutrients required for the human body including proteins, bioactive peptides, amino acids, fat, fatty acids, vitamins, and minerals. Some of the peptides in cheese are known as cardio-protective nutrients that also include blood pressure–lowering minerals such as calcium (van Mierlo *et al.,* 2006). In addition, it has been stated that the growth and development of the human body may be supported by the high concentration of essential amino acids found in most varieties of cheeses (Walther *et al.,* 2008). Fermented milk product including whey market is expanding yearly. In 2020, more than 16 million metric tonnes cheese was produced worldwide (Khanal *et al.,* 2019). This means 9 × 16 million metric tonnes whey was produced in that year as theoretically, for the production of each kilogram cheese 9-kilogram whey liquid was produced as residue (Pendon *et al.,* 2021).

This ocean of whey is major problem for the researchers to work upon. It nutritional value challenges us to reutilize the complete energy driven from the milk. Whey represents 85–

95% of the milk volume and almost 55% of total milk nutrition including about 5% lactose (Zotta et al., 2019). The very substantial polluting power of whey, with a BOD some 175-fold higher than typical sewage effluent (Smithers, G. W. 2008), has in the recent past prompted governments and other regulatory local communities to pressurized industries for disposing untreated whey notably in regional areas, has also forced dairy factories to reconsider how they manage the large and growing volumes of whey generated. Application of modern scientific disciplines, approaches and techniques has helped advance a greater understanding at molecular level of the chemical, physical, biological, and nutritional characteristics of whey components. This understanding has extended to an enhanced knowledge of the interactions between whey components, and the influence of these interactions on component functionality.

Lactose is a disaccharide consisting of the combination of two simple sugars, α/β-D-glucose and β-D-galactose, linked by a β (1–4) glycosidic bond and is the main carbon source in milk (Costa *et al.,* 2013). Utilization of lactose is mainly related to the fermentation processes by those micro-organisms which have genetics needed for lactose breakdown into its monomers and then subsequent production of ethanol or other value added compounds.

Saccharomyces cerevisiae is organism of choice and has long been used for fermentation propose industrially and in most of the fermentation related research projects due to its high yield, being safe for health and high ethanol tolerant capacity (Parapouli *et al.,* 2020). Though this organism is the main working horse for the ethanol production from various kinds of sugar substrate, it is unable to ferment lactose due to absent of lactose permease system (Russel, 1986). Nevertheless, due to probiotic nature (Pendón *et al.,* 2021) and having galactose utilizing genetics (Bro *et al.,* 2005) *Saccharomyces* becomes model organisms for the lactose fermented in food industries.

The LAB (Lactic Acid Bacteria) is heterogeneous group of organisms which ferment various carbohydrates predominantly into lactic acid. These groups of organisms have wide range of natural habitat including plants, meat and milk environment. They are involved in a large number of industrial food fermentations knowingly or unknowingly. Their primary contribution is in rapid acid production and acidification of foods which helps to preserve food products, but metabolic processes accompanying the growth of LAB impact also flavor, nutrition, and texture quality of a variety of fermented foods (Axelsson, L. (2004). *Bacillus megaterium, Bacillus subtilis* and *Lactobacillus spp.* having the good lactose metabolic

capacity as they produce β-galactosidase (M. J. Hill, 1983). Also these organisms show good probiotic nature and can improve gut health of the host there by regulating intestinal functions, colonic micro flora and boost immunity (Percival *et al.,* 2019). Probiotic bacteria in fermented products improve lactose digestion due to microbial β-galactosidase.

1.2 Current studies

According to the recent United Nations report, the global population is expected to rise by 2 billion people in the next three decades, from 7.7 billion currently to 9.7 billion in 2050 (UN 2019). To date, the fulfillment of these populations demand is largely reliant on petroleum-based entities. The intrinsic concerns related with fossil-based resources such as environmental, geopolitical, socio-economical, sustainability etc. eventually force to opt alternative resources. For instance, approximately 85% of the total global energy is derived from fossil fuels (Singh & Dwevedi 2019). Based on the latest World Bank's report, if immediate measures are not taken, global waste will increase by 70% from the current levels in 2050 (Silpa *et al.,* 2018). Our environment is therefore ubiquitously suffering huge burdens in one hand from the fossil fuels and, on the other hand, from various wastes. The notion to transform waste biomass into value-added products has got intriguing advantages as it directly and indirectly combats environmental problems, opens new avenue of revenues, and helps to reduce dependency to petroleum-derived products. Towards the end of 20th century to the present technology were involved in dairy industries to tackles the quality assurance of products along with better residue management. Complete characterization showed that the whey might work as a very useful cheap raw material and is not a threat any more. Science, technology and engineering have transformed the whey industry in the recent time. The modern whey and whey products industry has much to be proud of, but challenges loom and more opportunities abound. Full utilization of whey even with new technology has not been achieved. Industry has been slow to adopt whey processing schemes even though processes like ultrafiltration have been in commercial use since 1972 (Zall 1984). The research for complete utilization of whey is coming from long past history. During which different technologies were developed like ultrafiltration, membrane technology, reverse osmosis etc. which separate protein portion of the whey concentrate and commercialized. These techniques result often same amount of whey permeate as the lactose (the major contributor to the BOD of whey) portion remains unused and is often subjected to costly waste treatment processes (Terrell *et al.,* 1984). The

yeast *Kluyveromyces sps.* has been used, by several researchers in several countries, for production of yeast extract, Single cell proteins, ethanol production using whey as substrate (A.E. Ghaly & M.A. Kamal, 2004).

1.3 Objectives

The general objective of this study was to prepare ethanol free whey based Probiotic drinks by fermentation technology using whey as substrate.

The specific objectives were:

- Nutritional Characterization of whey.
- Production of mead from honey by fermentation (for fermentation & distillation optimization).
- Production of whey wine using co-culture of yeast and bacteria from whey as substrate.
- Optimization of ethanol distillation technology.
- Validation of probiotic strains and their stability in whey medium.

1.4 Scope

It is obviously not easy to shift from the well-established fossil-based economy to the emerging bio-economy. The underlining challenges include, but not limited to, decentralization of waste supply chain, technological barrier, dearth of large-scale production, and high initial investment. Development of effective and optimum waste biomass monitoring, logistic, pretreatment, and valorization techniques should be the central endeavor to overcome the existing encounters. Besides, the splendid research works we have witnessed so far in valuable utilization should move forward to large-scale operations. Implementing industrial–academic partnerships is one way to scale-up research findings for commercialization. Therefore, devoted collaboration from various sectors is crucial. Very recently, Liu *et al.* proposed cascade utilization as the efficient strategy to valorize waste according to its composition, characteristics, and nature (Liu *et al.,* 2019). Moreover, employing the concept of bio-refinery should be the vital part of bio-economy. Integrated bio-refinery model couples the production of low-value, high-volume commodities to the simultaneous production of low-volume, high-value products. The general concepts of petroleum- and bio-refineries are more or less similar, though the

former is well-established. Therefore, taking lessons from this and exploring the opportunities and advancements in biotechnology and other interdisciplinary fields, the quest towards green and sustainable economy will be realized sooner or later. The significance of our research is to establish the proof of concept that a bio-refinery for fuel ethanol production by using one of the cheapest substrate available and by developing energy saving distillation mechanisms for the production of viable probiotic drinks, conferring an added value to the process and providing an alternative to reduce environmental impact.

CHAPTER 2

LITERATURE REVIEW

2.1 Milk

Milk is a nutrient-rich liquid food produced by the mammary glands of mammals which has white color, mild taste and flavor. It contains all the required nutrition that need to sustain life specially, is a solo source of energy for young mammals including humans for the first few months after birth. However, it becomes the vital component of all age groups daily diet these days. It is consumed as fresh as well as in the form of various derived products such as yogurt, butter, ice-cream and cheese. It plays an incredible role in improvement of health of the consumers, along with the enhancement in the economic development of rural areas by improving their livelihoods by bringing the capital back from the city areas (Rasheed *et. al.,* 2016).

2.1.1 Composition of Milk

Table 1 Composition of Milk (Data compiled from Jost R., 2000).

Main constituents g/100g whole milk	Human	Cow	Buffalo	Goat
Water	87	86	82	88
Protein	1.1	3.3	4.0	3.7
Fat	4.0	3.8	8.0	3.9
Carbohydrates	7.1	4.8	5.1	4.8
Organic Acids	-	0.2	-	-
Minerals	0.2	0.7	0.8	0.8
Total energy (KJ/100g Milk)	290	280	450	290

Milk obtained from various species shows variations in term of composition and nutritional values. More than 10^5 different kinds of molecules including lactose proteins make colloidal system suspended in water (Jenness *et. al.,* 1988). Detail composition of milk components is summarized in table 1.

2.1.1.1 Lipid (Fat)

In milk lipids are mainly triglycerides, with much smaller amounts of free fatty acids, mono- and di-glycerides, phospholipids, sterols, and hydrocarbons. In general, milk fat consists of 65% saturated, 32% mono-unsaturated and 3% poly-unsaturated fatty acids with varying quantities of phospholipids, sterols, carotenoids, vitamins A, D, E and K (Creamer *et al.,* 1996).

2.1.1.2 Carbohydrate (Lactose)

Lactose is the main milk sugar with around 5 to 6% depending upon the source of animals and feeds (Costa et al. 2013). Besides lactose milk also contains monosaccharides, neutral and acid oligosaccharides, and glycosyl groups which are bound to proteins and lipids (Janness *et. al.,* 1988).

2.1.1.3 Protein

Casein and whey protein make milk protein in 4:1 ratio. Casein, a typical milk protein exists as casein micelles which are made up of calcium phosphate, are precipitate at pH 4.5 and are heat stable. In contrast to the casein, Whey proteins is water soluble protein consist of β - lactoglobulin and α - lactalbumin, bovine, serum albumin, immuno-globulins (mainly IgG1, IgG2 and IgM), lactoferrin, serum transferring, glycoprotein and enzymes. β – lactoglobulin (Creamer *et al.,* 1996).

2.1.1.4 Minerals

The mineral make-up of milk is crucial for stability of the physio- chemical equilibrium of milk. Although their concentration is less than 1 percent in milk, minerals significantly affect the technological behavior of milk. Detail composition of major minerals present in the milk is given in table 2.

Table 2 Composition of minerals in milk (Data taken from Rodriguez *et al.,* 2001).

Element	Concentration (mg/L)
Iron (Fe)	0.155
Cupper (Cu)	0.023
Zinc (Zn)	1.322
Calcium (Ca)	496
Magnesium (Mg)	34.2
Sodium (Na)	160
Potassium (K)	427

2.1.2 Milk production Worldwide

Naturally, thousands of mammalian diversities are present throughout the globe according to the natural habitats, only few are reared by farmers for milk production. Animal like buffalo, goat, camel, cow is the most abundant, easy to farm with high productive, economical that makes them trustable mammal farming for milk purpose. Among all farming mammals, cattle shared almost 80% of total milk production alone (Faye & Konuspayeva, 2012). In world market India is the leading nation for milk production whereas China is the world's largest importer of dairy products followed by New Zealand, UN and USA (FAS-USDA).

Table 3 worldwide milk production (From FAS-USDA, 2021).

Year	2019	2020	2021
Milk ('000 tonns)	379.883	388.546	323.498

This table shows the trend of milk production. World milk production is projected to grow at 1.6% p.a. over to 997 Mt by 2029, faster than most other main agricultural commodities (OECD-FAO Agricultural Outlook, 2018).

Figure 1 The trend of milk production and consumption in recent years and prediction for the future (OECD-FAO Agricultural Outlook 2020-2029)

2.1.3 Milk production in Nepal

Nepal covers a very small (around 0.03%) area of the globe with geographically less land available for farming. Though it adds almost 0.247% of total world's documented milk as around 2.05 metric tonnes milk was produced annually in Nepal (Shingh *et al.,* 2020). Milk available in Nepal is far below around 158.9 gm/day than that recommended value around 250 grams per day per person by WHO. With the constant population growth rate of 1.4% per annum at present, the annual milk production has to be increased at least by 9% from 3.4% at present to meet that requirement by the year 2020 (COMP-NDDB, 2017). Nepal being landlocked nation with most of the people here depends upon the agriculture and livestock. Nepal's Major National GDP (almost one forth, 28%) covered by the Agriculture and Livestock in which dairy sector alone covers more than 8% of the National GDP. Increasing rate in dairy sector benefited Nepalese with the chance of getting employment within their own locality and enhancing thousands of rural families' socioeconomic conditions by cash flow from urban to rural (Neupane *et al.,* 2018).

Table 4 Milk production in Nepal (From report of CASA-Nepal 2020).

Milking animals	Number of Animals	%	Milk production (metric tonnes)	% of Total milk Production
Cattle	1,026,135	43	643,807	35
Buffalo	1,355,384	57	1,210,422	65
Total	2,381,519	100	1,854,249	100

Most of the milk produced in the farm was consumed at the farmer level as diets; however, in recent time commercially processing of milk started and around 131 private dairies was registered in different district of Nepal (Timsina *et al.,* 2009).

2.1.4 Milk vs. Milk products

People these days preferred varieties in food. Though major portion of milk is consumed as fresh within the local areas after slightly pasteurized or fermented, delicious fresh products like yoghurt, desserts and custards as well preservative and distant market targeted products such as cheese, butter, and milk powders are well commercialized (Fox *et al*., 2017). To fulfill the international demands of the milk's nutrition different products are derived from milk and then traded. Reports suggest that only around 8% of total raw milk produced is transported without processing (OECD-FAO AGRICULTURAL OUTLOOK 2020-2029). Unprocessed liquid milk, which is practically not traded because most part of it is being water makes costly in transportation and due to high available nutrition chance of contamination and spoil is likely. Moreover, lactose intolerance, purpose of reducing fat content also encourages dairy industries to convert raw milk into different reliable and concentrate products. Statistically about 30% of raw milk is converted into milk products like cheese (second most consumed dairy product) butter, Skimmed milk powder (SMP), whole milk powder (WMP) with many more regional products (OECD/FAO 2020).

2.2 Cheese

2.2.1 Introduction

Naturally milk is unstable bulky items which are converted into useful nutritionally concentrated form. When raw milk is treated with some organic acids its protein portion specifically casein separates and cheese is formed, so the basic of cheese making is curdling of the milk and separation of the curds from the watery whey. It defines the ranges of dairy products which are either fermented by the microbial activities or by specific enzymes. Cheese is very versatile dairy products having diversity on forms, verities, textures and flavors (Rashidinejad *et al.,* 2017). To covers the overall varieties, it has been said that "there is a cheese for every taste preference and a taste preference for every cheese" (Olson, 1990). More than 2000 varieties of cheese were documented but the most famous are Cheddar, brick, cottage, cream cheese Mozzarella, ricotta etc. (Rasheed *et al.,* 2016).

Table 5 Composition of cheese (gm/100gm) (Data compiled from Rashidinejad *et al.,* 2017).

Type of cheese	Water	Fat	Protein	Carbohydrates	Cholesterols	Energy(kcal)
Cottage cheese	79.1	3.9	13.8	2.1	13	98
Mozzarella	49.8	21	25.1	Trace	65	289
cheddar	36	34.4	25.5	0.1	100	412
Ricotta	72.1	11	9.4	2	50	144

2.2.2 History of Cheese Making

History of cheese making suggested that it was a traditional practice of art. Cheese was believed to be evolved from Tigris and Euphrates rivers (current Iraq) 8,000 years ago when people started to domesticate plants and animals as a source of food whereas earliest cheese was made in Central Asia by Nomadic tribes (Fox, 2011). In these days' vast study and researches are going on to make cheese industry reliable with better value and quality. In Nepal Cheese is the pioneer product of dairy industries back to 1953 by Yak Cheese

factory located in Rasuwa which was helped by FAO (FAO). As the milk production increasing, the overall production of cheese will also estimate to increase. In Nepal, there are 15 small and medium scale cheese industries so far registered in the Department of Food Technology and Quality Control (DFTQC) and other offices under this department (DFTQC, 2020).

2.2.3 Why cheese??

2.2.3.1 Nutritional value

With increase in the scientific study and practices, evolution of technology and reduced land availability, dairy becomes most reliable source of energy to sustain world demand. Along with raw milk, Cheese plays major role to carry and distribute nutrition all over the globe. Almost 35 % of milk is converted into cheese industrially due to which about 19×10^6 tonnes per annum cheese produced worldwide and its production is still increasing (Fox *et al*., 2017). The manufacture of cheese is a form of milk preservation by which highly perishable and bulky raw milk is converted into a stable form cheese with concentrated nutrients, minerals such as calcium, phosphorus, long shelf life and having range of varieties (Bassette *et al.,* 1988). When compare with raw milk, cheese contains almost 30-40% protein and fat (almost 10 times more) with abundant fat-soluble vitamins (Khanal *et al.,* 2019) which make most famous and vitals milk products for energy distribution from rural to the urban areas where less farming is practiced.

2.2.3.2 To tackle milk holidays

Cheese manufacturing could be an attractive venture to overcome the milk holiday (days when milk is not procured from farmers) in the surplus season. Nepal was witnessed a milk holiday in past as dairies stopped buying milk from farmers amid a supply glut. Farmers were distressed with dairies declining to purchase their products. For example, in 2015 when most part of the Nepal was devasted by earthquake, National Dairy Development Board (NDDB) issued milk holiday by saying more than 1,200 tonnes of powdered milk and 1,400 tonnes of butter remains unsold. But the powdered making is not the solution as it has to be converted into liquid form, otherwise that would perish soon (Kathmandu post, 2015). Recently, in Ilam, Dairy Development Project, Biratnagar apply holiday in district on three days a week citing the milk products it has been producing were not getting the market along with insufficient chilling center and due to the transportation problem as continuous shutdown and global pandemic (RSS, Nepali headlines, 2020). So, to reduce the

loss to the farmer and uplift farmers' economy by providing an alternate solution to tackle milk holiday cheese manufacturing is the best and genuine option.

2.2.3.3 To resolve lactose intolerance

Dairy industries are also hit badly by lactose intolerance in the population due to the inability to synthesize lactase. Only 35% of the human population can digest lactose beyond the age of about seven or eight (Leonardi *et al.,* 2012). Most of the type of cheese are free from lactose as most of the lactose eluted along with whey and remaining portion (if any) is fermented by Lactic acid bacteria while ripening (Rasheed *et al.,* 2016). Cheese is also very important in various medical complications. The nutrients present in ripened cheese varieties preventing formation of acid like substances on teeth periphery along with the saliva stimulated during cheese consumption help to reduce dental problems (Akuzawa *et al.,* 2009).

2.3 Whey

2.3.1 Introduction

Whey, watery portion of the milk is the main by-product of dairy industry especially in cheese industries. Chhena and paneer are other source for whey as these dairy products are famous parts of diet in Asian countries including Nepal, India. This by-product represents about 85-95% of the milk volume and retains almost 55% of milk nutrients. It is the serum phase which contains mostly soluble components, including lactose, soluble salts, globular proteins and trace of fats, vitamins. It represents almost 6% of total solid present in raw milk of which more than 70% is lactose alone (Zall, 1984 & Siso, 1996).

2.3.2 Source and types

Conventionally, two main types of whey; acid whey and sweet whey are classify based on its processing conditions used for casein precipitation, were found to be studied most (Rocha-Mendoza *et al.,* 2020). Acid whey result from the soft and fresh cheese type dairy product such as cottage cheese, ricotta cheese in which organic acids were used as coagulates having pH less than 4.0 where as sweet whey produced as by-product of ripened hard cheese formed by using enzymatic activity with around 4.5 pH (Guimarães *et al.,* 2010).

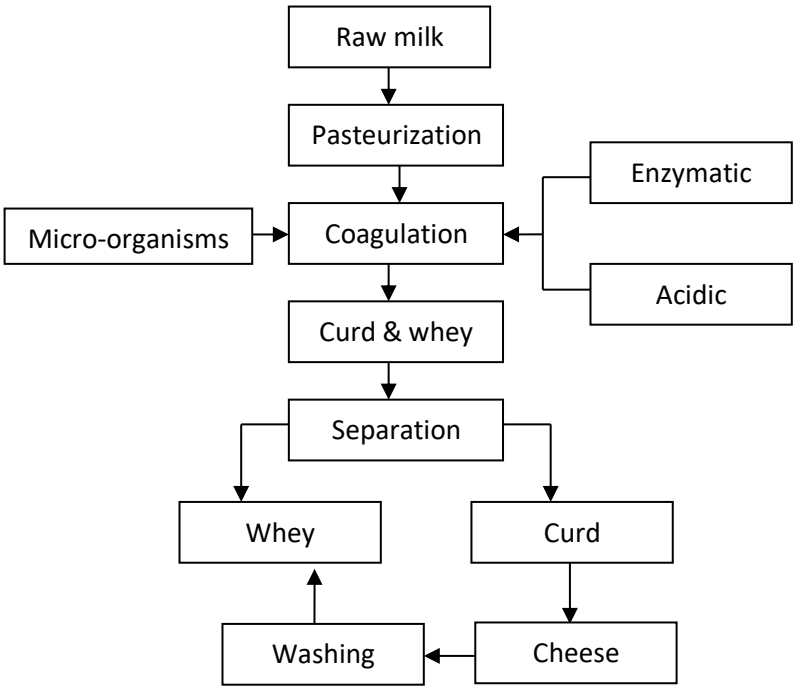

Figure 2 Schematic showing method of whey production

2.3.3 Composition

The chemical composition of whey is dependent upon chemical nature and quality of the milk, which varies with stage of lactation, feeding, breeding, individual animal differences, and climate. It also depends upon the milk processing methods. The main components of both sweet and acid whey, besides water, are lactose about 5%, whey proteins and minerals. The main differences between the two whey types are in the mineral content, acidity and composition of the whey protein fraction (Carvalho *et al.,* 2013).

Table 6 Details composition of whey components (Data compile from Casper, 1998 & Smithers, 2015).

Components	Sweet whey (%)	Acid whey (%)	Milk (%)
Agent for coagulation	Enzymes (Rennet)	Organic acids	-
Water	93-94	94-95	88-89
Total solid	6-6.5	5-6	12.8
Lactose	4.5-5	3.8-4.3	4.9
Fat	1.07	0.45	3.7
Total protein	0.8-1.0	0.8-1.0	3.5
Whey protein	0.6-0.65	0.6-0.65	0.7
Minerals	0.5-0.7	0.5-0.7	0.7
pH	6.2-6.4	4.6-5.0	6.5- 6.8
Acidity	0.1-0.2	>0.40	-
BOD (mg O_2 L^{-1})	30,000	35,000	140,000
COD (mg O_2 L^{-1})	60,000	80,000	218,000
Energy (KJ L^{-1})	1100	1100	1710

2.3.4 History of whey used

By definition whey is the byproduct of cheese industries, its history come along with curdling of the raw milk. Literature suggests that around 5,500 BC farmers unknowingly noticed the separation of liquid itself from curds during preservation of goat's milk (Fox,

2011). Since that time human linked with this by products. In the Middle Ages whey was applied as a pharmaceutical drug and as bathing alternate to water in the believe to cure skin problems (Hoffmann, 1961). In past with experience and believe whey was used as drinks and tropical uses in small scales. Evidences supports that Hippocrates, the father of modern medicine, treat and recommend to uses whey for patients with stomach and skin problems in around in 460 BC although the mechanism of action was unknown at the time (Siso, 1996). In middle of 17th century Whey houses where famous where peoples can enjoy whey drinks (Rail *et al.,* 1994). Bathing in sweet whey also became popular in the 19th century, health spas, based on its presumed skin healing and topical health-promoting properties, and the fact that it was far more economical than bathing in milk (Papademas & Kotsaki, 2019). Notably at Later on in 20th century, Italians popularize the methods for separating liquid whey from dairy in large volume which necessitates studying their nutritional values (Smithers 2015). There after dairy based product's demand increased worldwide resulting in an expansion in cheese production, and thereby an increase in whey volumes.

At this stage in history, whey became a threat and is considered nothing more than a potent bio-waste and a challenge that hinders the way of an even greater expansion in cheese manufacture (Smithers, 2008). In the recent time government from major nations apply number of polices and taking oath to treat the whey properly before disposing in nature. Reports suggested that the different agencies and organizations are involving actively to solve these problems (Smithers 2015).

2.3.5 Disposal of whey

The various means of whey disposal have been described in many reviews can be grouped into three broad categories;

2.3.5.1 Direct disposal

This is the most ancient whey disposal method in which whey is taken as an animal feed, so used whole or de-proteinated whey streams given as ingredients in foods or beverages without any processing, spray irrigation to land, and disposal to natural waterways or municipal sewer systems.

2.3.5.2 Direct stabilization (Application of technology)

In which the whey is treated by physical and/or chemical means to render it considerably more stable to microbial degradation. Techniques used include: protein recovery by ultrafiltration or heat denaturation, concentration by reverse osmosis and/or evaporation, crystallization of lactose, and drying.

2.3.5.3 Conversion processes (Biological treatments)

This is more controlled and most practiced method of whey management in which whey lactose is convened to another value added compound by microbial metabolisms (Mawson, 1994). Production of yeast biomass which is amply used by food and feed industries would be another possible use of whey; however, this is not a very interesting possibility from a commercial point of view, since the value of yeast is low because of its abundant supply from other sources, particularly breweries (Adam *et al.,* 2005).

2.3.6 Problem vs. opportunity

2.3.6.1 Problem

Ceaseless growth in human population led to high demand in everything. Currently, the world largely depends on petroleum-based scheme. On the other hand, depletion of fossil-based resources and their huge impact on environmental pollution have forced us to search for sustainable and eco-friendly alternative resources. If whey is dumped down the drain, a practice still common with many dairy processors, it constitutes the most potent of all dairy wastes. So, this byproduct demands effective and economical technology from researcher to make cheese industries competent with the other dairy products and does not prevent the industry from meeting the market demand for its products. Drying of whey is also considered by many cheese processors. This practice consumes considerable amount of already depleting energy resources mainly to remove water content. Spray dry whey to make powder of whey is a very good alternative to solve the puzzle but low pH makes difficult to dry as lactose present is converted into crystal form. Likewise, presence of minerals mostly calcium, phosphate damages the equipment (Rocha-Mendoza *et al.,* 2020). Farmers also used whey in low amount in their own diets adding in foods such as ice creams, cakes etc. Its excessive saline taste due to high concentration of mineral salts, low protein/ sugars ratio, low sweetening power (only 40% when compared to sucrose) and the

low solubility (18% in water at room temperature) make whey least choice for human food (Siso, 1996).

Whey is one of the strongest polluting substances among the dairy waste of all kind. Due to the requirement of high biological oxygen demand, around 32,000 mg O_2 per litter and chemical oxygen demand, around 70,000 mg O_2 per litter, ecosystems have to pay badly to dispose whey in the environment. These high oxygen demands for disposal are due to the elevated organic content mostly as lactose (Menchik *et al.,* 2019). Theoretically, the pollution load from a small cheese factory producing 4,000 L whey per day (for the production of 450 kg of cheese) would be equivalent to the sewage produced per day by 1,900 people (Tunick, 2008). These conditions are threating for the mankind, broadly to the entire ecosystem.

2.3.6.2 Opportunity

Traditionally, small producers discharge whey into creeks, lagoons, rivers and the ocean without or with little treatment through municipal sewage works. Some farmers are also used to feed animals. These types of practice solve the problem partially, they were all fraught with problems, including pollution, cost, odor, and low return. These practices support the dairy entrepreneur with a short-term out of sight, out of mind solution to the growing volumes of whey, but these methods were never going to provide sustainable management of whey streams. A major hindrance to the utilization of cheese whey is its relatively high lactose content compared to its protein content. New, high value whey products manufactured for direct sales to consumers must be developed for utilization of smaller quantities of whey. As researcher, farmer's whey disposal problem opens a way and resource to apply biotechnology to produce value added products with high demand.

Table 7 Worldwide Whey production (Data taken from USDA/NASS, 2021).

Year (‘1000 tons) / Leading cheese manufacturer	2018	2019	2020
European Union	10160	10210	10350
United States	5914	5959	6000
Russia	970	983	1035
Brazil	760	770	750
Canada	510	515	510
Nepal	311	-	-
Total cheese production (In Year)	18625	18437	18645
Total Whey production (9 times)	167625	165933	167805

Likewise, one way use of non-renewable energy causes the several energy crises in recent time. To overcome this problems researcher started to blend the ethanol with petroleum oils. So, the ethanol production in world increased (Licht 2003). Most of the ethanol industries these days used grain-based substrates to ferment which supposed to fulfill the demand for both drinkable beverage and as fuel. Whereas various organizations disagree and raise fuel vs. food issue and they asked scientist to seek alternative to grain-based ethanol production (Fang *et al.,* 2010). To resolve this problem cheese whey is one of the substrates for bioethanol production to look for.

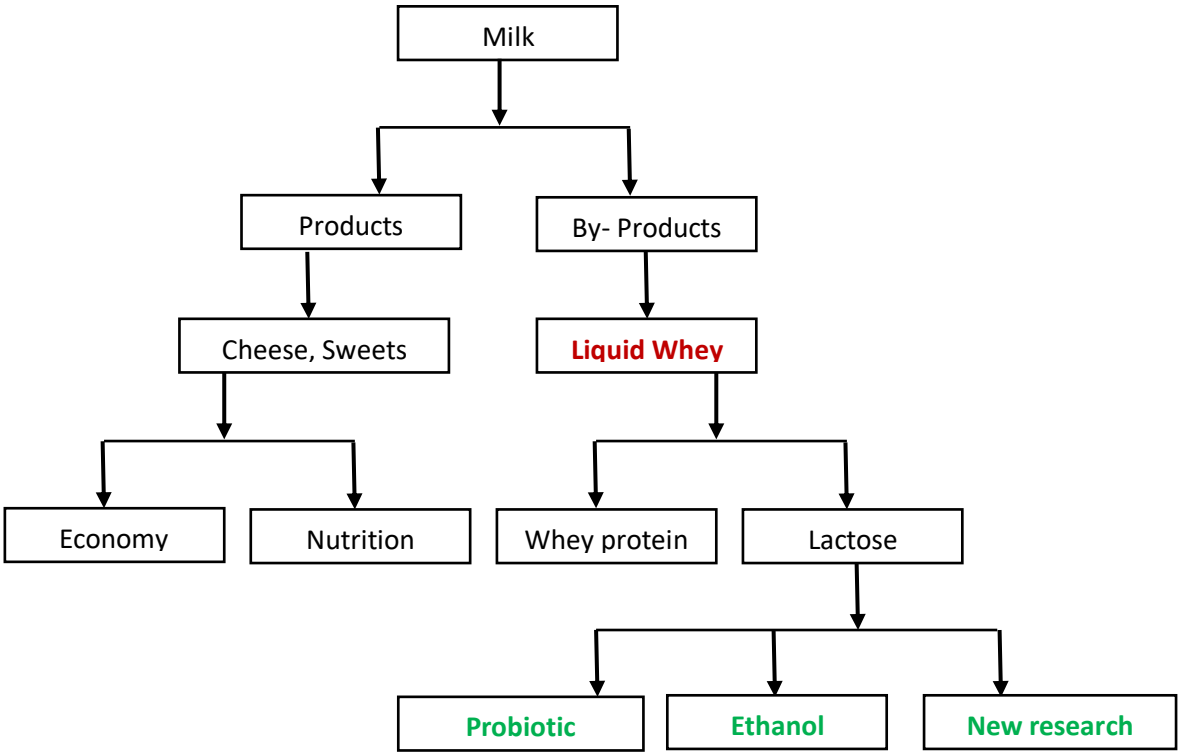

Figure 3 Charts showing trends and new aspects of Milk utilization.

Using whey as a substrate for the manufacturer to ferment ethanol, lactic acid, vitamins, antibiotics, or for feed-grade single cell protein though, the high protein content makes concern in digestion makes it not good choice for human (Ghaly & Kamal, 2004). Alcoholic beverage, whey-based wine remains laboratory curiosity for several years (Jelen, 1979). Fermentation is one of the best ways to utilize all the components of whey. To ferment the whey no sophisticated instruments were needed, that make easy for small cheese manufactures to produce alcoholic beverages as side product. This procedure is very effective as whole whey can be utilizing and addition of external any kind of energy is not needed.

Beside lactose other commercially and nutritionally very important biochemical like protein, salts, vitamins are also present, their low concentration makes their recovery uneconomical. That cheese whey is used for the international level products, and also used for biogas and electricity productions in scientific researches (Altuntas & Hapoglu 2019).

2.3.6.3 Why waste whey??

In general transformation of waste whey into value-added products could offer tremendous advantages such as environmental, economic and social advantages. Remarkably, production of value-added products from waste whey provides at least dual environmental advantages through preventing pollution by replacing petroleum-based synthesis and utilizing wastes that could otherwise have been release in atmosphere. Likewise, bio-based products are biodegradable and relatively non-toxic. Studies estimated that the world will need 70 to 100% more food by 2050 (Byerlee *et al.,* 2008). The ever increasing human population and consumption rate affect food production due to the evolving competition for land, water, and energy. Additionally, utilization of food crops for the production of valuable products aggravates this situation. Alternatively, exploitation of waste whey could possibly alleviate rising conflict between food and fuel or other bio-based products. Unlike fossils which may take millions of years to form, whey can be generated within a short period of time in a sustainable manner along with cheese production in dairy industries. More attractively, bio-waste abundance is not only described in terms of quantity but also accompanied with renewability, phenomenal characteristics which are the foremost shortcomings of fossil fuels. First of all, transformation of wastes for production of valuable products will harness land management in cities and rural areas by utilizing the waste that was supposed to be directly dispose to the environment. And second of all, it can also substitute grain based fermentation so as to avoid concerns of land use for food production vs. bio-based substrate production. Basically, in some metropolitan areas, utilization of waste mass is not just a matter of transformation it into valuable products but mainly a matter of land management due to the scarcity of dumpsites and the associated risks. Most of the reaction steps required to convert whey waste into a desired product require lower capital and energy costs than petro-chemical processes. Moreover, waste whey is far less expensive than other substrates in various aspects. Most nations of the world highly depend on imported fossil fuels from Middle East countries. This obviously resulted in tremendous rise in oil price leading to affect the economy of the several countries like Nepal. Fortunately, milk production resources are geographically more evenly distributed than the fossil fuels. Apart from being secure supply chain, domestic availability of bio-fuel could reduce transportation cost and also generate local as well as high-tech jobs. One of the major hurdles of manufacturing industries is controlling of the waste originate from it. On the other hand, these wastes can be converted or recycled into valuable products through

integrated bio-refinery process. Valorization of waste whey has very important socio-economic benefits to avoid unpleasant odors; problems associated public health risk and potential climate impact.

2.4 LACTOSE

2.4.1 Introduction

Lactose, a disaccharide which formed by a β-1→4 glycosidic linkage between galactose and glucose molecules (Guimarães *et al.,* 2010). The name Lactose comes from the Latin words Lac for milk and the suffix -ose used for sugars defines lactose as milk sugar. This sugar is undoubtedly one of the best-known sugars, owing its popularity to its abundance in the mammalian milk including humans. It is used as a supplement in baby milks since cow's milk contains 30% less lactose than human milk (Siso, 1996). In the pharmaceutical industry, lactose is used as excipient for most tablet drugs for its inert, non-hygroscopic, high purity and having good binding properties. Lactulose (formed by isomerization in alkaline solution) is a commercial product, used for treatment of constipation (Harper, 1992). Its plasticity, light flavor and reduced sweetening power as compared to sucrose or glucose make it appropriates for best choice in pill tablets (Spalatelu, 2012).

2.4.2 Whey lactose

It is noteworthy that the amounts of lactose available are rather significant from the milk products residue. As a simple exercise, if we take into account an annual world production of 167 million tons of whey in year 2020 (from table 2.3.6.2) with 5% lactose means 8 million tonnes of lactose deposited each year and somehow treated as waste or partially reutilized. Literature suggested that over the past 50 years, half of the worldwide whey production has not been transformed into sub-products but disposed of as waste effluent (Siso, 1996). Study of the market statistics suggest that below 5% of total lactose available in whey is purified and this is due to narrow and infamous market of pure lactose (Jelen, 1979). The low solubility of lactose presents problems in the concentration of whey for transportation, storage, and stock-food purposes. Furthermore, lactose is a hygroscopic sugar and has a strong tendency to absorb flavors and odors and can cause undesirable sandiness in frozen desserts. Further, excessive lactose in large intestine can lead to tissue dehydration due to osmotic effects, poor calcium absorption due to low acidity, and fermentation of the lactose by micro-flora resulting in fermentative diarrhea, bloating, flatulence, blanching and cramps, and watery diarrhea (Misselwitz *et al.,* 2013). Therefore,

alternative uses are being sought, with most of them based on the direct fermentation of lactose or the fermentation of the glucose and galactose obtained by hydrolysis of lactose. Nowadays, this microbial based valorization of cheese-whey is considered the most profitable, economical and best alternatives.

2.4.3 Lactose fermentation

Fermentation is one of the oldest forms of food preservation in the world. Human carry fermentation since Stone Age. Fermentation is a biological process in which a carbon compound serves as terminal acceptor of the electrons that are generated in the pathway in the course of converting the sugar metabolites to energy in the form of ATP. With the development of different biotechnological tools, fractionation of every component of whey is practiced and the constituents are converted into value added products. When protein is separated from whey by ultra-centrifugation lactose remains as the major compounds left in liquids with other micro-nutrients. In the same time scientists are seeking different substrate for producing ethanol, mainly environment researchers thereby managing sugar-based waste. These works can also decrease some burden to the fossils fuel which is competing alone with surging population in world. Ethanol production using Lactose can't compete with the other substrate like cane sugar-based fermentation, or with emerging second-generation technologies using lingo-cellulosic biomass as raw material (Guimarães *et al.,* 2010). However, ocean of whey being a waste, very cheap and a major dairy source of pollution creating threat to the ecosystem becomes obvious alternative as a substrate for fermentation to add few percentages of total ethanol production.

Nevertheless, commercial competence of this process is weak with well-established and commercialized fermentation substrate. Depleting fossils fuel, increasing demands of ethanol make whey, substrate of a choice though more profitable and optimized technology should be developed to enhance efficiency of the process.

Table 8 Commercial products from whey lactose (modified from Durham & Hourigan, 2007).

S.N.	Products	Organisms
1	Ethanol	*Saccharomyces cerevisiae*, (immobilised β-galactosidase) *Kluveromyces fragilis*, *Candida pseudotropicalis*, Recombined *Saccharomyces cerevisiae* (genes from *K. marxianus*)
2	Butanol/acetone	*Clostridium acetobutylicum*
3	Acetate	*Lactococcus lactis/Clostridium*
4	Galactitol Citric acid Gluconic acid	*Aspergillus niger*
5	Glycerol Single cell protein β-Galactosidase	*Kluveromyces marxianus*
6	Lactic acid	*Lactobacillus salivarius* *Lactobacillus lactis*
7	Succinic acid	*Anaerobiospirillum*

2.4.4 Lactose Fermenting Organisms

2.4.4.1 Yeast

Lactose can be utilized as a sole carbon source by a wide variety of micro-organisms having β-galactosidase and galactoside permease systems that involved in lactose metabolism

(Shaw *et. al.,* 2002). Lactose fermenting yeast are very rare due to the absence of Lac-Operon system (including *S. cereviseae*) though there are few wild yeast strains like *Kluyveromyces lactis*, *K. marxianus*, and *Candida pseudotropicalis* which are studied widely by several workers for the direct fermentation of lactose (Kosikowski & Wzorek, 1977). These days *Kluyveromyces* species are used successfully for various fermented products such as ethanol, lactic acids, glycerol etc. by using whey lactose as substrate. However, these strains have some major drawbacks including low ethanol tolerance about 5%, low yields, theoretically around 70 to 75% (Cote *et al.,* 2004) and very slow sugar conversion rate (Mawson, 1994). So, these technical and economic problems make very hard to produce lactose based fermented beverages using these strains in large industries. Clearly, improvements are needed in the conventional fermentation processes before a widely accepted method for whey lactose fermentation can be realized. To bypass the above-mentioned bottlenecks, we devise a scalable and cost-effective ethanol production strategy based on direct yeast fermentation of lactose by *S. cerevisiae*, an abundant sugar found in dairy by-products.

2.4.4.1.1 *Saccharomyces cerevisiae*

Yeasts are ubiquitous unicellular fungi widespread in natural environments colonizing from terrestrial, to aerial to aquatic environments, where the successful colonization is intimately related to their physiological adaptability to a highly variable environment. *Saccharomyces cerevisiae* is an organism of choice for fermentation and has long been used by the brewers, distillers, winemakers and bakers on a commercial scale. *S. cerevisiae* is a unicellular simplest eukaryote, possessing a nuclear genomic DNA of 12068 kilobases (kb) organized in 16 chromosomes. Their cells are round to ovoid, 5–10 µm in diameter and are reproduces by budding (Goffeau *et al.,* 1996). Like other heterotrophic organisms, in this yeast the energy and carbon metabolism are intimately interconnected, i.e., the anabolism coupled with catabolism. In one hand, the oxidation of organic molecules, as sugars, yields adenosine 5-triphosphate (ATP) that, in turn, is used as an energy resource for the cell (Boulton *et al*., 1999). On the other hand, such organic molecules can also be used as building blocks or to generate intermediary compounds for the synthesis of other compounds, some of which with high commercial value more precisely ethanol.

Naturally, yeast can metabolize wide range of organic compounds such as polyols, alcohols, organic acids and amino acids that can support their growth however, they prefer sugar

including fructose, galactose, maltose or sucrose if present (Rodrigues *et al.,* 2006). The utilization of carbohydrates by yeasts requires either their transport across the plasma membrane, or their external hydrolysis, followed by the uptake of the resulting products (Boulton *et al., 1999*). Mono-saccharides may enter the yeast cell via membrane proteins, so-called permeases, by a facilitated diffusion mechanism dependent on the sugar concentration gradient, or by an active mechanism dependent on metabolic energy (Spencer-Martins, I. 1994). All ethanol fermenting strains can use ammonia, urea, most amino acids, small peptides, and nitrogen bases as nitrogen sources likewise, assimilate di-hydrogen phosphate ion for phosphorus and sulfate ion for Sulfur. Some metals, like magnesium, iron, calcium, and zinc, are also required for good growth for cells while are prototrophic for vitamins (Rodrigues *et. al.,* 2006).

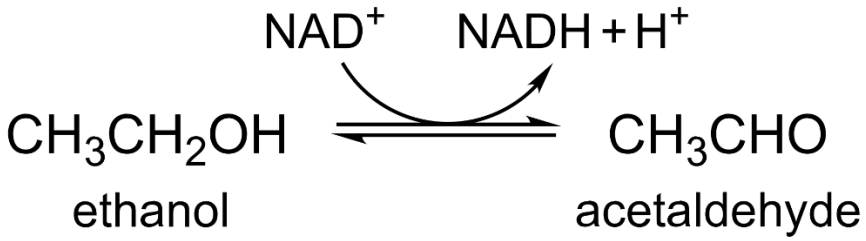

Figure 4 Biochemistry of ethanol production in yeast

Uptake and fate of the organic carbon depends upon the availability of oxygen. Aerobic condition is similar as in most of the eukaryotes where carbohydrates undergo glycolysis followed with Crab cycle. In contrast, in the absence of oxygen, yeast cells will take the pyruvate, end product of glycolysis and reduce it into acetaldehyde which is further reduced into ethanol along with the NAD+ co-enzymes that is needed for various metabolic processes of the yeast (Boulton *et al.,* 1999).

2.4.4.1.2 Why *Saccharomyces cerevisiae*

When researchers look for an organism to use in their studies, they look for several traits, in most case, size, generation time, accessibility, ease of manipulation, genetics, conservation of mechanisms, and potential economic benefit. *Saccharomyces cerevisiae* is the best studied eukaryote and a valuable tool for most aspects of basic research on eukaryotic organisms. This is due to its unicellular nature, which often simplifies matters, offering the

combination of the facts that presence of nearly all biological functions found in eukaryotes (Maicas, S. 2020). *S. cerevisiae's* biotechnological usefulness resides in its unique biological characteristics, i.e., its predictable and vigorous fermentation capacity, accompanied by the production of alcohol and CO_2 and its resilience to adverse conditions of osmolarity and ability to thrive in low pH between 2.8 and 4. As a single-cell organism, S. cerevisiae is small with a short generation time and can be easily cultured. Among the most prominent applications involving the use of *S. cerevisiae* are the ones in food, beverage especially wine and biofuel production industries. This feature is based on the Crabtree effect, which consists in the fact that *S. cerevisiae*, even under aerobic conditions does not use the respiratory machinery to metabolize saccharides and promote biomass growth, but instead, it produces ethanol and other two-carbon compounds, via pyruvate (Parapouli *et. al.,* 2020).

2.4.4.1.3 Dominance by *Saccharomyces*

Rapid high-level production of a toxic end product is one strategy to ensure dominance in the fermenting medium. Tolerance to high concentrations of ethanol is the principal feature of this yeast allowing survival in the fermenting medium. Production of heat as an end product and ability to survive warmer temperatures may have selected traits in *Saccharomyces* contributing to dominance of fermentations. Successful competition for limiting nutrients is also a factor contributing to the dominance of one species or genus over another in a mixed microbial fermentation. In *Saccharomyces*, pyruvate is converted to acetaldehyde, which serves as terminal electron acceptor generating ethanol. Even under aerobic conditions it does not use respiratory machinery and promote biomass growth.

2.4.4.1.4 Problem with *Saccharomyces cerevisiae* in whey lactose fermentation

Saccharomyces cerevisiae unable to ferment lactose because it lacks the cellular mechanisms responsible for lactose uptake and hydrolysis: a lactose permease system to transport lactose across the membrane and the intracellular enzyme for lactose hydrolysis, β-galactosidase, thus rendering it unable to ferment lactose directly into ethanol (RUSSELL, 1986). Most importantly, the efficiency of *Saccharomyces cerevisiae* to ferment ethanol is superior to that of direct lactose fermenting organisms like, *K. marxianus, K. lactis* (Terrell *et al.,* 1984). So, alternative methods were sought that make *Saccharomyces cerevisiae* potent to ferment lactose. Research papers suggested that to ferment with *Saccharomyces cerevisiae*, either lactose first hydrolyzed into Glucose and Galactose or genetically manipulate *Saccharomyces cerevisiae* cells.

2.4.4.1.5 Genetic manipulation in *Saccharomyces cerevisiae*

With the advance in molecular biology and engineering, researchers are able to clone the desire gene in the targeted cells specially in prokaryotes and often in higher cells too. To solve the problems related to lactose metabolism in *Saccharomyces cerevisiae,* recombinant DNA techniques are used and in which genes that coded β-galactosidase is expressed from *Kluyveromyces lactis* (Russel, 1986). This is very controlled and highly skilled mechanisms with lots of hopes but so far, no industrially applicable yeast strain has emerged from this approach. The efficient conversion of lactose requires adequate expression and stability of the foreign genes in the host yeast cells. Further, the products derived from genetically modified technology are not being highly accepted till date (Adam *et al.,* 1999). Besides this, the recombinant yeasts are slow growing and have reduced genetic stability, so yields are low even when specially designed bioreactors are used (Mawson, 1994 & Russel, 1986).

2.4.4.2 Lactic Acid Bacteria (LAB)

The term LAB relates to the metabolic capabilities of microorganisms to ferment various carbohydrates predominantly into lactic acid (Iskandar *et al.,* 2019). LAB is involved in a large number of industrial food fermentations are naturally found in a variety of natural habitats, including plants, meat and milk environment. LAB form a heterogeneous group of microorganisms that belong to the genera *Enterococcus, Lactobacillus, Streptococcus, Bacillus* etc. They are involved in a large number of industrial food fermentations where they are used as starter to enhance the quality of the products due to its ability of producing range of metabolites. During their growth in milk, lactose is used as the primary energy source thereby resulting glucose and galactose. *Bacillus* species are the interesting group of microbes having some unique characteristics like aerobic, thermostable, spore forming and resistance to high alkalinity (Natarajan *et al.,* 2012). Probiotic nature of Bacillus species is reviewed by different researchers. The mono-cistronic mbgA gene of *B. megaterium* encodes a β -galactosidase which plays major role in lactose utilization (Shaw *et.al.,* 2002).

2.4.4.2.1 Lactose and galactose metabolic pathway in LAB

In Gram-positive bacteria, lactose is internalized by the phosphoenolpyruvate (PEP)-dependent sugar-phosphotransferase system (PTS), yielding lactose-6- phosphate (Lac-6-P) which than hydrolyzed to glucose and galactose-6-phosphate (Gal-6-P) by a cytoplasmic

phospho- -galactosidase (LacG) where the D-galactose can directly enter the Leloir pathway and releasing galactose -6- phosphate outside the cells (Zeng *et al.,* 2010).

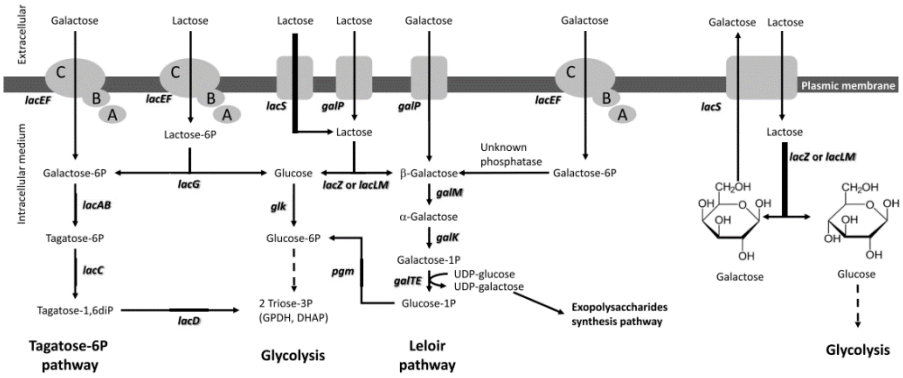

Figure 5(a) Schematic representation of the lactose metabolic pathways. The dotted line symbolizes several reactions (Iskandar et al., 2019).

Moreover, Lactose depending on which transporter internalized lactose into the cell, prior catabolism through the Leloir or the Tagatose-6P pathway, require hydrolysis by the action of a β-galactosidase or phospho β-galactosidase, respectively (Kandler, 1983). The end-product of the Leloir pathway is Glucose-1P, whereas the Tagatose-6P end products are triose-3 phosphates which subsequently enter the glycolysis pathway. One given bacterium may contain several types of glycosyl hydrolases allowing to utilize different types of carbohydrates. It can be noticed that β-galactosidase can be produced extracellularly in number of LAB strains (Iskandar *et al.,* 2019). Most of the LAB (including *B. megaterium*, *Lactobacillus*) utilizes lactose via Leloir pathway in which lactose is brought into the cell as the free sugar and cleaved by β-galactosidase than he glucose moiety of the sugar is further metabolized while the galactose moiety is released (Hottinger *et.al.,* 1991), one of the major aspects of this study. Approximately 1 mole of galactose was released into the medium for each mole of lactose utilized whereas no glucose molecules were detected in the growth medium after lactose utilization (Hickey *et al.,* 1986).

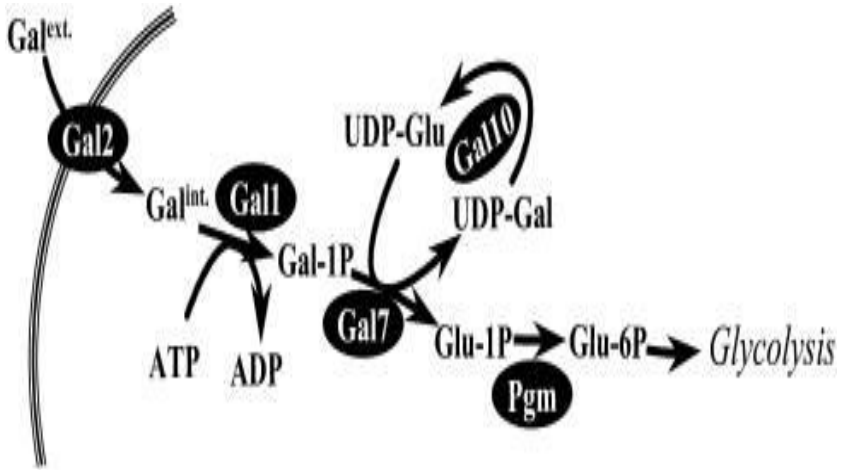

Figure 6 (b) Galactose utilization pathway in *S. cerevisiae* (For detail mechanism look at Bro *et al.,* 2005).

Galactose utilization is important at the industrial level where its accumulation in the product is a major problem for producers as well as for consumer's (Wu *et al.,* 2015). Galactose is a carbohydrate not only found in dairy products as a subunit of lactose molecules but also in many environments such as cereals, fruits, legumes, nuts, meats, seeds, and vegetables (Acosta & Gross, 1995). The ability of starter cultures to metabolize lactose and the resulting galactose is therefore a major aspect of starter selection.

2.5 Relation of LAB and *S. cerevisiae* in fermentation.

Generally, natural fermentations are carried out by yeast and lactic acid bacteria forming a complex micro-biota that acts in cooperation. Yeast has a prominent role in the production of beverages, due to the ability to accumulate high levels of ethanol and to produce highly desirable aroma compounds which are organoleptic whereas lactic acid bacteria are particularly important in fermentation as they produce desirable acids, flavor compounds, and peptides that inhibit the growth of undesirable organisms (Faria-Oliveira *et. al.,* 2015).

Beverage	Raw material	Microorganisms	Country
cauim	cassava, rice, peanuts	Lactic acid bacteria (LAB), *Saccharomyces cerevisiae*, other yeast	Brazil
caxiri	cassava	LAB, *Bacillus* spp., *S. cerevisiae* (predominant yeast), other yeast	Brazil
champús	maize	*S.cerevisiae*, other yeast	Colombia
chicha	maize	LAB, *S.cerevisiae* (predominant yeast), *Aspergillus* spp.	Peru
cachaça	sugarcane	*S. cerevisiae*; LAB, other yeast	Brazil

Figure 7 Some common products available in different countries where *S. cerevisiae* co-cultured with LAB.

2.6 Lactose Hydrolysis

Lactose can be hydrolyzed to its monomers glucose and galactose either by acids or by enzymes.

2.6.1 By Acids

Lactose can be hydrolyzed by using acids like sulfuric acid, hydrochloric acid at very low pH. Acid hydrolysis has been investigated both in the homogeneous phase with the acid in solution and in the heterogeneous phase with ion-exchange resins (Pasotti *et al.,* 2017). The process is well defined for pure glycosides, and further research is needed to optimize the process for acid whey. However, this procedure is a very harsh operational condition that causes possible protein denaturation, the appearance of a brown color due to Millard reactions and the formation of undesirable products so unacceptable for food grade procedures (Siso, 1996 & Jelen, 1979).

2.6.2 By enzyme

β-galactosidases (E.C 3.2.1.23) also called lactase, are metal-activated group of enzymes which hydrolyzes the milk sugar, lactose, to its component's and thus it is used for the treatment of milk and its derivatives for consumption by people who have lactose intolerance, for prevention of lactose crystallization in frozen and condensed milk products, for the reduction of water pollution caused by whey and also for increasing the sweetening properties of lactose (Konsoula *et al.,* 2007). Chemically, oxygen bridge i.e., glycosidic bond

of lactose molecules is cleaved and resulting in the two moieties, Glucose and Galactose (Natarajan *et al.,* 2012).

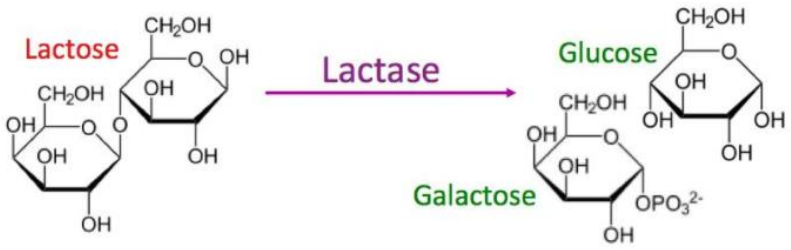

Figure 8 Lactase enzyme (β-galactosidases) hydrolyzed lactose into glucose and galactose.

While β-galactosidase has been found in numerous biological systems, microorganisms such as yeasts, molds and bacteria still remain the only commercially exploited sources (Panesar *et al.,* 2010). According to their sources, their properties differ markedly. β-galactosidases from bacterial sources have been widely used for lactose hydrolysis, as it shows the ease of fermentation, high enzymatic activity and good stability. Moreover, β-galactosidase from *Escherichia coli* has been well explored following the discovery of the lac operon for its use as recombinant genetic tool where β-galactosidase enzyme from this strains is hampered for industrial use by the fact that it is not considered safe for food applications though it is commercially available for analytical purposes (Percival *et al.*, 2019). Therefore, the production of β-galactosidase from probiotic microorganisms which are safer for human use becomes the need of the hour. In this regard, lactic acid bacteria include a diverse group of *Bacillus megaterium, Bacillus subtilis, Lactobacillus* and *Bifidobacterium* which are generally recognized as safe (GRAS) organisms, have been regarded as good sources of β-galactosidase, especially for functional food applications (Percival *et al.,* 2019).

Table 9 Microbial sources of β-galactosidase (Computed from Panesar *et al.,* 2010).

Source	Microorganisms
Bacteria	• *Bacillus acidocaldarius* • ***B. subtilis*** • ***B. megaterum*** • *B. stearothermophilus* • *Bifidobacterium bifidum* • *Escherichia coli* • *Klebsiella pneumonia* • ***Lactobacillus acidophilus*** • *L. bulgaricus* • ***L. lactis, L. themophilus, L. delbruecki*** • *Pseudomonas fluorescens*
Fungi	• *Aspergillus oryza* • *Neurospora crassa* • *Streptomyces violaceus*
Yeast	• *Candida pseudotropicalis* • *Saccharomyces anamensis, S. lactis* • *Kluyveromyces bulgaricus, K. lactis, K. marxianus*

Enzymatic hydrolysis is most practiced method for the utilization of lactose and is very important for elaboration of milk with sweetened flavor to reduce caloric content, of good acceptability, avoidance of lactose crystallization and the grainy aspect of ice-cream that

can be consumed by people with lactose intolerance (Kamel *et al.,* 2016). Many products of low lactose milk and whey find diversified applications, such as food and animal feed components, yogurts and ethanol production and in the production of the cheese, the industrial by-products include whey and permeate that can cause reduced environmental impacts when the lactose is removed (Mariotti *et al.,* 2008). The main problem associated with this method is to lack of ability of enzymes to complete hydrolysis of lactose due to unwanted polymerization, being expensive as it is intracellular enzyme and enzymatic instability (Coté *et al.,* 2004). Therefore, there is a definite need for β-galactosidase that is stable at different temperatures and could be approved as GRAS for hydrolysis of lactose. The resulted glucose and galactose from this hydrolysis then fermented with *Saccharomyces cerevisiae*.

2.7 Complete utilization of whey (Lactose)

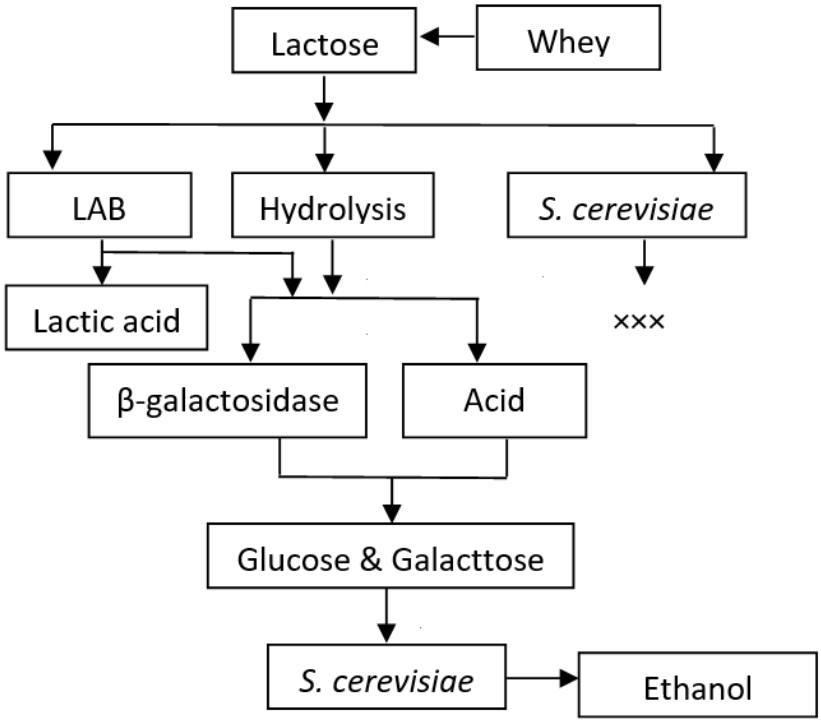

Figure 9 Diagrammatic representation of microbial utilization of whey lactose (Mawson, 1994).

2.8 Mead Fermentation

Mead is the perhaps the oldest fermented drinks in the world, yet it is difficult to find it commercially in markets. It is a traditional drink, containing 8–18% (v/v) of ethanol, which results from the alcoholic fermentation of diluted honey carried out by yeasts. Mead fermentation is a time-consuming process, often taking several months, and the fermentation rate depends on several factors, especially on honey variety, yeast strain, yeast nutrition, control of pH (Navrátil et al., 2001). Honey is a natural product, mainly composed of a complex mixture of carbohydrates (nearly 85–95% is fructose and glucose) and other minor substances, such as organic acids, amino acids, proteins, minerals, vitamins, and lipids (Pereira *et al.,* 2009). Development of honey-derived products appears to be a sound alternative to provide innovative alcoholic drinks to the consumers and to increase the profit of the beekeeping industry. From the fermentation of honey Different types of mead can be obtained having different flavors depending on the floral source of the honey, the yeast used in the fermentation and the presence of additives. The most common are metheglin (containing spices or herbs), melomel (with fruit juices), hippocras (with herbs and spices) and sack mead (produced with superior concentration of honey) (Gupta & Sharma, 2009). Consequently, complications such as a lack of uniformity of the final product arise, probably due to the variability of honey composition and to the occurrence of re-fermentations by yeasts or acetic and lactic bacteria, which may increase volatile acidity and promote abnormal ester production, affecting the sensory qualities of the final product (Pereira *et al.,* 2009).

Even though mead is perhaps the oldest fermented drink in the world, produced mainly in an empirical way, its production has suffered in recent years, partially due to the lack of scientific progress in this field. Even though there is not much scientific information regarding honey-must fermentations, it is generally accepted that the improvement of meads' quality includes the development of adequate additive formulations and the optimization of the fermentation conditions.

2.8.1 Favorable characteristics of a good wine yeast

- Conduct a vigorous fermentation.
- Conduct fermentation to dryness (Low to no residual fermentable sugar).
- Possess reproducible fermentation characteristics and behave predictably.

- Possess good ethanol tolerance.
- Possess good temperature tolerance.
- Produce no off-flavors or aromas.
- Be $S0_2$ tolerant.
- Flocculate so as to be easy to remove (particularly for secondary fermentation in sparkling wine production).

2.9 Probiotics

Nowadays, much attention has been devoted to the development of useful foods that have probiotic microbial strains responsible for health-promoting effects. Consumers are increasingly interested in maintaining health through food and dietary supplements. Use of evidence-based approaches to improve diets and lifestyles is a trend that continues to grow. This has generated an ever more varied market of foods and supplements, especially those containing probiotics. The word 'probiotic' is derived from the Greek meaning 'for life' (Fuller, R. 1992) which gives meaning exact opposite to ‘antibiotics’. WHO defines probiotics as “Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” and must have “defined contents, appropriate viable count at end of shelf life and suitable evidence for health benefits,” (ISAPP, 2018).

The micro-biota of the gastrointestinal tract in both humans and animals consists of microorganisms that flourish throughout the digestive tract. These living microorganisms form an enormous microbial community that includes both aerobic and anaerobic bacteria, as well as viruses, fungi and parasites. The intestinal micro-biota contributes to bowel health in the host by fermenting unused energy substrates, preventing growth of harmful pathogenic bacteria and assist host immunity (McNulty *et al.,* 1985). Various studies have indicated that probiotics may alleviate lactose intolerance; have a positive influence on the intestinal flora of the host; stimulate mucosal immunity; reduce inflammatory or allergic reactions; reduce blood cholesterol; possess anti-colon cancer effects; reduce the clinical manifestations of atopic dermatitis, diarrhea, and constipation, candidiasis, and urinary tract infections; and competitively exclude pathogens (Begley *et al.,* 2006). Considering this impressive list of potential health-promoting benefits, it is not surprising that there continues to be considerable interest in the use of probiotics as bio-therapeutic agents.

Many species of lactic acid bacteria, bifidobacteria and yeasts, representing most of the commercially available probiotic strains, are judged to be safe for use in foods and supplements. This is because they belong to genera and species with a documented history of safe use, either as probiotics or as starter cultures (Bourdichon *et al.,* 2018). Although lactic-acid bacteria like *Lactobacillus* are predominantly probiotic microorganisms, some yeasts such as *Saccharomyces* and *Kluyveromyces* and *Bacillus megaterium* and *Bacillus subtilis* bacteria of the Bacillus genus also have potentials as probiotic (Andriani *et al.,* 2017) and show potent antagonist nature to fungal and bacterial pathogens (Natarajan *et al.,* 2012). *Saccharomyces*, most commonly used for animal and human consumption in the food industry worldwide *(*Moradi *et al.,* 2018) shows enhanced digestion of a sucrose load in infants with sucrase deficiency when they consumed *Saccharomyces cerevisiae*, that contains the enzyme sucrase. This is a good example of a direct effect of a probiotic (Marteau *et al.,* 2001).

Providing consumers with foods and dietary supplements that meet applicable safety standards is a basic responsibility of probiotic manufacturers. Establishing that a specific probiotic is safe for use in foods and dietary supplements requires, as a starting point, proper identification to the strain level, and further documenting safe use through historical evidence or experimentation. Fermented beverages have progressed from traditional natural fermented products, to beverages formulated with functional ingredients to stimulate cardiovascular benefits, and then to ferment drinks that improve the gastrointestinal health. Considering the potential of whey, based on its nutritional value, the aim of this study was to define the growth and survival of probiotic bacteria in whey and the influence of prebiotic inulin addition on it, for possible production of a nutritive highly valuable whey drink. To enrich whey and therefore increase its nutritional value, probiotic microorganisms are one of the best choices for production of a fermented whey beverage. It is generally accepted that fermented milk products such as yogurt can efficiently improve lactose digestion in lactose mal-absorbers and therefore that they are well tolerated by most lactose intolerant subjects. There are several possible reasons for these effects. First, active microbial β-galactosidase in bacteria-containing fermented or unfermented milk products survives gastric passage and is released by bile salts into the small intestine, where it supports lactose digestion (De Vrese *et al.,* 2001)

Selection of microbes as elements of probiotics is an important step since the microbes have to meet certain criteria as candidates of probiotic microbes. Certain basic criteria that the candidates must possess are rapid growth and adequate extracellular enzyme. Furthermore, these probiotic bacteria should have excellent viability in order to survive the production, storage and preservation processes, as well as proven health benefits, resistance to acidity (low pH) and bile salts. These characteristics are essential in order for them to colonize and balance the micro-flora in the digestive system (Andriani *et al.,* 2017). The selection of potential probiotic strains that would be capable of performing effectively in the gastrointestinal tract is a significant challenge. Strain selection has generally been based on in vitro tolerance of physiologically relevant stresses: e.g., low pH, elevated osmolarity, and bile (Begley *et al.,* 2006).

CHAPTER-3

MATERIALS AND METHOD

3.1 Collection of whey samples

Different small diaries at Birgunj were visited during the study. Whey samples were collected from the local markets in polypropylene (plastic) bottles rinsed by 2% caustic soda and hot water.

3.2 Filtration and Pasteurization

At laboratory the sample was filtered by using muslin cloth to remove debris if present. The pasteurization technique carried by heating the whey to 60**°**C for 30 min in water bath, cooled down it to 0**°**C for 30 min using refrigerator and left it to stand at room temperature (21**°**C) for 24 h for any spore to germinate. The processes of heating, cooling and standing at room temperature was repeated three times to destroy any vegetative or spore cells present in the whey. The plate count test was performed to insure the effectiveness of this pasteurization technique. Then such pasteurized whey was immediately stored at refrigeration temperature (4^0C) until it required for the further work. Prior to placing the whey into the fermenter, it was allowed to completely thaw at room temperature for 24 h.

3.3 Characterization of Whey

3.3.1 Physical characteristics

Nature, color and odor of the whey samples were observed in laboratory.

3.3.2 pH

The pH was measured by using a Benchtop pH meter calibrated with buffer 4.0 and 7.0.

3.3.3 Lactose estimation

3.3.3.1 By Brix (Hadiwijaya et. al., 2020)

In this method, total soluble solid was measured in the term of °brix. °Brix of whey samples were measured by using ERMA hand refractometer having a range of 0-30°Brix at 20°C.

3.3.3.2 By DNS (Dinitro salicylic acid) method (Saqib et. al., 2011)

DNS test was performed to determine the reducing sugar in the whey samples. Reagent preparation (for 1000 ml): 10 gm of 3, 4-dinitrosalicylic acid was dissolved in 200 ml RO water under continuous stirring. In separate vessels 16 gm of NaOH pellets were dissolved

in 150 ml RO water and was mixed with first solution and incubated in 50 ˚C with continuous stirring until the solution became clear. In that solution 403 gm of potassium sodium tartarate tetrahydrate was mixed and filtered to remove residue and the final volume was made to 1000ml with RO water. So formed solution was stored in dark glass bottle at temperature below 20 ˚C for further use. Calibration Curve Preparation: 100mg/ml stock solution of lactose was prepared by dissolving lab grade lactose in distilled water. 11 clean and dry test tubes were taken and were labelled as blank and 10-100mg/ml. Dilutions were made of lactose standards with concentrations of 10, 20, 30, …. Up to 100mg/ml by transferring respective amount of lactose from the standard lactose solution (100mg/ml) and adjusting it to a total volume of 3ml by adding distilled water. In each tube 3ml of DNS reagent was added and after mixing properly tubes were boiled for 5 minutes and allowed to cool at room temperature. 900 µl of 40% Rochelle salt was added to the mixture and vortex mixing was carried. Then Spectrophotometric absorbance of each tube was taken at 540 nm wavelength. For samples: Whey Samples were prepared as same as standard and absorbance was taken. The concentration of lactose in whey samples were then calculated by using standard curve.

3.3.4 Nitrogen estimation

Amount of NH_3 was quantified by using Nessler's reagent (Zhao et. al., 2019) method. Stock solution of 1M ammonium hydroxide (NH_4OH) was prepared by dissolving 0.35gm of lab grade NH_4OH in 10 ml of RO water. In ten different tube concentration of 0mM-100mM were prepared by diluting the stock solution with distilled water. Then, the 2ml solution was made by adding 200 µl diluted sample, 100 µl Nessler's reagent and 1700 µl RO water. These mixtures were mixed well and left for 10 minutes at room temperature. Finally, absorbance was taken at 435 nm and by plotting these value standard curve prepared. Likewise, 200 µl of each whey samples were taken and followed the procedures same as above.

3.3.5 Protein Estimation

Total soluble protein can be estimated by the method explain in Lowry *et.al.,* 1951. Reagent Preparation: Solutions of different concentration ranges from 0.05 to 1 mg/ ml of BSA were prepared by mixing 1 mg/ ml stock BSA solution with required volume of water to make final volume 5 ml in each of the test tubes. Reagents, A was prepared by dissolving 50 ml of 2% sodium carbonate with 50 ml of 0.1 N NaOH solution and B by mixing 10 ml of 1.56%

copper sulphate solution with 10 ml of 2.37% sodium potassium tartarate solution. Then, analytical reagents were made by mixing 2 ml of reagent B with 100 ml of reagent A. Folin-Ciocalteau reagent solution was diluted an equal volume of water at the time of use. Standard Curve Preparation: Standard curve was made by taking 0.2 ml diluted protein solution to different test tubes. In each tube 2 ml of alkaline analytical reagent was added and mixed well followed with incubation at room temperature for 10 mins. Then 0.2 ml of freshly prepared Folin Ciocalteau solution was mixed to each tube and again incubate for 30 min. Spectroscopic absorbance were taken at 660nm and absorbance against protein concentration was plotted to get a standard calibration curve. For sample: 0.2ml of each whey samples were taken in test tube and followed the same procedure as standard and determine the concentration of the whey samples using the standard curve plotted above. For Blank measurement, deionized water was used instead of the sample.

3.3.6 Soluble Phosphate Quantification

Soluble Phosphate can be estimated by a single solution reagent method as carried in Murphy *et. al.,* 1958. Reagent Preparation: 125 ml of 5N H_2SO_4 was mixed with 37.5 ml 4% Ammonium molybdate in that solution 75 ml of 0.1M Ascorbic acid was added and final volume was made to 250ml with distilled water. 1mg/ml stock solution of phosphate was prepared by dissolving 1gm of Potassium dihydrogen phosphate in 1000ml of distilled water. For Standard curve 11 clean test tubes were taken and made concentration of solution range from 0(blank) to 10 µg/ml of volume 5ml by dilution taking 5 µl stock and remaining water. After that, 1ml of each solution was taken in clean test tubes and mixed with 1.25 ml of freshly prepared reagent. Then the mixture was left to boil for 30 minutes followed by cooling to the room temperature. Finally, the calibration curve was prepared by plotting the data taken from spectrophotometric analysis at wavelength of 827 nm. The concentration of soluble phosphate in whey samples was measured by comparing their absorbance in standard curve.

3.3.7 Calcium ions quantification using complexometric titration method

10 ml of whey sample was taken in a clean 250ml flask in which 40 ml of distilled water and 4 ml of 8M sodium hydroxide solution was mixed well and left solution to stand for about 5 minutes with occasional swirling. Then Patten and Reeder's indicator was added in very small amount. Titration was carried with 0.025mol/L EDTA till the sky-blue color was appeared. Blank titration was also carried with distilled water.

3.3.8 Vitamin's quantification (Indian pharmacopeia volume-1)

3.3.8.1 Vitamin B_2 (Riboflavin):

A stock solution of 50 mg/ml (0.05 gm/ml) of Riboflavin was prepared. 7 clean and dry test tubes were taken and, in each tube, working solution of concentration ranges from 0(for blank) to 1mg/ml were made by diluting the stock solution with RO water. From each working solution 1ml as a sample was taken in which 1ml NaOH of 2M was added followed by 20 ml water and 0.5 ml glacial acetic acid. Then, 4 ml of above solution was taken and mixed with 0.7 ml 1.4% (w/v) solution of Sodium acetate and the final volume was made to 40 ml. OD was taken at about 444nm and the calibration curve was constructed by plotting the data. Same procedure was followed for the whey sample and by comparing its absorbance with standard concentration of B_2 in whey was estimated.

3.3.8.2 Vitamin B6 (Pyridoxine)

For Standard Calibration curve, Pyridoxine hydrochloride powder was taken and a stock solution of 25 mg/ml (0.025 gm/ml) of Pyridoxine hydrochloride was made and working solution of concentration 0 (blank) to 1 mg/ml were made in 7 test tube by diluting with 0.1 M HCl solution, as in blank only 10 ml of 0.1 M HCl was taken. From each working concentration 10 ml solution were taken and 10 ml of 0.1 M HCl was added and subjected to water bath for 15 minutes with occasional swirling. The solution was cooled, and diluted to 20 ml with 0.1 M HCl. The solution was filtered discarding the first 4 ml of filtrate. From the remaining 1 ml of filtrate was taken and diluted to 20 ml with 0.1 M HCl. Absorbance reading was taken at 290 nm wavelength and curve was made. Whey sample was also followed with the same steps and by which concentration was measured.

3.3.9 Biological Oxygen Demand (BOD) Calculation of Whey (By Winkler 5-day BOD method)

3.3.9.1 Reagents Preparation

Manganese sulfate solution was prepared by dissolving 18.2gm of $MnSO_4.H_2O$ in distilled water and was filtered to get clear pink color. Alkaline Potassium iodide solution was prepared by dissolving 10gm potassium hydroxide and 5 gm of potassium iodide in 20 ml of distilled water. 1% pure Starch solution was prepared by dissolving starch in boiling water. Standard sodium thiosulfate titrant: 3.1gm of $Na_2S_2O_3$.$5H_2O$ was dissolved in distilled water

and 0.4 g solid NaOH was added and volume was made to 500ml. Concentrate solution of Sulfuric acid having specific gravity 1.84 was used.

For BOD, 40ml (50 times diluted with HPLC water) of Raw, non-sterile filtered whey samples were taken in two clean and dry BOD bottles with no air bubbles and labeled as D_0(initial) and D_5(5th day). Of which one bottle was taken for DO analysis for day 0 and another sample was incubated in incubator for 5 days at 20°C.

DO Determination

3.3.9.2 Sample preparation

Before preceding the DO calculation procedure, a de-chlorination chemical, sodium sulfite was added to the sample to make it free from chlorine and pH was adjusted at 6.5 - 7.5.

3.3.9.3. Sample treatment

Prepared sample was taken in BOD bottle and stopper was placed to ensure no air bubbles on working sample. In that sample 1 mL of alkali- iodide and then 1 mL manganous sulfate solution was added formed brown precipitate (If white precipitation was seen stop further works). Sample was mixed well by inverting number of times and left settles the precipitate, repeat again. When two phases were completely separated, conc. H_2SO_4 was added and mixed well by placing the cap on the bottle till the clear dark yellow solution was formed.

3.3.9.4 Titration

40 ml sample was taken in conical flask and titrated with standard sodium thiosulfate solution (0.025N) till the color changed from dark yellow to light yellow. Then few drops of starch solution as an indicator was added that formed blue color and continued to titrate till the color of the solution becomes colorless. The volume of 0.025N sodium thiosulfate consumed was noted. Blank titration was also carried. DO_0 value was then calculated.

After 5 days DO_5 value of incubated sample was then also calculated by using the same procedure.

$$\textbf{BOD of whey } BOD_5 = DO_0 - DO_5$$

3.4 Bacterial strains and growth conditions

Lactobacillus strain (LB-D1) was obtained from the market and the culture was maintained in lactobacilli MRS (De Man, Rogosa and Sharpe agar) broth. The organism was sub cultured

at 37°C once a week and stored in a refrigerator in 10 % glycerol stock. For growth determination and subsequent assays, MRS broth containing 2 % lactose was used.

Likewise, the novel probiotic strain *B. subtilis* 24 (BS-S4S1) and *B. megaterium13* (BM-13) was obtained from CDBT laboratory which was previously isolated and characterized by Miss Sabina Thapa Magar (M.Sc. Student of TU) and were revive in LB-media followed with their specific media with maleic acid and L-tryptophan respectively as sole carbon source.

Yeast strain, *Saccharomyces cerevisiae* (SS-D2) as brewer's yeast was purchased (From Bake King) as activated dried powdered form and was revived in SDA (Sabouraud dextrose agar) and The pure cultures were preserved on broth sabouraud by freezing at -70°C with 15% (v/v) of glycerol for longer preservation.

3.5 Characterization of organisms

3.5.1 Gram's staining

For the Gram's staining; microorganisms were first inoculated and smeared on the sterile glass slides with the help of sterile inoculating loop. The smears were allowed to air dry and then heat-fixed quickly. Primary stain i.e., Crystal violet was flooded over the smear on each slide and let stand for 1 minute and gently washed with the distilled water. Dilute solution of Gram's iodine was flooded gently over the smear and let stand for 1 minute and again washed with the distilled water and decolonization was done with an organic solvent, ethyl alcohol (95%) for 5-10 seconds. After washing with the distilled water, the slides were flooded with safranin to counter-stain and let stand for 45 seconds. Then the slides were washed with distilled water, allowed to dry and microscopic observations of the slides were performed.

3.5.2 Yeast staining

Yeast staining was carried by the procedure explained in Glenister, P. R., 1970. A smear of the yeast sample was prepared on a clean glass microscope slide and allowed to dry and fixed the cells by passing the slide quickly through the flame of a Bunsen burner about 20 time, take care for the probable distortion of the cells. After fixing cells in slides staining was done with methylene blue solution for four minutes, the smear was rinsed in a gentle stream of cold tap water for about 30 seconds. Then tannic acid solution was applied in the smear for two minutes followed with rinse as before. Finally, Stained the smear for one

minute in the safranin solution, rinsed as before allowed the smear to dry and observed the smear with the oil-immersion lens of the microscope, without a cover glass.

3.5.3 Biochemical test

3.5.3.1 Starch hydrolysis test

NA plates were supplemented with 1% (w/v) starch and the microbial cultures were inoculated by streaking on the agar plate using a sterile inoculating loop. The plates were incubated at 28°c for 24 hrs. Then the plates were flooded with Gram's iodine solution and a clear zone around the growth of bacterial colony indicated the starch utilization.

3.5.3.2 MR-VP test

The microorganisms were aseptically inoculated into the 2 test tubes labeled as the MR and VP medium. The tubes were incubated at 28°c for 48 hrs. After that, 5-6 drops of MR reagent were added to the test tube labeled as MR and color change of the media was observed. Similarly, for VP, Barritt's reagent A and B in the ratio of 3:1 (α-Naphthol: KOH) were added in the tubes and shaken to provide the oxygen. The changes in color were noticed after 20-30 minutes of incubation.

3.5.3.3 Citrate utilization test

The names of microorganisms were labeled in the slant of Simmon's citrate agar tube. Then the microorganisms were aseptically inoculated on the surface of the medium in a zig-zag way and incubated at 28°c for 24-48 h and the color change in the medium were observed.

3.5.3.4 Catalase test

Small inoculums of the microorganisms were mixed with a drop of 3% H_2O_2 using a sterile wooden stick (toothpick) and the effervescence marked by bubbles were observed.

3.5.3.5 Urease test

For this test the media used was urea broth media. The broth media was inoculated with a loopful of test organisms in the test tubes. The tubes were incubated at 28°C for 24 hrs. A positive urease test was indicated by the change in media color from yellow to pink.

3.5.3.6 Oxidative-fermentative test (OF)

For this test the media was hugh-leifson media. The media was prepared in separated in different test tubes and labelled as "O" for oxidative and "F" for fermentative.

Microorganisms were inoculated using sterile wire by stabbing straight. In order to maintain the anaerobic condition in the tube labeled as “F” small drop of heavy paraffin oil was added after the inoculation of microorganisms. Tubes were incubated at 28°C and change in media color from green to yellow was interpreted as positive test.

3.5.3.7 Sulphur Indole Motility test (SIM)

For this test the SIM media was prepared in the test tube and colonies were inoculated with the help of sterile inoculating loop. Inoculation was done by stabbing the colonies at the center of the media at the depth of 1-2 inches. After the inoculation of the organisms the tube was incubated at 28°C for 24 hrs. After incubation the tubes were observed for the production of hydrogen sulfide (H_2S) gas, formation of indole and the motility. After the observation of H_2S production and motility; formation of indole was tested by adding the Kovacs regent (3 drops) at the surface of the media and development of pink to red color was interpreted as positive indole test. A positive H_2S test was denoted by a blackening of the media along the line of inoculation. A positive motility test was indicated by a diffuse zone of growth flaring from the line of inoculation.

3.5.4 Tests for Lactose Fermenters

3.5.4.1 MacConkey Agar methods

Dehydrated form of MacConkey agar media was prepared by dissolving 5% of media in RO water and was dissolved completely by boiling. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes then cooled to 45-50°C, mixed well and was poured into sterile Petri plates aseptically. All four organisms were inoculated with top agar and incubated at 28°C for 24 hrs. MacConkey broth form was also used for inoculation and color changed during incubation period was observed.

3.5.4.2 By X-gal method

2% (W/V) stock solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was prepared in dimethylformamide (DMSO) at a concentration of 20 mg/mL in a polypropylene tube. Then, the NA plates with 0.5% lactose were made in which 50 µl X-gal solutions were infused. Organisms were inoculated by top agar methods and colonies were observed after 1 day.

3.7 Mead Production

The production of mead involves several steps that are presented in the diagram of (figure 10).

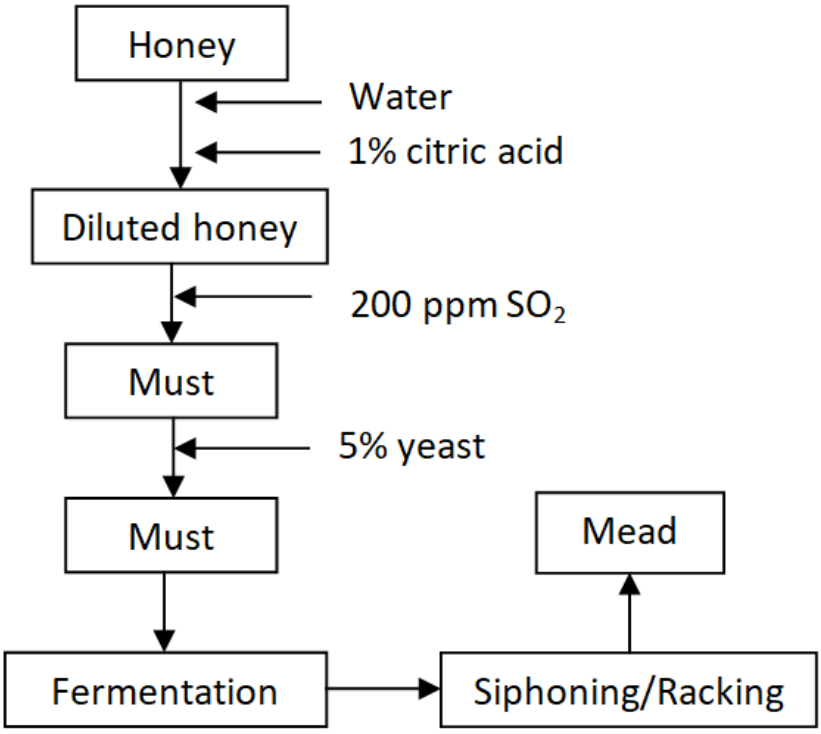

Figure 10 Schematic representation of Mead production

Sugar content of fresh honey (purchased from supermarket) was measured as °Brix with the help of hand refracto-meter. In general, the first step in mead fermentation was must preparation. In which honey is diluted with distilled water to make concentration 22 ° Brix that was explained in Pearson's square calculation.

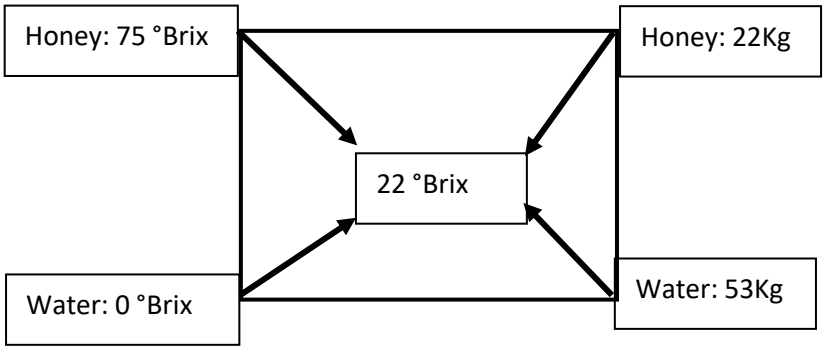

Figure 11 Pearson's square calculations.

By using above calculation, batch fermentation of batch size 2.0L was prepared in fermenter. After the must preparation in fermenter, its pH was monitored by using Benchtop pH meter and was maintained within a range of 3.7–4.0 by adding 1% solution of citric acid. In the next step whole set up and must was sterilized by using 200ppm Potassium metabisulfite (KMS) in order to reduce microbial load. Calculated amount of Yeast nutrients was added as additives. Dry yeast powder was added in the fermenter and whole set up was left in room temperature for fermentation. Air lock was adjusted after 12hrs to maintained anaerobic condition and to prevent from in-process contamination. After yeast inoculation, regular aseptic sampling was carried out for monitoring fermentation and growth parameters and ° Brix. Fermentation was carried for 18 days and was subsequently transferred to another fermenter by siphoning and was left for another 1 week for secondary fermentation. When the fermentation was complete, the young mead was filtered with cheese cloth and the un-dissolved residue and yeast removed, leaving the new mead. With the initial filtration, some turbidity remains. Then by, low temperature treatment this precipitates out as sediment and the clear part was transferred to another tank. Then filtered to produce a clear liquid mead.

3.7.1 Analysis of Mead

3.7.1.1 TSS (°Brix)

Brix reading of the mead samples was determined with the help of ERMA hand refractometer having a range of 0-32°Brix at 20°C.

3.7.1.2 pH

pH of the samples was recorded by using the pH meter of benchtop model.

3.7.1.3 Ethanol content

Ethanol in mead was determined by following process: Mead was placed in a flask in which the mead can be slowly boiled the mead to close to ½ its starting volume. It was important that can precisely return the level of mead to the pre-boil level by adding distilled water. The pre-boil amount should be close to 250mL in order to be able to properly take the specific gravity with a specific gravity bottle.

Specific gravity (SG) = (measured substance mass/volume)/ (distilled water mass/volume) both at 20°C. Then, with known value of water's specific gravity value density of ethanol can be calculated by:

Density of ethanol = Specific gravity of ethanol × Specific gravity of water.

By comparing the observed density of ethanol with the standard data ethanol concentration (V/V) can be calculated.

3.7.1.4 Methanol content

Methanol of mead was determined by spectrophotometric process: 50 mL of sample was taken in a simple still and distilled till 40 mL of distillate was collected. 1 mL of that distillate was diluted to 5mL with distilled water and shaken well. Then, 1 mL of that solution, 1 mL of distilled water (for blank) and 1 mL of methanol as standards was taken in to 50 mL stoppered test tubes and kept in an ice-cold water bath followed by the addition of 2 mL of $KMnO_4$ reagent in each tube and left aside for 30 min. After room incubation the solution was decolorized by adding a little sodium bisulphite and 1 mL of chromotropic acid solution was added and mixed well. Again, 15mL of sulphuric acid was added slowly with swirling and placed in hot water bath maintaining 80˚C for 20 min. during which violet color was changed to red. Finally, the solution was cooled and absorbance was measured at 575 nm using 1cm cuvette cell.

Calculations**:**

Methanol (%V/V) = Sample OD / Standard OD × 0.025 × dilution factor

3.8 Bacterial break down of whey lactose

Broth media was prepared by using cheese whey as a sole carbon sources. pH at 6.5 was adjusted by adding sodium carbonate (Detail composition was given in the appendix part). The media was autoclaved. Overnight culture of *Bacillus subtilis*, *Bacillus megaterium* and

lactobacillus was inoculated aseptically in which inoculation was carried individually and in combination of all three bacteria in 1-2 % of total medium. For 7 days, growth pattern was observed by taking OD at 600nm, pH change in broth was observed by pH meter and available carbon source was measured by using DNS (described above in section 3.3.3.2).

3.9 Whey fermentation

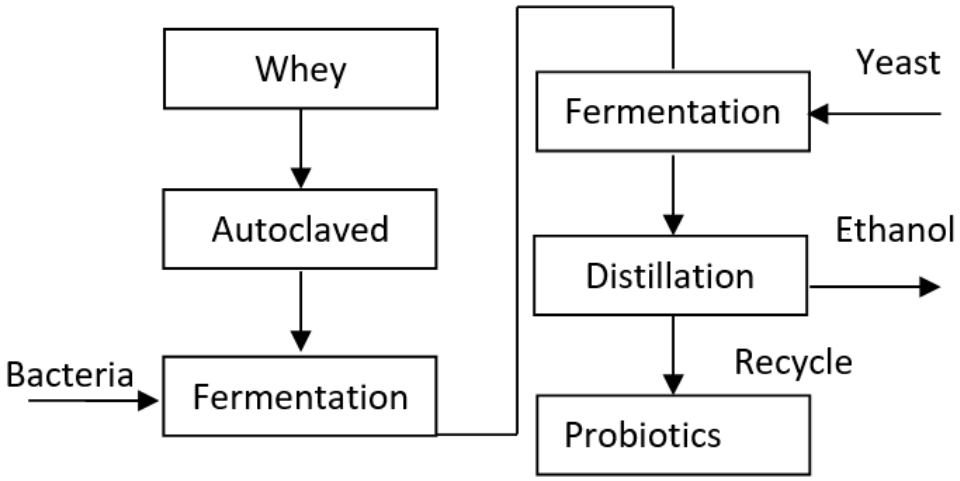

Figure 12 Details method of fermentation of whey using co-culture of bacteria and yeast.

3.9.1 Inoculum preparation

100µl of S. cerevisiae from glycerol stock was dissolved in 5 mL sterilized growth medium containing 1% yeast extract, 2% peptone and 2% dextrose. A loop of this solution was streaked on an agar medium, containing 1% yeast extract, 2% dextrose, 2% peptone and 2% agar, in a Petri dish. The Petri dish was then placed in a controlled environment incubator at 35°C and left until visual growth appeared. The yeast culture was then scooped from the surface of the agar into 100ml of pasteurized PDA in the sterilized Erlenmeyer flask. The Erlenmeyer flasks were then capped with nonabsorbent cotton plugs and mounted on a controlled environment shaker. The shaker was operated at a speed of 250 rpm for 48 h. Following the 48-h growth period, the yeast cultures were transferred to the fermenting broth. Yeast nutrients were used at appropriate amount to insure the necessary nutrients for the yeast. pH of the broth was adjusted around 4 with citric acid.

The fermenter and all accessories were chemically sterilized using a 2% potassium meta-bisulfite solution and washed with hot distilled–deionized water several times before starting the experiment in order to remove any chemical traces. The fermenter was filled with 1000 mL of whey media that was previously metabolized by bacteria in section 3.8 then; 100 mL of the active yeast inoculum (10% by volume) was added.

3.9.2 Sampling and analysis

Samples were drawn from the fermenters at 0 h and then every 12 h form the 48 h. The cell number, pH, temperature and ethanol content of the broth were monitored continuously.

3.9.3 Ethanol estimation by di-chromate method

Reagent potassium dichromate was prepared by dissolving 10gm of pure potassium dichromate in 100ml of 5M H_2SO_4. For standard curve, stock solution of 20 mg/ml was prepared by dissolving 2.537ml of 99% ethanol in 97.4626 ml of distilled water and different concentrations of ethanol working solution i.e., 0,1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/ml were prepared by diluting the stock solution with RO water. In fresh test tubes 1 ml of Tri-n-butyl phosphate (TBP) and 1 ml of each working solution of ethanol were mixed in a 2 ml Eppendorf tube and then vortexed vigorously using a vortex mixer for 12 minutes and it was subjected to centrifugation at 12000 rpm for 2 minutes. After phase separation, 750 µl of the solvent upper phase was transferred to a new Eppendorf tube to which 750 µl of dichromate reagent was added and vortexed vigorously for 12 minutes. It was then centrifuged at 12000 rpm for 2 min. and upper phase was completely discarded as far as possible using disposable pipette tips. After this, 500 µl of oxidized ethanol (chromium ions come to +3 state from its oxidation state +6; oxidized ethanol is green in colour) containing lower phase was pipetted out and optical density was measured and then standard calibration curve was constructed using optical density of oxidized dichromate by ethanol at 595nm with respect to concentration.

Sample's OD were also measured by following the same procedure as for standard curve and by comparing, ethanol concentration of the samples was then calculated by using standard curve.

3.9.4 Distillation

Ethanol distillation was carried at two different conditions dependent in temperature (heat applied). In first case, traditional technology of distillation was carried at 60-60°C

temperature and distilled was collected by condensation, whereas another set of distillation process was carried in which vacuum was applied in rotary evaporator apparatus and temperature was kept below 30°C under vacuum to collect distillate. Distillation was carried for complete removal of ethanol from whey wine and for this ethanol content in broth was examined by di-chromate ethanol estimation method explained in 3.9.3 to ensure zero ethanol in whey wine.

3.9.5 Cells viability test

Organisms that were used in this study were still present in the fermentation broth (whey wine) during the entire ethanol distillation process. To ensure viable cells in whey fermented broth, organism's colony count was done by top agar pour plate methods. For this specific media was prepared by using selective carbon source d-tryptophan for *B. megaterium*, maleic acid for *B. subtilis*, MRS media for *lactobacillus* and PDA with chloramphenicol for yeast (detail composition was given in index). Inoculum was taken from the fermentation broth and after serial dilution with the help of top agar poured in plate agar and colonies were counted after 48 hours.

3.10 In vitro test of the probiotic nature of organisms

Following tests were carried to examine the pre- assumed strain of bacteria and yeast was having probiotic natures. Single colonies of each of 4 organisms (3 bacteria and yeast) were taken from their respective specific media and were grown in LB media (for bacteria) and in SDB (for yeast) and were incubated for overnight for the following probiotic tests.

3.10.1 Survival at different temperatures

The effect of temperature on the growth of strains was examined by adding 100 µl of the overnight cultures into broth and was incubated at 25, 30, 37 and 42°C for 24 hours and viability was measured by pour plating method.

3.10.2 Bile tolerance

100 µl of the active overnight cultures were added into broth with different concentrations (1%, 2%, 3%, 4% and 5% w/v) of oxgall. The bile tolerance of strains was evaluated by estimating the number of viable cells after 0, 24, and 48h incubation at 37°C.

3.10.3 Survival at low pH

The growth at acidic pH was evaluated by inoculating of 100 µl of overnight cultures into broth by initial pH of 1.5, 2.0, 2.5, 3.0, 3.5, 4 and 5.0. The media were incubated at 37°C and the number of viable cells was determined after 0, 24, and 48h incubation.

3.10.4 Survival in gastric juice

To determine the viability of strains in the presence of pepsin, simulated gastric juice was prepared by suspending 3 mg/mL pepsin in sterile NaCl solution (0.9% w/v) and adjusted by HCl to achieve pH 2.3 and was inoculated with 20 µl overnight cultures. After 90 min of incubation at 37°C, 10 µl of the suspension was added to broth media (LB for bacteria and SDB for yeast) and incubated at 37°C. Then, the viable organism's cells were enumerated on agar plates after 48 h of incubation.

3.10.5 Stability test

To examine the stability of presumptive probiotic organisms, the fermented whey wine containing consortium of all four organisms were charged on stability chamber in, both RT and RH chamber and their viability was tested by pour plating top agar method after 100 times dilution and colonies forming units (CFU) were counted.

3.10.6 Pathogen test of probiotics (IP volume-1, 2014 38-50 P.N.)

After the stability check of the fermented whey wine drinks for viable count of probiotic organisms, checking of cross contamination of pathogens in drinks becomes compulsory for possible candidates of probiotics WHO prioritized pathogenic test was carried by the method explained in IP (Indian Pharmacopeia).

3.10.6.1 *Pseudomonas aeruginosa* test

Presumptive probiotic culture was inoculated in 10 ml of Casein Soyabean digest broth and was incubated at 30° for 24 h. After that subculture on a plate of Cetrimide agar was carried and again incubated at 30° for 48 h and colonies were observed.

3.10.6.2 *Staphylococcus aureus* test

Presumptive probiotic culture was inoculated in 10 ml of Casein Soyabean digest broth and was incubated at 30° for 24 h. After that subculture on a plate of Mannitol salt agar (MSA) was carried and again incubated at 30° for 48 h and colonies were observed.

3.10.6.3 *Escherichia coli* test

Presumptive probiotic culture was inoculated in 10 ml of Casein Soyabean digest broth and was incubated at 30° for 24 h. After incubation the broth was shaken well and 1 ml broth was transferred to 100 ml of MacConkey broth and was incubate at 44°c for 24 hrs. Finally, loop full of inoculum was taken and then sub cultured on MacConkey Agar plate. Colonies were observed after 48 h incubated at 30°c.

3.10.6.4 *Salmonella* test

Presumptive probiotic culture was inoculated in 10 ml of Casein Soyabean digest broth and was incubated at 30° for 24 h. After incubation the broth was shaken well and 1 ml broth was transferred to 100 ml of Rappaport Vassiliadis Salmonella enrichment broth and was incubate at 30°c for 24 hrs. Finally, loop full of inoculum was taken and then sub cultured on Wilson and Blair's BBS agar plate. Colonies were observed after 48 h incubated at 30°c.

3.10.6.5 *Shigella* test

Presumptive probiotic culture was inoculated in 10 ml of Casein Soyabean digest broth and was incubated at 30° for 24 h. After incubation the broth was shaken well and 1 ml broth was transferred to 100 ml of GN broth and was incubate at 30°c for 24 hrs. Finally, loop full of inoculum was taken and then sub cultured on Xylose lysine deoxycholate Agar medium. Colonies were observed after 48 h incubated at 30°c.

CHAPTER-4

RESULTS AND DISSCUSION

4.1 Collection of whey samples

10 different samples of whey were collected from the different locally available small dairies in Birgunj Metropolitan city. These dairies were producing cheese, sweets, paneer as results residual liquid, whey was produced. Field observation in those dairies showed that in most of the cases whey was considered as normal valueless left out liquids and were threw simply into the gutter without any treatment as waste.

4.2 Whey characterization

4.2.1 Physical

Depending upon the type of milk used and the methods by which milk was coagulated, variable pH and color was observed. Samples taken from sweets shops were white in color with pH 5.5 in average whereas golden-yellow color whey having pH around 3.5 was observed for cheese and paneer whey samples. The major reasons for the variations in color and the pH is due the methods of coagulating the milk and the sources of the milk.

Figure 13 Sweet (left) and Cheese whey.

Whey is highly perishable due to its high organic load, needs to be collected hygienically and utilized soon after manufacture. In our work, whey samples were first clarified by sieving to remove solids if presence and autoclaved to remove if any organisms present. These steps were taken to enhance the life of the whey and thus, can be used as starter medium in a wide range of applications.

4.2.2 Lactose estimation

4.2.2.1 By °Brix

All 10 whey samples were tested in this method and average °Brix was found to be 5.5. Standard data in Casper,1998 suggest that 6-6.5 TSS present in whey. The measurement was done by dripping the liquid whey on the detector. During wine making the total sugar present in the broth was measure by this method as major portion of solid in liquid is sugar. This result indicates that the sugar content was roughly 5% in the whey samples as TSS value mostly equivalent with the amount of sugar present in the solution with very small portion of soluble proteins, amino acids and other organic materials (Hadiwijaya *et. al.,* 2020). Moreover, lactose is the only dominant sugar present in the milk (Costa et al. 2013) and its major portion was come along with the whey during cheese making.

4.2.2.2 By DNS method:

Calibration curve was constructed by plotting the absorbance taking from the set of solutions having known lactose concentration.

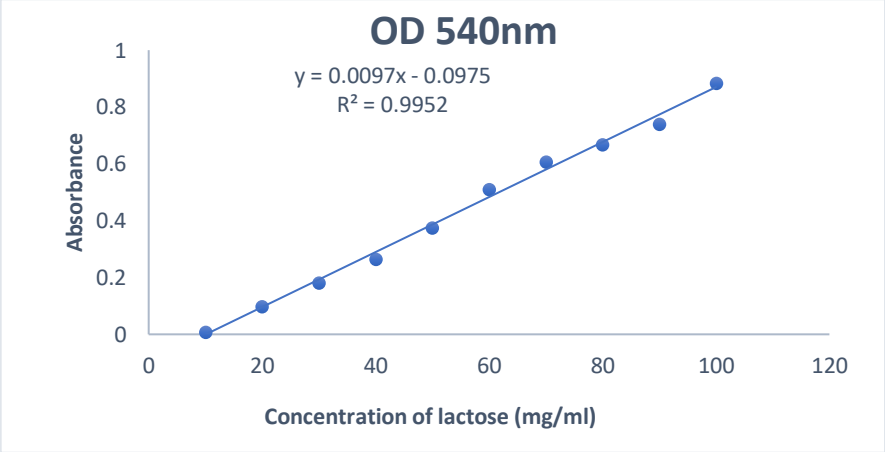

Figure 14 Calibration curve for DNS test

The concentrations lactose in unknown whey samples were then derived from the standard curve by taking absorbance at 540nm wavelength. The average lactose concentration in whey samples were found to be 4.1% (41mg/ml) which is seemed to be accurately measured as it 4.14% in most of the literatures (Smithers, 2015). In milk total sugar is around 5.2% (https://foodstruct.com/compare/milk-vs-whey). This method is used extensively in biochemistry for the estimation of reducing sugars. It detects the presence of

free carbonyl group, aldehydes or ketone of reducing sugars. During this reaction 3,5-dinitrosalicylic acid (DNS) is reduced by carbonyl group of reducing sugar to 3- amino-5-nitrosalicylic acid (ANSA) which under alkaline conditions converted to a reddish brown colored complex which has an absorbance maximum of 540 nm.

This results indicates that most of the carbohydrate present in the milk is washed in whey during cheese manufacturing.

4.2.3 Nitrogen estimation

In solution, nitrogen is present in the dissolved form as NH_4OH. By calculating the concentration of ammonium hydroxide in whey, total nitrogen content can be estimated. Standard curve was constructed by Nessler's method using the solution with the known concentration of NH_4OH in distilled water. R^2 value was found 0.988 which indicates the prepared graph is reasonably accurate as standard calibration curve.

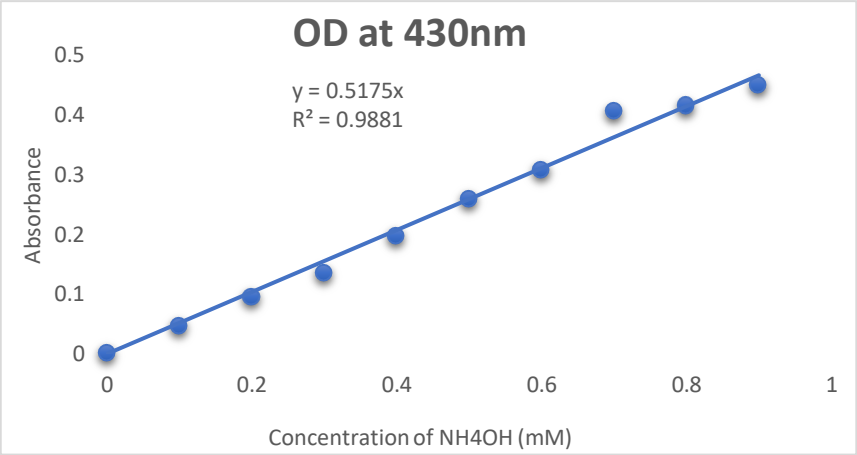

Figure 15 Standard calibration curve for Nessler's test.

Absorbance of all the whey samples was taken by the same procedure as standard and the concentrations were calculated by using equation y = 0.5175x (from figure 4.2.3). Average nitrogen as ammonium hydroxide was found to be 7.26mM.

$$2[HgI_4]^{2-} + NH_3 + 3OH^- \longrightarrow Hg_2ONH_2I + 7I^- + 2H_2O \quad \text{(eq. 1)}$$

K_2HgI_4 and KOH are the working component of the Nessler's reagent in which iodide and mercury ions react with ammonia under alkaline conditions to produce a reddish-brown

complex Hg_2ONH_2I as shown in equation (1), which absorbs strongly at 420 nm. The absorbance of the resulting reddish-brown complex is directly proportional to the ammonia concentration.

4.2.4 Protein estimation

Total protein present in the solution can be calculated by Lowry's protein estimation method. Standard curve was constructed with the absorbance against concentrations of standard protein. 0.99 R^2 value validates the standard calibration curve by this method for estimation of protein concentration. An average concentration of proteins in whey samples was fond to be 0.98%. Protein content is very low in whey samples as compared with the raw milk which is around 3.5% as this data gives the total protein present in sample as most of the protein portion of milk is separate by coagulation (Casper, 1998 & Smithers, 2015).

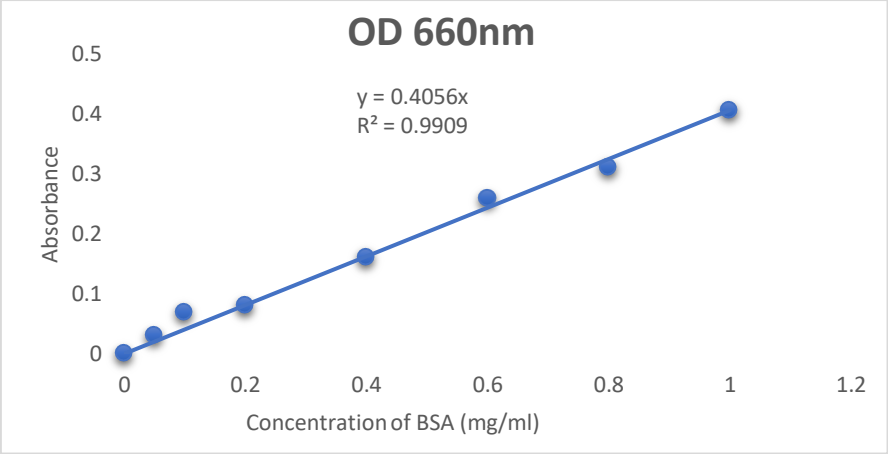

Figure 16 Calibration curve for Protein estimation

In this method, the peptide nitrogen reacts with the copper ions under alkaline conditions and the subsequent reduction of the Folins reagent to hetero poly-molybdenum blue by the copper-catalyzed oxidation of aromatic acids. This method is sensitive to pH and low concentrations of protein. To nullify the pH limit we had taken less pH adjusted volume which have no effect in reaction mixture.

4.2.5 Phosphate estimation

Soluble phosphate was calculated by the single- solution reagent method in which sulphuric acid, ammonium molybdate and ascorbic acid react simultaneously. By using potassium dihydrogen phosphate as standard, calibration curve was constructed by taking absorbance

at 827nm. By following the same procedure as standard solution, absorbance of all the whey samples was calculated. Putting those absorbed values in standard curve and taking average of all the calculated data phosphate concentration in whey samples were found 4.59 µg/ml.

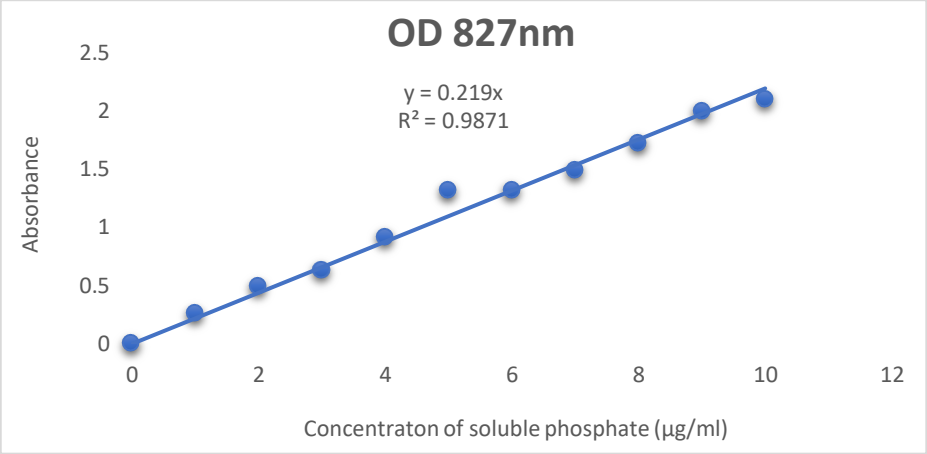

Figure 17 Standard curve for Phosphate estimation.

4.2.6 Calcium estimation

Titration was carried with EDTA using Patton-Reeder as indicator and by taking the reading of volume of EDTA consumed to reach end point, calcium content in whey was calculated. In an average 0.39 g/L calcium content was observed in whey samples. In milk around 0.49 g/L (Rodriguez *et al.,* 2001) is present and standard of whey calcium is 0.365 g/L (Wong *et al.,* 1978).

Calculation:

$$Ca^{2+} \text{ ions concentration (g/L)} = \frac{\text{Molarity of EDTA} \times \text{volume of EDTA consumed (L)} \times 40.08}{\text{Sample taken (L)}}$$

$$= 0.39 \text{ g/L}.$$

The reaction is:

$$Ca\text{-}PR + EDTA^{4-} \rightarrow PR + [Ca\text{-}EDTA]^{2-}$$

In this method, EDTA molecules form blue colored complexes with calcium ions. A blue dye called Patton and Reeder's indicator (PR) is used as the indicator. This blue dye also forms a complex with the calcium ions changing color from blue to pink/red in the process, but the dye–metal ion complex is less stable than the EDTA–metal ion complex. As a result, when the calcium ion–PR complex is titrated with EDTA the Ca^{2+} ions react to form a stronger complex with the EDTA. The precise concentration of Ca^{2+} in the sample solution may well vary considerably depending on the nature and source of the sample. To obtain good results, the average titer volume should ideally be between 10 and 30 ml.

4.2.7 Vitamin quantification

4.2.7.1 Vitamin B_6 quantification

Standard curve was constructed by using known solutions of pyridoxine hydrochloride as standard substance. The calibration curve shows good accuracy by the method explained in IP. The same procedure was followed and the absorbance of the whey samples were taken. By computing those in calibration curve, concentration of B_6 vitamin in whey found as 0.0098 mg/ml. Around 0.037mg/100gm is present in milk whereas standard data in whey was found as 0.031 mg/ 100gm (https://foodstruct.com/compare/milk-vs-whey). This results indicates some error in procedure though we used standard protocol.

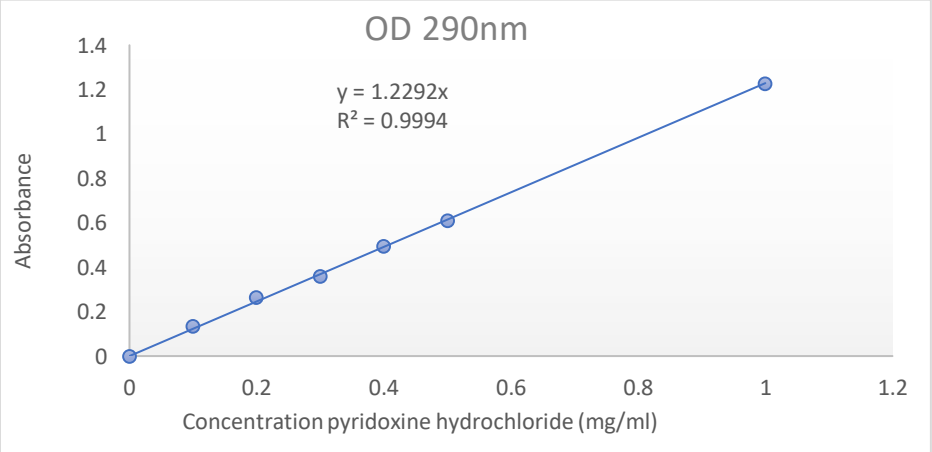

Figure 18 Standard curve for Vitamin B_6.

4.2.7.2 Vitamin B2 quantification

By using pure amorphous riboflavin known sets of solutions were used in the reactions and by taking their absorbance standard curve was plotted. 0.99 R^2 value of this curve validates

it as standard calibration curve. By using this curve, concentrations of the all samples of whey were calculated and by taking their average value, the concentrations of Vitamin B2 in whey samples were found as 0.0016 mg/ml. This data suggests that significant amount of vitamins present in whey. Comparatively, 0.00158mg/gm this vitamin was recorded in papers (https://foodstruct.com/compare/milk-vs-whey).

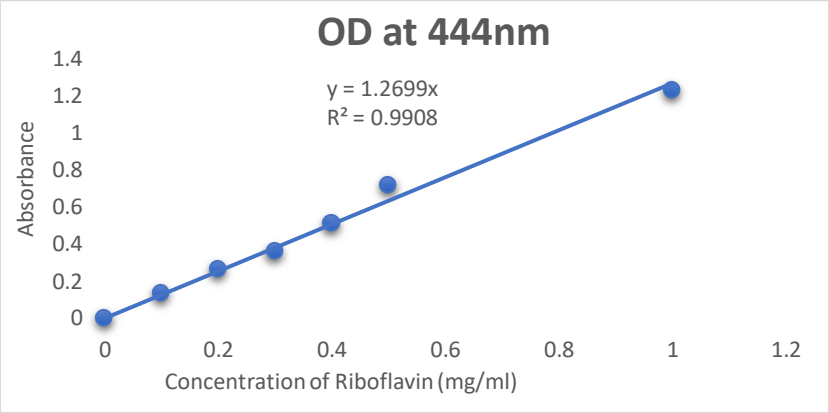

Figure 19 Standard curve for vitamin B_2.

4.2.8 Biological oxygen demand (BOD) calculation of whey samples

Biological oxygen demand of whey samples was calculated by using Winkler 5 day's method.

Chemistry involved in DO calculation:

$2Mn^{2+}$ (aq.) + $4OH^-$ (aq.) + **O_2** (aq.) ⟶ **$2MnO_2$** (s) + $2H_2O$ (L) ——— (1)

$2MnO_2$ (s) + $2I^-$ (aq.) + $4H^+$ (aq.) ⟶ Mn^{2+} (aq.) + **I_2** (aq.) + $2H_2O$ (L) ——— (2)

I_2 (aq.) + **$2S_2O_3^{2-}$** (aq.) ⟶ $S_4O_6^{2-}$ (aq.) + $2I^-$ (aq.) ——— (3)

Here, the equations (1), (2) & (3) gives the chemistry involved in the calculation of dissolved oxygen in the samples. The highlight compounds are involved in which number of oxygen present was determined by titration with sodium thiosulfate in alkaline iodine and manganese dioxide solution using starch solution as indicator.

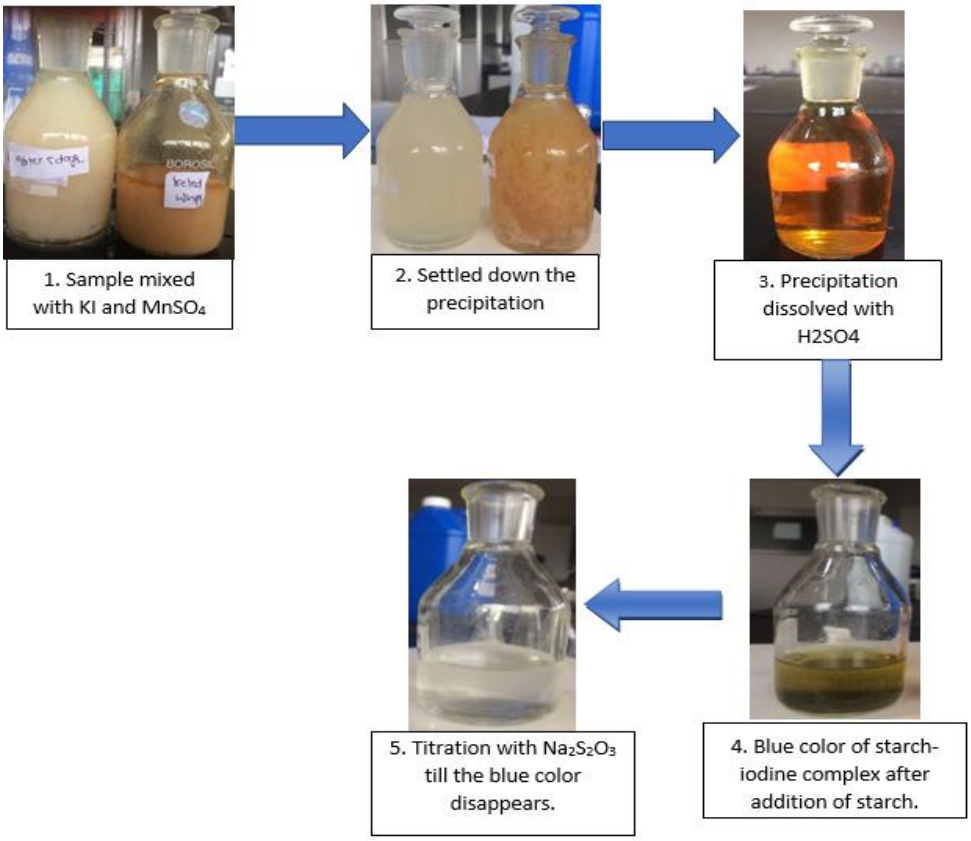

Figure 20 Steps involved in DO calculation of whey.

Calculation:

Dissolved oxygen at initial day (D_0):

Temperature of whey = 11°C

Volume taken for analysis = 40 ml (50 times diluted)

Volume of $Na_2S_2O_3.5H_2O$ used in titration = 11 ml ± 0.1 ml

$Na_2S_2O_3.5H_2O$ solution made from 1.45gm of $Na_2S_2O_3.5H_2O$ made up to 100.00 ml.

i.e. 0.0584 mole/ L.

Now, concentration of $Na_2S_2O_3.5H_2O$ in solution utilized = mole/volume

Mole of $Na_2S_2O_3.5H_2O$ Utilized = volume utilized × concentration of $Na_2S_2O_3.5H_2O$

= 11/1000 L × 0.0584 = 6.4×10^{-4} Mole.

Equations (1), (2) and (3) indicates that presence of one molecule of oxygen titrate 4 moles of $Na_2S_2O_3.5H_2O$ so,

Mole of oxygen = 6.4×10^{-4} Mole / 4

= 1.6×10^{-4} Moles.

Now, $\mathbf{D_0}$ = mass of solute / volume taken

= $1.6 \times 10^{-4} \times 32.00 \times 50 / 0.04$

= 6.4gm O_2 per liter.

Again the same procedure was followed to calculate D_5 after 5 days' incubation at 20°C and was found to be 0.9gm O_2 per liter.

Thus, **BOD of whey** = $\mathbf{D_0 - D_5}$

= (6.4-0.9) gm O_2 per liter

= 5.5 gm O_2 per liter

= 5500 mg O_2 per liter

BOD is the amount of oxygen consumed by microorganisms in the decomposition process. Average BOD of our whey samples was found 5500 mg O_2 per liter. This result indicates that the whey is the reach source of different organic compounds.

Table 10 Summary of analysis result of parameters of whey samples.

Characteristics	**Value in whey**
Color	White & golden-yellow
pH	3.5
°Brix	5.5
Lactose	**4.1%**
Protein	0.98%
Soluble nitrogen	7.26 mM

Soluble phosphate	4.59 µg/ml
Calcium	0.39 g/L
Vitamin B_2	0.0016 mg/ml
Vitamin B_6	0.0098 mg/ml
BOD	**5500 mg O_2 per liter**
Total calorific value	32Kcal

The complete characterizations of whey samples were carried. Results explain whey as one of the competent residue left out from the dairy industries. Its high nutrition content in addition with the presence of all required minerals and vitamins opens it as a good candidate for the microbial growth substrate. Its high BOD and huge production quantity always possessing hugs management and environmental challenge for the researchers. Whey is a very interesting product due to its components. Their properties, functions and chemistry structure make whey a great base for the creation of a series of new products or an ideal alternative compound to more traditional ones. In terms of whey utilization there are so many things that can be done instead of treating whey as a waste.

Irrespective, the origin and types of whey, it presents different challenges due to its mineral composition, protein and lactose contents. However, careful analysis of whey composition, properties, and potential leads us to believe that it has very high value that has yet to be tapped. Perhaps the way to commercialization and valorization of whey rests a fundamental development in which a more scientific and fundamental understanding of whey as a whole matrix or on each of its components is needed. For example, a better understanding of the role of lactose in nutrition for probiotic organisms or how the whey would help a variety of fermentations to produce high-value components like bioethanol. Commercialization of whey based processes has been the hallmark of whey utilization, and this process has taught us valuable lessons on the environmental impact of valorization strategies as we wait for better solutions for the new processes to develop. We propose, in conclusion, that whey components, novel processes, and knowledge of their effects in nutrition and complete

probiotic media and new substrate for bio-ethanol will show us the way to its optimal utilization, valorization, and concomitant benefit to the dairy industry.

4.3 Organisms' isolation and characterization

In our work, bacterial strains were selected with the initial thought of their probiotic nature and having the ability to utilize lactose as sole carbon source. Likewise, yeast strain was selected for the bioethanol production and probiotic capability.

In lab, *Bacillus megaterium* & *B. subtilis, Lactobacillus spp.* and *Saccharomyces cereviseae* were revived in their selective carbon sources and then in LB where they showed good growth condition.

Figure 21 Revive colonies of Presumptive probiotic organisms in their specific growth media.

4.3.1 Gram's staining

The Gram staining is done in initial stages of the study to confirm the type of bacteria in the inoculums and to characterize the initial features of the organism present in the inoculums. All three bacterial strains hold the crystal voilet stain and purple blue rod shaped cells were observed which indicates Gram's positive bacterial strains. Where as cells of yeast which were in good condition stain a bright blue with methylene blue.

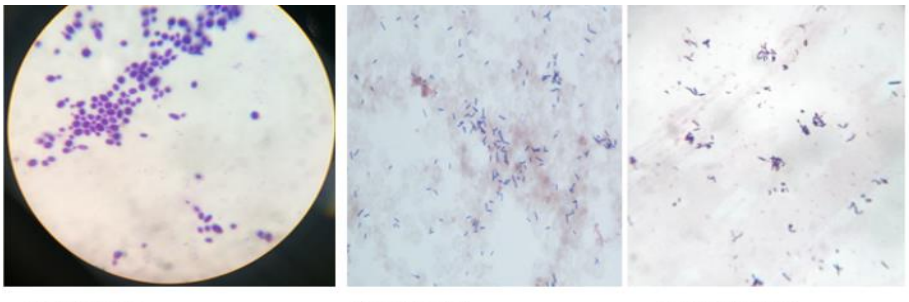

Figure 22 Gram's staining of organisms seen in light microscope.

Table 11 Cell morphology of BM- 13, BS- S4S1 and LB- D1.

Label	Shape	Gram	spore
BM- 13	Rod	Positive	Sporous
BS- S4S1	Rod	Positive	Sporous
LB- D1	Rod	Positive	Non-sporous

Staining is the one of the reliable and quick initiative first step either for validating or conforming the microbial strains. BM- 13 and BS- S4S1 is spore forming bacteria which can withstand extreme nature. Vegetative propagation in yeast cells were seen in 100X magnification.

4.3.2 Biochemical test of the bacterial strains

Table 12 Result of biochemical test of BM-13, BS-S4S1 and LB-D1.

S.N.	Test	BM-13	BS-S4S1	LB-D1
1	Starch hydrolysis	+ve	+ve	-ve
2	MR	+ve	+ve	-ve
3	VP	+ve	+ve	-ve
4	Citrate	+ve	+ve	-ve
5	Catalase	+ve	+ve	-ve
6	Urease	+ve	-ve	-ve
7	OF	+ve	+ve	+ve
8	Sulphur	-ve	-ve	-ve

9	Indole	-ve	-ve	-ve
10	Motility	+ve	+ve	-ve

The cultures were characterized through a number of microbiological and biochemical tests. The colonies re-identified as catalase-positive, Gram-positive, endospore-forming rods as BM-13 (*Bacillus megaterium*) though exception is in VP test. Likewise, urease negative spore forming strain ensured as BS- S4S1 (*Bacillus subtilis*) with exception in MR test and the inoculum with colonies showing non- sporous, gram positive and these entire test negative except OF test validate as LB-D1 (*Lactobacillus)* strain.

4.3.3 Lactose utilization test

In our project whey was taken as sole nutrient source in which lactose is the major carbon source. To show the lactose utilizing ability of our organisms we carried following test.

4.3.3.1 MacConkey test

This is a selective and differential culture medium designed to selectively isolate enteric bacteria and differentiate them based on lactose fermentation (MacConkey, A., 1905). The media detects lactose fermentation by bacteria with the pH indicator neutral red.

Figure 23 Growth of organisms in MacConkey agar plates.

Lactose fermenting organisms show pink colonies due to the presence of neutral red dye. Colonies were observed after 24 h incubation though color change was not observed. Good growth turbidity was also observed in MacConkey broth. The inability of our presumptive probiotic strains to change color in MacConkey was may be due to the slow lactose

fermenter. The result in this section does not provide us a clear nature of our strains ability on lactose fermentation. For this we carried another set of experiment in which we grow organisms in NA media diffused with X-gal.

4.3.3.2 X-Gal utilization

Nutrient agar medium supplemented with X-Gal was used for conforming the β-galactosidase producing organisms there by observing the lactose fermenting nature. Incorporation of the chromogenic substrate (X-Gal) into screening media was reported for selecting various β-galactosidase producing microorganisms (Kamel *et.al.,* 2016). All three bacterial strains BM-13, BS S4S1 and LB-D1 grow in NA without any problems and colonies were observed blue in color indicates lactose fermenting nature. Likewise, colonies of yeast SC-D2 was also observed with same color as that of control.

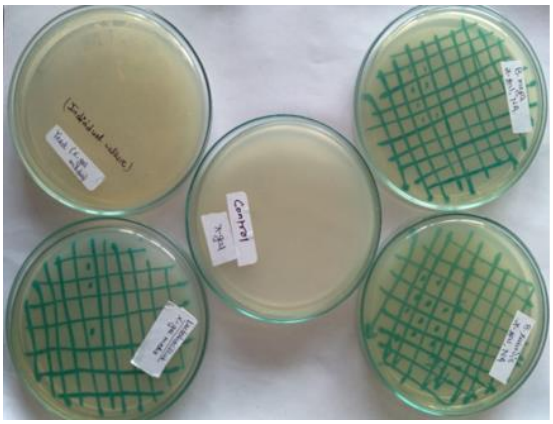

Figure 24 Growth of organisms in NA with X-Gal.

4.4 Mead fermentation

Batch fermentation of honey was successfully carried in lab. Regular monitoring was carried. Visual observation showed that the initial days were quite may be due to the stress environment for yeast cells. Rapid fermentation starts after the start of second week and then it continued till the end of the third week of fermentation. After that the constant microbial activities was observed.

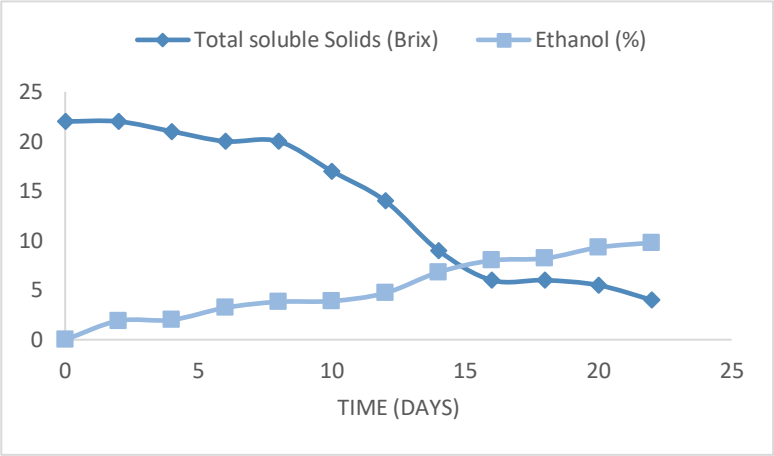

Figure 25 Graph showing the changes in °Brix with fermentation progress and ethanol production.

Monitoring the pH in mead fermentation is the crucial factor which depends upon the acids in the whey and the level of carbonates in water used. However, the honey and water mixture is poorly buffered, and its fermentation can quickly drop the pH to a level below which the yeast are inhibited. In my work, 1% citric acid was used for the balancing the pH initially and once, the pH of broth was controlled then no acidic stress was observed during the entire fermentation process.

In process analysis of pH and °Brix was carried to observe the fermentation progress. Once, the fermentation was completed and processes like racking, siphoning and filtration were carried, yield, °Brix, color, taste, final pH, methanol and ethanol analysis was done.

4.4.1 Ethanol estimation

Weight of empty specific gravity bottle W_1=25.865gm

Weight of specific gravity bottle with distilled water W_2 =76.11 gm

Weight of specific gravity bottle with ethanol W_3=75.45 gm

Weight of water = $W_2 - W_1$

=76.11-25.865

=50.242 gm

Weight of ethanol =$W_3 - W_1$

=75.45-25.865

=49.585 gm

Specific gravity of ethanol = Mass of ethanol / Mass of water

= 49.585/ 50.242

= 0.987

Specific gravity of water= 0.997 at 20°C

Density of ethanol = Specific gravity of ethanol × Specific gravity of water

= 0.987 × 0.997

=0.984g/ml at 20°C

From comparing this density of ethanol on standard data 0.984g/ml value is equal to around 11% ethanol by [v/v] 20°C.

4.4.2 Methanol estimation

Intense red color was observed for standard sample whereas faint red color was seen for test sample and after 50 times dilution for each absorbance was observed under 575nm wavelength which gave 0.17 reading on an average for sample and 0.7 for standard.

Methanol (%V/V) = Sample OD / Standard OD × 0.025 × dilution factor

= 0.17 / 0.7 × 0.025 × 50 = = 0.29% (V/V) at room temperature.

Table 13 Analysis result of mead after 3 weeks of fermentation.

S.N.	Parameter	Result
1.	Yield	2L
2.	°Brix	4.7
3.	Color	Clear yellowish
4.	pH	3.8
5.	Ethanol	11% (V/V)
6.	Methanol	0.29% (V/V)

Quality of mead depends on the source of honey, yeast and additives used. Heating of honey during must preparation has influenced the aroma and taste of the mead. Unheated dilute experimental honey solution fermented with inoculated yeast for 21 days at room temperature produced mead acceptable to sensory assessor. Commercial yeast strain used in our experiment shown good fermentation capability with good sugar-ethanol conversion rate. Yield was found to be around 82% and probable reason for this comparatively less yield was due to the high acidic condition of honey, wild microbial load in honey and environmental stress. Ethanol concentration in final mead was found to be 11% which is excellent in lab practice. In most of the cases ethanol content in fermented product depends upon the nature of yeast strain, batch and anaerobic condition and the acidity of the broth as high percent of organic acid like lactic acid, acetic acid which may be produced due to the wild organisms that present in honey as in our case we used raw honey without sterilization to keep intact the honey's wild flavor in mead. Low alcoholic compounds like methanol were also produced in fermentation mead fermentation as by products. Spectroscopic method was implanted to estimate methanol content and was found to be 0.29% which is in acceptable concentration as 0.4% (v/v) methanol at 40% alcohol (V/V) provides a greater margin of safety (Paine *et. al.,* 2001). It is documented that mead has been prepared from the time of imomemorial, but barring a few investigations other appear to be that of home scale wine.

4.5 Break down whey lactose

Characterization of whey already give us nutritional values of whey and thus presumptive probiotic organisms were cultured in probiotic media in which whey was the major portion. Details of composition of probiotic media was mentioned in index section.

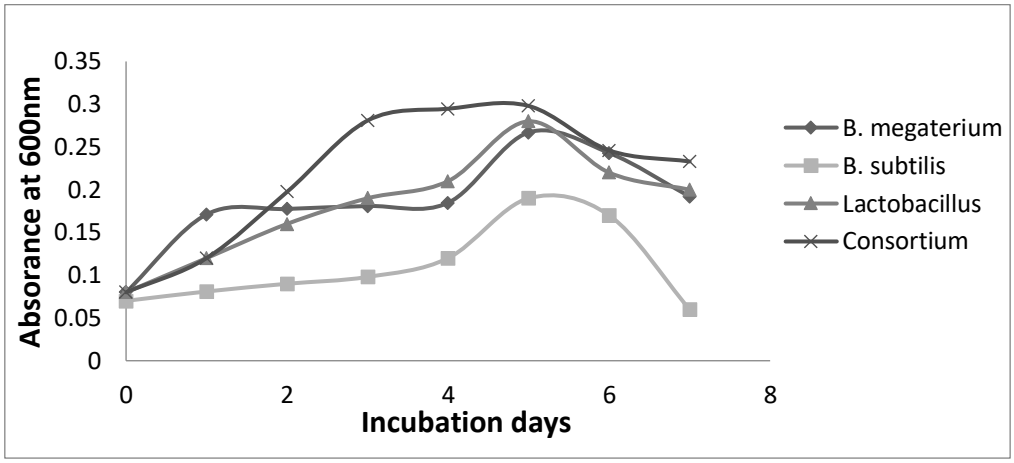

Figure 26 Curve showing the growth pattern of organism in whey media.

OD was taken at 24 h interval at 600nm and plotted in graph. These results indicate that all three organisms show good growth in whey. Among all three *lactobacillus* show maximum growth probably due to having dairy habitat naturally. Comparatively, consortium of all three bacterial growth pattern was highest than that of individual organisms. To breakdown and utilize the lactose sugar from the media, organisms must have β- galactosidase coding genes (Shaw *et. al.,* 2002).

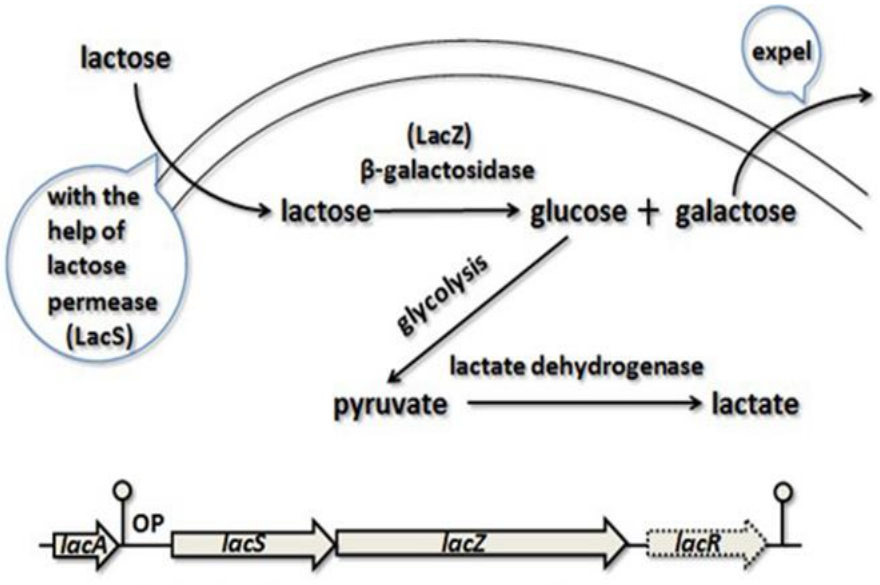

Figure 27 Lactose metabolisms in LAB (Adopted from HIT- Harbin Project- 2011).

This metabolism explain β-galactosidase derived lactose breakdown in LAB. In yeast *S. cerevisiae,* this whole system is not present due which unable to metabolize whey lactose alone. Thus, with series of trials and working experiments we established the co-culture method in which LAB bacteria and yeast strain was grown together in a fermentation batch. In this model, first LAB strains that we used, BM-13, BS S4S1 and LB-D1 were cultured in whey broth break down that lactose sugar into glucose and galactose by the mechanism explained in figure 4.5 (b). Then in the same broth the fermentation by *S. cerevisiae* was taken place as result bio-ethanol was produced.

4.6 Fermentation of whey wine

In this experiment, batch fermentation of size 1000ml was carried under controlled condition in laboratory with high aseptic condition. Due to the initial LAB activity in whey formed broth pH was already gone below 4 and was around 3.5 so, pH optimization for fermentation was not needed. Yeast nutrient was added to ensure no possible nutrition arrested condition during fermentation period. In process analysis of ethanol production and total carbon present in medium was carried during entire process.

4.6.1 Ethanol estimation

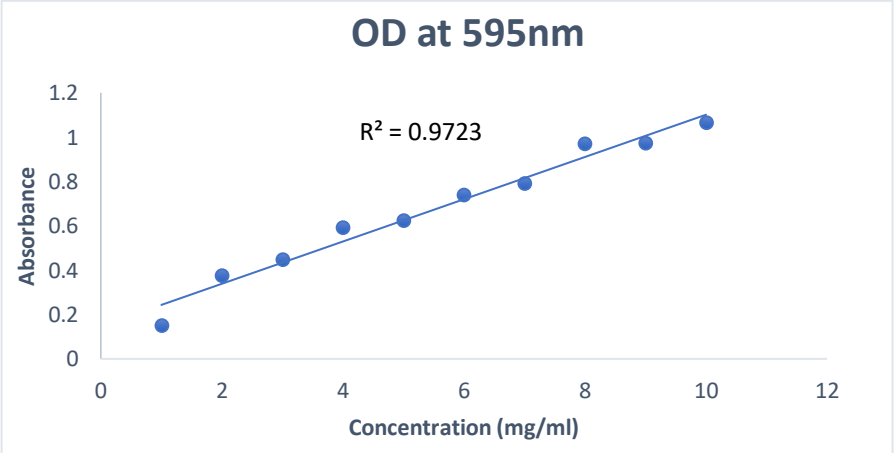

Figure 28 Standard curve for ethanol estimation by Di-chromate method.

Fermentation became stable after starts of the second week of the yeast inoculation. After the completion of fermentation ethanol, sugar, yield, pH, flavor and BOD was tasted. Standard curve was prepared by using the solution with known concentration of ethanol using Potassium dichromate method and distilled water is taken as blank. Samples were taken and compared samples absorbance with the standard curve which gives 0.92% ethanol in final whey wine. Overall ethanol yield (70-80%) of the theoretical value has been achieved by using whey lactose as fermentation substrate.

Table 14 Summary result of whey fermentation by co-culture.

S.N.	Parameter	Result	
		Before (Whey)	After (Whey wine)
1.	Yield	-	72%
2.	°Brix	5.5	1.6
3.	Color	Clear yellowish	Turbid white
4.	pH	3.8	3.5
5.	Lactose	4.1%	1.2%
5.	Ethanol	-	0.92% (V/V)
6.	**BOD**	**5500 mg O_2 per liter**	**1200 mg O_2 per liter**

Whey wine parameters mentioned in table was carried by the same procedure that we followed while characterized whey. This result indicates the sugar ethanol conversion rate of the yeast is 72%. The amount of ethanol produced per unit of sugar during the wine fermentation is of considerable commercial importance. The theoretical conversion of 180 g of sugar into 88 g of CO2 and 92 g of ethanol (or 51.1 % on a weight basis) could only be expected in the absence of any yeast growth and loss of ethanol as vapor (R. B. Boulton *et al.,* 2013). According to this concept whey fermentation by yeast should produce around 2% (V/V) ethanol but not in our case as only 0.92% ethanol was observed by di-chromate method. This probably due to the reason of unfavorable condition, competition for sugar source in co-culture inoculum and low lactose content in broth. The question as to how much yeast should be used in the fermentation is important in, terms of the time and economy of the fermentation. On the basis of the average number of grams of lactose fermented per gram of yeast per hour of elapsed fermentation time, it was established that a maximum amount of yeast corresponding to 2 per cent of the weight of the lactose

initially present is sufficient to ensure a satisfactory rate of fermentation (Rogosa *et. al.,* 1947).

Likewise, BOD calculation showed that only 1200 mg O_2 per liter in final wine broth that indicates that around 80%. Thus, this method can relief the environmental problem associated with the whey management. Around 1% of lactose was seen to be still present in the broth at the end of the fermentation is assume to support probiotic organism's nutrition.

From the percentage of ethanol produced in this method and total lactose available in worldwide contest, only in 2020 (near about 8 million tons) with 72% conversion efficiency could yield about 5 million tons of ethanol can be produced which is significant for the world fuel markets.

The transformation of the lactose in dairy whey into bioethanol is barely reasonably competitive with the at present recognized processes, utilizing sugarcane and cornstarch as raw substrates, or with rising second-generation technologies utilizing lignocellulosic biomass as substrate. But, being a waste effluent represents a benefit of whey over food feedstock's, like corn, for ethanol production. Furthermore, the ease of use of varied solutions for whey bioremediation is important, so that each dairy industry can assess, according to its own problems, and customize accordingly.

Feedstock costs are a major component to overall biofuel production costs. In most of the ethanol producing industries, feedstock accounts for as much as two-thirds of the total cost. Both sugar and starchy materials are consumed as human food and are thus potentially more expensive than a third alternative: cellulosic materials such as wood and fibrous plants however, breaking down cellulosic materials into sugar, however, are still expensive, and the cost of harvesting and transporting cellulosic feedstock to ethanol plants can also be high (Claassen *et al.,* 1999). So, by this method stable, good flavored, clear wines from whey are produced which may give a good alternative substrate for bio-ethanol production which is waste and easily renewable along with helping pollution reduction and providing a relatively inexpensive and agreeable beverage. The present study has indicated such a method in which all, or almost all, of the lactose in whey is fermented and bio-ethanol was produced.

4.6.2 Distillation

After the complete analysis of the whey wine, ethanol portion of the wine was distilled. Ethanol distillation of fermented whey wine was carried in lab successfully in varying temperature. The major difference in working conditions and observed results were summarized in table 4.7.

Table 15 Comparative results obtained during distillation of whey wine.

S.N.	Parameters/ results	Distillation methods	
		Conventional	Vacuum rotary evaporator
1.	Temperature (°C)	62-65	28-30
2.	Vacuum	-	Created
3.	Time consumed (for 1 liter mead)	20 minutes	More than 2 hrs.
4.	Condition of whey wine	More dark yellow	No change
5.	Cells viability	Nil	Viable

The low temperature in vacuum distillation ensures the presumptive probiotic organisms still viable in the fermented whey broth. In the fermented broth solution, ethanol estimation was carried by di-chromate method several times during the entire process of distillation to ensure zero ethanol to make it ethanol free whey fermented drinks by co-culture of probiotic (presumptive) and *s. cerevisiae*.

In most of the fermentation sugar is converted by yeast in which broth contains 6-12% of ethanol with trace of other compounds like aldehydes ketones, fossils oils, and methanol. The final step distillation to make water free ethanol consumes almost 50-80% of total energy consumed in ethanol fermentation process. The energy potential of traditional distillation technique always cited on criticizing the potential of ethanol production for bio-fuel. Likewise, Energy consumption greatly increased with decrease in ethanol

concentration in the feed below 4% ethanol (as in whey wine distillation), since a disproportionately large portion of feed must be vaporized to get same amount of ethanol products (Ladisch *et al.,* 1979). Designing highly efficient distillation processes is decisive in the competitiveness of bioethanol against fossil fuels. Among all operations involved in the production of fuel grade ethanol, the separation process takes up a large fraction of the total energy requirements. The application of optimization methods to the design of ethanol distillation allows exploring simultaneously the configurations and operating conditions that provide the best economic projections.

Vacuum distillation can be used for the production of anhydrous ethanol from aqueous ethanol. It is known that the concentration of ethanol in the ethanol–water azeotrope varies as the pressure changes [Black *et al.,* 1972]. The concentration of ethanol in the ethanol–water azeotrope increases with decrease in pressure. Below about 11.5 kPa, ethanol and water do not form an azeotrope and theoretically the components can be separated. High pressure operation also breaks the azeotrope but it leads to thermal decomposition. At high temperature chemical reaction occurs between the residual sugars present in the broth with the nitrogen compound and damaged the wash.

Fermentation creates waste by-products, such as spent fermentation liquor or stillage from alcohol production. These require further treatment to recover biomass for use as stock feed, or wastewater treatment prior to disposal (Durham *et al.,* 2007). The residue in the wash is either discharged to waste or evaporated which obviously need huge energy. Hence, this work was to establish the proof of concept that yeast along with well-known probiotic (presumptive) strains with probiotic features in the fermentation broth can be obtained as residue after fermentation followed by distillation as a by-product from the dairy industry, namely the conversion of lactose into ethanol**.**

4.6.3 Viability test

In this experiment, colonies from whey fermented broth were inoculated in LBA, SDA and NA plates diffused with X-Gal. In all agar plates TMTC colonies were seen. Blue colonies were observed in X-Gal indicating the strains were β-galactosidase producing presumptive probiotic organisms.

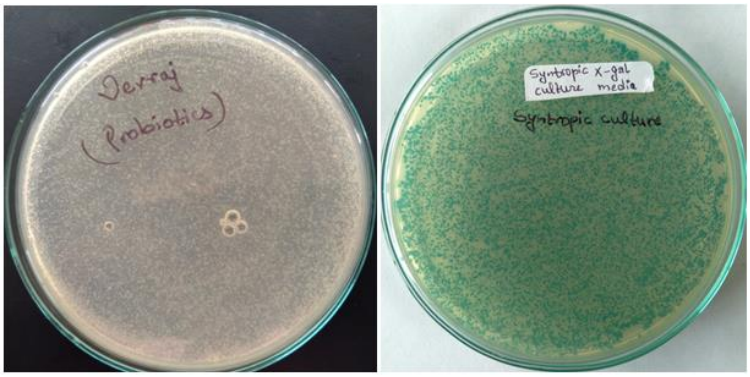

Figure 29 Mix colonies of probiotic organisms in LB plate (left) and NA with X-gal (right).

This result indicates the advantages of the distillation processes we adopted in which high temperature was cutoff. In ordinary distillation method wine was treated under temperature beyond 55°C to 60°C at which organisms might die.

4.7 Probiotic tests

4.7.1 Temperature tolerance test

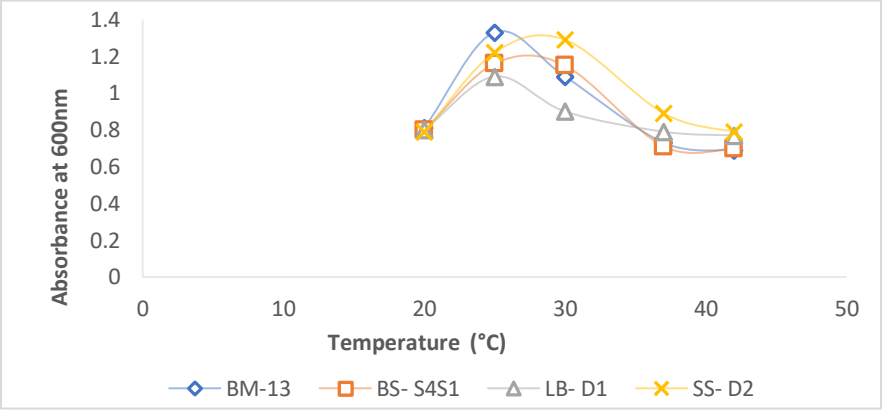

Figure 30 Effect of incubation temperature in growth of organisms.

The effect of different incubation temperature (25, 30, 37 and 42°C) on growth of the selected strains was shown in figure 30. All data points are the means of three replicates taken after 48 h of incubation. Generally, all of our presumptive strains showed good ability to grow at temperatures of 25-42°C. However, the growth of was reduced at 42°C.

4.7.2 Low pH tolerance test

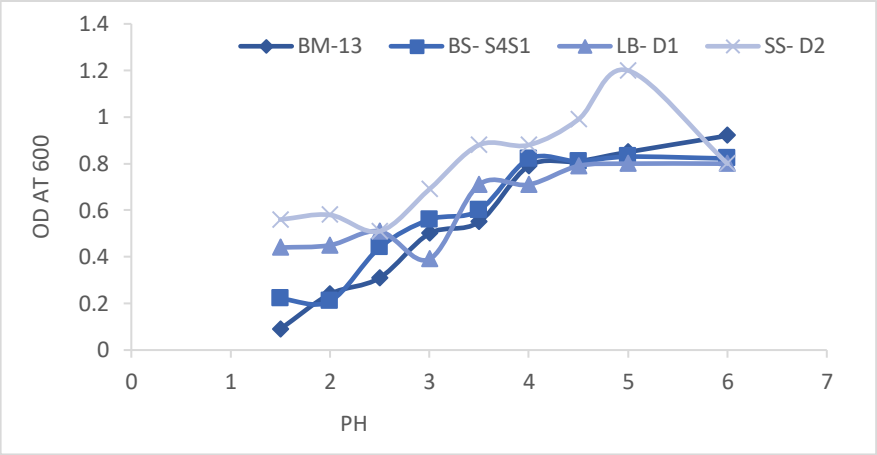

Figure 31 Ability of the selected probiotic strains to tolerate low pH.

4.7.3 Bile salt tolerance test

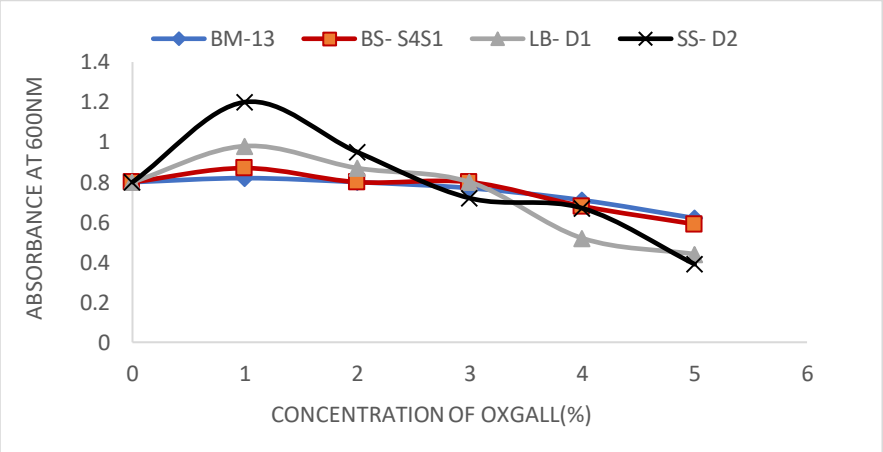

Figure 32 Survival and growth of the selected probiotics strains under high concentrations of bile salts.

All data points are the means of three replicates. Readings were taken after 48hrs of incubation.

4.7.4 Gastric juice tolerance test

Plates were observed after 48 h in which good viability of cells were seen as shown in figure 4.7.4. This means presumptive strains have good tolerance of gastric juice and can

successfully survive in gastro-intestinal track of human being and can colonize in the intestinal cells there by maintain the good healthy environment for host body.

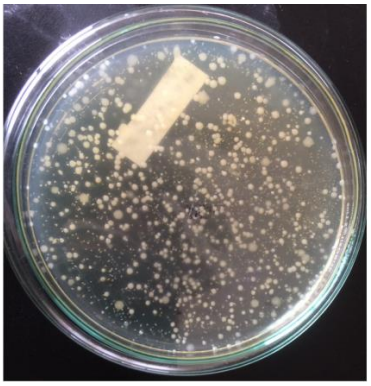

Figure 33 Mix colonies of organisms in NA plate

4.7.5 Stability test

After testing all the parameters of probiotic nature of the presumptive strains, their viability test in whey fermented broth was carried by treated the broth in RT (relative temperature) and RH (relative humidity) chamber in Alpha agro R & D laboratory to ensure the stability in fermented broth.

Figure 34 Mix colonies in LB plate after 28 days in RT and RH chamber

Colonies were observed after 10^{-5} time's dilution and more than 10^{8} CFU/ml cells were viable in the broth. For further validating the pure presumptive probiotic cultures without any contamination of pathogens, broth was checked by using pathogenic test.

4.7.6 Pathogenic test

Table 16 Result for pathogen test in presumptive probiotic broth.

S.N.	Pathogens	Media Used	Result/ Observation	Remarks
1.	*Staphylococcus aureus*	Casein soyabean digest broth, MSA plates	Absence of yellow/ white colonies with yellow zones	Absence of *Staphylococcus aureus* in the broth.
2.	*Pseudomonas aeruginosa*	Casein soyabean digest broth, Cetrimide agar	Absence of greenish color colony.	Absence of *Pseudomonas aeruginosa* in the broth.
3.	*Escherichia coli*	Casein soyabean digest broth, MacConkey broth and MacConkey agar	Absence of pink, non- mucoid colony	Absence of *Escherichia coli* in the broth.
4.	*Salmonella*	Casein soyabean digest broth, Rappaport Vassiliadis Salmonella enrichment broth And Wilson and Blair's BBS agar.	Absence of uniformly black colonies surrounded by a dark zone and metallic sheen	Absence of *Salmonella* in the broth.
5.	*Shigella*	Casein soyabean digest broth, GN broth and Xylose lysine deoxycholate medium.	Absence of red colored translucent colony without black center.	Absence of *Shigella* in the broth.

We carried major pathogenic analysis which was prioritized by WHO. The fermented broth which passes the stability test was inoculated in each specific media for pathogens test. Different indicators were analyzed as explained Indian pharmacopeia. All the test results

negative indicating no in-process cross contamination was present in the fermented broth and can be considering as safe fermented probiotic drinks for use. Our results showed the lab scale fermented stable probiotic drinks with zero ethanol.

Table 17 Summary of results of presumptive probiotic strains.

Organisms	Parameters	Temperature (20-42°C)	pH (6.0 -1.5)	Salt (Oxgall 5%)	Gastric juice
BM-13		++	++	++	++
BS-S4S1		++	++	++	++
LB-D1		++	++	++	++
SS-D1		++	++	++	++

Adapting the specific conditions of gastrointestinal tract is a crucial property for considering a microorganism as probiotic. Therefore, the isolates were surveyed for tolerance against different temperatures, high concentration of bile salt. The potential ability of the 4 mentioned strains to withstand during the passage through the human gastrointestinal tract as assayed indirectly in vitro is demonstrated and results were given in table 4.7.

The selected strains were able to grow under stressful environments, showing resistance to acid pH and high concentrations of bile salts. With respect to the effect of oxgall concentration, all the 4 selected strains were able to grow even when the bile salts concentration was as high as 5 % (w/v); however, increased bile salt concentration in medium was associated with a gradual decrease in the rate of growth. In this work, medium inoculation at various initial acidic pH values (1.5, 2.0, 3.0 and 5.0) resulted in survival of all the strains at pH 3.0 and 5.0 after 96h incubation (figure 4.7.2). However, after incubation in low pH (1.5 and 2.0), cell counts of all strains were reduced. In other words, the least amount of growth was detected in pH 1.5.

This observation emphasizes that these isolates could serve as efficient probiotic candidates. Foods with supplementary probiotic microorganisms not only need to be safe at

the expiration of shelf-life, but must also keep their functional characteristics throughout the same period. In this respect, the ability of growth and survival of the probiotic strains in dairy products (fermented whey wine) during shelf-life and stored period could be monitored. According to the WHO/FAO definition, a standardized probiotic food must contain a minimum of 10^6 CFU/g active and live organisms at the time of consumption (WHO). To assure their survival, we have shown that the strains are capable of growth into the whey wine and maintaining cell counts exceeding 10^6 CFU/ml. In this study, no contamination of any pathogens were observed this might be due to the identified strains would inhibit the growth of any pathogen as the same fact of probiotic strains were reviewed and standardized for the probiotic products (Donkor *et al.,*2007). Thus, based on the growth curve results, microscopic observations, biochemical test and probiotic criterion tests on *B. megaterium (BM-13), B. subtilis (BS-S4S1), Lactobacillus (LB-D1)* and *Saccharomyces cerevisiae,* it is concluded that all these strains are potential for use as probiotic.

CHAPTER 5

SUMMARY

Ceaseless growth in human population led to high demand in everything. Currently, the world largely depends on petroleum-based all material synthesis scheme. On the other hand, depletion of fossil-based resources and their huge impact on environmental pollution have forced us to search for sustainable and eco-friendly alternative resources. In this context, the notion to utilize waste biomass could possibly provide environmental and economic benefits. So in our study, from different small dairies 10 different whey samples were collected and preserved. By using various analytical methods characterization of whey samples was carried. Total calorific value and presence of necessary minerals and vitamins make whey a good substrate for microbial growth medium. Co- culture of presumptive probiotic strains (LAB bacteria and *S. cereviseae*) was optimize in lab in whey media as a result whey wine was produced. 72% yield was observed in whey fermentation with 0.9% ethanol produced. This technology reduced the environmental threat possess as high BOD due to organic compounds present in whey by 80%. Mead with 11% ethanol was prepared using natural honey to validate distillation process by conserving the natural flavor in both distilled ethanol and the fermented broth post distillation. Zero ethanol fermented probiotic drinks was prepared from whey waste by distillation of the fermented whey wine. Probiotic nature of the presumptive organisms, their stability in whey media and possible contamination in broth was carried. Ethanol production from whey is potable and so can find appropriate markets such as food and beverages, pharmaceutical formulations, and personnel hygiene. Likewise, probiotic organisms enhance the healthy host condition and would be useful for the lactose tolerant populations. In addition, it would provide nutrition security through enrichment of high quality probiotic drinks and uplift farmers' economy besides providing an alternate solution to tackle milk holiday originated whey management.

CHAPTER 6

CONCLUSIONS AND RECCOMENDATIONS

6.1 Conclusions

The conclusions of this study are as follows:

a) Nutritional values of waste whey were characterized and its TCV was found to be 32kcal.

b) Mead was prepared having 11% (v/v) ethanol content to validate fermentation and distillation process in laboratory.

c) Co-culture of yeast and bacteria was carried in whey media and found reduction in BOD level by around 80%.

d) Whey wine was made with 0.9% ethanol and ethanol form wine was distilled successfully without damaging the broths compositions.

e) Lab scale fermented probiotic drinks was prepared and tested successfully by using whey as sole substrate.

f) New concept for valorization of whey was initiated in lab scale.

6.2 Recommendations

For the further work, following suggestions are recommended.

a) Low ethanol concentration was observed in whey wine. Hence, lactose concentration can be optimized for better yield.

b) More optimization should be done in distillation process for sludge management and to make whole distillation process more economical for large scale industrial purpose.

c) In-vitro analysis of probiotic strains should be done for reliability.

d) Recommended that a biological test be conducted on the usage in probiotic form in animal model with respect to immunity response and growth.

e) Complete nutritional analysis of whey fermented probiotic drinks can be carried for industrial trial.

REFERENCES

a Waste, W. (2018). 2.0: A Global Snapshot of Solid Waste Management to 2050/Silpa Kaza, Lisa Yao, Perinaz Bhada-Tata and Frank Van Woerden. World banc group.

Adam, A. C., Prieto, J. A., Rubio-Texeira, M., & Polaina, J. (1999). Construction of a lactose-assimilating strain of baker's yeast. Yeast, 15(13), 1299-1305.

Adam, A. C., Rubio-Texeira, M., & Polaina, J. (2005). Lactose: the milk sugar from a biotechnological perspective. BFSN, 44(7-8), 553-557.

Akuzawa, R., Miura, T., & Kawakami, H. (2009). Bioactive components in caseins, caseinates, and cheeses. Bioactive Componentsin Milk and Dairy Products, 217.

Altuntas, S., & Hapoglu, H. (2019). Kefir-Type Drinks from Whey. In Non-Alcoholic Beverages (pp. 185-226). Woodhead Publishing.

Andriani, Y., Rochima, E., Safitri, R., & Rahayuningsih, S. R. (2017). Characterization of Bacillus megaterium and Bacillus mycoides bacteria as probiotic bacteria in fish and shrimp feed. KnE Life Sciences, 127-135.

Axelsson, L. (2004). Lactic acid bacteria: Classification and physiology. In S. Salminen, A. von Wright, & A. Ouwehand (Eds.). Lactic acid bacteria: Microbiological and functional aspects (pp. 1–66).

Bassette, R., & Acosta, J. S. (1988). Composition of milk products. In Fundamentals of dairy chemistry (pp. 39-79). Springer, Boston, MA.

Begley, M., Hill, C., & Gahan, C. G. (2006). Bile salt hydrolase activity in probiotics. Applied and environmental microbiology, 72(3), 1729-1738.

Bennett, R. J., & Johnston, K. A. (2004). General aspects of cheese technology. In Cheese: chemistry, physics and microbiology (Vol. 2, pp. 23-XIII). Academic Press.

Black, C., & Ditsler, D. E. (1972). Dehydration of aqueous ethanol mixtures by extractive distillation.

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (1999). Yeast and biochemistry of ethanol fermentation. In Principles and practices of winemaking (pp. 102-192). Springer, Boston, MA.

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (2013). Principles and practices of winemaking. Springer Science & Business Media.

Bro, C., Knudsen, S., Regenberg, B., Olsson, L., & Nielsen, J. (2005). Improvement of galactose uptake in Saccharomyces cerevisiae through overexpression of phosphoglucomutase: example of transcript analysis as a tool in inverse metabolic engineering. Applied and Environmental Microbiology, 71(11), 6465-6472.

Bourdichon, F., Alper, I., Bibiloni, R., Dubois, A., Laulund, S., Miks, M., et al. (2018). Inventory of microbial food cultures with safety demonstration in fermented food products. Bull. Int. Dairy Federat. 495, 5–71.

By, S. (2018). OECD-FAO Agricultural Outlook.

Byerlee, D., De Janvry, A., Sadoulet, E., Townsend, R., & Klytchnikova, I. (2008). World development report 2008: agriculture for development (No. 41455, pp. 1-384). The World Bank.

Casper, J. L., Wendorff, W. L., & Thomas, D. L. (1998). Seasonal changes in protein composition of whey from commercial manufacture of caprine and ovine specialty cheeses. Journal of Dairy Science, 81(12), 3117-3122.

Carvalho, F., Prazeres, A. R., & Rivas, J. (2013). Cheese whey wastewater: Characterization and treatment. Science of the total environment, 445, 385-396.

Champagne, C. P., & Goulet, J. (1988). Growth of Bakers' Yeast (Saccharomyces cerevisiae) in Lactose-Hydrolyzed Cheese Whey Ultrafiltrate. Canadian Institute of Food Science and Technology Journal, 21(5), 545-548.

Claassen, P. A. M., Van Lier, J. B., Lopez Contreras, A. M., Van Niel, E. W. J., Sijtsma, L., Stams, A. J. M., ... & Weusthuis, R. A. (1999). Utilisation of biomass for the supply of energy carriers. Applied microbiology and biotechnology, 52(6), 741-755.

Coté, A., Brown, W. A., Cameron, D., & Van Walsum, G. P. (2004). Hydrolysis of lactose in whey permeate for subsequent fermentation to ethanol. Journal of Dairy Science, 87(6), 1608-1620

Costa, Simone & Natália, & Rossi, Porfírio & Maldonado, Rafael. (2013). Evaluation of Lactose in Milk and Dairy Products. International Journal for Innovation Education and Research. 1. 10.31686/ijier.vol1.iss3.115.

COMP-NDDB, (2017). Study on cost of Milk production, Final report, NEPC Research Pvt. Ltd.

Creamer, L. K., & MacGibbon, A. K. (1996). Some recent advances in the basic chemistry of milk proteins and lipids. International Dairy Journal, 6(6), 539-568.

Curry, A. (2013). Archaeology: The milk revolution. Nature News, 500(7460), 20.

Dessie, W., Luo, X., Wang, M., Feng, L., Liao, Y., Wang, Z., ... & Qin, Z. (2020). Current advances on waste biomass transformation into value-added products. Applied microbiology and biotechnology, 104(11), 4757-4770.

De Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., & Schrezenmeir, J. (2001). Probiotics—compensation for lactase insufficiency. The American journal of clinical nutrition, 73(2), 421s-429s.

Dr Narendra K Nayak Department of Livestock Products Technology College of Veterinary Science & A.H., Mhow.

Donkor, O. N., Nilmini, S. L. I., Stolic, P., Vasiljevic, T., & Shah, N. P. (2007). Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. International Dairy Journal, 17(6), 657-665.

Durham, R. J., & Hourigan, J. A. (2007). Waste management and co-product recovery in dairy processing. In Handbook of waste management and co-product recovery in food processing (pp. 332-387). Woodhead Publishing.

Fang, X., Shen, Y., Zhao, J., Bao, X., & Qu, Y. (2010). Status and prospect of lignocellulosic bioethanol production in China. Bioresource technology, 101(13), 4814-4819.

Faye, B., & Konuspayeva, G. (2012). The sustainability challenge to the dairy sector–The growing importance of non-cattle milk production worldwide. International dairy journal, 24(2), 50-56.

Food, J., & Agriculture Organization/World Health Organization Working Group. (2002). Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. Geneva: World Health Organization.

https://foodstruct.com/compare/milk-vs-whey

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. (2017). Cheese yield. In Fundamentals of cheese science (pp. 279-331). Springer, Boston, MA.)

Fuller, R. (1992). History and development of probiotics. In Probiotics (pp. 1-8). Springer, Dordrecht.

Fuquay, J. W., McSweeney, P. L., & Fox, P. F. (2011). Encyclopedia of dairy sciences. Academic Press.

Ghaly, A. E., & Kamal, M. A. (2004). Submerged yeast fermentation of acid cheese whey for protein production and pollution potential reduction. Water Research, 38(3), 631-644.

Glenister, P. R. (1970, May). Some useful staining techniques for the study of yeast, beer, and beer sediments. In Proceedings. Annual Meeting-American Society of Brewing Chemists (Vol. 28, No. 1, pp. 163-167). Taylor & Francis.

González, S. (1996). The biotechnological utilization of cheese whey: A review. Bioresource Technology, 57(1), 1-11.

Guimarães, P. M., Teixeira, J. A., & Domingues, L. (2010). Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnology advances, 28(3), 375-384.

Gupta, J. K., & Sharma, R. (2009). Production technology and quality characteristics of mead and fruit-honey wines: A review.Smithers, G. W. (2015). Whey-ing up the options–Yesterday, today and tomorrow. International Dairy Journal, 48, 2-14.

Harper, W. J. (1992). Lactose and lactose derivatives. In Whey and lactose processing (pp. 317-360). Springer, Dordrecht.

Hadiwijaya, Y., Putri, I. E., Mubarok, S., & Hamdani, J. S. (2020, February). Rapid and non-destructive prediction of total soluble solids of guava fruits at various storage periods using handheld near-infrared instrument. In IOP Conference Series: Earth and Environmental Science (Vol. 458, No. 1, p. 012022). IOP Publishing.

Hickey, M. W., Hillier, A. J., & Jago, G. R. (1986). Transport and metabolism of lactose, glucose, and galactose in homofermentative lactobacilli. Applied and environmental microbiology, 51(4), 825-831.)

Hoffmann, K. F. (1961). On the history of whey cures, especially in the 17th, 18th, and 19th centuries. Medizinische Monatsschrift, 15, 411-414.

ISAPP (2018). Minimum Criteria for Probiotics [Online]. Sacramento, CA: International Scientific Association for Probiotics and Prebiotics.

Iskandar, C. F., Cailliez-Grimal, C., Borges, F., & Revol-Junelles, A. M. (2019). Review of lactose and galactose metabolism in Lactic Acid Bacteria dedicated to expert genomic annotation. Trends in food science & technology, 88, 121-132.

Jelen, P. (1979). Industrial whey processing technology: An overview. Journal of Agricultural and Food Chemistry, 27(4), 658-661.

Jenness, R. (1988). Composition of milk. In Fundamentals of dairy chemistry (pp. 1-38). Springer, Boston, MA.

Liu, Y., Nie, Y., Lu, X., Zhang, X., He, H., Pan, F., ... & Zhang, S. (2019). Cascade utilization of lignocellulosic biomass to high-value products. Green Chemistry, 21(13), 3499-3535.

Jost, R. (2000). Milk and dairy products. Ullmann's Encyclopedia of Industrial Chemistry.

Jung, B., & Hoilat, G. J. (2021). MacConkey Medium.

Kamel, Z., Mohamed, N. M., & Farahat, M. G. (2016). Optimization of Culture Conditions for Production of B-Galactosidase by Bacillus Megaterium NM56 Isolated from Raw Milk. RESEARCH JOURNAL OF PHARMACEUTICAL BIOLOGICAL AND CHEMICAL SCIENCES, 7(1), 366-376.

Khanal, B. K. S., Pradhan, M., & Bansal, N. (2019). Cheese: Importance and Introduction to Basic Technologies. Journal of Food Science and Technology Nepal, 11, 14-24.

Konsoula, Z., & Liakopoulou-Kyriakides, M. (2007). Co-production of α-amylase and β-galactosidase by Bacillus subtilis in complex organic substrates. Bioresource Technology, 98(1), 150-157.

Kosikowski, F. V., & Wzorek, W. (1977). Whey wine from concentrates of reconstituted acid whey powder. Journal of dairy science, 60(12), 1982-1986.

Ladisch, M. R., & Dyck, K. (1979). Dehydration of ethanol: new approach gives positive energy balance. Science, 205(4409), 898-900.

Leonardi, M., Gerbault, P., Thomas, M. G., & Burger, J. (2012). The evolution of lactase persistence in Europe. A synthesis of archaeological and genetic evidence. International Dairy Journal, 22(2), 88-97.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of biological chemistry, 193, 265-275.

MacConkey, A. (1905). Lactose-fermenting bacteria in faeces. Epidemiology & Infection, 5(3), 333-379.

Maicas, S. (2020). The role of yeasts in fermentation processes. Microorganisms, 8(8), 1142.

Marteau, P. R., Vrese, M. D., Cellier, C. J., & Schrezenmeir, J. (2001). Protection from gastrointestinal diseases with the use of probiotics. The American journal of clinical nutrition, 73(2), 430s-436s.

Martínez-García, C. G., Rayas-Amor, A. A., Anaya-Ortega, J. P., Martínez-Castañeda, F. E., Espinoza-Ortega, A., Prospero-Bernal, F., & Arriaga-Jordán, C. M. (2015). Performance of small-scale dairy farms in the highlands of central Mexico during the dry season under traditional feeding strategies. Tropical Animal Health and Production, 47(2), 331-337.

Mawson, A. J. (1994). Bioconversions for whey utilization and waste abatement. Bioresource technology, 47(3), 195-203.

McGee, Harold (2004) [1984]. "Milk and Dairy Products". On Food and Cooking: The Science and Lore of the Kitchen (2nd ed.). New York: Scribner. pp. 7–67. ISBN 978-0-684-80001-1.

McNulty, C. A., & Wise, R. (1985). Gastric microflora. British Medical Journal (Clinical research ed.), 291(6492), 367.

Menchik, P., Zuber, T., Zuber, A., & Moraru, C. I. (2019). Composition of coproduct streams from dairy processing: Acid whey and milk permeate. Journal of dairy science, 102(5), 3978-3984.

Moradi, R., Nosrati, R., Zare, H., Tahmasebi, T., Saderi, H., & Owlia, P. (2018). Screening and characterization of in-vitro probiotic criteria of Saccharomyces and Kluyveromyces strains. Iranian Journal of Microbiology, 10(2), 123.

Murphy, J. A. M. E. S., & Riley, A. J. (1958). A single-solution method for the determination of soluble phosphate in sea water. Journal of the Marine Biological Association of the United Kingdom, 37(1), 9-14.

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

Neupane, N., Neupane, H., & Dhital, B. (2018). A socioeconomic view of status and prospects of goat farming in rural areas of Nepal. Journal of the Institute of Agriculture and Animal Science, 35(1), 1-8.

Paine, A. J., & Dayan, A. D. (2001). Defining a tolerable concentration of methanol in alcoholic drinks. Human & experimental toxicology, 20(11), 563-568.

Panesar, P. S., Kumari, S., & Panesar, R. (2010). Potential applications of immobilized β-galactosidase in food processing industries. Enzyme research, 2010.

Papademas, P., & Kotsaki, P. (2019). Technological utilization of whey towards sustainable exploitation. Adv. Dairy Res, 7(4), 231.

Pendón, M. D., Madeira, J. V., Romanin, D. E., Rumbo, M., Gombert, A. K., & Garrote, G. L. (2021). A biorefinery concept for the production of fuel ethanol, probiotic yeast, and whey protein from a by-product of the cheese industry. Applied Microbiology and Biotechnology, 105(9), 3859-3871.

Pereira, A. P., Dias, T., Andrade, J., Ramalhosa, E., & Estevinho, L. M. (2009). Mead production: Selection and characterization assays of Saccharomyces cerevisiae strains. Food and chemical toxicology, 47(8), 2057-2063.

Percival, G. C., Chamundeeswari, M., Lovlyna, R. F., Seethalakshmi, R., & Sreekumar, G. (2019). Production and partial purification of β-galactosidase enzyme from probiotic Bacillus subtilis SK09.

Rail, T., & Thomas, B. (1994). Joseph Priestley's Journal While at Daventry Academy, 1754'. Enlightenment & Dissent, 13, 49-113.

Rasheed, S., Qazi, I. M., Ahmed, I., Durrani, Y., & Azmat, Z. (2016). Comparative study of cottage cheese prepared from various sources of milk. Proceedings of the Pakistan Academy of Sciences, 53(4), 269-282.

Rashidinejad, A., Bremer, P., Birch, J., & Oey, I. (2017). Nutrients in Cheese and Their Effect on Health and Disease. In Nutrients in Dairy and their Implications on Health and Disease (pp. 177-192). Academic Press.

Rocha-Mendoza, D., Kosmerl, E., Krentz, A., Zhang, L., Badiger, S., Miyagusuku-Cruzado, G., ... & García-Cano, I. (2020). Invited review: Acid whey trends and health benefits. Journal of Dairy Science.

RODRIGUEZ, E. SANZ ALAEJOS M. DIAZ ROMERO C. 2001. Mineral concentration in cow's milk from the Canary Island, 419-430.

Rogosa, M., Browne, H. H., & Whittier, E. O. (1947). Ethyl alcohol from whey. Journal of Dairy Science, 30(4), 263-269.

RUSSELL, J. (1986). Will a recombinant DNA yeast be able to solve whey disposal problems? Trends in biotechnology (Library edition), 4(5), 107-108.

Saqib, A. A. N., & Whitney, P. J. (2011). Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono-and di-saccharide sugars. Biomass and bioenergy, 35(11), 4748-4750.

Shingh, S., Kalwar, C. S., Poudel, S., Tiwari, P., & Jha, S. (2020). A Study on Growth and Performance of Dairy Sector in Nepal. International Journal of Environment, Agriculture and Biotechnology, 5(4).

Singh, D. P., & Dwevedi, A. (2019). Chapter 2-production of clean energy by green ways. Solutions to Environmental Problems Involving Nanotechnology and Enzyme Technology; Dwevedi, A., Ed, 49-90.

Siso, M. G. (1996). The biotechnological utilization of cheese whey: a review. Bioresource technology, 57(1), 1-11.

Spalatelu, C. (2012). Biotechnological valorisation of whey. Innovative Romanian Food Biotechnology, 10, 1.

Smithers, G. W. (2008). Whey and whey proteins—from 'gutter-to-gold'. International dairy journal, 18(7), 695-704.

Sneath, P. H., Mair, N. S., Sharpe, M. E., & Holt, J. G. (1986). Bergey's manual of systematic bacteriology. Volume 2. Williams & Wilkins.

Terrell, S. L., Bernard, A., & Bailey, R. B. (1984). Ethanol from whey: Continuous fermentation with a catabolite repression-resistant Saccharomyces cerevisiae mutant. Applied and Environmental Microbiology, 48(3), 577-580.

Timsina, K. P., & Regmi, P. P. (2009). Poduction and marketing dynamics of milk: A case study of Chitwan districts, Nepal. J. Inst. Agric. and Anim. Sci, 30, 33-43.

van Mierlo, L. A., Arends, L. R., Streppel, M. T., Zeegers, M. P. A., Kok, F. J., Grobbee, D. E., & Geleijnse, J. M. (2006). Blood pressure response to calcium supplementation: a meta-analysis of randomized controlled trials. Journal of human hypertension, 20(8), 571-580.

Walther, B., Wong, N. P., LaCroix, D. E., & McDonough, F. E. (1978). Minerals in whey and whey fractions. Journal of dairy science, 61(12), 1700-1703., K. (2008). Cheese in nutrition and health. Dairy Science and Technology, 88(4-5), 389-405.

Wong, N. P., LaCroix, D. E., & McDonough, F. E. (1978). Minerals in whey and whey fractions. Journal of dairy science, 61(12), 1700-1703.

Wu, Q., Cheung, C. K. W., & Shah, N. P. (2015). Towards galactose accumulation in dairy foods fermented by conventional starter cultures: Challenges and strategies. Trends in Food Science & Technology, 41(1), 24–36.

Yang, H. Y., Bodyfelt, F. W., & Larson, P. K. (1976). Utilization of cheese whey for wine production. In Proceedings of Sixth National Symposium on Food Processing Waste, USA (p. 180).

Zall, R. R. (1984). Trends in whey fractionation and utilization, a global perspective. Journal of dairy science, 67(11), 2621-2629.

Zeng, L., Das, S., & Burne, R. A. (2010). Utilization of lactose and galactose by Streptococcus mutans: transport, toxicity, and carbon catabolite repression. Journal of bacteriology, 192(9), 2434-2444.

Zhao, Y., Shi, R., Bian, X., Zhou, C., Zhao, Y., Zhang, S., ... & Zhang, T. (2019). Ammonia detection methods in photocatalytic and electrocatalytic experiments: how to improve the reliability of NH3 production rates? Advanced Science, 6(8), 1802109.

Zotta, T., Solieri, L., Iacumin, L., Picozzi, C., & Gullo, M. (2020). Valorization of cheese whey using microbial fermentations. *Applied Microbiology and Biotechnology*, *104*(7), 2749-2764.

https://www.canterbury.ac.nz/media/documents/science-outreach/calcium.pdf for calcium ion quantification.

http://2011.igem.org/Team:HIT-Harbin/Project

https://www.clal.it/en/?section=forecast_me

https://ec.europa.eu/info/sites/default/files/food-farming-fisheries/farming/documents/world-production-main-dairy-products_en.pdf

https://nepaliheadlines.com/milk-holiday-costs-dear-to-dairy-farmers/

https://kathmandupost.com/money/2015/08/24/chances-of-milk-holiday-high

APPENDIX

Appendix I

Table a) Composition of YPD agar media

YPD Agar	Ingredients(g/L)
Yeast extracts	3
Peptone	5
Glucose	10
Agar	20
Distilled water	1

Table b) Composition of Probiotic growth media (1000ml)

Sodium carbonate	0.25 gm
Ammonium chloride	0.15 gm
Potassium chloride	0.06gm
Mineral mix.	10 ml
Whey	13%
Distilled water	Remaining volume

Table c) Composition of SDA media (100ml)

Dextrose	4 gm
Peptone	1 gm
Agar	2 gm

Table d) Composition of Nutrient agar media (100 ml)

Peptone	0.5 gm
Beef extract	0.3 gm
Agar	1.5 gm
Sodium chloride	0.5 gm

Table e) Composition Tryptophan media (for 1000ml).

L-tryptophan	0.05 mM
$(NH_4)_2SO_4$	1 gm
K_2HPO_4	0.8 gm
KH_2PO_4	0.2 gm
$MgSO_4.7H2O$	0.5 gm
$CaSO_4.7H2O$	0.05 gm
Mineral mix	10 ml
Vitamin mix	10 ml

Table f) Composition of Luria–Bertani medium (100ml)

Tryptone	1 gm
Yeast extract	0.5 gm
NaCl	0.05 gm

Table g): Composition of SIM media.

Ingredients	Composition
1. Enzymatic digest of Casein	20.0 g/L
2. Enzymatic digest of animal tissue	6.1 g/L
3. Ferric Ammonium citrate	0.2 g/L
4. Sodium thiosulfate	0.2 g/L
5. Agar	3.5 g/L

Appendix II

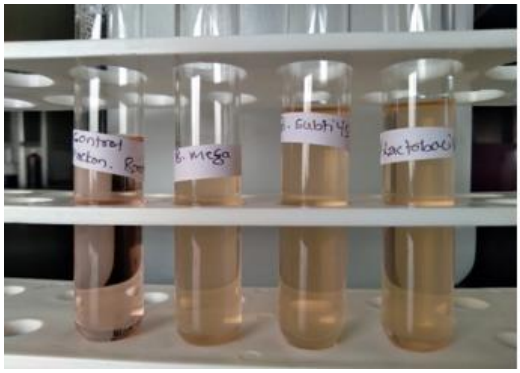

Figure a) Growth of probiotic organisms in MacConkey broth.

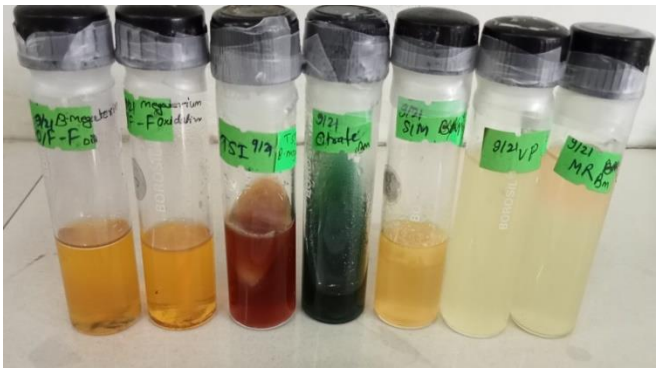

Figure b) *Biochemical test of Bacillus megaterium*.

Figure c) Biochemical test of *Lactobacillus.*

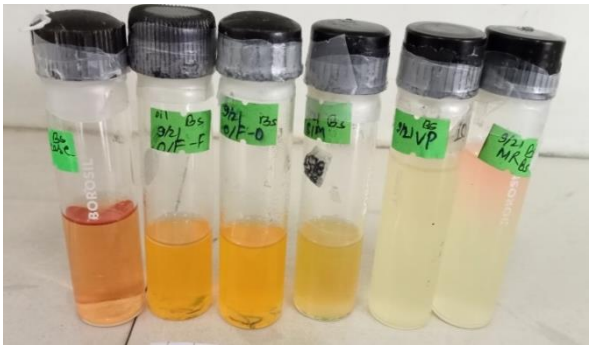

Figure d) Biochemical test of *Bacillus subtilis.*

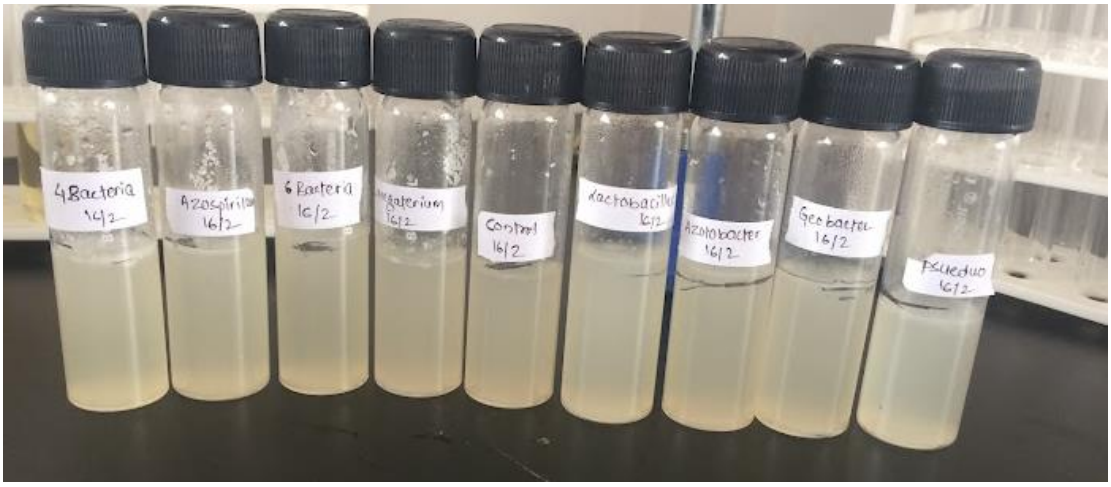

Figure e) Growth of different bacterial strain in Whey broth.

Figure f) Fermentation of Mead.

Figure g) Final Mead from Honey

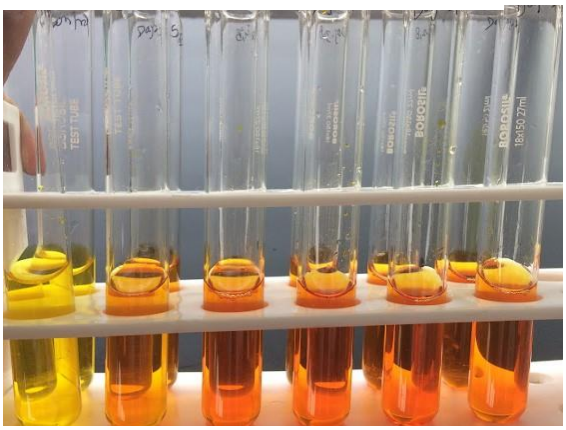

Figure h) Nessler's test

Figure i) Fermentation of Whey broth

Figure j) Rotary evaporator for Distillation

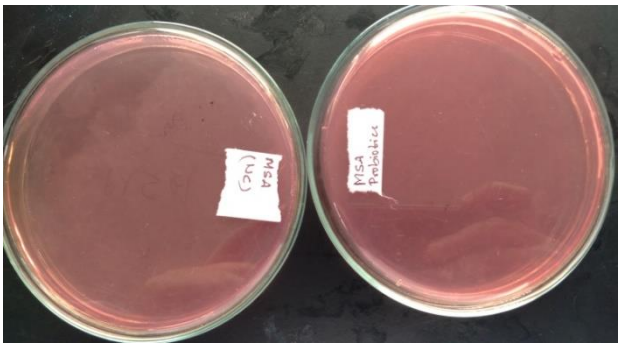

Figure k) MSA plate for *Staphylococcus aureus*

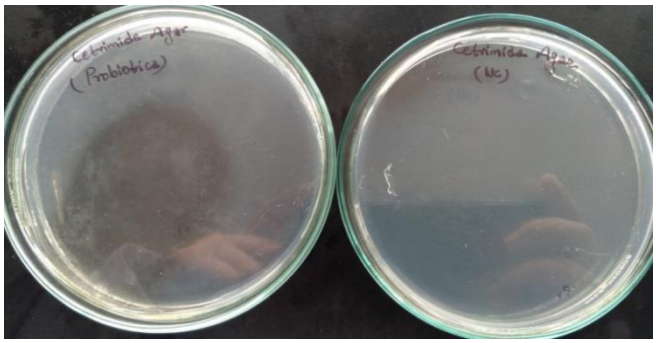

Figure l) Centrimids Agar for *Pseudomonas aeruginosa*

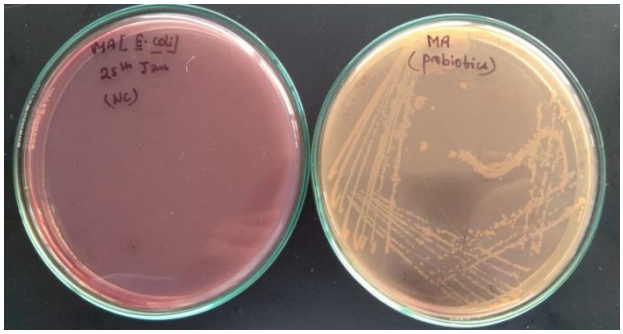

Figure m) MA for *E. coli*

Figure n) Wilson and Blair's BBS agar for *Salmonella.*

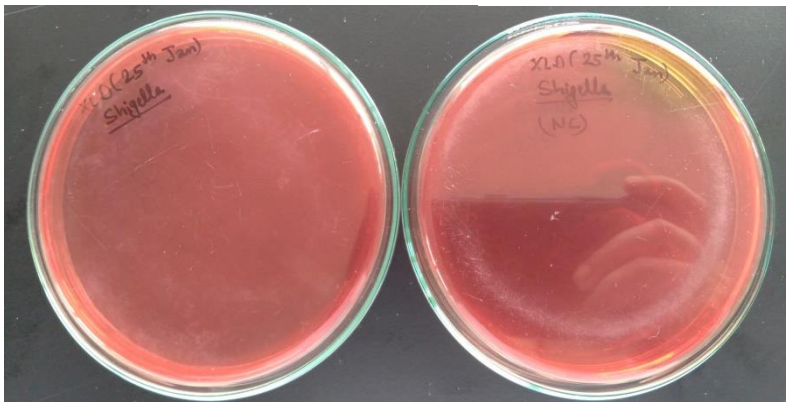

Figure o) XLD Agar for *Shigella.*