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This is to certify that the thesis prepared by Agurash Adane, entitled "Lactic acid production from agro-industrial wastes using lactic acid bacterial isolates" submitted in partial fulfillment of the requirements for the Degree of Masters of Science in Biotechnology complies with the regulations of the university and meets the standard with respect to originality and quality.

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LACTIC ACID PRODUCTION FROM AGRO-INDUSTRIAL WASTES USING LACTIC ACID BACTERIAL ISOLATES

BY: AGURASH ADANE

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ABBRIVATION

AHA	Alpha Hydroxyl Acid
AOAC	Association of Official Analytical Chemists
BNP	Banana peel
BSG	Brewers' spent grain
CAPD	Continuous Ambulatory Peritoneal Dialysis
DM	Dry Matter contents
DNS	Dinitrosalcylic acid
FAO	Food and Agricultural organization
GRAS	Generally Recognized As Safe
LA	Lactic Acid
LAB	Lactic Acid Bacteria
MRS	De Man, Rogosa and Sharpe
PLA	Poly Lactic Acid
SGB	Spent Grain of Beer
SPSS	Statistical Package for Social Science
US	United States
UV	Ultra Violate

ABSTRACT

Lactic acid is a chemical compound that plays an important role in food, pharmaceutical, cosmetics, chemical and polymer industries. It can be produced either through fermentation or chemical synthesis methods. Hence, the objective of this study was production of lactic acid from the fermentation of spent grain of beer and banana peels using indigenous lactic acid bacteria isolated, screened and selected during this study. The effects of temperature, pH, carbon sources, nitrogen sources, and incubation period and inoculum size on lactic acid production during the fermentation processes were also studied. From a total of 90 lactic acid bacteria isolated from ergo and siljo, 66 (84.4%) and 14 (15.6%) LAB isolates were found producing 7.30 to 15.91 and 16-25.2 g/l *LA at 48 hrs from 5 ml of MRS broth, respectively. Out of the 14 isolates AS1S that produced the highest amount LA (23.35g/l and 25.20 g/l at 24 and 48 hrs, respectively) from 5 ml MRS broth was selected and subjected to further analysis of LA from produced spent grain of beer (SGB) and banana peel (BNP) during this study. The moisture, ash, protein, fat, fiber and carbohydrate contents SGB and BNP were shown as 7.38% and 5.08%, 3.85% and 5.09%, 4.70% and 6.65%, 3.11% and 1.70%, 4.41% and 12.65%, and 76.56% and 66.59% respectively. The proximate analysis result exhibited that the substrates were nutritionally rich enough to be used as medium to grow lactic acid bacteria to produce LA. During this study the optimum conditions for production of the maximum amount of LA (*27.73 and 25.98 g/l *from SGB and BNP, respectively), and were obtained at a temperature, pH, nitrogen content, carbon source, and inoculum size of 37 ^{o}C, 5.5, 15 g/l $NH4)_2SO_4$, 50 g/l glucose and 5% at 72 hrs in that order. The results of this study displayed the potential use of indigenous microbial resources for producing high value biotech products like lactic acid from locally available agro-industrial wastes.*

Key words*:* lactic acid, banana peel, spent grain of beer, lactic acid bacteria

1. Introduction

1.1. Background

Lactic acid has major potential applications in food, cosmetic, pharmaceutical, and chemical industries (Nesryia, 2018). Lactic acid is classified as generally regarded as safe (GRAS) for use as food additive by the US Food and Drug Administration (FDA) (Umesh and Preethi, 2014). Lactic acid is widely used in every segment of the food industry, which serves as flavoring, pH regulator, improving microbial quality, and mineral fortification (Vijayakumar *et al*., 2008). It is also used commercially in the processing of meat and poultry industries, to provide products with an increased shelf life, better control of food-borne pathogens, and enhancing flavor (Vanessa, 2015). Due to its slight acidic taste, it is also used as an acidulant in salads and dressings, pickled vegetables, beverages and baked goods (Mudaliyar and Kulkarni, 2011). In the pharmaceutical industry it is used as an electrolyte in many parenteral/intraviolat/ I.V. solutions that are intended to replenish the bodily fluids or electrolytes. Examples of bodily fluids include Lactated Ringer's or Hartmann's solutions, continuous ambulatory peritoneal dialysis (CAPD) solution, and dialysis solution for conventional artificial kidney machines, in a wide variety of mineral preparations, which include tablets, prostheses, surgical sutures, and controlled drug delivery systems (Abd Alsaheb *et al.*,2015;and Wee *et al.*,2006). In chemical industry it is used as a descaling agent, pH regulator, neutralizer, chiral intermediate, solvent, cleaning agent, slow acid-release agent, metal complexing agent, antimicrobial agent, and humectants (Azhar, 2014; Pikul-ngoen, 2010 and John *et al*., 2007). Presently, the main growing application of lactic acid is in the production of biodegradable and renewable product such as poly lactic acid (PLA) polymers (Chaisu *et al*., 2014). Poly lactic acid (PLA) is biodegradable aliphatic polyester produced from renewable sources, due to its excellent physical and chemical properties and compatibility to the environment, which is considered the best to replace petroleum-based plastics (Xavier, 2010). It has a wide range of applications ranging from medical devices, such as suture treads and scaffolds, to commodity products like bottles and films for food packaging (Yonas, 2016).

Lactic acid can be obtained by either chemical synthesis or microbial fermentation (Pikul-ngoen, 2010).The chemical synthesis of lactic acid is based on lactonitrile as an intermediate. It involves

the base-catalyzed addition of hydrogen cyanide to acetaldehyde to produce lactonitrile. The crude lactonitrile is then recovered and purified by distillation and hydrolyzed to lactic acid using either concentrated hydrochloric or sulfuric acid, producing the corresponding ammonium salt as a by-product (Krishna *et al*., 2018). Then the crude lactic acid is esterified with methanol, producing methyl lactate. The latter is recovered and purified by distillation and hydrolyzed by water under acid catalysts to produce lactic acid (Waswar, 2005). On the other hand, microbial fermentation is a biochemical process in which carbohydrate molecules, (e.g. glucose and sucrose) are converted into energy, lactate, and other by-products depending on the type of microorganism involved in the fermentation process (Ameen, 2017). Fermentative production of lactic acid has advantages in the utilization of renewable carbohydrates. Currently, more than 95% of industrial production of lactic acid is based on fermentation, due to the increasing demand for naturally produced lactic acid (Azhar, 2014 ; Rashid, 2008). Furthermore, a typical lactic acid bacterium cultured under standard conditions (not limited by glucose, growth nutrients, or oxygen) is a non-taxonomic group of gram-positive, non-spore forming, catalase negative, aerotolerant (some species are strict anaerobes), acid tolerant, non-motile, chemoorganotroph and produces lactic acid from various fermentable carbohydrates (Vanessa, 2015). Genera of lactic acid bacteria which can be used for lactic acid production include *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*, (*Bifidobacterium* is are sometimes included) (Yonas, 2016; Hofvendahl and Hahn-Hligerdal, 2000).

Industrial scale production of lactic acid needs the availability of sustainable cheap raw materials with minimum level of contamination, rapid fermentation rate and high lactic acid yield (Oh *et al.*, 2005). These raw materials which used as a substrate for fermentation found to be in the form of starch (corn, wheat, potato, banana, cassava, rice, sweet sorghum) and lignocelluloses (corn cobs, waste paper, brewers' spent grain and woody materials) (Bayitse,2015). Thus, lactic acid (LA) can be produced from environmentally friendly, easily available and cheap agricultural and industrial wastes as resources (Schieber and Saldana, 2009). For example peels of banana (peels of *Musa sapientum*) are released as a waste to the environment. But it has a protein component, carbohydrate which is important to produce organic acids, enzymes and other chemicals by means of fermentation (Chandrasekaran, 1996). Brewers' spent grain (BSG) is also the major by-product of the brewing industry, representing around 85% of the total by-products generated and it is a lignocellulosic material (Mussatto *et al.,* 2006). Recent advances in

biotechnology ensure that brewery spent grain (BSG) is no longer regarded as a waste but rather a feedstock for producing several products. Based on this, it is an undeniable fact that BSG has its own potential for sustainable reuse through biotechnological processes. In Ethiopia 26,722.8 ton per year of brewery spent grain was generated from 12 brewery factories in 2016/2017 (Getu *et al*., 2018). It is partly used as animal feed and the rest of it damped directly into the environment rather being used for product creation (Emru and Ojitu, 2020). As a result it is also used as a substrate to produce lactic acid in addition to animal feed (Aliyu and Bala, 2011). On the other hand, in Ethiopia, banana is cultivated in large amount which covers, about 68.72% (37,076.85 hectares) of land and about 77.53% (370,784.17 tones) of the production (Zinabu *et al*., 2019).

Global LA demands were estimated to be 1,947.2 kilo tons in 2018 and are expected to grow annually by 16.2% from 2019 to 2025 as a result of increasing sales of medicines and perfumes in different countries (Abdel-Rahman, 2019). The demand for lactic acid is dependent on the development of the manufacturing sector, particularly the pharmaceuticals, food, and soft drinks sub sectors (Nesryia, 2018). According to Ethiopian Revenues and Customs Authority 2017 data, the requirement of LA in Ethiopia is entirely met through import.

. Only few attempts were so far done to produce LA using fermentation method in Ethiopia (Yonas, 2016; Edris, 2017; Nesriya, 2018; Yohanns, 2018). Moreover; very little attention has been given to utilizing banana peel from juices houses and spent grain of beer from brewery industry for the production of lactic acid using indigenous lactic acid bacterial isolates. Therefore, this study was aimed at isolating, selecting and using indigenous microbial resources, wastes from juice houses and industry, optimizing substrates and producing high value multipurpose biotech product, lactic acid. Unlike the other studies mentioned above, during this study the two substrates (banana peels and spent grain of beer) were utilized and indigenous lactic acid bacterial isolates were isolated, screened, selected and used for production of lactic acid.

1.2. Significance of the study

As we know LA has various applications in different industries. But, except the attempt by Yonas Abebe to produce LA using bulla as source of carbon and MRS as yeast extract, meat extract, peptone and glucose source using *Lactobacillus plantarium* (Yonas, 2016), there is no any other

attempt so far done to produce LA using either synthetic or fermentation method in Ethiopia. Hence, the echo-friendly LA production from locally available cheap substrate will be a good alternative in waste management and developing indigenous biotech skill and technique in producing the product. If LA is produced in this manner and linked with local similar chemical factories in the country, in the near future it will be available to local market at significantly reduced cost; which in turn reduce the cost of production of industrial products that utilize the product in significant amount (pharmaceuticals, food, and polymers like PLA and beverage products). It is also my hope that the consumers of products from industries that will utilize cheaper LA will get the products in relatively cheaper costs.

Generally, the proposed study signifies:

- Increased agricultural productivity
- Produce LA from industrial and household waste material
- Support and initiate biotech production industrial products
- Alleviating environmental problems by managing waste to be damped to the environment otherwise
- Develop indigenous cost-effect biotech skill and technique

1.3. Objectives

1.3.1. General objective

- The general objective of this study was to produce lactic acid from agro-industrial wastes (beer spent grain and peels of banana) using indigenous lactic acid bacterial isolates

1.3.2. Specific objectives

The specific objectives were to**:**

- isolate and screen LAB that has the potential of producing higher amount of LA from locally available fermentable wastes.
- carry out proximate analysis of locally available agro-industrial waste resources (spent grain of beer and peels of banana).

- ♠ evaluate and optimizing agro-industrial waste resources (beer spent grain and peels of banana) for production of LA using selected LAB isolates.
- ♠ estimate lactic acid yield produced from an optimizing beer spent grain and peels of banana by using the selected isolates

2. Literature Review

2.1. Lactic acid and its properties

Lactic acid (2-hydroxypropionic acid, $CH_3CHOHCOOH$) is the most widely occurring hydroxyl carboxylic acid. It was first isolated in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component (Narayanan, 2004). Lactic acid was first commercially produced around 1880 from sour milk. It exists naturally in two optical isomers: D (-) lactic acid and L (+) lactic acid (Habova *et al*., 2004). It is colorless, sour in taste, odorless and soluble in all proportions in water, alcohol and ether but insoluble in chloroform and it is also a weak acid with low volatility (Rashid, 2008).Lactic acid is one of the most important organic acids which are being widely used around the world in a range of industrial and biotechnological applications. From its very old history to date, many methods have been introduced to improve the optimization of lactic acid to get highest yields of the product at industrial scale (Ghaffar *et al.,* 2014).

Pure anhydrous lactic acid is a white crystalline solid and has a melting point of 53^{o}C and normally appears in the form of diluted or concentrated aqueous solution, as syrupy (Jawad *et al*., 2013). It is considered as a stable and a combustible substance as well. Lactic acid is compatible with strong oxidizing agents (Rashid, 2008).

Lactic acid is colorless to yellow liquid after melting, sour in taste, odorless and soluble in all proportions in water, alcohol and ether but not in chloroform (Narayanan, 2004). It is a weak acid with low instability. The two functional groups (hydroxyl and carboxyl) present in lactic acid permits a wide variety of chemical reactions for it. The main classes of these reactions include oxidation, reduction, condensation and substitutions (Rashid, 2008).In solutions with roughly 20% or more lactic acid, self-esterification occurs because of the hydroxyl and carboxyl functional groups and it may form a cyclic dimmer (lactide) or more linear polymers (Holten *et al.,* 1971).

2.2. Import of lactic acid

The quantity and value of LA imported in the past sixteen years was presented in Table 1. The table tells us the increasing demand for LA despite some fluctuations (Table 1). So the value and the quantity of LA seem to be increased from year to year.

Table 1**:** Imported data of lactic acid from 2002-2017

Year	quantity (Tons)	Value (Birr)
2002	0.45	34,692
2003	0.30	35,163
2004	1.35	171,036
2005	1.28	113,474
2006	3.44	285,137
2007	4.34	300,352
2008	3.10	326,434
2009	3.40	267,541
2010	4.73	359,661
2011	8.20	1,243,590
2012	9	1,500,123
2013	10	2,243,490
2014	11.09	3,432,211
2015	13.66	5,321,123
2016	15.67	8,214,984
2017	16.83	9,321,432

Source: Ethiopian Revenues and Customs Authority, 2017

2.3. Application of lactic acid

Lactic acid is used in food, pharmaceutical, cosmetic, chemical, textile and polymer industry, schools and research centers (Azhar, 2014 and Boontim *et al*., 2018). Since the lactic acid has gained increasing importance and it has been used in a great variety of forms as salt, ester and many other derivatives (Champomier-Vergs *et al*., 2002). The uses of lactic acid can be broken

down by grade and by lactic acid derivatives. Some of the important applications of lactic acid are given below (Yonas, 2016).

2.3.1. Food industry

Lactic acid is widely used in almost every section of the food industry, where it serves in a wide range of functions. In U.S, the major use of lactic acid in food and food-related applications accounts for approximately 85 % of the demand and the rest of around 15 % are for non-food industrial applications (Pikul-ngoen, 2010). It also occurs naturally in many food products and hence it is used as an acidulant (does not introduce a foreign element into the body), preservative and pH regulator for quite some time (Dusselier *et al*. 2013); regulates the micro flora of the food (Mudaliyar and Kulkarni, 2011); and in the processed meat and poultry industries, to provide products with an increased shelf life, improved flavor, and better control of food-borne pathogens (John *et al*., 2007). Another potential application of lactic acid in the food industry is the mineral protection of food products (Vijayakumar *et al*., 2008). Sometimes a combination of lactic acid and acetic acid are used for their greater bactericidal activity (Datta and Henry, 2006).

2.3.2. Pharmaceutical industry

Lactic acid (L (+) lactic acid but not D (-) because the D (-) isomer is not metabolized by the human body) used in pharmaceutical industry as an electrolyte in various parenteral/intravenous solutions (Krishna *et al.*, 2018). These parenteral solutions are prepared to supplement bodily fluids. In addition to this, lactic acid comes into play in pH-regulation, chiral intermediate and metal sequestration, mineral preparations, tablets, prostheses, controlled drug delivery system, surgical sutures, and in preparation of dialysis solutions for dialysis processes like Continuous Ambulatory Peritoneal Dialysis using artificial kidney machines (Wee *et al*., 2006). Lactic acid, along with its salts, acts as an intermediate in the manufacture of pharmaceuticals, to adjust the pH of preparations. Salts of lactic acid such as calcium, iron, sodium, and other salts are used in pharmaceutical industry because of their anti-tumor activity (Abd-Alsaheb *et al*., 2015).Therefore, the current applications of lactic acid in the pharmaceutical industry are under the following categories: Parenteral/I.V. (intravenous), dialysis solution, controlled drug delivery system, ammonium lactate for dry skin disorders, mineral lactate formulations for diseases such as anemia, hypertension, and osteoporosis and for chiral synthesis (Mudaliyar and Kulkarni, 2011).

2.3.3. Cosmetic industry

In cosmetic industries it is used as natural ingredients of moisturizers and pH regulators, but it possesses multiple other properties such as antimicrobial activity, skin lightening, and skin hydration (Krishna *et al.*, 2018). The moisturizing effect is related directly to lactate's water retaining capacity, and the skin-lightening action of lactic acid is produced by the suppression of the formation of tyrosinase (Wee *et al*., 2006). Since it is natural ingredients of the human body, lactic acid and its salt fit perfectly into the modern trend towards natural and safer formulations of cosmetic products, and thereby it produces such effects as skin lightening and rejuvenation which makes it very useful as active ingredients in cosmetics (Avinc and Khoddami, 2009). Lactic acid is popularly known as an alpha hydroxyl acid (AHA) in the cosmetics industry. It is widely used as a milder alternative to glycolic acid. It is primarily used as an anti-aging chemical claimed to soften lines, reduce photo damage from the sun, improve skin texture and tone and improve overall appearance (Vanessa, 2015).). However, precautions should be taken when using lactic acid as a cosmetic ingredient, because it can increase sensitivity to the sun's UV radiation (Vijayakumar *et al*., 2008).

2.3.4. Chemical industry

Currently, lactic acid is considered the most potential feedstock monomer for chemical conversions; this is because it contains two reactive functional groups, a carboxylic group and a hydroxyl group (Vijayakumar *et al*., 2008). Lactic acid can undergo a variety of chemical conversions into potentially useful chemicals, such as propylene oxide by hydrogenation, acetaldehyde through decarboxylation, acrylic acid by means of dehydration, propionic acid via reduction process, 2,3-pentanedione using condensation reaction, and dilactide via self-esterification) (Dusselier *et al.*, 2013). In the chemical industries, lactic acid is used in the dyeing of silks and other textile goods, as a harsh in the printing of woolens, in the bating and dropping of leathers, in the deliming of hides, in vegetable tanning, and as a flux for soft solders (Martinez *et al*., 2013).. Lactic acid functions as a descaling agent, pH regulator, neutralizer, chiral intermediate, solvent, cleaning agent, slow acid-release agent, metal complexing agent, antimicrobial agent, and humectant (Yonas, 2016). Natural lactic acid has an emerging use as an excellent and safe solvent, which can serve as an alternative in many fine mechanical cleaning applications. Moreover, due to the high solvency power and solubility of lactic acid, it is an excellent remover of polymer and resins (Wee *et al*., 2006).

2.3.5. Polymer industry

Nowadays, lactic acid is used as a monomer for producing poly lactic acid (PLA) which has wide range of application as biodegradable plastic (Champomier-Vergs *et al*., 2002). This kind of plastic is a good option for substituting conventional plastic produced from petroleum oil because of low emission of carbon dioxide that it causes global warming (Vanessa, 2015). The polymerization process can be applied to obtain PLA after lactic acid is produced (commonly through fermentation). PLA has also numerous uses such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap, and short shelf-life trays (Avinc and Khoddami, 2009 and Srivastava, 2018)

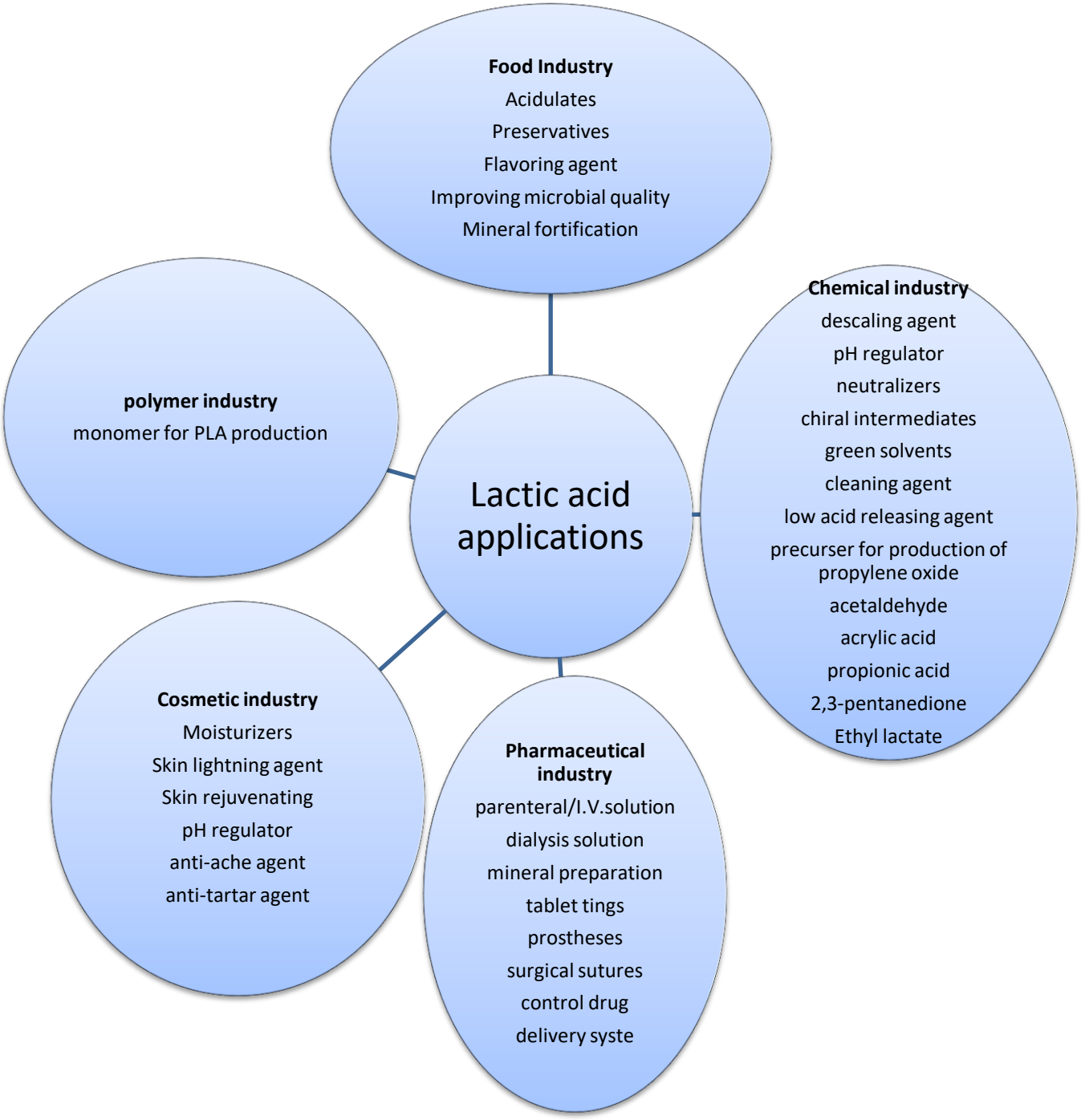

Figure 1: application of lactic acid in different industry

Source: AbdAlsaheb *et al* (2015) and Nawaz *et al* (2017)

2.4. Lactic acid Production process

Lactic acid is a naturally occurring organic acid that can be produced by fermentation and chemical synthesis (Vijayakumar *et al.*, 2008). However, it is more commonly produced from renewable resources via fermentation process. In fermentation processes, bacteria or other microorganism produce lactic acid as they metabolize carbon-containing (e.g. carbohydrate) raw material but in chemical synthesis it is based on the hydrolysis of lactonitrile, derived from acetaldehyde and hydrogen cyanide (Rashid, 2008).

2.4.1. Chemical synthesis

The commercial chemical synthesis process for lactic acid production is based on lactonitrile (Narayanan, 2004). It involves the addition of hydrogen cyanide to acetaldehyde to produce lactonitrile with base-catalyzed reaction. This is a liquid-phase reaction and occurs at atmospheric pressures. The crude lactonitrile is then recovered and purified by distillation and is hydrolyzed to lactic acid using either concentrated HCl or H_2SO_4, producing the corresponding ammonium salt and lactic acid (Holten *et al.*, 1971). The crude lactic acid is esterified with methanol, producing methyl lactate. The latter is recovered and purified by distillation and hydrolyzed by water under acid catalysts to produce lactic acid, which is further concentrated, purified, and shipped under different product classifications and methanol, which is recycled (Wasewar,2005).

2.4.2. Fermentation

Fermentation processes are characterized by biological degradation of substrate (commonly starch or cellulosic materials) by a population of microorganism (biomass) into products such as ethanol, citric acid and lactic acid (Farooq *et al.*, 2012). The most common fermentation processes divided based on the reactors used are (batch, repeated batch, fed-batch and continuous) (Krishna, 2018). Batch and fed-batch cultures producing higher lactic acid concentration than that of continuous culture, which has higher productivity (Rashid, 2008). In addition to higher productivity, the continuous process can also be continued for a longer period of time (Bayitse, 2015). And also based on the characteristic state of matter in the system it is divided submerged and solid substrate fermentations. Mostly in liquid (submerged) fermentation system the bioreactors are operated in batch, fed batch and continuous system to culture different types of microorganisms producing a wide range of products (Paulova *et al.*, 2013).In solid state

fermentation the process is carried out in solid matrix and bioreactors also operated in batch, fed batch and continuous system, but it is mostly operated in fed batch mode (Kumar and Lonsane, 1987).

2.4.2.1. Solid state Fermentation

Solid-state fermentation (SSF) is the fermentation process in which microorganisms develop on solid substrates without the presence of free liquid in which they use a natural substrate or inert substrate as solid support (Sadh *et al.,* 2018). The aim of SSF is to bring microorganism in tight contact with the insoluble substrate and to achieve the highest nutrient concentration from the substrate for fermentation (Bhargav *et al.*, 2008). In this fermentation technique, the substrates are utilized very gently and steadily, so the same substrate can be used for long fermentation periods (Subramaniyam and Vimala, 2012). SSF is used in lactic production by using solid substrates like wheat bran, rice and rice straw, fruit and vegetable waste, paper pulp, and bagasse (Qi and Yao, 2007).

The preferable substrate for SSF fermentation process are filamentous fungi, mainly due to their abilities to grow on substrates with reduced water activity, to penetrate their hyphae into the solid substrate, to produce exoenzymes (for example, amylolytic and cellulolytic enzymes), which decompose the polysaccharides (the main carbon source often present in solid substrates) (Paulova *et al*., 2013).

2.4.2.2. Submerged Cultivation

Submerged cultivation also called a liquid fermentation, it is the type of fermentation in which the substrate is liquefied or put off in a water source (Sadh *et al.,* 2018). Submerged cultivation of microbial cells in bioreactors guarantees a controlled environment for the efficient production of high-quality end products like lactic acid and to achieve optimum productivity and yield (Subramaniyam and Vimala, 2012). Industrial bioreactors operated in batch, fed-batch, or continuous mode are utilized by using different types of microorganisms to produce a wide range of products (Paulova *et al*., 2013).

2.4.2.3. Batch Fermentation

Batch is the simplest and most common operation mode for fermentation. At the beginning of the fermentation, the reactor is filled with the all carbon substrates, culture medium and other components are added at the same time. But neutralizing agents for pH control are added during

fermentation. After microbial inoculation, a lag phase is generally observed until the biological reaction begins. The main advantages of this mode are the reduction of the contamination risks because the bioreactor is closed and the high lactic acid concentrations obtained in comparison to other processes (Hofvendahl and Hahn-Hligerdal, 2000). For lactic acid industrial production, the processes are mainly implemented in batch mode. However, batch fermentation suffers from low productivities, due to either substrate and/or product inhibitions (Rashid, 2008).

2.4.2.4. Fed-batch Fermentation

In fed-batch fermentation, all the necessary raw materials includes carbon source, nitrogen source, and other required components are added during fermentation process at regular intervals of time without removal of fermentation broth (Krishna, 2018). The fed-batch fermentation of production of LA is used high substrate concentrations that caused microbial inhibitions, such as cell lysis or long lag phases (Paulova *et al*., 2013). This kind of inhibition results decrease the growth the biomass, decrease production and sugar consumption rates (Ding & Tan, 2006). Thus, low substrate concentrations can be maintained in the reactor using a fed-batch process. In this case, the substrate is fed continuously to the fermenter. There is no remove of output, so the broth volume in the reactor gradually increases during the fermentation. This leads inhibition of the product due to the accumulation the substrate (Abdel-Rahman *et al*., 2013).

2.4.2.5. Continuous Fermentation

In a continuous fermentation, fresh medium is added into the reactor with the same rate as the product exits from the fermenter. A steady state is established in this operation.

Product inhibition that is present in batch or fed-batch fermentation may be avoided in this case (Yang and Sha, 2019). At steady state, the cells are maintained at constant physiological state and growth rate. The efficiencies and advantages of continuous process over the batch processes; stability, ease of control and increase in the productivity, make the continuous process more attractive for the industry than a simple batch process. Nevertheless, continuous charge of the nutrients and substrate may lead to substantial losses that will add to the cost of the final product (Nesryia, 2018). The advantages of continuous fermentation are to prolong the microbial exponential growth phases during cultivation compared with batch processes, and thus reduce the processing time, ensuring high levels of the production of the final products; remove inhibition by substrates and/or byproducts, as the final products (Li *et al.*, 2014). The disadvantages of continuous fermentation processes can be summarized as; increase the risks of

contamination of the whole system; maintenance of stability of the fermentation and biological system over a long period of time is a challenge; the concentration of final product(s) obtained from continuous fermentation is usually lower than that from fed-batch processes, thus increasing the cost of the downstream processing; suffer from the need for complicated equipment and process flow sheet design (Verbelen *et al*.,2006).

2.5. Fermentation using Lactic Acid Bacteria

Lactic acid bacteria (LAB) are the most widely used bacterial group for industrial production of lactic acid, because of their long history in industrial scale production and they are safe to the consumers and production workers (Vijayakumar *et al*., 2008). LAB are gram-positive microorganisms widely present within plants, meat, and dairy products, they produce lactic acid as an anaerobic product of glycolysis with high yield and productivity. Most of the LAB are anaerobic, but some of them, for example some *Lactobacillus* species are facultative anaerobes; they can also grow in presence of oxygen (Yonas, 2016). Commercially important LAB strains, such as *Lactobacillus* strains, are particularly useful because of their high lactic acid yield, high acid tolerance and their ability to be metabolically engineered (Ghaffar *et al*. 2014).

Lactic acid bacteria are also classified as homofermentative or heterofermentative depending on the nature and concentration of the fermentation products of sugars. Homo-fermentative lactic acid bacteria produce lactic acid as a main end product.The homofermentative process takes place in two steps. In the former step, called glycolysis or Embdene-Meyerhof-Parnas-pathway, glucose is transformed into pyruvic acid, while in the latter is reduced to lactic acid by the reducing power previously produced in the form of NADH (Martinez *et al*., 2013). The homofermentative LAB includes *Lactobacillus delbrueckii, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus helveticus, Lactobacillus casei, Streptococcus lactis, Streptococcus cremoris, Streptococcus faecalis, Streptococcus thermophilus and Pediococcus cerevisiae* (John *et al*., 2007). The heterofermentative LAB include *Leuconostoc mesenteroides, Lactobacillus cremoris, Lactobacillus brevis and Lactobacillus fermentum* (Patel and Parikh, 2016). Heterolactic fermentation is characterized by the formation of other co-products such as CO_2, ethanol and acetic acid in addition to lactic acid (Srivastava, 2018). The first step of glucose degradation, which is called pentose phosphate pathway, leads to glyceraldehyde 3-phosphate, acetyl-phosphate and CO_2. Glyceraldehyde 3-phosphate enters the glycolysis through

which it is transformed into lactic acid, while acetyl-phosphate is converted to acetic acid and/or ethanol (Martinez *et al.*, 2013).

2.6. Identification method of lactic acid bacteria

Especially in industrial or applied microbiology units, phenotypic tests are still being used on a routine basis for the identification of (food-associated) LAB. These methods include morphological physiological and biochemical characterization, carbohydrate fermentation patterns. Some of phenotypic methods that are commonly used are gram staining, catalases test, carbohydrate fermentation test, morphological identification, and bile salt tolerance.in addition to this there is also genotypic or molecular identification in strain level (Temmerman *et al*., 2004). It is found that gram-positive, catalase-negative, non-sporing, anaerobic, but aero tolerant cocci or rods, acid-tolerant and strictly fermentative producing lactic acid as the major end product of carbohydrate-fermentation. (Karsma, 2015).

2.7. Factors affecting Lactic Acid Production

The productivity and yield of lactic acid production is influenced by numerous factors. The production of lactic acid is not possible without good fermentation parameter knowledge and effective quality control (Endris, 2017). Critical control points (CCP) during the lactic acid production process must be identified to ensure optimum lactic acid production. Of the many factors that are responsible for lactic acid fermentation process, the effect of temperature, pH, incubation time and inoculum size are the most dominant one (Hofvendahl and Hahn-Hägerdal, 2000).

2.7.1. Effect of pH

pH is an easily manipulated variable in the process and it has a very strong impact on the cellular metabolism and lactic acid production (Zhang,2015). From the production standpoint, pH control is absolutely required to achieve the lactic acid concentrations essential for an economical process (Pikul-ngoen, 2010). In general, LAB can tolerate pH values between 3.4 and 8.0, but growth and production mostly occur between pH 5.4 and 6.4, with the optimum pH being strain-dependent. The decrease in pH with time during the fermentation process may be attributed to the production of lactic acid from sugars (Abdel-Rahman *et al.,* 2013).

2.7.2. Effect of temperature

Temperature is one of the important factors that affect the growth of microorganism and the production rate. The effect of temperature on the growth and production rate is different for LAB strains (Pikul-ngoen, 2010). Most lactic acid bacteria which are responsible for the conversion of sugar to lactic acid are classified as thermophilic or mesophilic bacteria and usually have an optimum growth between 20 ºC and 40 ºC (Rashid, 2008). The yield of lactic acid production increases with each increase at temperature level of fermentation between 30 to 40 ºC, but above 45 ºC the lactic acid production decreases (Vanessa, 2010 and Zhang, 2015).

2.7.3. Effect of carbon source

A number of different substrates have been used to fermentation production of lactic acid by lactic acid bacteria. A wide variety of carbon sources are used for producing lactic acid, and these include molasses, fruits waste, glucose, sucrose, fructose and lactose (Ghaffar *et al* .,2014.

Literatures reported on the capability of several bacterial strains to produce lactic acid using molasses, whey, starchy, and cellulosic materials (Wee *et al*., 2006). The starchy materials used for lactic acid production include wheat, corn, sago, potato, rye, sweet sorghum, tapioca, potato, rice, and barley as a carbon source (John *et al*, 2007).

2.7.4. Effect of nitrogen source

Different sources of nitrogen including yeast extract, peptone, ammonium sulphate, corn step liquor and urea are added during lactic acid production (Vijayakumar*et al*., 2008). Yeast extracts are the most commonly used nitrogen source in lactic acid fermentation as it provides convenient growth for lactic acid bacteria (Altaf *et al*., 2005). However, the high cost of yeast extract imparts its negative impact on the economics and its uses in industrial scale processes (De Lima *et al.*, 2010). In order to improve the economic parameter of lactic acid fermentation by using cheap and comparable nitrogen sources corn step liquor, peptone, urea, and ammonium sulphate can be used (Nancib *et al*., 2001).

2.7.5. Effect of incubation time

Fermentation time is one of the critical environmental parameters affecting lactic acid production, its molecular mass, and the sugar composition (Azhar, 2014). Utilization of total sugar and the production of lactic acid increased as the fermentation time increased, thus reducing the available sugar content in the media but above optimum value of the fermenting time the production rate decreased because growth of the culture entered to the stationary phase and as a consequence of it slows down the metabolic activity (Farooq *et al.*, 2012, Sarkar and Paul, 2019)

2.7.6. Effect of inoculum size

Inoculum size of the LAB has an effect on the production of lactic acid. When it increased to optimum number, the production also increased and decreased when the inoculum size increased above optimum level (Nagarjun, 2015). For lactic acid fermentation the size of inoculum varies from 1- 10 % (v/v) Sheeladevi and Ramanathan, 2011). According to (Patel and parikh, 2016) the influence of inolculum size varies from 1-5% v/v.

2.8. Substrate used for lactic acid production with fermentation

Several carbohydrate materials have been used for the commercial production of lactic acid by fermentation. Sugar and starch also have food value and their sources are limited. Several by-products or raw materials have been evaluated as potentially inexpensive substrates for lactic acid production (Rashid, 2008). As indicated in Figure 2 below wheat straw, corn cob, corn stover, apple waste, banana stalk, corn stalk, banana peel, and orange peel are some of the substrates used for lactic acid production. Agro-industrial wastes such as spent grain of beer and peels of banana are an example of such type of raw materials which are inexpensive, readily available and those have positive impacts as green industry and on agro-industrial waste management system are used in this study.

Figure 2: Lignocellulosic substrates for lactic acid production.

Source: Ghaffar *et al.*, 2014

2.9. Availability of spent grain in Ethiopia

Currently, there is a rapid growth of brewery industries in Ethiopia. There are about eleven breweries in full function and production. There are also some breweries being expanded and

expected to be completed for the next few years. In Ethiopia around 263,736 kg barley spent grain is generated daily. The spent grain from Ethiopian breweries, now a days is only used for animal feed (Yohanes 2018).

Table 2: Capacity of spent grain generated from Ethiopian brewery industries,

No	Name of Brewery in Ethiopia	Capacity of spent grain generated Kg per day
1	Dashen(Gonder)	31,000
2	Dashen (Debrebrhan)	21,830
3	Saint George Hawassa	32,980
4	Saint George Addis Ababa	33,592
5	Saint George Kombelcha	32,640
6	Meta	21,930
7	Bedele	21,930
8	Harar	22,950
9	Waliya	31,980
10	Habesha	21,780
11	Raya	20,500
Total	**263,736**	

2.7. Lactic acid recovery

Product recovery is an important step in lactic acid production that is associated to separation and purification of lactic acid from fermentation broth (Li *et al.*, 2016). Fermentation broth contains a number of impurities such as residual sugars, color, nutrients and other organic acids, as part of cell mass (Li *et al.*, 2016). These impurities must be removed from the broth by different techniques in order to reduce lactic acid losses and achieve more pure lactic acid (Ghaffar *et al.*, 2014). Recently a number of different downstream processing methods for lactic acid recovery from fermentation broth have been studied such as: precipitations with calcium hydroxide, ultra- and nanofiltration, ion-exchange/adsorption, reactive distillation, electrodialysis (Komesu *et al.*, 2017), crystallization and extraction (Bishai *et al.*, 2015). Most of these separation technologies have either shown low selectivity or low recovery yield. Crystallization

for example shown high selectivity, yet, its low recovery of mother liquor restrained its application for large-scale purpose. Separation by precipitations with calcium hydroxide is conventional primary purification step for LA production by fermentation (Olszewska-Widdrat *et al*., 2019). Hence, the tendency for chromatographic techniques rose due to its low cost, high selectivity and simplicity of operation (Bishai *et al*., 2015). Chromatography method mostly depends on ion-exchange or adsorptive properties of resin to the products. It is a well-established operation for the final purification of lactic acids from aqueous solution, especially for refining of the products (Li *et al*., 2016). Among the various categories of chromatographic techniques, ion exchange chromatography is extremely selective, gives product recovery at very low cost within a short period of time, analyze the end product purity, increase the product yield, moderate operational conditions, generation of minimum amount of wastes and having low processing steps, chemical requirement and energy consumption (Ghaffar *et al*., 2014). Advances in membrane-based separation and purification technologies, particularly in microfiltration, ultrafiltration and electrodialysis (ED), have led to the beginning of new processes for lactic acid production that do not produce a salt waste. Successful commercial development of bipolar ED membranes has recently occurred. These membranes can split and separate water to protons (H+) and hydroxyl (OH−) ions and the membranes can operate at about 80% of the theoretical thermodynamic efficiency. This technology advance now enables the H+ to transport to the acid anion to form the free acid and the OH− ion to transport to the cation compartment to form the free base (Datta and Henry, 2006).

3. Materials and methods

3.1. Description of the study area

The experiment was conducted at Addis Ababa University Arat Killo compass Biomedical laboratory at time period between December 2017 upto May 2019

3.2. Sample collection

Six samples of ergo and siljo were collected from kebena Arat kilo, Amst killo and kasanchis of Addis Ababa by using sterilized bottles.and then the samples were labled and kept under refrigeration at 4°C until analysis

3.3. Isolation of lactic acid bacteria

The lactic acid producing lactic acid bacteria used in this study were isolated from samples of fermented products (*ergo* and *siljo*). To isolate LAB 10 g of sample (*ergo* and *siljo* separately) was suspended in 90 ml sterile distilled water and serially diluted. From the diluted samples, 0.1 ml of each of 10^{-3} to 10^{-6} dilution was aseptically spread evenly on MRS (De Man Rogosa and Sharpe) agar plates. Then, plates were incubated at 37 °C for 24 and 48 hrs. From countable plates some representative colonies were picked and streaked on fresh MRS agar plates to obtain pure colonies. This procedure was repeated in order to purify the isolates.

3.4. Screening of lactic acid bacteria for higher lactic acid production

The purified and cultivated LAB cultures were inoculated into 5 ml MRS broth medium and incubated for 48 hrs at 37 °C. Then, the growth was separately centrifuged and the supernatant was separated from the pellet. The supernatant (known volume) was taken into the flask and 2 drops of phenolphthalein was added to it. After shaking, it was titrated by using 0.1 N NaOH as titrant. Then, the volume of NaOH standard solution consumed was recorded as titratable acid and used to calculate the concentration of lactic acid produced (Chen *et al*., 2017; Khalil and Anwar, 2016). Then each of the LAB isolates that were good titer was further analyzed at 24 hrs. Finally, the isolate that were found producing good titer of LA from our stock were activated in 5 ml of MRS broth, and made ready for further analysis. The fermentation method was batch submerged fermentation method.

3.5. Biochemical and morphological characterization of good titer LAB isolates

3.5.1. Catalase test

The catalase reaction was determined by adding a drop of 3% H_2O_2 in to a clean microscopic slide. Then, 24 hrs incubated pure colony of isolates were transferred in to slide and the presence or absence of bubbles or foam formation was observed (Kimaryo *et al.* 2000).

3.5.2. Gram staining and microscopic examination

To test gram staining technique isolates were smeared on clean microscopic slide and heat-fix by quickly passing the slide through the Bunsen burner (upper part of flame). The smear stained with a drop of crystal violates and after 1 minute rinsed with tap water. Then, it was immersed into iodine solution for 1 minute and washed with tap water and decolorized with 95% alcohol for about 20 seconds and washed with tap water. Then, after counter stained with safranin for 60 second, finally rinsed with tap water, then allowed to air dry. The slides examined with (100x) objective lens microscope using oil immersion: Gram-positive cells appeared purple, while gram-negative ones appeared as pinkish-red (Toth *et al*., 2013).

3.6. Collection and preparation of the substrate

The two types of substrates those were used in this study were spent grain of beer and banana peel. The spent grain was obtained from Debre Berhan Dashn beer factory which is produced 21,830 Kgs brewers' spent grain daily (Yohanes, 2018) and 1,832.6 tons annually (Getu, 2018), which is actually being removed as animal feed and waste. Banana peels were collected from fruit juice producing kiosks in Addis Ababa. The banana peels were cut into pieces of about 3-5 cm length for drying and grinding. Then, the substrates were dried separately in oven at 60 °C for 72 hrs to obtain easily crushable material. After drying, each of the samples was milled separately to cut complex lignocellulosic biomass in order to increase surface area to volume ratio of the substrates by means of mechanical pretreatment. The particle size was reduced to 1.2-1.6 mm or was ground into powder form.

3.7. Substrates composition analysis

3.7.1. Moisture content determination

The moisture content of each sample was determined by oven drying method as described by AOAC (2005). In this process 3 g of the sample was dried in a hot air oven for 24 hrs at 102 ± 2 °C. The loss of weight was determined and recorded as the moisture content and expressed as;

$$Moisture\ content = \frac{W2 - W1 \times 100}{W2}$$

Where;

W1 = Initial weight of the sample

W2 = Weight of the dried sample

3.7.2. Determination of ash contents

The ash content was determined by the direct heating method as indicated in AOAC (2005). In this method, 3 g of each of the sample was measured into a crucible of known weight; the sample was burned to ash in a muffle furnace for 5 hrs at 550 °C. It was then cooled in a desiccators and the percent of ash was finally determined as follows;

$$\%Ash\ = \frac{W2 - W1 \times 100}{W}$$

Where:

W1 = Weight of crucible

W = Weight of sample test portion

W2 = Weight of dish and dry sample

3.7.3. Protein content determination

The macro Kjeldahl method as described by AOAC (2005) was used to determine the crude protein content. From each sample, 2 g was introduced into the digestion flask. Then after 10 g of copper sulphate and sodium sulphate in the ratio of 5:1 and 25 ml of concentrated sulphuric acid were added to the digestion flask. The flask was placed in a digestion block in fume cupboard and heated until frothing ceased given a clear and light blue coloration. The mixture was allowed to cool and diluted with distilled water until it reaches 25 ml of volumetric flask. Then after, 10 ml of the mixture was poured into the distillation apparatus and 10 ml of 40% sodium hydroxide was added. The released ammonia by boric acid was allowed to continue until 10 ml of boric acid is treated with 0.02 M of hydrochloric acid and the green color change to purple. Finally, the percentage of nitrogen in the sample was determined and calculated as the following:

$$\%\text{Crude protein}\ = \frac{10 \times 1000 \times \text{sample weight (2 g)}}{\%\text{Nitrogen} \times 6.25}$$

3.7.4. Fat content determination

The soxhlet solvent extraction method as described by AOAC (2005) was used to determine the fat content. In this method 2 g of the sample was weighed in a flat bottom flask of known weight with the extractor mounted on it. The thimble was held half way into the extractor and the weighed sample was carefully transferred into the thimble, and the thimble was plugged with cotton wool. The extraction was carried out at the temperature of 40-60 °C for 8 hrs. The solvent was removed by evaporation and then, the remaining part in the flask was dried in the oven at 80 °C for 30 minute and then cooled in desiccators. The flask was reweighed and the percentage fat was calculated as follows;

$$\%\text{fat} = \frac{\text{weight of extracted fat} \times 100}{\text{weight of sample}}$$

3.7.5. Determination of crude fiber

Acid-Base digestion method was used to determine the crude fiber content. Two gram sample was weighed in 600 ml beaker, 200 ml 1.25% H_2SO_4 was added and the mixture was boiled for 30 minutes on hot plate in the fume hood. After 30 minutes, 20 ml of 28% KOH was added and the mixture again was boiled gently for further 30 minutes, while stirred occasionally. The hot solution was quickly filtered under suction. The residue was washed several times with hot distilled water and then washed by 1% H_2SO_4 and filtered after rinsing the residue with distilled water; it was washed with 1%NaOH. The crucible containing the residue was once again washed with distilled water and finally by 1% acetone. The washed residue was dried in an oven at 100 °C to constant weight and cooled in a desiccators and weighed (C1). The weighed residue was ashed in a muffle furnace at 550 °C for 2 hrs, cooled in desiccators and reweighed (C2). Crude fiber content was expressed as percentage loss in weight on ignition (AOAC, 2005).

$$\%\ \text{Crude fiber} = \frac{\text{C1} - \text{C2} \times 100}{\text{weight of original sample}}$$

3.7.6. Determination of carbohydrate

Carbohydrate was estimated by difference:

% Total carbohydrate = 100 - (% moisture + %Ash + % fat + % Protein + % Fiber) (AOAC, 2005)

3.8. Pretreatment of the substrates

3.8.1. Steam Pretreatment

The separate powder of each substrate (10 g of each substrate powder placed in a conical flask with 200 ml of distilled water) was autoclaved at 121 ℃ for 15 minute. After completing autoclaving, samples were allowed to cool; and then the soluble form was separated from the non-soluble portion with filtration (Wondale, 2012). Then after, the non-soluble component was treated with acid(1% HCl)

3.8.2. Acid Pretreatment (acid hydrolysis)

Dilute acid pretreatment of each separate substrate was performed by adding dilute acid (100ml of 1% HCl) into the non-soluble component (which was separated with steam pretreatment). Each substrate into which the dilute acid added was heated at temperatures of 120 ℃ for 20 minutes and the soluble was separated with Whatmann filter paper following the protocol s(Wondale 2012).

3.8.3. pH Adjustment

The pH of the hydrolysate developed from acid post hydrolysis was adjusted with 1 M NaOH to 4.5-6.5. (Yohannes ,2018).

3.8.4. Fermentation set up

The fermentation method that were used in this study was batch submerged fermentation.The fermentation process was conducted by adding 200 ml of each of pretreated hydrolysate separately and 20 ml of MRS broth into 250 ml flask as indicated by Kiros Haddish (2016). Different concentration (3-8%) of each of selected potential LA producing LAB was inoculated in to each separate flask containing the mixture of pretreated hydrolysate and MRS. Then, the fermentation was carried out for four days at temperatureo range of 30 ℃ -45 ℃, pH (between 4.5 and 6.5).carbon source of 25-70g/l glucose and with nitrogen source of 5-15 g/l of $NH_4(SO_4)$, while it was being mixed in rotary incubator (180 rpm) following the protocol indicated by John, *et al.* (2008).Then after, production of LA and amount of reducing sugar were examined with titration and DNS method respectivly.

3.9. Analysis of factors that affect LA production

3.9.1. Effect of temperature

To study the effect of temperature on lactic acid production by AS1S (with inoculum size of 5%), the production medium (220ml of hydrolyzed substrates and MRS medium) was set at different

temperatures (30, 37, and 45 °C). The culture was incubated for the time of 24, 48, 72 and 96 hrs, at pH 5.5 and at constant nitrogen source and carbon source. Then, the amount of lactic acid produced was calculated with titration on daily bases. .

3.9.2. Effect of pH

The prepared substrates medium were placed in three separate 250 ml flasks and the pH in each flask was adjusted to 4.5, 5.5 and 6.5 pH using 1M HCl and 1N of NaOH. Then each flask was separately inoculated with 5% of 24 hrs old inoculum (AS1S) and kept in a shaker incubator at 180 rpm for 4 days at temperature of 37°C the procedure repeated for the second substrate. Then the amount of lactic acid was estimated on daily bases..

3.9.3. Effect of nitrogen

220 ml ready substrate medium were placed in three separate 250 ml flasks and the amount of ammonium sulphate was added in the value of 5, 10 and 15 g/l. Then, 5% 24 hrs cultured AS1S was inoculated in the separate flasks. Then after the temperature was adjusted at 37°C and pH of 5.5.The procedure repeated for the second substrate. Finally, incubated in a shaker incubator at 180 rpm for four days and the amount of lactic acid and reducing sugar were estimated in daily with 24 hrs gap.

3.9.4. Effect carbon sources

220 ml prepared substrate medium was placed in three 250 ml flask. To determine the effect of carbon source on the production of LA, different concentration of glucose (25,50 and 70 g/l) was added to the flask Then, 5% 24 hrs old AS1S was added on it. The procedure repeated for the second substrate. It was incubated in shaker incubater at 180 rpm with different incubation time regiments, namely 24, 48, 72 and 96 hrs at a pH of 5.5 and temperature of 37 °C. At the end the amount of lactic acid and reducing sugar were estimated daily with 24 hrs interval.

3.9.5. Effect of incubation Period

To find out the optimal incubation time for the maximal lactic acid production, 220 ml of prepared substrate medium was placed in three 250 ml flask and inoculated with 5% 24 hrs cultcure AS1S isolate and repeated for the other substrate then it was incubated in a shaker incubator at 180 rpm for different time intervals of 24, 48, 72 and 96 hrs at a pH of 5.5 and temperature of 37 °C as indicated by Patel and Parikh (2016). Lastly, the amount of lactic acid and reducing sugar was estimatedon daily.

3.9.6. Effect of inoculum size

220 ml of the prepared substrate medium was placed in three 250 ml flask. To know the influence of inoculum size on the lactic acid production, different inoculum levels (3, 5 and 8% v/v) were inoculated to each flask. Then, pH arranged at 5.5 and temperature of 37 °C. The procedure repeated for the other substrate and the fermentation stayed for four days. Lastly, the amount of lactic acid and reducing sugar was estimated.

3.10. Process optimization for maximum lactic acid production

220 ml of ready substrate medium was placed in a 250 ml flask. To examined the optimization product of lactic acid the factors of optimum value (optimum pH value, temperature, carbon source, time, nitrogen source and inoculum sizes) were combined together and incubate in shaker incubator at 180 rpm. Then the amount of lactic acid produced was estimated

3.11. Assessment of the utilization of substrates during fermentation

The residual reducing sugar content of the fermentation broth was determined using DNS (Dinitrosalcylic acid) Colorimetric method.

3.11.1. Preparation of DNS reagent

DNS reagent (solution) was prepared from sodium hydroxide (2 g/100 ml), DNS (2 g/100 ml), sodium potassium tartrate (4 g/100 ml), sodium sulfite (0.5 g/100 ml) and phenol (0.2 g/100 ml). These ingredients were dissolved in 100 ml of distilled water gently in 200 ml flask.

3.11.2. Preparation of standard curve

To determine the standard curve, 100 mg of anhydrous glucose was dissolved into 100 ml of distilled water in 500 ml flask. Appropriate volume of reagents was aliquoted in to each test tube. The content of the test tubes were mixed well and then placed in a boiling water bath for 5 minutes. The tubes were cooled in ice bath thoroughly and then 7.0 ml of distilled water was added to each tube. The optical density of the colored solutions was recorded at 540 nm using spectrophotometer using the solution in tube 1 as a blank.

Table 3: preparation of standard curve with DNS for estimation of reducing sugars

Tube No	Aliquot of stock solution (ml).	Distilled water (ml)	Dinitrosalicylic acid reagent (ml)
1(blank)	0.0	1.0	2.0
2	0.2	0.8	2.0
3	0.4	0.6	2.0
4	0.6	0.4	2.0
5	0.8	0.2	2.0
6	1.0	0.0	2.0

3.11.3. Estimation of reducing sugar in the fermentation

The estimation was done by mixing 1 ml of each sample with 2 ml of DNS reagent (DNS used to stop the reaction) into each test tube. Then, each test tube was kept in boiling water bath for 5 minutes and was cooled in cold water or waited until being cooled. Subsequently, its absorbance was measured at 540 nm using a spectrophotometer (Nwogwugwu *et al.*, 2016).the amount of reducing sugar from the sample calculated as follows.

$$\text{Reducing sugar content} = \frac{\text{absorbance of unknown sample} - \text{y\_intercept slope}}{\text{slope}}$$

3.12. Evaluation of the production of lactic acid

LA production was done by titrating the fermentation medium against 1M NaOH by using phenolphthalein as indicator using titratable acidity method (Section 3.2.).The amount of lactic acid in fermentation broth was determined by transferring the supernatant of fermented SGB and BNP hydrolate (which was centrifuged and filtered) into 500 ml flask. Then, 3 drops of phenolphthalein indicator was added into the flask. This was titrated with 1 M NaOH and the appearance of pink color marked to indicate the end point to record the volume of 1M NaOH. The titratable acidity was calculated as w/v following Sheeladevi and Ramanathan (2011) as shown below.

$$\text{g/l LA} = \frac{\text{Volume of NaOH consumed for titration x Gram Eq. wt of LA x N NaOH}}{\text{Volume of fermentation broth used for titration}}$$

3.13. Data analysis

Data analysis was performed by using SPSS version 23 software and Microsoft excels 2010. Results were summarized and presented using tables, and graphs. The comparisons for each mean was performed by using Tukey's Studentized Range test (HSD)

4. Results

4.1. Isolation, screening and biochemical identification of lactic acid bacteria

A total of 90 isolates of LAB were isolated from two fermented products of food samples (*ergo* and *siljo*), .. As shown in Table 3, out of 90 LAB isolates, 10 (11.1%), 66 (73.3%) and 14 (15.6%) isolates produced lactic acid with the range of 7-9 g/l, 10-16 g/l and 16-25.2 g/l at 48 hrs, respectively (Table 5). The characteristics of the 14 LAB were further studied. All of them were found Gram positive, catalase negative and most of them were found to be *Bacilli* (Table 4).

Table 4: screening of LAB for high acid producing ability

Samples	Number of LAB isolates per sample	Number of LAB isolates produced LA (7-9 g/l)	Number of LAB isolates produced LA(10-16 g/l)	Number of LAB isolates produced LA (16-25.23 g/l)
Siljo 1	15	1	11	3
Siljo 2	15	4	9	2
Siljo 3	15	2	13	-
*Ergo*1	15	3	7	5
Ergo 2	15	-	11	4
Ergo 3	15	-	15	-
Total	90	10	66	14

Table 5: morphological and biochemical characterization of the isolate

LAB isolates	Gram staining	Catalase test	Shape	Arrangement
AS1S	+	-	Co	S
AS2S	+	-	Co	S
AS3S	+	-	Co	S
AS7S	+	-	B	C
AS8S	+	-	B	C
E1AA	+	-	B	C
E2AA	+	-	B	C
E3AA	+	-	B	C
E1AG	+	-	B	P
E2AG	+	-	B	C
E4AA	+	-	B	C
E5AA	+	-	B	C
E6AA	+	-	B	C
E7AA	+	-	B	C

-: negative test, +: positive test, Co: *Cocci*, B: *Bacilli*, C: Cluster and P: Pair

The 14 pure LAB isolates were shown to produce good titer (16-25.23 g/l) of LA at 48 hrs as compared to others. Further study also conducted on these 14 isolates at 24 hrs as indicated in Appendex 2. On the basis of producing more titer of LA, isolate AS1S was selected for further analysis. It was characterized as Gram positive, catalase negative, coccus in shape, and dominantly arranged singly and pairs under a microscope (Table 4).

4.2. Proximate analysis of substrates

The results of proximate analysis of raw materials, the spent grain of beer (SGB) and banana peel (BNP) used for production of lactic acid are presented in Table 5. The moisture, ash, protein, fat and fiber content of SGB and BNP examined were shown as 7.38% and 5.08%, 3.85% and 5.09%, 4.70% and 6.65%, 3.11% and 1.70% and 4.41% and 12.65%, respectively (Table 5). The carbohydrate content was found as 76.56% and 66.59% of SGB and BNP in that order.

Table 6: Composition analysis of the substrate

Composition analysis	SGB	BNP
Moisture (%)	7.38±0.23	5.09±0.12
Ash (%)	3.85±0.52	7.32±0.44
Crude protein (%)	4.70±0.25	6.65±0.21
Crude fat (%)	3.11±0.18	1.70±0.14
Crude fiber (%)	4.41±0.23	12.65±0.21
Carbohydrate (%)	76.56±0.36	66.59±0.41

SGB: Spent grain beer; BNP: banana peel

4.3. Effect of factors on lactic acid production

4.3.1. Effect of temperature on lactic acid production

The optimum temperature for maximum amount of lactic acid production (24.1 g/l from SGB and 22.11 g/l from BNP) by AS1S was shown at 37 °C and 72 hrs (Figure 3).

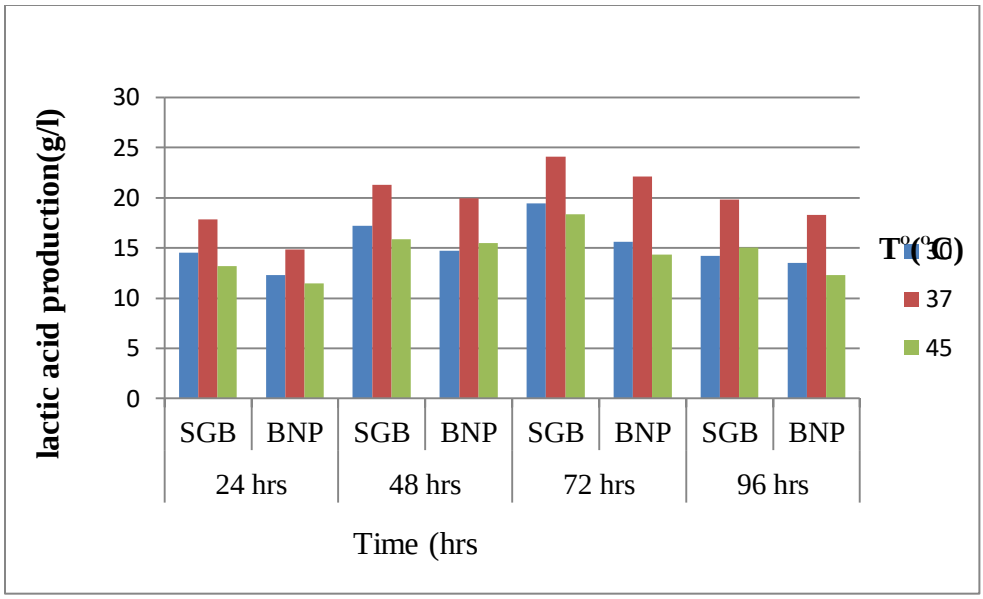

Figure 3: Effect of temperature on lactic acid production

Key=SGB, Spent grain beer; BNB, Banana peel

4.3.2. Effect of pH on lactic acid production

. The optimum pH for lactic acid production by AS1S was found at 5.5 (24.3 and 22.72 g/l from SGB and BNP, respectively).The amount of lactic acid at pH of 5.5 was shown as 17.85 g/l and 16.2 g/l at 24 hrs; 21.7 g/l and 19.63 g/l at 48 hrs; 24.3 g/l and 22.72 g/l at 72 hrs and finally at 96 hrs it was found out as 20.34 g/l and 18.85 g/l from SGB and BNP substrate, respectively at 37 °C . The highest amount of LA was produced by AS1S at pH 5.5, 72 hrs and 37 °C from SGB (Figure 4).

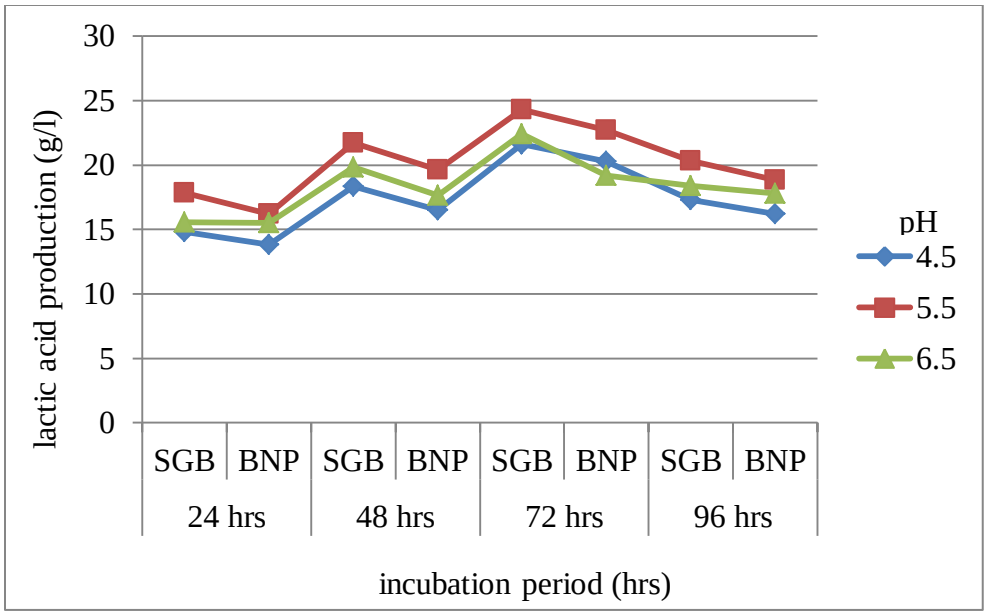

Figure 4: Effect of pH on lactic acid production

Key=SGB, Spent grain beer; BNB, Banana peel

4.3.3. Effect of carbon (glucose) on lactic acid production

The isolate AS1S produced the maximum amount of LA (27.54 g/l and 26.14 g/l from SGB and BNP, respectively) at 50 g/l of glucose concentration enrichment at 72 hrs of fermentation (Figure 5).

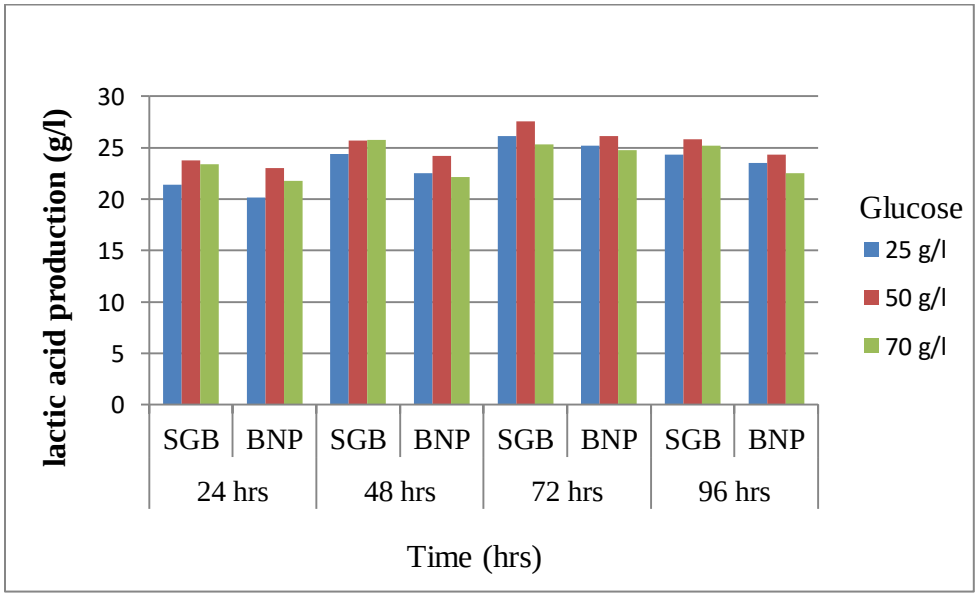

Figure 5: effect of glucose on lactic acid production

SGB: Spent grain beer; BNB: Banana peel

4.3.4. Effect of nitrogen on lactic acid production

The effect of nitrogen source on the activity of AS1S isolate and the production of LA was studied by adding different concentration of ammonium sulphate (5 g/l, 10 g/l and 15 g/l) into the fermentation hydrolyte. The isolate AS1S produced the maximum amount of LA (24.92 g/l and 23.85 g/l from SGB and BNP, respectively) at 15 g/l ammonium sulphate as nitrogen source with incubation time of 72 hrs, in that order (Figure 6).

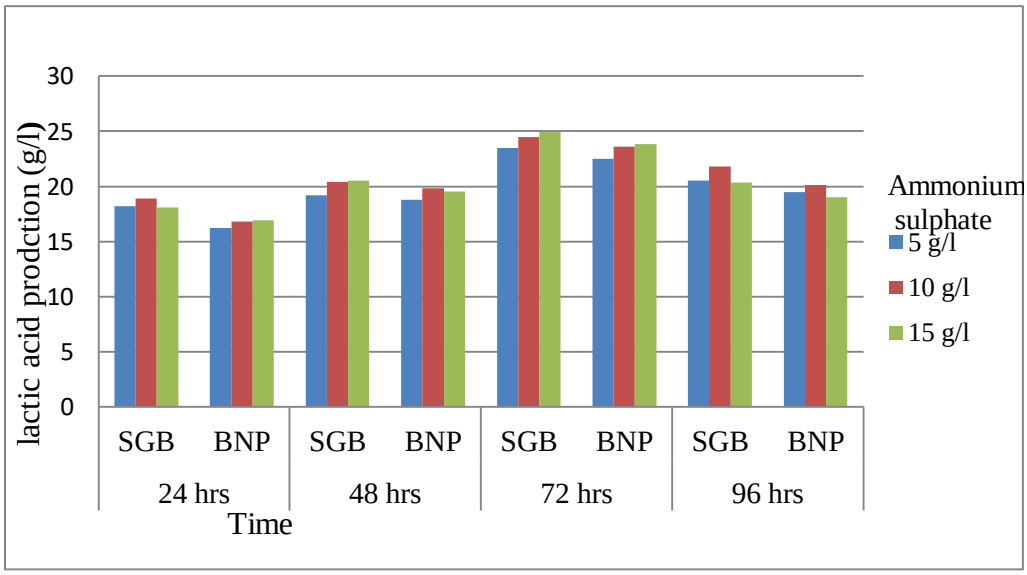

Figure 6: Effect of nitrogen on lactic acid production

Key=SGB, Spent grain beer; BNB, Banana peel

4.3.5. Effect of inoculum size

The effect of inoculum size of the selected LAB isolate, AS1S, was studied to optimize the inoculum size for maximum lactic acid production using the two substrates as nutrient source. A different amount of inoculum size (3, 5 and 8%) was studied. A maximum amount of lactic acid was produced (23.57g/l from SGB and 21.11g/l from BNP) when inoculum size of 5% was used (Figure 7).

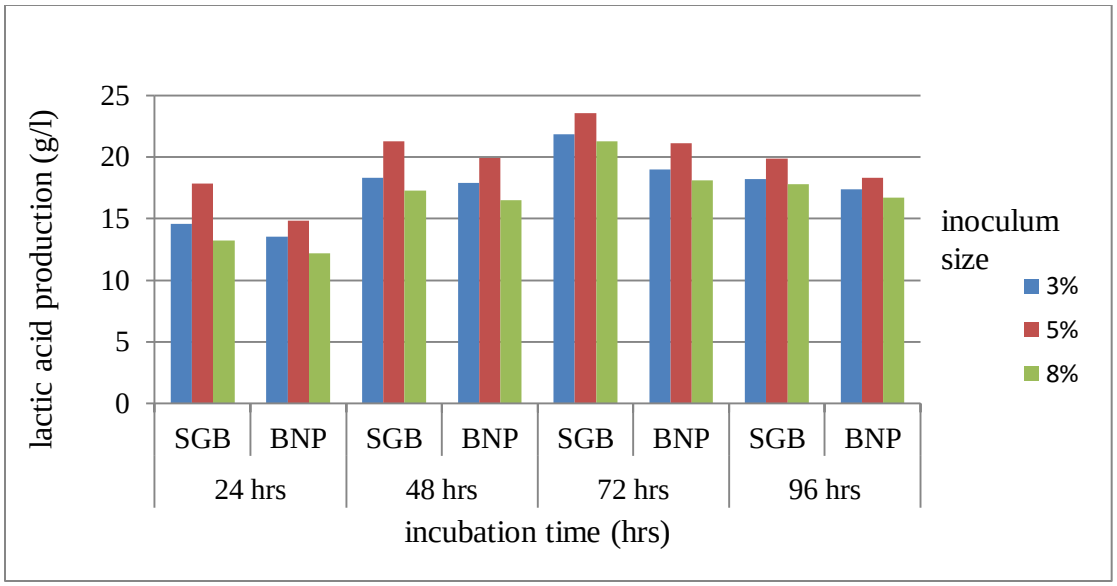

Figure 7: Effect of inoculum size on lactic acid production

SGB: Spent grain beer; BNB: Banana peel

4.4. Optimization of parameters for maximum lactic acid production

In this study the amount of lactic acid produced from the combining factor of 50 g/l of glucose (as a carbon source), 15 g/l ammonium sulphate (nitrogen source) at 37 ^{o}C, pH 5.5, 5% of inoculums size and for 72 hrs of fermentation time were 27.73 and 25.98 g/l from SGB and BNP in that order. In all parameters high amount of lactic acid was produced from SGB hydrolyte substrates.

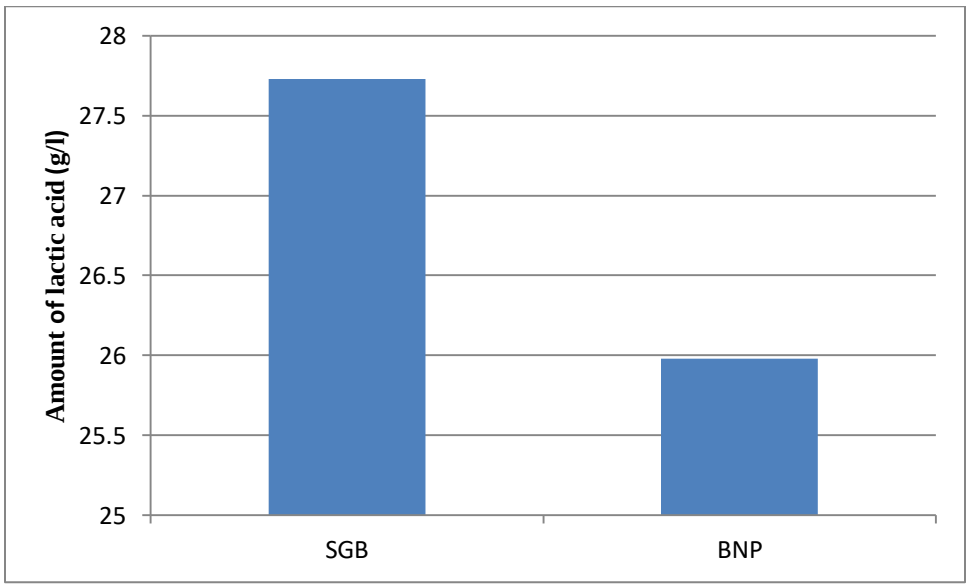

Figure 8: Optimized lactic acid amount

4.5. Estimation of reducing sugars (analysis of substrate consumption)

As shown in Figure 8, the amount of reducing sugar in the fermentation broth was reduced when the incubation time was extended from 24 hrs to 96 hrs (Figure 9)

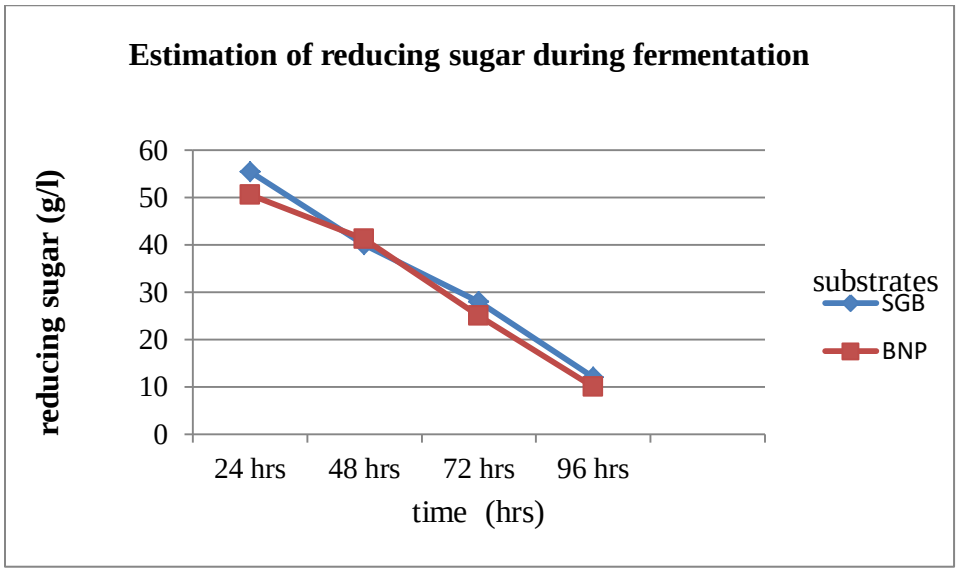

Figure 9: Estimation of reducing sugars

SGB: Spent grain beer; BNB: Banana peel

5. Discussion

The high acid producing LABS were tested based on titration of the supernatant.Similar study also stated that the high acid producing LAB were selected based on the amount of acid produced (Chen *et al*., 2017; Khalil and Anwar, 2016). Based on cultural and microscopic characteristics of AS1S, it was shown that, Gram-positive coccus, dominantly arranged singly or pairs and catalase negative. Generally, lactic acid bacteria commonly grow in/on MRS medium and produce lactic acid as their sole product (Yohannes, 2018).

In this study, the proximate analysis (Table 4) , enrichment and optimization studies (Figure 3 to Figure 6) of the two substrates (SGB and BNP) indicated that enrichment of the two substrates make them nutritionally rich enough to support the growth of LAB and produce LA. Similarly, Farooq., *et al* (2012) stated that agroindustrial wastes rich in carbon source have been considered as attractive nutrient source for industrial lactic acid production. The results of this study

displayed that the moisture (7.38% and 5.08%), ash (3.85% and 5.09%), protein (4.70% and 6.65%), fat (3.11% and 1.70%) fiber (4.41% and 12.65%) and carbohydrates (76.56% and 66.59%) contents of SGB and BNP, respectively. Comparable to the result of this study Ravindran *et al.*, (2018) have indicated that the composition of BSG as 79.9%, 3.3%, and 2.4% of carbohydrate, crude fiber, and protein, respectively. Similarly, Kieran *et al* (2016) have stated that the ash content of BSG ranges from 2-5% and lipid from 3-10%. Regarding to the compositional analysis of banana peel, different studies also indicated comparable results with of this study. Pyar and Peh (2018) have stated that 8.8 % of ash, 5.3% of protein, 1.6% of lipid and 19.2 % of fiber from banana peel. In agreement with this study, Hassan *et al* (2018) have also reported 9.60 % of ash, 1.95 % of crude protein, 5.93% of crude lipid and 8.37% of crude fiber.

Optimization of the production of LA via fermentation process need to be done by optimizing the most important parameters in the process that favors and support the growth of LAB (Yonas 2016).Temperature is one of the environmental parameters that affect lactic acid production (Nesryia, 2018). In this study the optimum temperature for the highest amount of lactic acid production (24.1 g/l from SGB and 22.1 g/l from BNP) was recorded by isolate (AS1S) at 37°C (Figure 3). Reports from different works in the field indicated that maximum amount of lactic acid production by LAB has been shown to be at 37°C (Yao and Qi, 2007; Panesar *et al*., 2010; Sheeladevi and Ramanathan, 2011; Hadish, 2016; Patel and Parikh, 2016; Boontim *et al.*, 2018 Shindo and Tachibana (2004)). Comparable to the result of this study, Rashid (2008) has stated that the highest lactic acid production was obtained at 37°C and the yield obtained was 28.73 g/l and when the temperature was increased to 45°C the lactic acid production reduced to 26.79 g/l yield. Like the result of this study, Rashid (2008) has demonstrated that a further increase of incubation temperature to 50°C resulted in further decrease of lactic acid production to 20.53 g/l. The optimum pH for LA production with isolate (AS1S) was shown to be at 5.5 (Figure 4). Similar studies showed that the optimum pH value for production of LA was 5.5 by using LAB species (Nagarjun, 2015; Sheeladevi and Ramanathan, 2011, Yao and Qi, 2007; Ghaly *et al*. ., 2004). Studies conducted by Nesrya (2018) and Endriss (2017) have shown that the maximum lactic acid production of 17.4 g/l and 16.94 g/l was obtained at pH of 6 using *Lactobacillus plantarum*. Among different carbon sources, glucose was used in this study due to limitation of resource. As shown in Figure 5, the isolate (AS1S) produced the maximum lactic acid at 50 g/l of glucose concentration during different incubation period. Nagarjun (2015) and Toptas *et al*,

(2014) have stated that the maximum lactic acid yield was noticed when commercial glucose was supplemented as carbon source at the concentration of 50 g/l by *lactobacillus* spp. Other studies ,Sheeladevi and Ramanathan (2011); Hassan (2014) have indicated that glucose was used as a substrate for lactic acid production using LAB at 60 g/l of glucose; but the glucose concentration above this value was shown decreasing. on the other hand the application of 40 g/l carbon source (glucose) showed further decrease in the value of lactic acid concentration (Sheeladevi and Ramanathan (2011); Hassan (2014)). The nitrogen source is another factor that has a great influence on the production of lactic acid by LAB. This is because lactic acid bacteria are nutritionally fastidious, requiring nitrogen sources for growth and product synthesis (Gomez-Gomez *et al*., 2015). Among the different sources of nitrogen, $(NH_4)_2SO_4$ was considered as feasible and inexpensive alternative nitrogen sources for lactic acid production. In this study the maximum lactic acid production was observed when 15 g/l of $(NH_4)_2SO_4$ was used under the optimized pH (5.5) using the two substrates (SGB and BNP) with the isolate AS1S (Figure 6). Arasaratnam *et al* (1996) have proposed that whey with total sugar of 30 g/l supplemented with 10 g/l of $(NH_4)_2SO_4$ resulted in 22 g/l of lactic acid at 48 hrs incubation time. Another study also revealed that supplementing with 5.65 g/l of $(NH_4)_2SO_4$ produced 18.68 g/l of LA (De Lima *et al.,* 2010).

In this study, maximum of lactic acid production (23.57 g/l from SGB and 21.11 g/l from BNP) was observed when inoculum size of 5% applied (Figure 7). Lower inoculum size resulted in insufficient biomass formation and the lower LA production and this was due to the fact that the need of relatively prolonged time for the organism to build enough biomass to increase the production of LA (Nagarjun, 2015). Whereas, at high concentration of inoculum, the bacteria build heavy mass in short period of time and the nutrients present in the media may become insufficient to support the increased number of bacteria and making it very difficult for them to produce the desired product (Panesar *et al*., 2010). The result of this study is agreement with by the report of Nagarjun (2015) in which 5% (v/v) inoculum size was recorded as being the optimum for maximum lactic acid production of 0.9 g/g. In addition, the optimum length of time for production of maximum amount of LA was observed at 72 hrs of incubation. Similarly, Sheeladevi and Ramanathan (2011), Patel and Parikh (2016) and Toptas *et al.* (2014) have showed the production of higher titer of lactic acid at the fermentation period of 72 hrs as compared to 24, 36, 48 and 96 hrs. However, at 96 hrs of incubation, the amount of lactic acid

reduced due to the depletion of nutrient and the death of microbes. On contrary, Haddish (2016), Umesh and Preethi (2014) have stated that the immense reduction of LA content after third day was decreased because of the limiting amount of reducing sugars in the batch medium and it was an indication of the reaching of the bacterium into the stationary phase and then after to the decline phase.

The amount of reducing sugar was reduced when the time of incubation was extended and this was due to the fact that the bacteria utilize the glucose to lactic acid and into other byproducts. As indicated by Haddish (2016) and Umesh and Preethi (2014), reducing sugar analysis according to DNSA method revealed a gradual decrease in the residual reducing sugar content due to the consumption of the sugars by the starter culture as fermentation process proceeded. Nesryia (2018) stated that the amount of reducing sugar was decreased when the incubation time of the fermentation increased to 30 hrs.

6. Conclusion and recommendation

6.1. Conclusion

. The optimized conditions for maximum amount of LA production using the two substrates (27.73 and 25.98 g/l from SGB and BNP, respectively) were shown at temperature (37 °C), pH (5.5), carbon source (50 g/l glucose), nitrogen source (15 g/l of $(NH_4)_2SO_4$), incubation time (72 hrs) and inoculum size (5%). Therefore, the results of this study have clearly exhibited that the production of LA using the two enriched substrates and optimized important parameters will hopefully facilitate the possible utilization of agro-industrial byproducts, managing the waste and thereby reducing the level of environmental pollution due to disposing the wastes into the environment. It will also serve initiating and introducing local biotechnological knowledge and skills for production of different products using local resources and skills. The results of this study has the potential of being promoted to large scale production (industrial scale) and is hoped to reduce the amount of lactic acid imported into the country and thereby saving foreign currency

6.2. Recommendation

This study would like to suggest the following recommendation.

- ♠ Further study should be conducted for different factors like agitation, different carbon sources, and different nitrogen source in combination and separately to study their effects on the production of lactic acid.

- ♠ Evaluating more than one LAB cultures (mixed cultures of LAB) for production of LA.
- ♠ Molecular identification of the LAB isolate should be done in order to identify the selected isolate to strain level.
- ♠ Testing the potential use of other wastes generated from home, restaurant, juice house, hotels, cafeterias, industries and chips frying shops for production of lactic acid in order to find the cheapest resource and best substrate for production LA using LAB isolates.

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APPENDIX

Appendix table 1: LAB isolates and screening of them

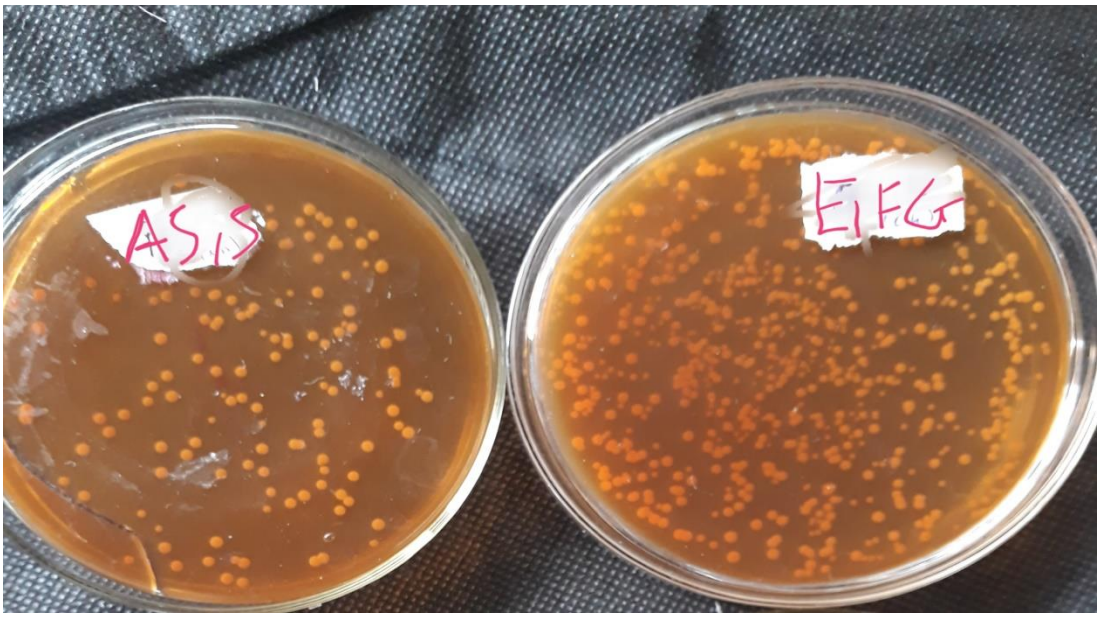
isolates	Lactic acid (g/l)	isolates	Lactic acid (g/l)
AS18	7.70±0.11	AS11S	15.15±0.21
AS22	8.11±0.66	AS12S	13.96±0.35
AS26	7.60±0.35	AS13S	13.31±0.41
AS27	8.30±0.46	AS14S	14.91±0.43
AS28	8.50±0.97	AS15S	11.80±1.13
AS33	8.70±0.42	E10A	13.10±0.98
AS34	7.30±0.14	E11A	13.01±0.43
E14A	8.70±0.06	E12A	14.70±0.56
E15A	8.91±0.14	E13A	15.31±0.14
E19A	8.66±0.56	E16A	13.05±0.21
AS10	11.91±0.84	E17A	10.81±0.98
AS11	13.05±0.63	E18A	14.15±0.49
AS12	10.82±0.80	E20A	10.70±0.28
AS13	14.90±0.99	E21A	13.65±0.50
AS14	15.61±0.62	E22A	13.25±0.63
AS15	13.52±1.20	E23A	14.30±0.21
AS16	13.02±0.53	E24A	13.87±0.48
AS17	15.53±0.50	E25A	13.87 ±0.48
AS19	13.21±0.60	E26A	10.87±0.90
AS20	15.91±0.20	E27A	14.62±0.035
AS21	13.41±1.10	E28A	15.40±0.28
AS23	14.90±0.99	E29A	15.35±0.63
AS24	15.65±0.49	E30A	14.45±0.21
AS25	14.15±0.92	E31A	11.80±0.70

AS29	13.95±0.49	E32A	13.11±0.64
AS30	11.90±0.84	E33A	13.11±0.49
AS31	14.85±0.92	E34A	13.24±0.46
AS32	12.20±0.49	E35A	15.95±0.71
AS35	10.75±0.64	E36A	14.82±0.16
AS36	15.10±0.28	E37A	12.92±0.81
AS37	15.30±0.98	E38A	15.71±0.12
AS38	14.65±0.49	E39A	13.26±0.18
AS39	15.11±0.13	E3AG	15.40±0.11
AS4S	13.91±0.78	E4AG	13.85±0.92
AS5S	11.85±0.76	E5AG	11.11±0.27
AS6S	11.95±0.49	E6AG	15.50±0.28
AS9S	14.35±0.91	E7AG	12.10±0.35
AS10S	15.45±0.78	E8AG	13.90±0.53

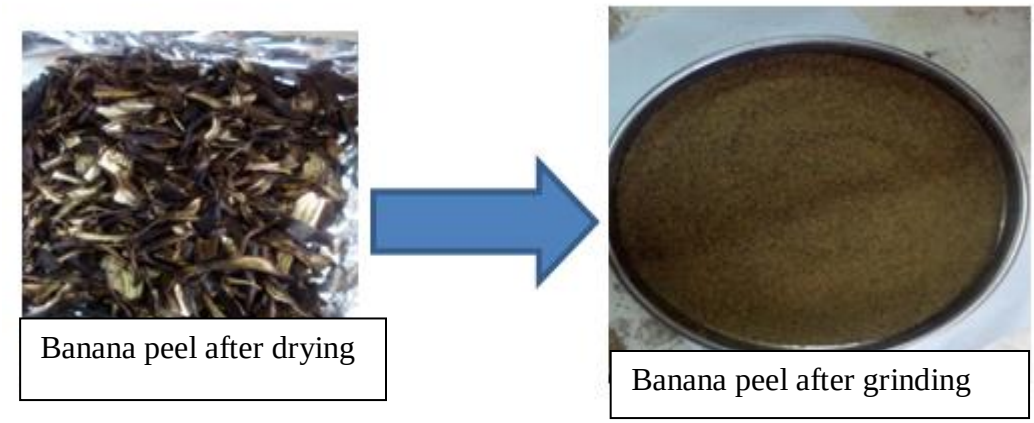
Appendex table 2: good titer of lactic acid production

LAB Isolates	LA (g/l) at 24 hrs	LA(g/l) at 48 hrs
AS1S	23.35±0.43	25.20±0.14
AS2S	20.70±0.56	23.25±0.35
AS3S	19.9±0.43	24.15±0.21
AS7S	19.70±0.70	21.08±0.74
AS8S	22.75±0.77	24.00±0.00
E1AA	16.55±0.63	19.02±0.03
E2AA	14.51±0.72	16.64±0.06
E3AA	16.85±0.63	18.45±0.63
E1AG	15.90±0.42	18.50±0.70
E2AG	14.25±0.35	16.65±0.64
E4AA	15.5±0.42	17.14±0.06
E5AA	17.20±0.14	17.96±0.19
E6AA	17.90±0.14	18.40±0.55
E7AA	17.45±0.35	18.82±0.31

Appendex figure 1: Lactic acid bacteria on MRS media

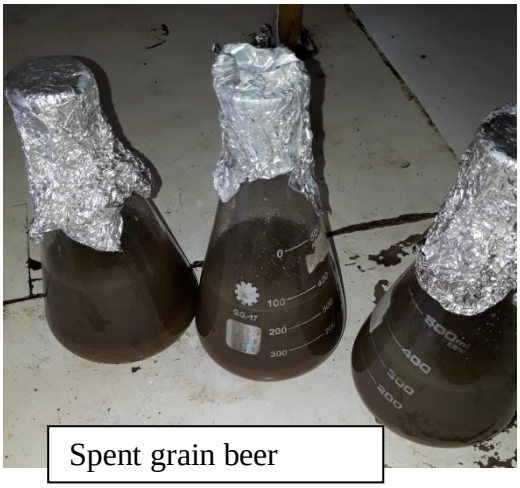


Appendix 4: over view of the substrates



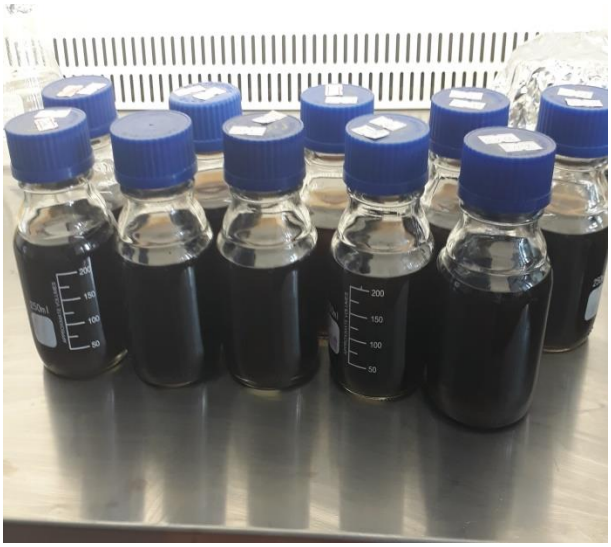
Appendex figure 2: pretreatment of the substrate

A. steam pretreated substrates



B. acid hydrolysis of the substrate

Appendex figure 3:Fermentation set up



a) Hydrosylate ready for fermentation

b) after fermentation

Appendex 4: After centrifugation ready for titration