PURIFICATION OF MICROBIALLY PRODUCED CA-L(+)-LACTATE WITH A NOVEL TECHNIQUE: DRYING INDUCED ELUTION OF IMPURITIES

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ABSTRACT

PURIFICATION OF MICROBIALLY PRODUCED CA-L(+)-LACTATE WITH A NOVEL TECHNIQUE: DRYING INDUCED ELUTION OF IMPURITIES

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For the production of calcium L-(+)-lactate (Ca-L(+)-Lactate) fermentation was performed by using *Lactobacillus casei*. Production with inexpensive raw materials and low cost purification of lactic acid (LA) as Ca-L(+)-Lactate was aimed because LA production from renewable materials is limited due to high production and purification costs. Fermentation conditions were 37°C for temperature, pH was kept at 5.5 by adding $CaCO_3$ as neutralizer and stirring was adjusted at 120 rpm. Fermentation medium consisted of 7-10% carbohydrates from different substrates, 0-2.9% yeast extract and was supported by minerals and working volume of fermentation was 100 ml in small scale and 70 L for large scale. After fermentation was done solid particles were filtered off and the filtrate was evaporated to obtain 10% Ca-L(+)-Lactate. The color of the filtrate was light brown due to soluble proteins, trace metals and various other biochemical materials within. As an initial purification step, crystallization was done. The filtrate containing minimum 10% Ca-L(+)-Lactate was crystallized at 4°C and the crystals obtained were separated from liquid phase under vacuum. Nevertheless, the Ca-L(+)-Lactate cake obtained still

had protein and trace metals in the inclusions. The cake was light yellow in color and the color deviation from white was treated as the impurity indicator since pure Ca-L(+)-Lactate is white in color. Its color difference from white was 267 ΔE in dry basis, it had still 0.1 g/L protein and 269 ppm Zn, 809 ppm Fe, 22 ppm Cu. Since the cake still had protein and trace metals further purification technique which is drying induced elution of impurities was experimented. Air flow rate and temperature were selected as 1 m/s and 55 °C and it had been observed that repeating more than four times of drying was unnecessary since there had been no further color change between bottom parts of samples. During drying part of purification step initial Ca-L(+)-Lactate had been 90.8 % in dry basis and became 98.3 % after four drying stages 90.2 % of the coloring compounds were removed. However, there was still 34 ppm protein, 97 ppm trace metals (Fe, Zn, Cu) and probably other undetermined materials in the final Ca-L(+)-Lactate powder.

Keywords: Calcium Lactate, Fermentation, Purification

ÖZ

MİKROBİYEL OLARAK ÜRETİLEN KALSİYUM L-LAKTATIN ÖZGÜN BİR TEKNİKLE SAFLAŞTIRILMASI

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Kalsiyum L-(+)-laktat üretimi için *Lactobacillus casei* suşu kullanılarak fermantasyon gerçekleştirilmiştir. Geri dönüştürülebilir kaynaklardan laktik asit üretimi üretim ve saflaştırma maliyetinin çokluğundan dolayı kısıtlıdır bu yüzden bu çalışmada laktik asidin L-(+)-laktat olarak ucuz hammaddeler kullanılarak üretimi hedeflenmiştir. Fermantasyon 37 °C'de gerçekleşmiş, pH $CaCO_3$ eklenerek 5.5'ta tutulmuştur ve karıştırma devri de 120'dir. Fermantasyon ortamı farklı substratlarla oluşturulan %7 ila %10 karbonhidrat, %2,9'a kadar maya özütünden elde edilen protein ile birlikte minerallerle desteklenmiştir ve çalışma hacmi küçük ölçekli deneyler için 100 ml ve büyük ölçekli deneyler için 70 L'dir. Fermantasyon sonucunda ortamdaki katı maddeler filtrelenip sıvı faz %10 Kalsiyum L-(+)-laktat oranına ulaşana kadar buharlaştırılır. Bu sıvı fazın rengi içinde bulundurduğu çözünmüş protein, eser metal ve bazı bilinmeyen maddelerden dolayı açık kahverengidir. Kalsiyum laktatı sıvı fazdan ayırmak için ise kristalizasyon uygulanır. %10 kalsiyum L-laktat içeren sıvı faz 4°C'de bir gün boyunca kristalleştirilir ve elde edilen kristaller sıvı fazdan vakumla süzme yoluyla ayrılır. Ancak yine de elde edilen kalsiyum laktat katısı içerisindeki nemde daha az da olsa protein ve metal içerir. Bu katı uçuk sarı renktedir ve saf kalsiyum laktat beyaz

renkte olduğu için beyaz renkten farklılığı bir saflaştırma indikatörü olarak kabul ediyoruz. Bu katının renk olarak beyazdan farkı kuru maddede 267'dir, hala içerisinde 0,1 g/L protein, 269 ppm Zn, 809 ppm Fe, 22 ppm Cu bulunduruyordu. Bu katı halen biraz protein ve bazı eser maddeler içerdiği için ileri bir saflaştırma yöntemi olan "drying induced elution" a ihtiyaç duyuldu. En geçerli hava akış hızı ve sıcaklığı 1 m/s ve 55 °C olarak belirlendi ve aşamalı kurutmanın dört defa yapılmasının bu yöntemle daha fazla saflaştırma gerçekleştirilememesi nedeniyle yeterli olduğu görüldü. Saflaştırmanın dört aşamalı kurutma kısmında kuru maddede %98,3 kalsiyum laktat elde edilmiştir ki ilk numune kuru maddede %90,8 kalsiyum laktat içeriyordu, ayrıca rengin de %90,2'si uzaklaştırıldı. Ancak, son üründe bile kuru maddede 34 ppm protein, 97 ppm metal (Fe, Zn, Cu) ve bazı bilinmeyen maddeler vardı.

Anahtar kelimeler: Kalsiyum laktat, fermantasyon, saflaştırma

To my family

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Q = air flow velocity

AAS = atomic absorption spectroscopy

CIE = *Commission internationale de l'éclairage* (International Commission on Illumination)

°C = degree celcius

MRS = De Man Rogosa and Shapes

d = density

D- = *dexter* (on the right)

ø = diameter

DC = direct current

db = dry base

et al. = *et alii* (and others)

g = gram

g/L = gram per liter

g/mL = gram per mililiter

HPLC = high performance liquid chromatography

h = hour

inc. = incorporation

LA = lactic acid

LAB = lactic acis bacteria

LSW = lactose from sweetened whey

L- = *laevus* (on the left)

L = length

MD = malt dust

m/s = meter per second

mL = milliliter

mm = millimeter

min = minute

M = molarity

nm = nanometer

ppm = part per million

% = percentage

PLA = Polylactic acid

PME = powdered malt extract

pH = power of hydrogen

Q_v = productivity

rpm = revolution per minute

SPYE = spent Brewer's yeast extract

T = temperature

TM = total metal

UV = ultraviolet

(v/v) = volume by volume

(w/v) = weight by volume

WP = whey powder

YE = yeast extract

CHAPTER 1

1. INTRODUCTION

1.1. Significance of the study

Lactic acid (LA) polymers are promising to be major biodegradable plastics with many applications. LA production is limited by the cost of production and purification. Low cost raw materials and low cost processes for purification are sought. LA can be produced either by chemical synthesis or by fermentation; however, biotechnological fermentation is preferable because of high enantiometric purity, possible utilization of renewable sources as raw materials. Although low cost production are searched for LA production, few studies have been published for low cost purification of LA. This study focuses on developing an economic process for producing Ca-L(+)-Lactate and a novel process for the purification of solid Ca-L(+)-Lactate.

1.2. Objectives and hypothesis of the study

The main objectives of this study are to produce pure Ca-L(+)-Lactate obtained from biotechnological fermentation of low cost raw materials and to use a novel purification step. A series of processes were practiced in which crystallization, crystal separation, elution of impurities through surface and mechanical removal of impurities took place to obtain nearly pure Ca-L(+)-Lactate.

1.3. Scope of the study

This study involves investigation of LA production starting from fermentation process followed by Ca-L(+)-Lactate crystallization and recovery of pure solid Ca-L(+)-Lactate. The ultimate objective of the whole thesis is to decrease the production cost of high grade LA. Fermentation was performed using inexpensive raw materials or waste materials as nutrients. In addition, recovery of high grade LA was investigated by purifying solid Ca-L(+)-Lactate with drying it from one surface while forcing the moisture within to travel through the distance perpendicular to it. This forced convective motion of the solvent cause transport of solute impurities alongside. This purification technique which we named as "Drying Induced Elution of Impurities" is a novel process and has the potential of being low cost.

1.4. Expected Results

- High LA yield from fermentation processes under the optimum conditions will be obtained.

- Solute transport of impurities through surface during drying will be achieved.

- Successful recovery of purified solid Ca-L(+)-Lactate from fermentation broth using our novel purification technique will be realized.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Lactic Acid

LA was first discovered in sour milk in 1780 by C. W. Scheele, initially it was considered a milk component. In 1789, this milk component was named as “acide lactique” by Lavoisier, who originated current terminology for LA. In 1857; however, Pasteur discovered that it was a fermentation metabolite generated by certain microorganisms and not a milk component. Later, a French scientist named Fremy produced the LA through fermentation, which triggered its industrial production in 1881. In 1895, Boehringer Ingelheim started its commercial production in Germany. (Vijayakumar, Aravindan, & Viruthagiri, 2008). From that time on LA production increased and now the worldwide demand for LA is roughly estimated to be 130,000 to 150,000 tons per year (Farooq et al., 2012).

Ca-L(+)-Lactate is a calcium salt of LA with a chemical formula of $C_6H_{10}CaO_6$. Ca-L(+)-Lactate has an increasing demand while L-lactate used as food ingredients for teeth and bone health; on the other hand, calcium D-lactate was used in polylactic acid (PLA) production because D-form increases the melting point of the copolymer. Ca-L(+)-Lactate salts are soluble in water and water miscible organic solvents but insoluble in other organic solvents (Narayanan, 2004).

Table 2.1 Physical Properties of LA and Ca-L(+)-Lactate *

Properties	Lactic Acid	Ca-L(+)-Lactate
Empirical Formula	$C_3H_6O_3$	$C_6H_{10}CaO_6$
Chemical name	2-hydroxypropanoic acid	Calcium 2-hydroxypropanoate
Molecular weight	90.08 g/mol	218.218 g/mol
Melting temperature, ℃	L: 53 ℃ / D: 53 ℃	120 ℃ (pentahydrate)
	D/L: 16.8 ℃	
Density, d_{420}, g/ml	1.1	1.494

* (Datta, Tsai, Bonsignore, Moon, & Frank, 1995)

Ca-L(+)-Lactate exists as two optical isomers as LA. One is known as Ca-L(+)-Lactate and its mirror image is calcium D-lactate. Figure 2.1 shows the two optical isomers of LA, and some physiochemical properties of LA are shown in Table 2.1.

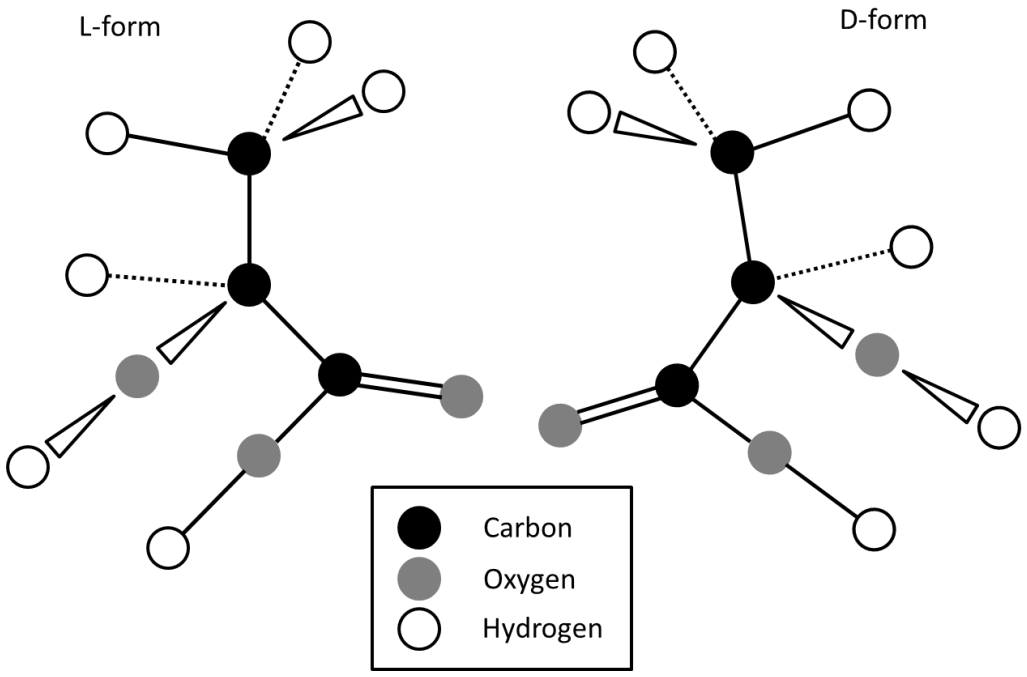


Figure 2.1 Optical Isomers of Lactic Acid

"LA can be polymerized to produce a high crystalline PLA through the serial reactions of polymerization, depolymerization, and ring opening polymerization that is suitable for commercial uses." (Vijayakumar et al., 2008) Both isomers of LA can be used for PLA production; however, optical purity of LA is essential since pure L- and D-polymers are more stable than amorphous, racemic polymers (Qin et al., 2009). These polymers are transparent and properties of PLA may be adjusted by the ratio of the L- and D-PLA. Thus its physical property approaches petroleum derived plastics besides it is biodegradable thermoplastics. The resultant polymer, PLA, has several uses in a wide range of applications, such as food packaging, protective clothing, trash bags, mulch film, and short-life trays.

It is easy to obtain nearly pure L- and nearly pure D-lactate by microbial fermentations since fermentative production has the advantage that by choosing a strain of LAB that is capable of producing only one of the isomers. Therefore, most of commercial LA is prepared by fermentation of carbohydrates by bacteria, using homolactic microbes such as a variety of modified or optimized strains of the genus *Lactobacilli*, which produce only LA. "Other biological agents capable of producing LA are also used such as strains of *Rhizopus*, *Escherichia*, *Bacillus*, *Kluyveromyces* and *Saccharomyces."* (Maas et al., 2008)

2.2. Industrial Lactic Acid Production

2.2.1. Chemical Synthesis

LA can be synthesized industrially either chemically or by microbial fermentation. LA is commercially synthesized based on lactonitrile. Hydrogen cyanide is added to acetaldehyde to produce lactonitrile in the presence of a base at high atmospheric pressures. Then lactonitrile is hydrolyzed to lactic acid by H_2SO_4 to produce lactic acid and ammonium salt. Obtained LA is then esterified by using methanol. "The chemical synthesis method produces DL-lactic acid as a racemic mixture. This process is showed by the following reactions." (Narayanan, 2004)

(a) Addition of Hydrogen Cyanide

CH_3CHO + HCN → $CH_3CHOHCN$

Acetaldehyde Hydrogen cyanide Lactonitrile

(b) Hydrolysis by H2SO4

$CH_3CHOHCN + H_2O + ½H_2SO_4$ → $CH_3CHOHCOOH + ½(NH_4)_2SO_4$

Lactonitrile Sulfuric acid Lactic acid Ammonium salt

(c) Esterification

$CH_3CHOHCOOH$ + CH_3OH → $CH_3CHOHCOOCH_3 + H_2O$

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H2O

CH3CHOHCOOCH3 + H2O → CH3CHOHCOOH + CH3OH

Methyl lactate Lactic acid Methanol

2.2.2. Microbial Lactic Acid Production

Biotechnological fermentation has the advantages of obtaining relatively higher optical purity. Commercially pure LA can be produced by microbial fermentation from the carbohydrates such as glucose, lactose and sucrose. It is also possible to use renewable resources as substrates which do not give any net contribution of carbon dioxide to the atmosphere. “The preference of feedstock depends on its availability, price, and the respective costs of LA recovery and purification. Biomass of lignocelluloses is a low-cost and extensively available renewable carbon source as an alternative to conventional feed-stocks such as molasses, beet sugar, barley malt and whey that has no challenging food value.” (Pang, Zhuang, Tang, & Chen, 2010)

Fermentation is considered as an energy yielding process whereby organic molecules serve as both electron acceptors and electron donors. The molecule being metabolized does not have all its potential energy extracted from it. Hence, LAB are mostly used as a low cost method by fermentation and generally little energy is required during the fermentation since optimal growth temperature of LAB is low. Yeast extract obviously exhibits the most significant effect on LA production, especially at the beginning of growth, with the LA concentration increasing almost linearly with an increase in yeast extract level.

2.2.2.1. Microbial LA Producers

"LA can be produced by several microorganisms classified into bacteria, fungi, yeast, cyanobacteria, and algae. Each biocatalyst has achieved one or more improvements over the others, such as a broader substrate range, improved yield and productivity, reduction of nutritional requirements, or improved optical purity of lactic acid." (Abdel-Rahman, Tashiro, & Sonomoto, 2013) Bacteria that produce LA can be divided into four main groups, namely, LAB, *Bacillus* strains, *Escherichia coli*, and *Corynebacterium glutamicum*.

LAB are nonsporulating rods and cocci, Gram-positive which produce LA as the main fermentation product under proper conditions. "Most of the LAB are anaerobic and they grow at temperatures from 25-45 °C, and not surprisingly are tolerant to acidic conditions. Most strains are able to grow at pH 4-6. The fastidious LAB have limited biosynthetic ability, requiring performed purines, pyrimidines, amino acids, B vitamins, and typically a sugar as energy source. These multiple requirements restrict their habitats to areas where the required compounds are abundant." (Boontawan, 2010)

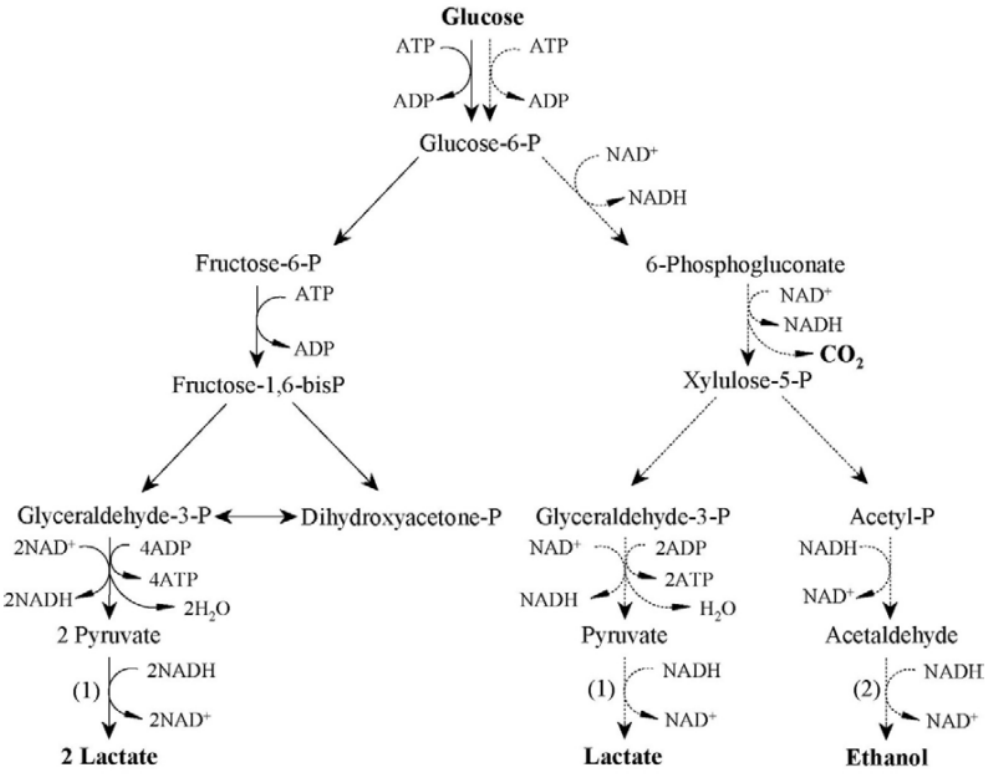


Figure 2.2 Metabolic pathways of homo-fermentative (left) and hetero-fermentative (right) LAB (Wee, Kim, & Ryu, 2006)

LAB ferment sugars either homofermantatively or heterofermantatively. Homofermantative LAB produce LA as the main product while hetero- produce also ethanol, acetic acid, formic acid and CO_2. Although it is sought to divide LAB into homo- and heterofermentative strains, the division is not that straightforward as the actual metabolism is dependent on both fermentation conditions and the nature of the C/energy substrate. LAB used in LA production should be homofermentative as shown in left side Figure 2.2 and this pathway is called Embden-Meyerhof-Parnas (EMP).

Either D- or L- producing microorganisms selected for recent investigations of fermentation of LA are listed in Table 2.2. In this study, *Lactobacillus casei* is used for L-lactic acid production. It is a homofermentative LAB that is used

generally to ferment lactose. Whey is one of the most widely used substrates for LA production by this organism. It is also known this bacterium could ferment glucose and sucrose.

Table 2.2 Microorganisms used in recent investigations of the fermentation of LA

Microorganism	Lactic Acid (g/L)	Yield Y (g/g)	Productivity Q_v (g/L.h)
Enterococcus faecalis	95.7	0.94	4.0
Lactobacillus delbrueckii NC1MB8130	90.0	0.97	3.8
Lactobacillus delbrueckii IFO 3202	60.3	0.95	3.4
Rhizopus oryzae	93.8	0.77	1.4
Lactobacillus paracasei	88-106	0.91-0.95	3.3-3.7
Enterococcus faecalis RKY1	102	0.97	4.9
Lactobacillus amylophilus GV6	76.2	0.7	0.8
L. pentosus ATCC 8041	21.8	0.77	0.8
L. plantarum ATCC 21028	41.0	0.97	1.0
Lactobacillus lactis	109	0.93	1.1
L. bulgaricus NRRL B-548	38.7	0.9	3.5
L. helveticus ATTC 15009	65.5	0.66	2.7
L. acidophilus R	8.6	0.17	
S. thermophiles	18.0	0.5	5.9
L. casei NRRL B-441	82.0	0.91	5.6

Source: (Vijayakumar et al., 2008)

2.2.2.2. Nutrients Required for the Lactic Acid Fermentation

All LAB require a source of nutrients for metabolism. The fermentative bacteria require carbohydrates, such as glucose, fructose and starch or cellulose. Their growth can stop when available substrate is limiting. It is necessary to

supplement the fermentation media with sufficient nutrients for rapid LA production. “If small amounts of other nutrients are supplemented to the process, then the efficiencies of LA fermentation is improved significantly. The higher are the purity of the substrates the higher is the purity of the LA produced.” (Boontawan, 2010)

2.2.2.2.1. Carbon Sources

Raw material cost is one of the major factors in the economic production of LA. The purer the substrate are used the purer the LA are produced. However, this is not economical, because pure sugars are more expensive than LA at least in Turkey. “Thus, various materials have been considered as alternative substrates and renewable resources, including byproducts of agricultural industries, food industries, and natural unutilized biomasses such as starchy biomass, lignocellulosic biomass, whey, glycerol, and algal biomass.” (Abdel-Rahman et al., 2013) “Among these, starch and cellulosic materials are currently receiving a great deal of attention, because they are cheap, abundant, and renewable. Consequently, inexpensive starch, starch derivatives or starch containing waste materials as substrates would offer advantages when combined with minimal preprocessing and supplementation.” (Wee et al., 2006)

Starchy Materials & Lignocellulosic Biomass

Starch from crops or wastes is used as a common substrate for LA production. Different pretreatments and saccharifications of raw materials are applied according to the type of raw material and these methods are the bottle-neck processes for LA production when starchy and/or lignocellulosic materials are used. There is also studies for direct fermentation of starchy materials; however, further developments are necessary since these processes are not commercially feasible according to John, G.S., Nampoothiri, & Pandey (2009).

Lignocellulosic biomass is another carbohydrate source for LA production and it has drawn a lot of interest since it would help to overcome environmental problems. However, it is most difficult to ferment lignocellulosic biomass into LA. This is because the lignocellulosic biomass contains cellulose, hemicellulose, lignin etc. which have to be degraded to small sugar molecules by the help of pretreatments and multi-enzyme reactions (Abdel-Rahman et al., 2013).

Dairy Products

The most common substrates for LA fermentation is whey from dairy industry. It is a byproduct from cheese production and its disposal is a pollution problem for the dairy industry. It mainly contains lactose, proteins, some lipids and salts; however, for LA fermentation they would not be enough so it is supplemented with some of the yeast extract, peptone, corn steep liquor or soy flour.

Sugar Molasses

Molasses is a byproduct of the sugar manufacturing process and it is used as a substrate for baker's yeast production, an animal feed, and ethanol production. It could be also used for LA production due to its high concentration of sucrose, glucose, and fructose; however, it is a product laden with a lot of impurities.

Glycerol

Glycerol is a byproduct of biodiesel production and it is colored and thick solution. Therefore effective glycerol utilization as a cheap raw material would be both economic and solve some environmental problems (Abdel-Rahman et al., 2013).

Microalgae

Microalgae have been recently used for microbial LA production as the substrate. In comparison to lignocellulosic biomass, microalgae do not contain lignin (Abdel-Rahman et al., 2013) which is a major problem for LA production from lignocellulosic biomass. For example, the green microalga contain nearly 50% reducing sugar and it can easily be grown and be harvested in 1-10 days (Schenk et al., 2008).

2.2.2.2.2. Nitrogen Sources

The LA production is influenced by the type and the concentration of the nitrogen sources. “Different nitrogen sources and their combinations are used for LA fermentation. For example; MRS medium, which contains yeast extract, meat extract and peptone, is superior to yeast extract, which in turn is better than malt extract.” (Hofvendahl & Hahn–Hägerdal, 2000)

Biotechnological production of LA requires an amount of nitrogen source and the most common used one is yeast extract. “When a yeast cell is inactivated, a natural digestion process called “autolysis” starts. During this process the yeast's own enzymes breakdown proteins and other parts of the cell. This causes the release of peptides, amino acids, vitamins and other yeast cell components which, once the insoluble components have been removed, is called “Yeast Extract”.” (Boontawan, 2010)

“Yeast extract is rich in nitrogen, vitamins and other nitrogenous growth factor stimulating compounds, and therefore is used as an ingredient in media for the cultivation of microorganisms. Moreover, all published reports have shown that LA production increases with the concentration of the supplement (especially yeast extract).” (Lund, Norddahl, & Ahring, 1992) “The typical composition of yeast extract is 8 -12 % total nitrogen content (organic and inorganic compounds), 50-75 % protein content, 3-5.2 % fermentable nitrogen content, 4-13 % total carbohydrate content, and very little lipid content.” (Rivas, Moldes, Domínguez, & Parajó, 2004)

2.2.2.3. Factors Affecting LA Production

The optimization of fermentation processes requires profound knowledge of the factors determining microbial metabolism, and the influence of process parameters. It was known that temperature, pH and agitation speed were crucial for LAB when batch fermentation was preferred.

2.2.2.3.1. Effect of Temperature

Temperature is one of the key environmental parameters that affect the fermentation process directly (Yuwono & Kokugan, 2008). For *Lactobacillus casei* the optimal temperature was reported as between 37 ℃ to 42 ℃ (Vijayakumar et al., 2008).

2.2.2.3.2. Effect of pH

The fermentation pH is either controlled by neutralizer or it is set at the beginning and then left to decrease due to LA production. Research indicates that controlled pH fermentations resulted in higher or equal LA concentration and yield in comparison with no pH control (Cavazzoni, Manzoni, & Craveri, 1988). The optimal pH for LA production varies between 5.0 to 7.0 and a pH below 5.5 was only for *Lactobacillus* strains (Kashket, 1987).

2.3. Lactic Acid Purification

LA is sold in different grades, and better grades require that well purified substrates be utilized in the medium in order to decrease the levels of impurities which cannot be separated from the LA without difficulty. “One of the commercial grades of lactic acid, “crude” or “technical” grade is a colored product prepared for commercial usage at mass fraction in water of 22, 44, 50, 66 and 80 %. It is prepared by employing sulfuric acid to remove the calcium from the Ca-L(+)-Lactate derived

from the heated and filtered fermentation broth, followed by filtration, concentration, and refiltration to remove additional calcium sulfate. Thus, this grade of lactic acid contains many of the impurities from the fermentation medium, and it finds many industrial uses where purity of the product is not essential as, for example, in the deliming of hides in the leather industry." (Vijayakumar et al., 2008) The 'edible' grade of LA is snow colored and is marketed at 50–80 % strengths. Thus, it receives additional purification over that of technical LA. "Colorless, high purity lactic acids are the plastic grade, marketed at 50–80 % strength, and "U.S.P". Lactic acid marketed at 50–80 % strengths. Other commercial preparations of lactic acid are Ca-L(+)-Lactate, sodium lactate, and copper lactate. The final recovered yields of technical and edible-grade lactic acids, based on the original carbohydrate of the medium, are approximately 85–90% and 80%, respectively. Plastic and U.S.P grades are prepared by further refining of technical grade lactic acid and therefore, slight to moderate yield losses are incurred during this refining" (Vijayakumar et al., 2008)

Fermentation broth contains a number of impurities such as residual sugars, color, nutrients and other organic acids, as part of cell mass. These impurities must be removed from the broth in order to achieve purer LA. To recover the LA produced from the microbial fermentation media efficiently and economically, commercial procedure is used mostly.

The current commercial method for LA production can be described as

Fermentation and Neutralization:

$$C_6H_{12}O_6 + 2CaCO_3 \rightarrow (CH_3CHOHCOO^-)\,Ca^{2+} + 2CO_2$$

Carbohydrate + Calcium Carbonate → Calcium lactate

Acidification by H_2SO_4:

$$(CH_3CHOHCOO^-)\,Ca^{2+} + H_2SO_4 \rightarrow 2CH_3CHOHCOOH + CaSO_4$$

Calcium Lactate + Sulfuric Acid → Lactic Acid + Calcium Sulfate

Esterification:

$$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOH_3 + H_2O$$

Lactic Acid + Methanol → Methyl Lactate

Hydrolysis by H_2O:

$$CH_3CHOHCOOH_3 + H_2O \rightarrow CH_3CHOHCOOH + CH_3OH$$

Methyl Lactate → Lactic Acid + Methanol

In this method, the broth containing calcium lactate is filtered to remove cells, excess calcium carbonate and other insoluble particles, then the filtered liquor is carbon treated in order to remove color from liquor. Evaporated liquor is acidified with sulfuric acid to get LA and calcium sulfate which is removed by filtration since it is insoluble. Obtained LA is esterified with methanol and hydrolyzed, then methanol is distilled to obtain pure LA (Wasewar, 2005).

For the recovery of LA some other applications are sought to be alternative to conventional process such as: ion exchange chromatography, adsorption, and liquid-liquid extraction. Ion exchange chromatography is used among the variety of alternative downstream operations. It is a procedure which ion exchange membranes are used for removing ions from an aqueous solution under driving force of electrical field and it is applied to remove salts from solutions (Wasewar, 2005). LA may also be recovered by the adsorption which is a process suitable for recovering substances produced in small concentrations and in complex solutions, such as fermentation broth. Synthetic polymeric sorbents have been used in the recovery of carboxylic acids from fermentation broth and this polymeric sorbents have also been

used to extract LA and control pH during fermentation (Evangelista & Nikolov, 1996). However, it was further concluded that adsorption requires regeneration of ion exchange resin and adjustment of feed pH to increase the sorption efficiency requiring large amounts of chemicals (Wasewar, 2005).

"LA can also be extracted from aqueous solution using n-butanol as an extractant. Factors such as pH, mixing time, initial concentration of lactic acid, and volume ratio between the organic and the aqueous phase affect the extraction of lactic acid. Degree of lactic acid extraction and distribution coefficient increases when the pH of aqueous solution is decreased. The pH effect is considerably marked when the pH of the aqueous solution is less than 1. Initial concentrations of lactic acid and organic-to aqueous volume ratio appear to have positive effect on the degree of extraction and distribution coefficient. As the n-butanol is miscible partially in water, so integration of aqueous phase into organic phase in the extraction has a great organic-to-aqueous volume ratio." (Chawong & Rattanaphanee, 2011) Lactic acid can be separated and substantially purified from fermentation broths by several membrane-based unit operations as shown in the Figure 2.3.

In another procedure, the free LA is solvent extracted with isopropyl ether directly from the heated and filtered fermentation broth. "This is a counter current continuous extraction, and the lactic acid is recovered from the isopropyl ether by further counter-current washing of the solvent with water. An older procedure, not utilized commercially to any extent today, involves direct high-vacuum steam distillation of the lactic acid from the fermentation broth, but decomposition of some of the lactic acid occurs." (Ghaffar et al., 2014) "As a result extraction is a closed loop process and proper combination of extractant and diluent and proper choice of back extraction process yields high productivity. Also practically all data of extraction is available for commercial design. In extraction, most of the extractant works efficiently at low pH while most microbes give higher productivity at higher pH. Also most of the solvents are toxic towards microbes, so further improvement in

the extractant-solvent and microbes is needed i.e. immobilization of microbes and development of extractant-solvent system." (Wasewar, 2005)

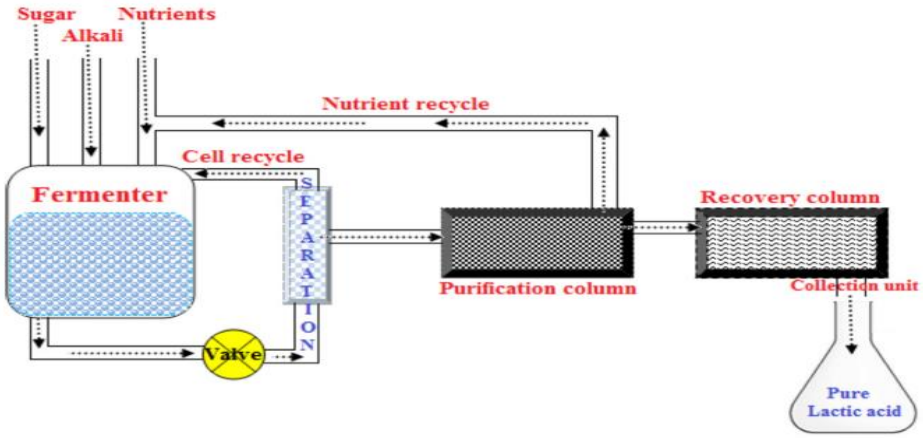


Figure 2.3 Substantial purification of lactic acid from fermentation broth by several membrane based unit operations (columns) (Ghaffar et al., 2014)

2.3.1. Crystallization

Numerous processes are employed for the recovery and purification of the lactic acid. "In one procedure similar to this study, the heated and filtered fermentation broth is concentrated to allow crystallization of calcium lactate, followed by addition of sulfuric acid to remove the calcium as calcium sulfate. The lactic acid is then re-crystallized as calcium lactate, and activated carbon is used to remove colored impurities. As an alternative to the latter step, the zinc salts of lactic acid are sometimes prepared because of the relatively lower solubility of zinc lactate." (Vijayakumar et al., 2008)

2.3.2. Drying Induced Elution of Impurities

In this work we are proposing the removal of the impurities by causing their migration to the surface. The movement is achieved by water which is transport to the surface due to the drying. It is novel using drying process for purification of

organic acid salts. It can be summarized as the accumulation of impurities within cake at the surface by the help of evaporating water during drying thus liberating the body of form of them. Solid calcium lactate crystals contain interspatial moisture even after filtration of the crystals from the crystallization medium. Most of the impurities are contained in this phase. When we transport water towards the surface some of the impurities also migrate towards the surface.

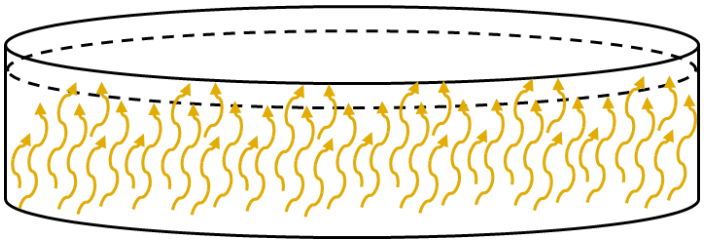
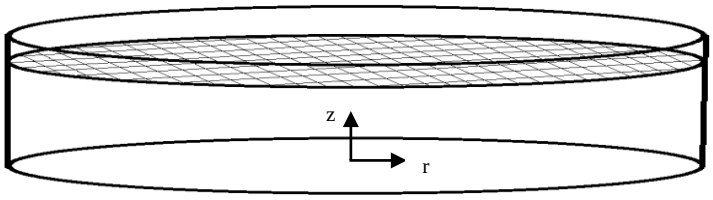


Figure 2.4 Motion of impurities along with moving water during drying

Predicted displacement of impurities are shown in Figure 2.4. We are assuming bulk motion of water within impurities is taking place through surface during drying. It can be explained with the "equation of continuity for species" as;

$$\frac{\partial C_i}{\partial t} + v_r\left(\frac{\partial C_i}{\partial r}\right) + \frac{v_\theta}{r}\left(\frac{\partial C_i}{\partial \theta}\right) + v_z\left(\frac{\partial C_i}{\partial z}\right) = D_i\left(\frac{\partial}{r\partial r}\left(r\frac{C_i}{\partial r}\right) + \frac{1}{r^2}\frac{\partial^2 C_i}{\partial \theta^2} + \frac{\partial^2 C_i}{\partial z^2}\right) + Rv_i$$

In defining the convective and diffusive fluxes of impurities (i) one must select an average velocity for mixture.



v_r & $v_\theta = 0$ Because only top surface is in contact with desiccant air and we can assume there were no gradients in the r and θ directions. Even though there was no reaction occurring between the calcium lactate and impurities, there was probably some ionic interaction which impeded their free migration.

For a system where there is one gradient only in one direction and when no reaction is taking place the diffusion equation reduces to;

$$\frac{\partial C_i}{\partial t} + v_z\left(\frac{\partial C_i}{\partial z}\right) = D_i\left(\frac{\partial^2 C_i}{\partial z^2}\right)$$

where C_i is the concentration of impurity (g/cm^3), D is the diffusion coefficient of impurity in Ca-L(+)-Lactate (cm^2/min), v_i is the pore-water velocity (cm/min), z is the distance (cm), and t is time (min). In this equation the interaction between the impurities and matrix can be included as a "reaction" term if volume-averaging technique is employed; if not the interaction would be included as a boundary condition.

In our case a concentration gradient is formed in the positive z-direction. The convective motion is also in the same direction whereas the diffusion of the impurities will be in the negative z-direction. Under these conditions, the convective motion is several orders of magnitude larger than the diffusive effects thereby dominates the whole process. Thus, impurities will be accumulated at the surface.

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemical Agents and Nutrients

All chemicals used for analyses were of analytical grade, and are listed in Table 3.1 with the producers of the chemicals.

Table 3.1 Chemicals and their producers

Chemical	Producer	Chemical	Producer
L-Lactic Acid	Sigma	Potassium phosphate (dibasic)	Sigma
Lactose	Sigma	Zinc sulfate	Merck
D-Glucose	Sigma	Sodium Acetate	Sigma
Sucrose	Merck	Tween 80	Merck
Glucose, L-Lactic acid standard	YSI	Copper sulfate	Merck
Calcium carbonate	Merck	Sodium carbonate	Merck
Yeast extract	Merck	Sodium hydroxide	Merck
MRS Broth	Merck	Folin Reagent	Fluka
Magnesium sulfate	Merck	Sulfuric Acid	Sigma
Manganese sulfate	Horosan Kimya	Bovin Serum Albumin (1 mg/ml)	Sigma
Potassium dihydrogen phosphate	Sigma	Nitric Acid	Merck

3.1.2. Strain and Growth Medium

Homofermentative lactic acid bacterial strain of the genus of *Lactobacillus: L. casei* NRRL B-441 was used for the production of LA. MRS is a growth medium which is so-named by its inventors: de Man, Rogosa and Sharpe. Developed in 1960, this medium was designed to favor the luxuriant growth of *Lactobacilli*. It contains sodium acetate, which suppresses the growth of many competing bacteria. This medium has a clear brown color. It typically contains 1.0% peptone, 0.8 % egg extract, 0.4 % yeast extract, 2.0 % glucose, 0.5 % sodium acetate trihydrate, 0.1 % polysorbate 80 (also known as Tween 80), 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.02 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate. Bacteria were stored in MRS broth with glycerol at – 80 °C, and precultures were grown in MRS broth at 37 °C for 18h. At least three generations of precultures were required before fermentation (Figure 3.1).

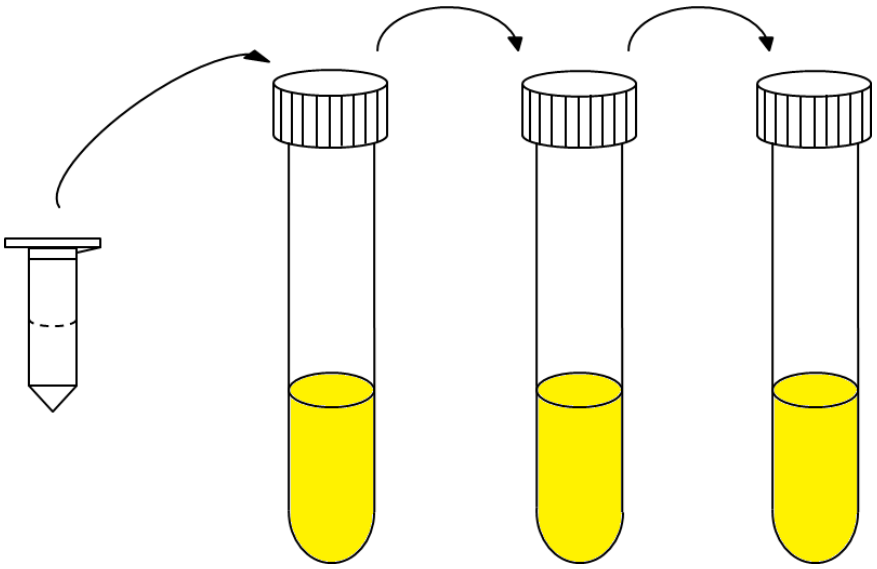


Figure 3.1 Inoculation procedure for small scale production

For large scale of production due to production of Ca-L(+)-Lactate MRS medium was not used. It was prepared with 10% sugar (whey and/or sucrose syrup), 0.5-2% yeast extract from spent Brewer's yeast, 4% calcium carbonate, 0.5% sodium acetate, 0.5% dipotassium hydrogen phosphate, 0.05% magnesium sulfate heptahydrate, 0.005% manganese sulfate tetrahydrate.

3.1.3. Fermentation Media

3.1.3.1. Carbon Sources

3.1.3.1.1. Sweetened Whey Powder & Whey

ENKA Dairy, Inc (Konya, Turkey) supplied the sweetened whey powder used in this study. Lactose concentration of sweetened whey was 80% and amount added to fermentation broth was adjusted according to the desired initial sugar concentration of the medium.

Açkar Dairy Products, Inc (Ankara, Turkey) supplied whey used in this study. Lactose concentration of whey was 4.4% and it was supplemented with another carbon source to obtain desired initial sugar concentration when necessary.

3.1.3.1.2. Starch Hydrolysate

Commercial wheat starch from Sunar Group (Adana, Turkey) was used. Prior to fermentation, starch was processed with two distinct steps by enzymatic hydrolysis. The liquefaction by α-amylase (Orbamil, İstanbul, Turkey) was used with the concentration of 1 ml/kg starch at 85 °C for 24 h. "The α-amylase breaks down long-chain starch, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and limit dextrin from amylopectin. Secondly, the saccharification step for liberation of glucose molecules was carried out using gluco-amylase (Orbamil) with the working concentration of 1 ml/kg starch at 55 °C for 24 h. For the preparation of hydrolyzed starch, 100 g of starch was produced equivalent to 95 g of glucose." (Boontawan, 2010)

3.1.3.2. Nitrogen Sources

3.1.3.2.1. Whey Powder

ENKA Dairy, Inc (Konya, Turkey) supplied the whey powder (WP) used in this study. Its total protein content was 35% and the amount added to fermentation broth was adjusted for the fermentation medium.

3.1.3.2.2. Powdered Malt Extract

Powdered malt extract's (PME) producer was Hambleton Bard Ltd. (Chesterfield, UK). It contains about 6% protein (8% db), as well as an abundance of free amino acids, vitamins, and minerals. It was used in small amounts for the LA fermentation.

3.1.3.2.3. Spent Brewer's Yeast Extract

Spent brewer's yeast was collected from Anadolu Efes Brewer's company. Hundred gram of yeast slurry with a solid content of 40% (w/v) resuspended to 20% (w/v) with tap water and the slurry was autolyzed for 24 h at 50 °C with stirring. The autolyzate was filtered with ceramic tubular filter and the clear supernatant was then autoclaved at 121 °C for 15 min to sterilize. It was used in fermentation directly.

3.2. Methods

3.2.1. Fermentation and Neutralization of LA

All laboratory scale fermentations were performed in a 500 ml of flasks containing 100 ml of fermentation medium. The operating temperatures of 37 °C for *L. casei* was set for all experiments with the agitation speed of 160 rpm in order to ensure the homogeneity of the system. Fermentations were carried out in a temperature controlled flask shaker (Infors HT, Switzerland). All substances were

used for fermentation were sterilized as a whole. Sterilization temperature and time were 121 °C and 15 minutes, respectively. The flasks were inoculated with 3rd generation of stock culture with the inoculum size of 5% (v/v). All experiments were performed at optimal growth temperature at 37 °C for *L.* casei, pH was adjusted to 6.0 at the beginning of the fermentation by adding $CaCO_3$. The pH was daily measured by a pH sensor (Mettler Toledo, Switzerland) but not controlled. Fermentation ended when either glucose was completely consumed or no net change in its concentration for more than 12 h. Samples (1 ml) were withdrawn aseptically at regular intervals for further analysis.

3.2.1.1. Effect of the Type of the Substrate

L. casei was tested for its capacity of utilizing synthetic glucose, lactose from sweetened whey (LSW), starch hydrolysate, and LSW and glucose (GLSW) mixture. All fermentations were carried out in shake flasks and initial sugar concentrations were 80-100 g/L. For each of four sugar utilization experiments, fermentations were performed with same concentrations of minerals and nutrients.

3.2.1.2. Effect of the Sources of the Nutrients

Fermentations were carried out with five different nutrient sources which were yeast extract (YE), whey powder (WP), powdered malt extract (PME), malt dust (MD) and spent Brewer's yeast extract (SBYE). Minerals and $CaCO_3$ content were the same for all experiments. In Table 3.2 nutrient sources are listed for glucose with the total protein content.

Table 3.2 Nutrient sources and total protein content for the substrate glucose

Substrate	**Nutrients**	**Total Protein Content (%)**
Glucose	2.9% YE	2.0
	5.5% WP	2.0
	2% WP & 2% YE	2.0
	10% WP	3.52
	10% WP & 1.4% YE	4.50
	10% WP & 2.9% YE	5.48
	10% WP & 1% PME	3.58
	10% WP & 2% PME	3.64
	10% WP & 1% PME & 1.4% YE	4.56
	10% WP & 2% PME & 1.4% YE	4.62
	10% WP & 1% PME & 2.9% YE	5.54
	10% WP & 2% PME & 2.9% YE	5.60

Nutrients and protein content of the substrates lactose from sweetened whey (LSW) and whey & sucrose (WS) are listed in Table 3.3.

3.2.1.3. LA Fermentation in Fermenter

Large scale of production of LA as Ca-L(+)-Lactate which was used for purification studies was conducted in a 100 L fermenter with 70 L working volume. The heating jacket around a fermentation tank provided the temperature control. Agitation was provided by DC motor at the top of the fermenter. The system was equipped with PT-100 and pH sensor (Mettler-Toledo, Switzerland). Temperature, agitation and pH was observed by screen of the control panel and temperature and agitation controlled by the software. Scheme of the reactor is shown in Figure 3.2.

Table 3.3 Nutrient sources and total protein content for the substrate LSW and SLSW

Substrate	Nutrient	Total Protein Content (%)
Lactose from Sweetened Whey (LSW)	3.7% WP	2.0
	1% YE 1.5% WP	2.0
	1.7 % YE	2.0
	2.9% YE	2.7
	2% PME	0.82
	1% PME & 1.4% YE	1.74
	2% PME & 1.4% YE	1.8
	2% PME & 2.9% YE	2.82
Whey & Sucrose	0.9% SBYE	0.9
	0.7% SBYE	0.7
	0.5% SBYE	0.5
	0.4% SBYE	0.4
	0.2% SBYE & 2% MD	2.0
	0.14% SBYE & 3% MD	2.0
	0.08% SBYE & 5% MD	2.0
	5% MD	2.0

For large scale production whey and starch hydrolysate used as substrates and SBYE used as nitrogen source. Fermentation media in the shake flasks a total of 7 liters were used as the inocula for the fermenter. pH was adjusted to 6.0 at the beginning of the fermentation and was not allowed to decrease below 4.5 by adding solid $CaCO_3$ manually.

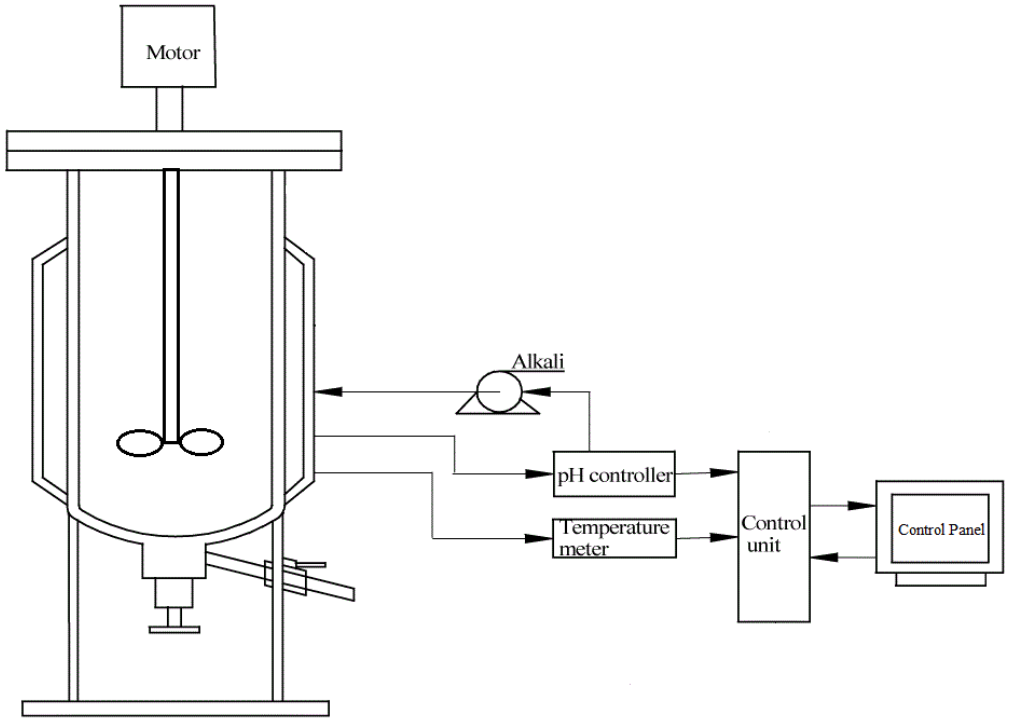


Figure 3.2 Schematic diagram of fermenter

3.2.2. Sugar and LA Analysis of Fermentation Medium

During fermentation, 10-ml samples were collected and centrifuged at 10,000 rpm for 2 min and the supernatant was analyzed by HPLC. The supernatant was diluted and filtered through 0.20 μm membrane filter (Merck Millipore, Germany). Ten microliters of the sample was automatically injected into the HPLC system (Agilent Technoogies, USA) equipped with a fast acid column (Phenomenex Inc., USA) and RI detector. The column temperature was maintained at 25 °C. The mobile phase was 0.05 M H_2SO_4 at flow rate of 0.6 mL/min. Standard curves of sugars and LA are in Appendix C.

3.2.3. Purification of Lactic Acid as Ca-L(+)-Lactate

3.2.3.1. Crystallization of Ca-L(+)-Lactate

Biomass and other insoluble materials are removed from fermentation medium via ceramic tubular membrane. Filtrate contained Ca-L(+)-Lactate, soluble protein, residual sugar and trace metals and this filtrate was evaporated to induce crystallization for removing impurity rich part, the other soluble compounds, within the liquid phase. Crystallization was done in a glass beaker until crystals were observed within minimum 24 hours. Solubility of Ca-L(+)-Lactate at 4 °C is 40.06 g/L according to Cao, Lee, Yun, & Koo (2001) so liquid phase of crystals had still remarkable amount of Ca-L(+)-Lactate thus this could be recycled for other applications. However, it was not recycled in this study.

After proper crystallization, a supernatant was separated from Ca-L(+)-Lactate crystals by vacuum filtration and very thick Ca-L(+)-Lactate solution was obtained. Vacuum filtration was done by using Buchner funnel and flask with a stainless steel mesh to separate lactate crystal from its supernatant.

3.2.3.2. Drying Induced Elution of Impurities from Ca-L(+)-Lactate

Solid Ca-L(+)-Lactate clump was dried with a novel technique which involves the drying the slabs of Ca-L(+)-Lactate crystals from one surface. The continuous removal of the moisture from one end causes the migration of the moisture in the slab material. This convective motion of the moisture causes the impurities in the slab move against their own gradient and accumulate at the surface since the moisture at there is continuously removed by evaporation (Figure 3.3).

Drying was processed in a tray drier with different relative humidities, air flow temperatures and air flow velocities. Initial moisture content and weight of Ca-Lactate were fixed as 85% and 100 g respectively and drying area did not change since all drying experiments were done using petri dishes (ø=9 cm, L=1.2 cm). A plastic mesh was placed 2 mm below top edge for ease removal of impurity rich top

part from bottom part which was more pure than initial sample. Drying lasted until no net weight change was measured for one hour and was repeated by moisturizing the bottom part with distilled water until the purity of bottom part was not higher than the previous sample.

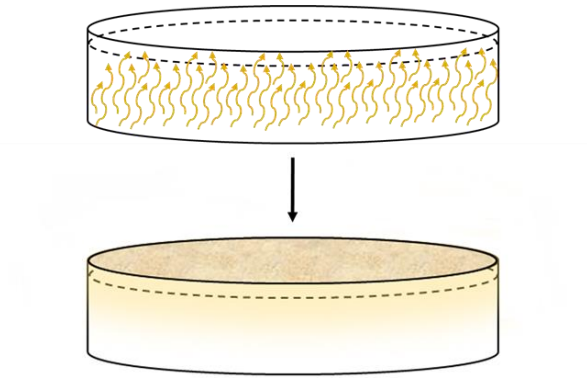


Figure 3.3 Drawing of accumulation caused by convective motion

3.2.4. Analysis of Ca-L(+)-Lactate

3.2.4.1. Lactic Acid and Ca-L(+)-Lactate

The Ca-L(+)-Lactate was dissolved in water and filtered through 0.20 µm membrane filter. 20 microliters of the sample was injected into the HPLC system (Agilent Technologies, USA) equipped with an organic acid column (Phenomenex Inc., USA) and RI detector. The column temperature was maintained at 25 °C. The mobile phase was 0.05 M H_2SO_4 at flow rate of 0.6 mL/min. Results are measured as lactic acid in percentages and Ca-L(+)-Lactate percentage is calculated by multiplying LA percentage with 1.211 (molecular weight of Ca-L(+)-Lactate/ molecular weight of LA).

3.2.4.2. Protein

Lowry Protein Assay

"The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. Most other methods are sensitive to the amino acid composition of the protein, and absolute concentrations cannot be obtained." (Sapan, Lundblad, & Price, 1999) The procedure of Lowry, Rosebrough, Farr, & Randall is no exception, but its sensitivity is moderately constant from protein to protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude extracts are involved. "The method is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphor-molybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acid. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content and this method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations in the range 0.01–1.0 mg/mL of protein." (Waterborg & Matthews, 1984)

3.2.4.3. Color

Color measurements were performed by using Spectrophotometer CM-5 (Konica Minolta, Inc., Japan). White and black calibration was preferred as instrument standardization. Pure water was used as the blank material. The diluted samples (minimum 20 folds) were placed into quartz cuvettes and measured at 740 nm. L^*, a^*, b^* color space was used for the measurement. When a color is expressed in CIELAB, L* defines lightness, a* denotes the red/green value and b* the yellow/blue value.

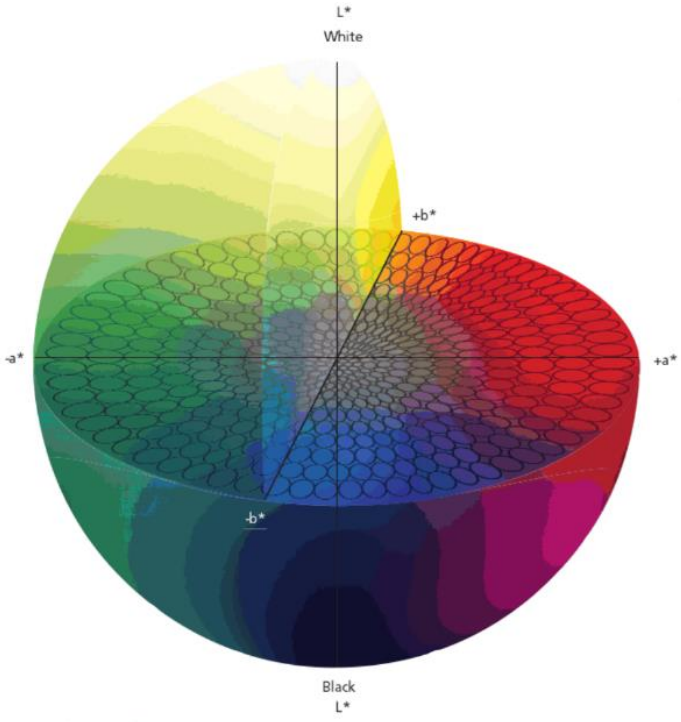


Figure 3.4 Color plotting diagram for L*a*b* (Gundlach, 2015)

Figure 3.4 shows the color-plotting diagrams for L*a*b*. The a* axis runs from left to right. A color measurement movement in the +a direction depicts a shift toward red. Along the b* axis, +b movement represents a shift toward yellow. The centre L* axis shows L = 0 (black or total absorption) at the bottom and L = 100 or white at the top. In between are greys. All colors on this axis can be considered as neutrals as they are not colored in any particular direction.

Assessment of color is often more than a numeric expression. Usually it is an assessment of the color difference (delta) of a sample relative to a known standard. CIELAB (L*a*b*) and CIELAB (L*C*h) are used to compare the colors of two objects. Total color change (ΔE*) was calculated as:

$$\Delta E^* = \sqrt{(L^* - L^*_{ref})^2 + (a^* - a^*_{ref})^2 + (b^* - b^*_{ref})^2}$$

Distilled water was used as a reference liquid since it is colorless and $L^*_{ref} = 100$, $a^*_{ref} = 0$ and $b^*_{ref} = 0$.

3.2.4.4. Residual Metal

AAS

Atomic absorption spectroscopy (AAS) is a widely used technique for determining a large number of metals. "In the most common implementation of AAS, an aqueous sample containing the metal analyte is aspirated into an air-acetylene flame, causing evaporation of the solvent and vaporization of the free metal atoms. This process is called atomization. A line source (hollow cathode lamp) operating in the UV-visible spectral region is used to cause electronic excitation of the metal atoms, and the absorbance is measured with a conventional UV-visible dispersive spectrometer with photomultiplier detector. The narrow spectral lines of atomic samples necessitate the use of a line source as well as a high-resolution monochromator. This helps to prevent interference from adjacent spectral lines of other atomic species present in the sample matrix. In this experiment, AAS in conjunction with flame atomization will be used to determine specific metals in a solid sample. The availability of a spectrometer equipped with a lamp turret (allowing several line sources to be used in sequential fashion) will facilitate the measurement of multiple metals in the sample." (Small, 2009)

CHAPTER 4

4. RESULTS AND DISCUSSION

In this section, experimental results obtained from batch fermentation of L(+)-LA from different carbon sources by using *L. casei* are presented. All fermentations were performed in optimum temperature and pH value of strain which were obtained from literature. For *L. casei* the glucose and LA concentrations are shown in charts. The maximum productivity values which were obtained from some of the experiments were reported.

In addition, purification results are also reported in this section. Purification is done for solid Ca-Lactate by using a novel method; Drying induced elution of impurities through the surface. The technique involves the drying the slabs of Ca-lactate crystals from one surface. The continuous removal of the moisture from one end causes the migration of the moisture in the slab material. This convective motion of the moisture causes the impurities in the slab move against their own gradient and accumulate at the surface since the moisture there continuously removed by evaporation. Initial moisture content and weight of Ca-Lactate were fixed for all experiments as 85% and 100 g respectively and drying area did not change since all dryings were done by using petri dishes (ø=9 cm). Thus experimental results includes required maximum Ca-lactate thickness possible, the minimum number of required drying stages. While determining air flow temperature and velocity and number of drying stages, only the color of Ca-lactate solution was considered as a purification indicator. The degree of purification achieved was further determined by HPLC, AAS analysis and Lowry protein assays.

All purification experiments were done with two replicates and mean values are reported.

4.1. L-(+)-Lactic Acid Fermentation

In this study, to produce optically pure L(+)-LA the strain *Lactobacillus casei* was used. While inocula was prepared with MRS medium, fermentation medium which consisted of 70-100 g/L total sugar from whey, whey powder, glucose and starch and salts were prepared, to which 0-29 g/L of yeast extract from spent Brewer's yeast or commercial yeast extract was added as a nitrogen source, for some studies whey powder, powdered malt extract and malt dust was used as a supplement.

4.1.1. Effect of the Type of the Substrate

The fermentative production of LA is interesting due to the anticipation of using readily available, and cheap raw materials. Experiments concerning the impacts of various carbon sources were investigated with the objective of determining an inexpensive fermentation medium. The influence of substrate type on the production of LA as a calcium salt using 2% protein from YE, salts and $CaCO_3$ was investigated. Four different carbohydrate sources were used in amounts of 8-10% of the initial substrate, the temperature was 37 ℃ and the pH was in the range of 4.5-6.0.

Figure 4.1 shows the production of the LA by using glucose, lactose from sweetened whey (LSW), glucose from starch and glucose-whey (GLSW) mixture. Figure 4.2 demonstrates the productivity values obtained from the fermentations.

The production of LA in the case of the fermentation conducted with lactose from sweetened whey was the best alternative for *L.casei* NRRL-41 since it had the highest productivity value with an 8% LA concentration. While glucose from starch

hydrolysate was the worst substrate for LA production using *L.casei* NRRL-41, glucose or GLSW can be used as substrate for it.

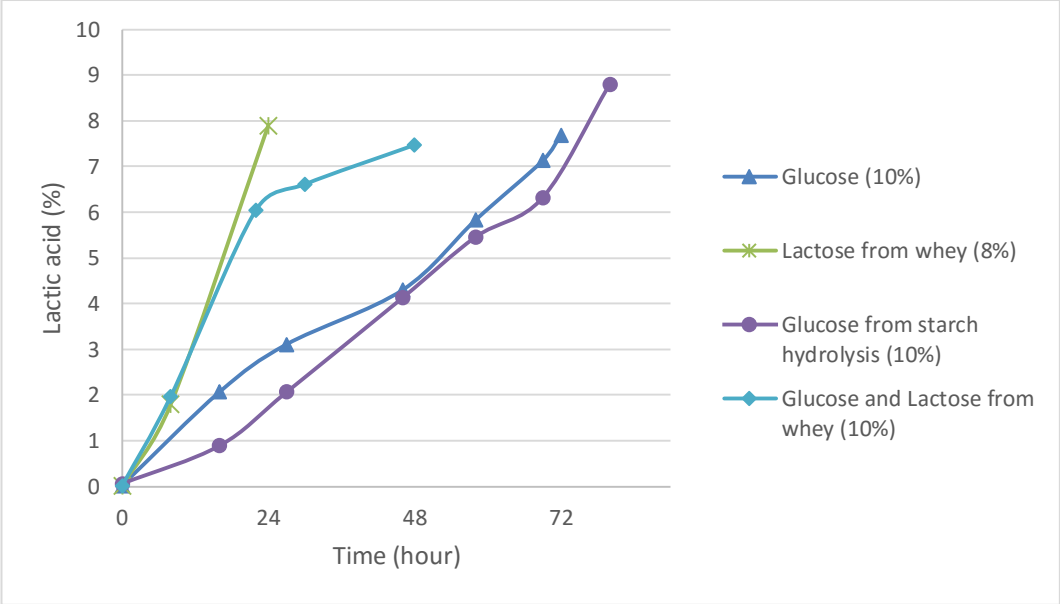


Figure 4.1 Effect of the substrate type on LA production

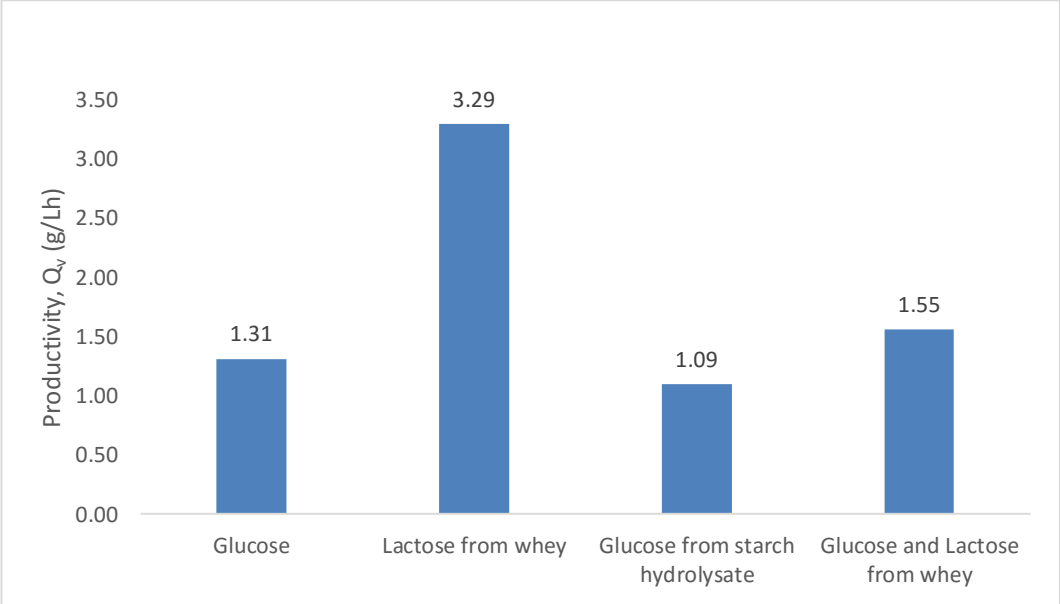


Figure 4.2 Productivity values obtained at different substrate type and combinations

4.1.2. Effect of the Type of the Nitrogen Source

In order to develop an economical fermentation, cheap and effective nitrogen source is also necessary to minimize the fermentation cost. Supplementation of nitrogen source into the fermentation medium is essential in order to achieve a high yield and productivity. In this study, nutrients have a considerable impact on the cost of the final product. In order to minimize the nutrient cost five different nutrients were used in different amounts in the fermentation with different substrates.

In the case of glucose as a substrate, YE, WP and PME were used as nutrient with different concentrations and combinations. Table 4.1 demonstrates LA production, yield and productivity (Q_v) for different nutrients with different combinations.

Results show that LA production using glucose as a substrate must require YE as a nutrient; however, it can be used in small amounts with a fortification of WP and/or PME. Productivity was definitely influenced by YE concentration because, higher the YE concentration in the medium, the shorter the fermentation lasted. Since it is a high cost raw material, YE can not be used in 2.9% for fermentation for economical production of LA.

On the other hand, results also show that WP and PME were not sufficient as a nutrient alone because yields were too small in comparison to nutrients with YE. Thus the cheapest alternative for glucose as a substrate was 10% WP, 1% PME, 2.9% YE nutrient mixture considering yield and productivity together.

In the case of lactose from sweetened whey (LSW) as a substrate, YE and PME were used as nutrient with different concentrations and combinations. Table 4.2 demonstrates LA production, yield and productivity for different nutrients with different combinations.

Table 4.1 Effect of the type of nutrient on the LA yield and productivity for glucose as a substrate

Substrate	**Nutrients**	**Total Protein Content (%)**	**LA g/L**	**$Y_{LA/tot}$ g/g**	**Q_v g/Lh**
Glucose	2.9% YE	2.0	76.9	0.80	1.31
	5.5% WP	2.0	15.2	0.15	0.11
	2% WP & 2% YE	2.0	62.4	0.62	0.75
	10% WP	3.5	36.2	0.34	0.47
	10% WP & 1.4% YE	4.5	58.0	0.58	0.81
	10% WP & 2.9% YE	5.5	74.7	0.79	1.55
	10% WP & 1% PME	3.6	37.2	0.37	0.52
	10% WP & 2% PME	3.6	35.9	0.36	0.51
	10% WP & 1% PME & 1.4% YE	4.6	61.4	0.61	0.85
	10% WP & 2% PME & 1.4% YE	4.6	73.8	0.74	1.03
	10% WP & 1% PME & 2.9% YE	5.5	86.3	0.86	1.98
	10% WP & 2% PME & 2.9% YE	5.6	88.3	0.88	2.02

Results show that LA production using LSW as a substrate must require YE as a nutrient; however, it can be used in small amounts with a fortification of PME. Productivity was again influenced by YE concentration. Results also show that PME was a good nutrient but not sufficient as a nutrient alone because yields were too small in comparison to YE&PME mixture. Thus the cheapest alternative for LSW as a substrate was 2% PME and 1.4% YE nutrient mixture considering yield and productivity together.

Table 4.2 Effect of the type of nutrient on the LA yield and productivity for LSW as a substrate

Substrate	Nutrients	Total Protein Content (%)	LA g/L	$Y_{LA/tot}$ g/g	Q_v g/Lh
LSW	-	2.0	32	0.32	0.22
	1% YE	2.0	34	0.34	0.23
	1.7% YE	2.0	82	0.82	0.57
	2.9% YE	2.7	79	0.99	3.29
	2% PME	0.9	56	0.58	0.77
	1% PME & 1.4% YE	1.7	69	0.72	2.85
	2% PME & 1.4% YE	1.8	72	0.82	2.99
	2% PME & 2.9% YE	2.8	85	0.89	3.53

For lowering the substrate cost, whey was used with a fortification of sucrose syrup and nutrients were SBYE and MD due to their inexpensive cost. SBYE concentrations were changed between 0.4% and 0.9% and MD was used as a supplement when SBYE concentration were decreased further. In Table 4.3 LA production, yield and productivity are shown with nutrient concentration. SBYE was the controlling nutrient for productivity again; however, yield was not influenced by SBYE. If process conditions were suitable for long time fermentation, 0.14% SBYE and 3% MD mixture would be the best alternative for economic production of LA. In addition, for half of this fermentation time 0.9% SBYE would be enough as a nutrient for economic production of LA.

Table 4.3 Effect of the type of nutrient on the LA yield and productivity for whey and sucrose as substrates

Substrate	**Nutrients**	**Total Protein Content (%)**	**LA g/L**	**$Y_{LA/tot}$ g/g**	**Q_v g/Lh**
Whey & Sucrose	0.9% SBYE	2.0	44	0.63	0.92
	0.7% SBYE	2.0	40	0.57	0.83
	0.5% SBYE	2.0	37	0.53	0.77
	0.4% SBYE	2.0	36	0.51	0.74
	0.2% SBYE & 2% MD	2.0	62	0.62	0.37
	0.14% SBYE & 3% MD	2.0	69	0.69	0.41
	0.08% SBYE & 5% MD	2.0	63	0.63	0.37
	5% MD	2.0	38	0.38	0.23

4.2. Purification of Solid Ca-L(+)-Lactate

A number of approaches are used for separation of lactate salt from fermented medium including extraction by ion-exchange separation, adsorption, and organic solvents. Purification steps in this study are shown in Figure 4.3. For purification studies, fermentation was conducted in a 100 L fermenter with 70 L working volume using whey as a substrate and SBYE as a nutrient. After fermentation, fermentation liquid was collected and subjected to ceramic tubular membrane filtration to remove the insoluble parts. The filtrate was collected and subjected to crystallization. Then the crystals precipitated were filtered out under vacuum and obtained crystals had 85% moisture in them. Hence, the solid Ca-L(+)-Lactate cake were dried under hot air current due to migrate impurities into surface by the help of evaporating water. While moisture content were decreasing during drying, impurities were accumulated in the surface by the help of migrating and evaporating water. The surface, impurity rich part, was manually separated from bottom part which was moisturized for further drying and this step was repeated several times to give white crystals of Ca-L(+)-Lactate. To do this, distilled water was added to bottom parts and the powder and distilled water were mixed at 50°C.

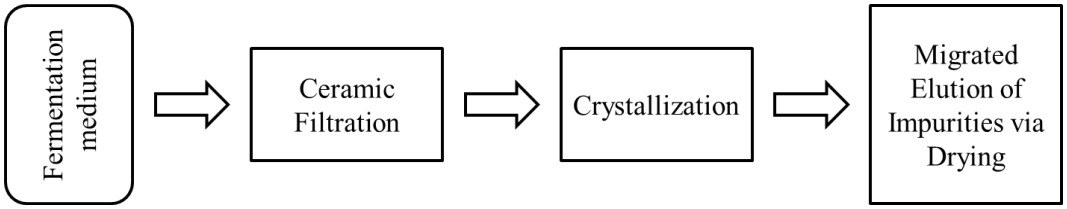


Figure 4.3 Flow chart of the purification

4.2.1. Crystallization of Ca-L(+)-Lactate

Crystallization was done to obtain solid Ca-L(+)-Lactate cake from fermentation medium prior to purification. Filtered fermentation medium contained 8.5% lactic acid and some other impurities like soluble proteins and trace metals and the color of the filtrate was brownish. The filtrate containing minimum 10 % Ca-L(+)-Lactate was crystallized at 4°C for 24 h and the crystals obtained were separated from liquid phase under vacuum. Nevertheless, the Ca-L(+)-Lactate cake obtained still had some protein and trace metal within the liquid phase it contained. The cake was light brownish in color and the color deviation from white color was treated as the impurity indicator since pure Ca-L(+)-Lactate is white in color. Its color difference from white was 267 in dry basis (CIE color system), it had still 0.104 g/L protein (Lowry protein assay) and 269 ppm Zn, 809 ppm Fe, 23 ppm Cu (AAS). Thus, there was still requirement of further purification.

4.2.2. Drying Induced Elution of Impurities from Ca-L(+)-Lactate

Filtration after crystallization was not enough for removing all of the water from Ca-L(+)-Lactate so solid Ca-L(+)-Lactate obtained from crystallization was dried with a novel technique which involves the drying of Ca-L(+)-Lactate crystals from one surface. The continuous removal of the moisture from one end causes the migration of the moisture in the slab material. This convective motion of the moisture causes the impurities in the slab move against their own gradient and

accumulate at the surface since the moisture at there is continuously removed by evaporation until crust formation. The symbolic representation of drying induced elution of impurities is shown in Figure 4.4.

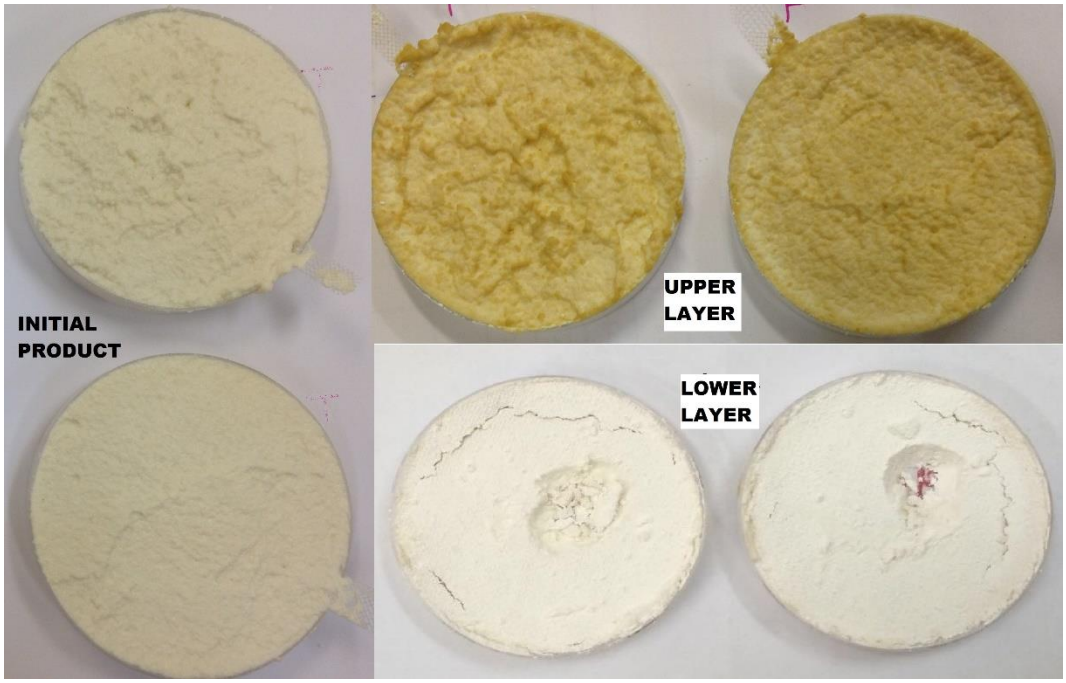


Figure 4.4 Picture of Ca-L(+)-Lactate Before and After Drying

4.2.2.1. Determination of Drying Characteristics of Ca-L(+)-Lactate

During the drying process crust was formed in trial experiments. Since crust formation would cause to slow down the evaporation, drying experiments were done at different temperature and relative humidity. Ca-L(+)-Lactate was dried both at 55 °C and 35 °C and the values of free moisture at these temperatures are shown in Figure 4.5.

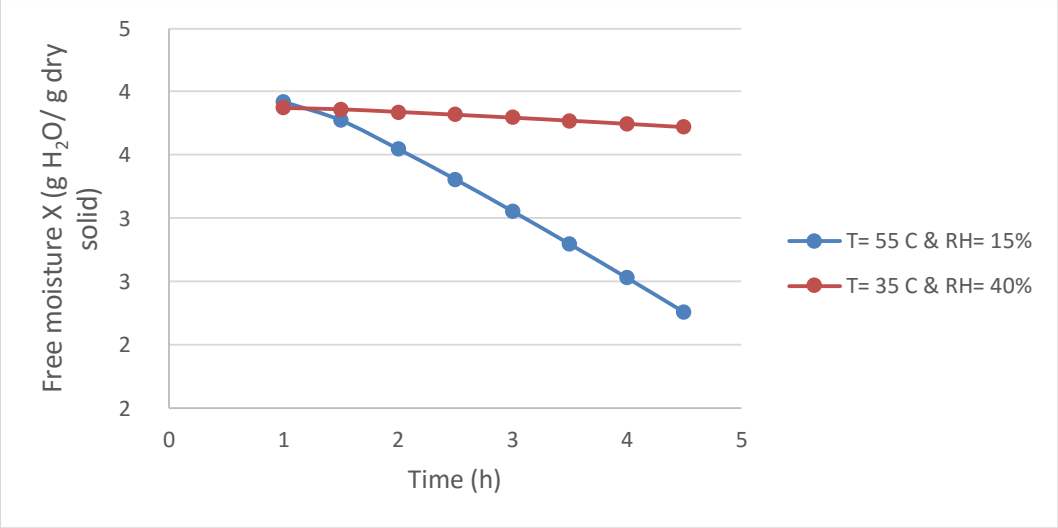


Figure 4.5 Free Moisture of Ca-L(+)-Lactate Cake at 35 ℃ and 55 ℃

While temperature was set to 35 ℃, maximum relative humidity was 40%. Solid Ca-L(+)-Lactate cake with an initial moisture of 85% was dried in these condition until crust formation. Drying lasted nearly five hours, moisture dropped to 75% and drying rate of cake is shown in Figure 4.6.

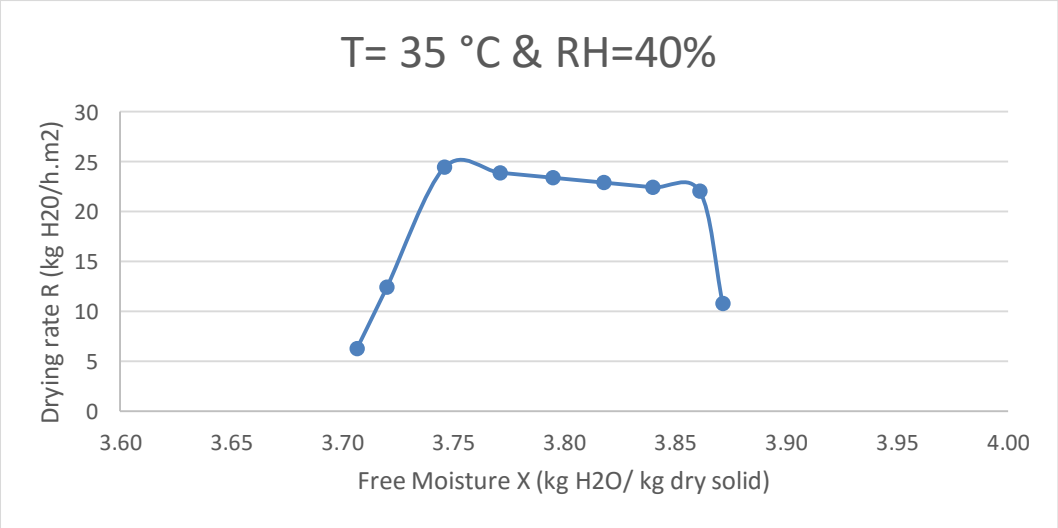


Figure 4.6 Drying Rate of Ca-L(+)-Lactate Cake When Dried at 35 ℃

While temperature was set to 55 °C, maximum relative humidity was obtained as 15%. Solid Ca-L(+)-Lactate cake with an initial moisture of 85% was dried in these condition until crust formation. It lasted nearly five hours, moisture dropped to 66% and drying rate of cake are shown in Figure 4.7.

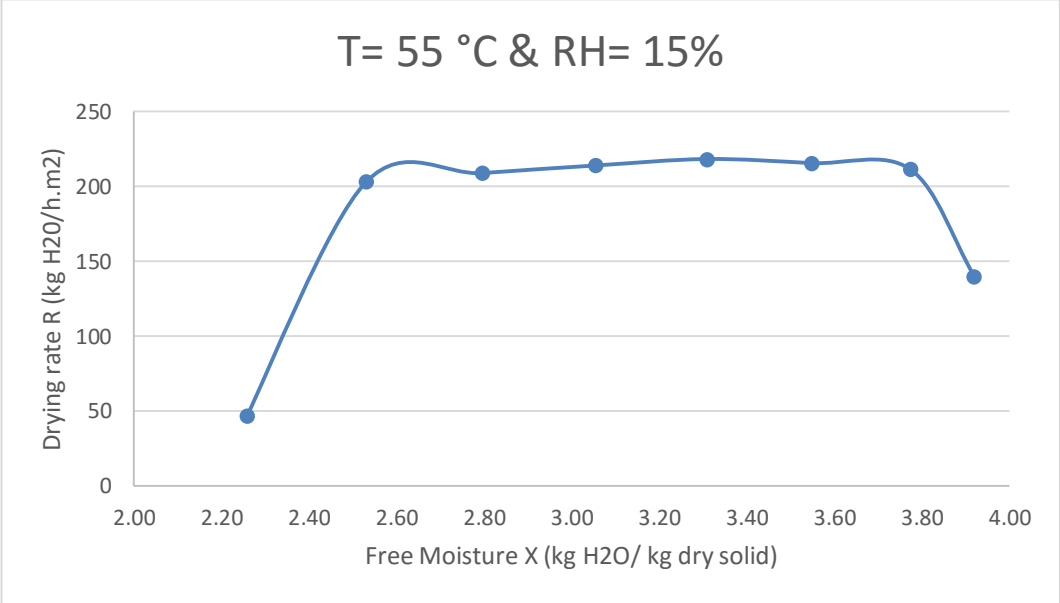


Figure 4.7 Drying Rate of Ca-L(+)-Lactate Cake When Dried at 55 °C

In the experiment when relative humidity was kept 15% and temperature was 55 °C, drying rate was relatively higher than the other experiment. We aimed that crust formation would not occur when relative humidity high; however, in both cases crust formation occured. Thus we concluded that relative humidity was not a dependent variable for purification studies, yet temperature was.

4.2.2.2. Effects of Drying Parameters

4.2.2.2.1. Effect of Air Flow Velocity and Temperature

Solid Ca-LA was prepared with the separating plastic mesh which easily separates top part from bottom part after drying has a moisture content of 65-70%. For drying studies a tray dryer which has a maximum temperature capacity of 55 °C and maximum air velocity 2 m/s was used. Thus, three different temperatures and three different velocities are tried in this experiment. Temperature and velocity values are tabulated in Table 4.4.

Table 4.4 Temperature and Air Velocity values of Drying

T/Q_{air}	1 m/s	1.4 m/s	2 m/s
35 °C	T1Q1	T1Q2	T1Q3
45 °C	T2Q1	T2Q2	T2Q3
55 °C	T3Q1	T3Q2	T3Q3

For each of the 9 samples drying lasted until the weight of sample was constant for 1 hour. After drying was complete, top surfaces of the samples were removed from the bottom part which has less impurity than the original sample by the help of separating mesh placed beforehand. Both the top and the bottom parts were diluted 20 folds for color determination.

For Top Surface;

Initial color of the Ca-L(+)-Lactate was 267 ΔE and the difference between color of the top surface after drying and color of the initial sample were reported to understand how much impurity would accumulate at the surface after drying.

Table 4.5 Color differences between top surface of the sample after drying and initial sample

Sample	**T1Q1**	**T1Q2**	**T1Q3**	**T2Q1**	**T2Q2**	**T2Q3**	**T3Q1**	**T3Q2**	**T3Q3**
ΔE*	43.11	83.70	38.85	62.88	111.36	44.57	102.82	108.20	57.80

*$\Delta E_{top\ surface} - \Delta E_{initial\ sample} = \Delta E$

According to results obtained from drying with different air flow velocities and air temperatures, highest impurity accumulation was achieved when temperature and air flow velocity were set as 45 °C and 1.4 m/s, respectively. However, as it can be seen from the Table 4.5 there is no trend between either temperatures or air flow velocities.

For Bottom Part,

The difference between color of the bottom part after drying and the color of the initial sample were tabulated in Table 4.6 in order to analyze color deviation during drying according to temperature and air velocity of desiccant air. Results showed that preferring the maximum air flow velocity and temperature gave the highest absolute value of color difference between bottom part and initial sample. However, when color differences between top surface and bottom part evaluated together by simply adding them, it was seen that choosing T3Q3 was not reasonable. As a result of this, the trial T3Q1 (T= 55 °C and Q= 1 m/s) was chosen as an optimum for further experiments.

Table 4.6 Color differences between bottom part of the sample after drying and initial sample

Sample	**T1Q1**	**T1Q2**	**T1Q3**	**T2Q1**	**T2Q2**	**T2Q3**	**T3Q1**	**T3Q2**	**T3Q3**
ΔE*	-12.90	-29.25	-14.18	-15.37	-19.37	-12.25	-33.32	-13.34	-47.71

*$\Delta E_{bottom\ part} - \Delta E_{initial\ sample} = \Delta E$

4.2.2.2.2. Effect of the Number of Drying Stages

During drying stage, since crust formation could not be stopped, one drying stage was not enough for removal of moisture within impurities. When first drying stage was completed, Ca-L(+)-Lactate still had 60-70% moisture in it. Thus most of the impurities were still in moist Ca-L(+)-Lactate. As a result of this, dried bottom part of Ca-L(+)-Lactate containing less impurities than initial Ca-L(+)-Lactate were moisturized and dried again. In this experiment, necessary number of drying stages were examined. Tray dryer was run at 55 °C with an air flow of 1 m/s. After each step of drying bottom part of the sample was moisturized with distilled water at 50 °C and drying was repeated like this for five times.

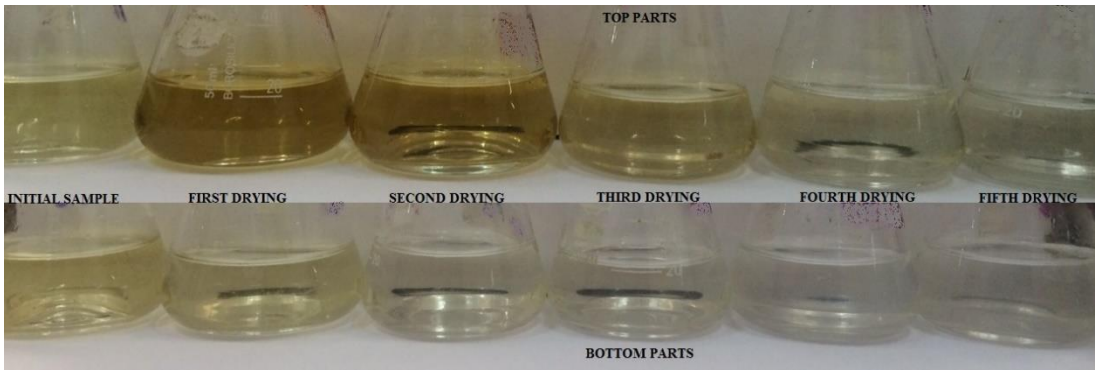


Figure 4.8 Picture of Diluted Top and Bottom Parts of Ca-L(+)-Lactate for Five Drying Stages

For color determination samples were dissolved and diluted 20 fold and were measured as liquid. In Figure 4.8, diluted top and bottom parts are demonstrated with diluted initial part. First and second drying stages were the most effective drying stages and top of the samples from first and second drying stages were not used for this study because they were dirtier than initial sample and would cause the accumulation of impurities. However, in further drying stages top of the samples can be recycled. As it can be seen in Figure 4.9, since there was a little change between fourth and fifth dryings optimum number of drying stage was decided as four.

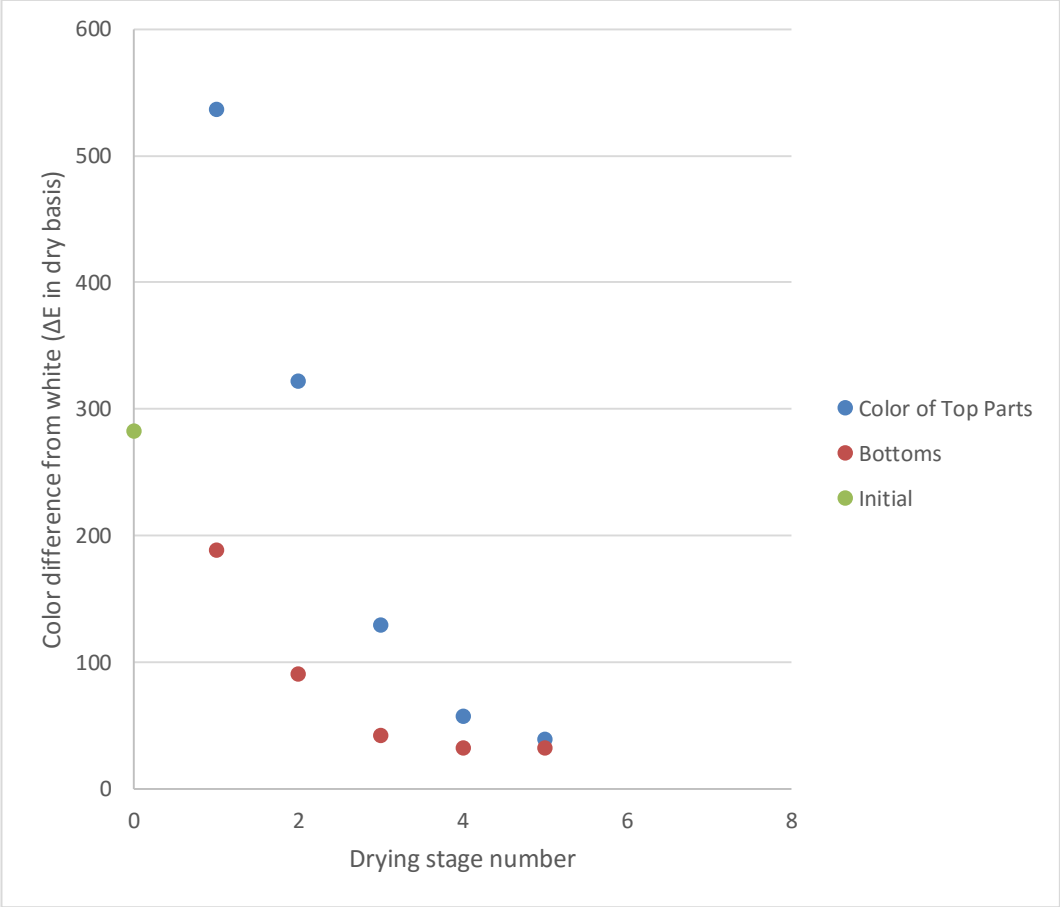


Figure 4.9 Color differences from white vs. repetitive drying stages

4.2.2.3. Indication of Solid Ca-L(+)-Lactate Purification

In this section solid Ca-L(+)-Lactate was again dried to purify with guidance of previous drying studies; however, for this study protein and trace metal distribution in layers was also analyzed in addition to the color deviation for observation of purification grade. Most reasonable air flow and temperature values had been selected as 1 m/s and 55 °C and it had been observed that repeating the drying stages more than four times of drying was unnecessary since there had been no color change between bottom parts of samples. The scheme of this process is demonstrated in Figure 4.10.

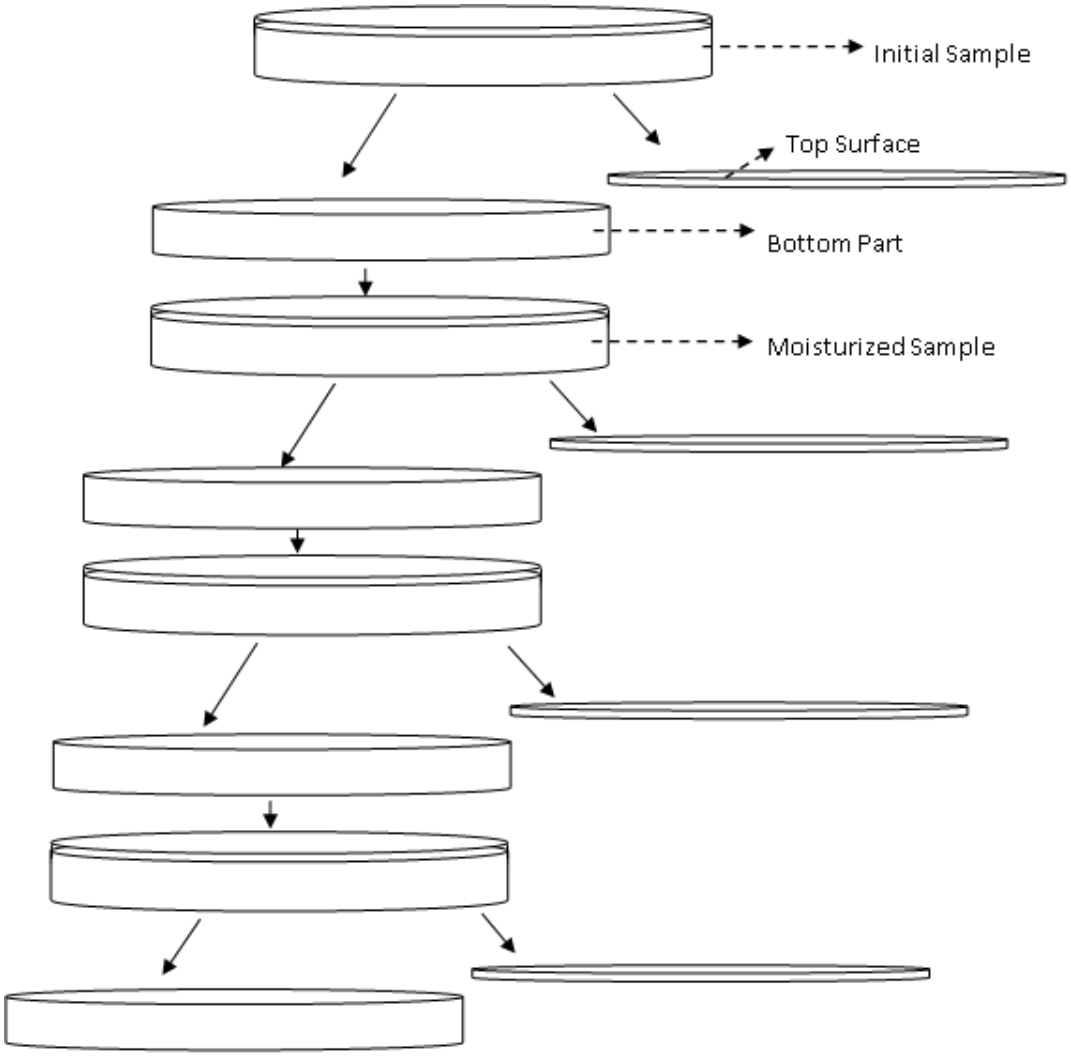


Figure 4.10 Scheme for drying of Ca-L(+)-Lactate

After all drying stages were completed samples were ground to make possible homogeneous sampling. Powdered Ca-L(+)-Lactate was dissolved in distilled water and if there was any cloudiness arising from insoluble particles, it was centrifuged to obtain a clear solution. These Ca-L(+)-Lactate solutions was subjected to color spectroscopy and obtained results were recalculated in dry basis considering moisture content. The change in color difference from white in dry basis are shown in Figure 4.11 and it was again seen the color of Ca-L(+)-Lactate was

decreasing to some point as in the study of determination of necessary drying stage. However, it was not known how impurities such as proteins and trace metals distribute during drying stages. Thus, additional analyses were done.

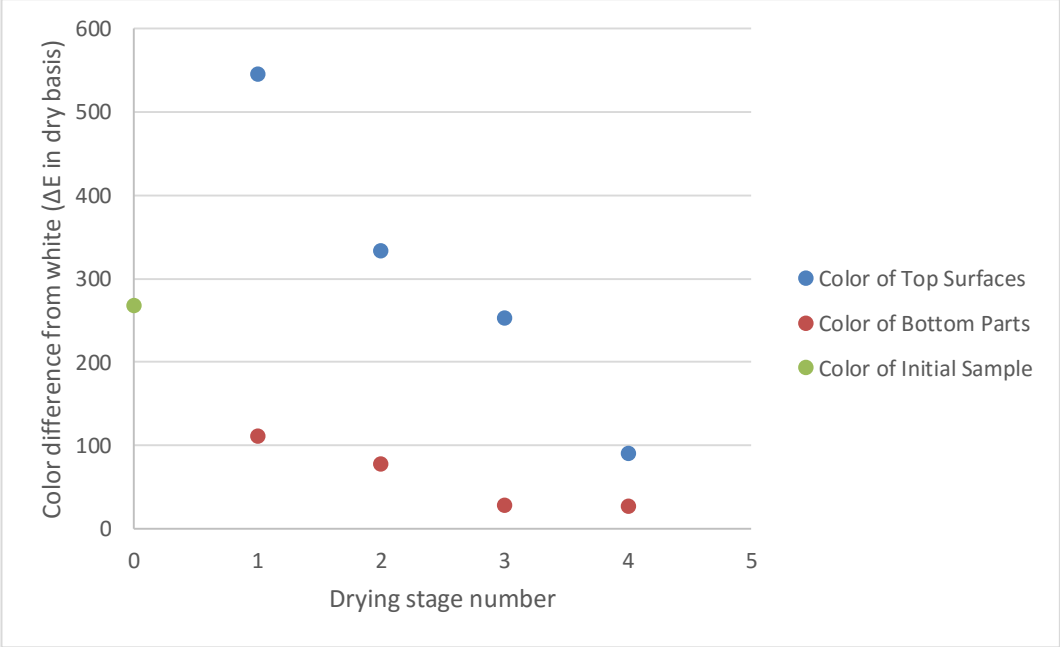


Figure 4.11 Color Differences from White During Dryings

Lowry protein assay was applied to ground Ca-L(+)-Lactate samples to determine the protein distribution during drying. The Ca-L(+)-Lactate that was dissolved in distilled water was diluted to make Lowry protein analyses and results are shown in dry basis to normalize the dilution factor. Results indicated that the protein concentrations and color distributions followed the same trend. Protein content of dried Ca-L(+)-Lactate are decreasing in each drying for bottom parts of samples, this means proteins were also removed via drying induced elution. However, in third drying protein accumulation at the surface was lower than expected. Since the initial moisture content and the weight of the samples are equal, the possible reason of this could be the drying time. Although temperature and air flow velocities were equal, third drying lasted shorter.

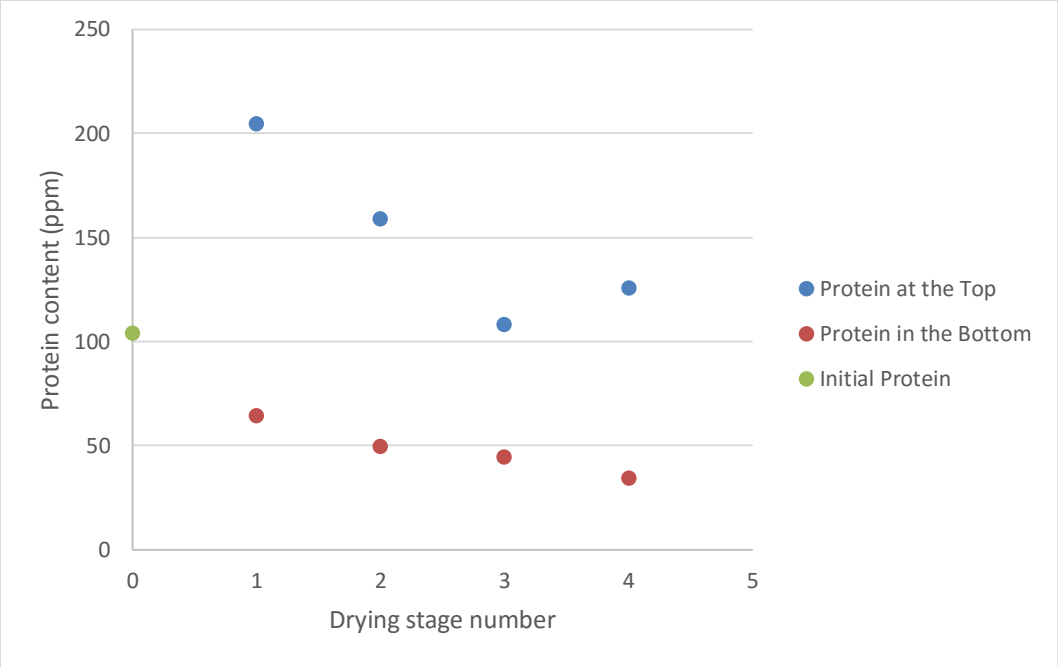


Figure 4.12 Protein Distributions Resulting After Drying Experiments

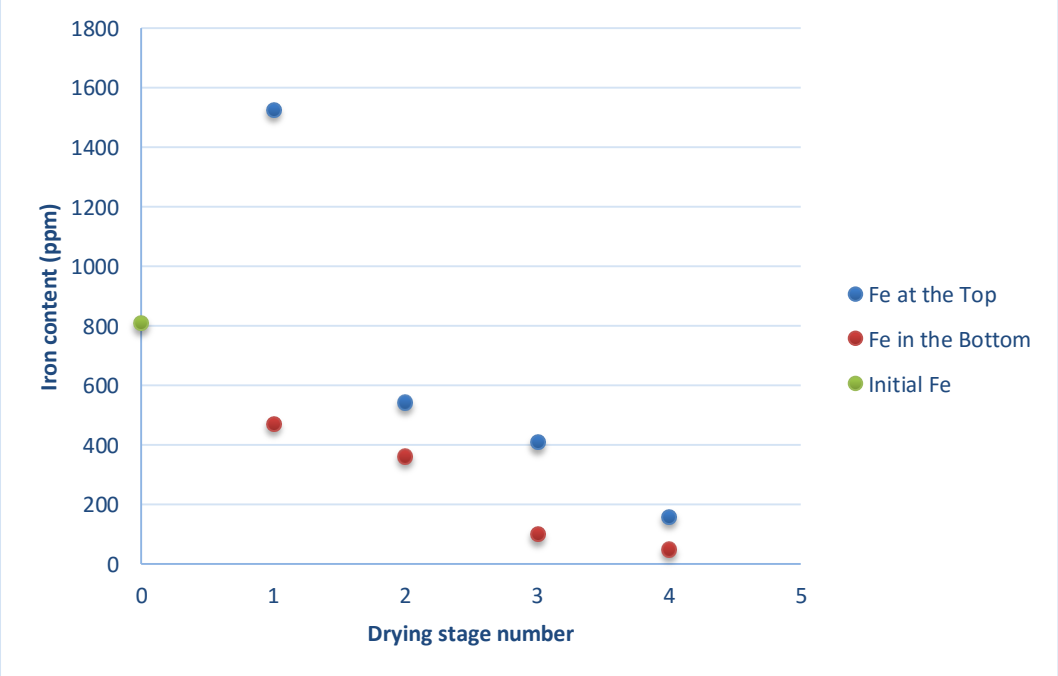


Figure 4.13 Iron Content Distributions Resulting After Drying Experiments

Besides protein distribution (Figure 4.12) contamination with metals within the Ca-L(+)-Lactate samples were mapped during the purification procedure using AAS. Iron, copper and zinc distributions were reported and results showed that initial Ca-L(+)-Lactate consisted 809 ppm iron, 23 ppm copper, 269 ppm zinc and respectively 94%, 78% and 84% of them was removed off. All of these three metal anions had the same purification trend (Figure 4.13, 14 & 15) with color, thus considering the removal of metals during drying it should be concluded that drying induced elution of impurities through surface provided recovery of Ca-L(+)-Lactate from metal anions.

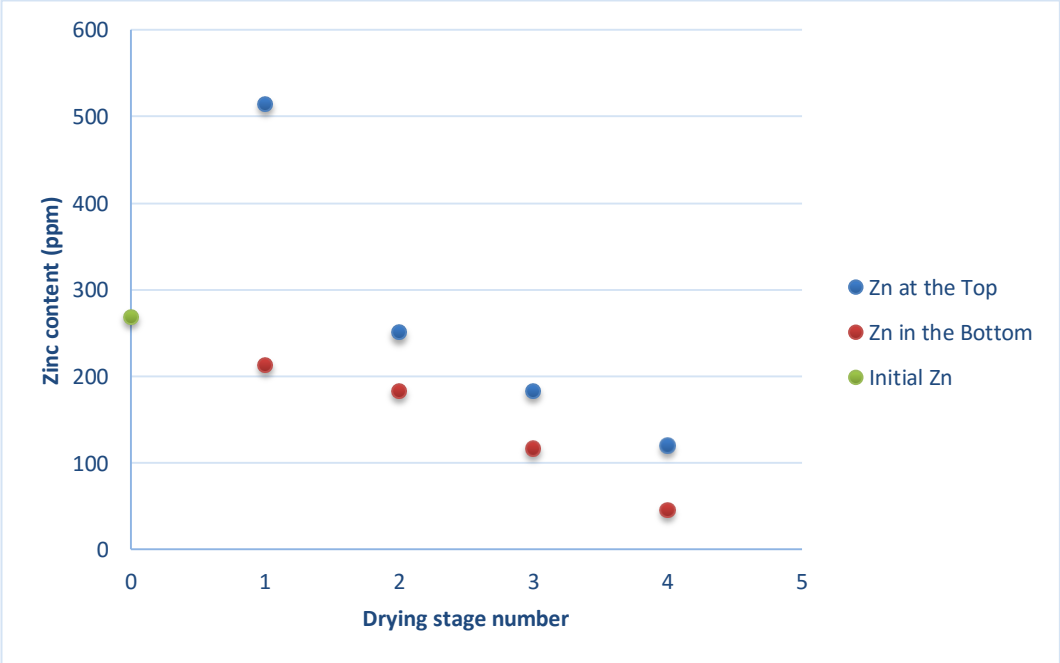


Figure 4.14 Zinc Content Distributions Resulting After Drying Experiments

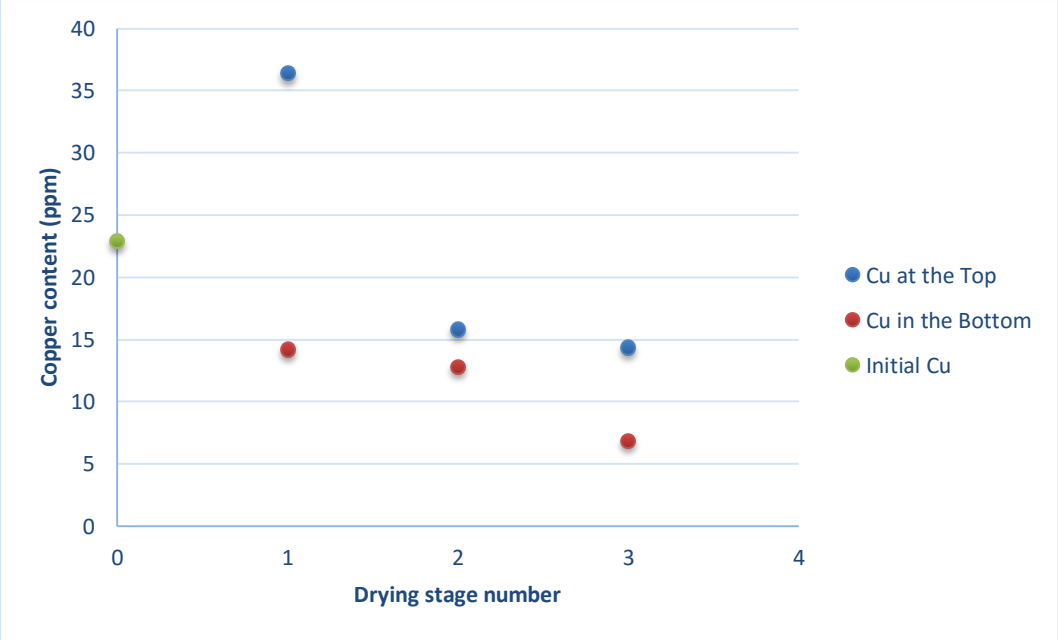


Figure 4.15 Copper Distributions Resulting After Drying Experiments

Table 4.7 Color deviation, amount of Ca-L(+)-Lactate, protein and trace metals (Fe, Zn and Cu) in dry basis for each fraction of dryings

Fractions	**Color (ΔE)**	**Ca-L (g/L)**	**Protein (g/L)**	**TM (ppm)**
Initial Sample (I)	267	909	0.104	1101
Top of I (T1)	545	871	0.205	2079
Bottom of I (B1)	111	926	0.065	699
Top of B1 (TB1)	333	886	0.159	810
Bottom of B1 (B2)	77	950	0.050	557
Top of B2 (TB2)	252	944	0.108	610
Bottom of B2 (B3)	28	964	0.042	226
Top of B3 (TB3)	90	943	0.126	279
Bottom of B3 (B4)	26	983	0.034	97

When all purification analyses were examined together it was concluded that color, protein and iron have the same purification trend. In addition, 67% of protein and 93% of iron were removed from Ca-L(+)-Lactate cake by the help of drying. Ca-L(+)-Lactate concentrations were demonstrated in Table 4.7 with color values, protein and trace metal content for each fraction of dried samples. Initial Ca-L(+)-Lactate was 90.8% on dry basis and became 98.3% after four drying stages while color degraded 90.2%. However, there were still 34 ppm protein, 97 ppm trace metals (Fe, Zn, Cu) and possibly some other unknown materials in the final Ca-L(+)-Lactate powder (B4).

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATIONS

"LA obtained by biotechnological process is preferred for industrial applications, especially, bioplastic industry." (Boontawan, 2010) Thus production of both optically and chemically pure LA is essential for this approach. LAB are the preferred organisms for LA fermentation. *L. casei*, a homo-fermentative LAB was used extensively in this study. It exhibited 99 % lactic acid production yields which are desirable for industrial application for mainly L(+)-producing strain. The intention of the present investigation was to study lactic acid production and to improve the lactic acid production for industrial purposes. The nutrients traditionally used in most of the fermentative media, particularly yeast extract and peptone, are costly; thus whey products as carbon source and SPYE as nitrogen source were used in this work.

Recovery of lactate from fermentation broth was investigated in order to pave the way for applying in the fermentation system. Ca-L(+)-LA was used for purification experiments since it was produced in high volume without any trouble. LA which was a calcium salt within the fermentation medium was removed from it by the help of crystallization and after that it was purified as Ca-L(+)-Lactate under hot air current by accumulating impurities at the surface which is named drying induced elution of impurities. In this process it was observed that evaporating water would carry the impurities against concentration difference of the impurities thus factors effecting this accumulation was investigated. Firstly, air flow temperature and velocity was chosen as dependent variables and there different air flow

velocities and air flow temperatures were selected and among these nine trials T3Q1 (T= 55 °C & Q= 1 m/s) was the most reasonable one because total color change was the highest in this trial. These values for temperature and air flow velocity were selected as independent variables for further experiments. After determining temperature and velocity of a desiccant air the effect of the number of drying stages was investigated. Ca-L(+)-Lactate was dried five times separating impurity rich top surface from the bottom and it was observed that fifth drying was unnecessary because no color change between fourth bottom part and fifth bottom part was observed. When all variables were set experiment was proceeded in higher amount to analyze what were the causes of impurities. The preliminary experiments were carried on by observing the color changes only. After deciding the ideal conditions experiments in which protein and metal ion contents were monitored along with the color changes. Lowry protein analyses, HPLC and AAS were done for determination of protein, LA and trace metals for final purification experiment. Finally results may show that the causes of impurity and color were mostly protein and metal ions especially iron within the solid Ca-L(+)-Lactate cake. They demonstrated almost the same trend with color deviations during drying.

For purification of lactic acid, different techniques had been introduced; such as, ion exchange chromatography, adsorption, strong-acid anion exchange resins, foam separation, nanofiltration, solvent extraction and esterification (Ghaffar et al., 2014). In this work we show that the combination of crystallization and drying together is a promising downstream process, which separated other organized impurities from lactic acid. It has still the disadvantages such as hard to handle drying parts of purification step and loss of Ca-L(+)-Lactate with impurity rich top part.

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APPENDICES

APPENDIX A

Lowry Protein Assay and Calibration Curve

Materials:

1. Complex-forming reagent: Prepared immediately before use by mixing the following stock solutions in the proportion 100:1:1 (by volume), respectively:

   Solution A: 2% (w/v) Na_2CO_3 in distilled water.

   Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water.

   Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

2. 2 N NaOH.

3. Folin reagent (commercially available): Used at 1 N concentration.

4. Standards: Bovine serum albumin containing 0.1 mg/mL protein in distilled water, stored at 4°C.

Table A. 1 Table for standard sample preparation

Stock Solution (µl)	0	25	50	100	150
Distilled Water (µl)	500	475	450	400	350
Protein Concentration (µg/ml)	0	5	10	20	30

Method

1. Since the sample is a precipitate, was dissolved in 2 N NaOH

2. Hydrolyzed at 100°C for 10 min in a heating block or boiling water bath and carried 0.5-mL aliquots of the hydrolysate.

3. The hydrolysate was cooled to room temperature and added 2 mL of freshly mixed complex-forming reagent. The solution stood at room temperature for 10 min.

4. Added 0.2 mL of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30 min.

5. After color formation finished, samples were centrifuged to remove precipitates.

6. The absorbance were read at 750 nm.

7. The standard curve of absorbance as a function of initial protein concentration was plotted and demonstrated in Figure A.1.

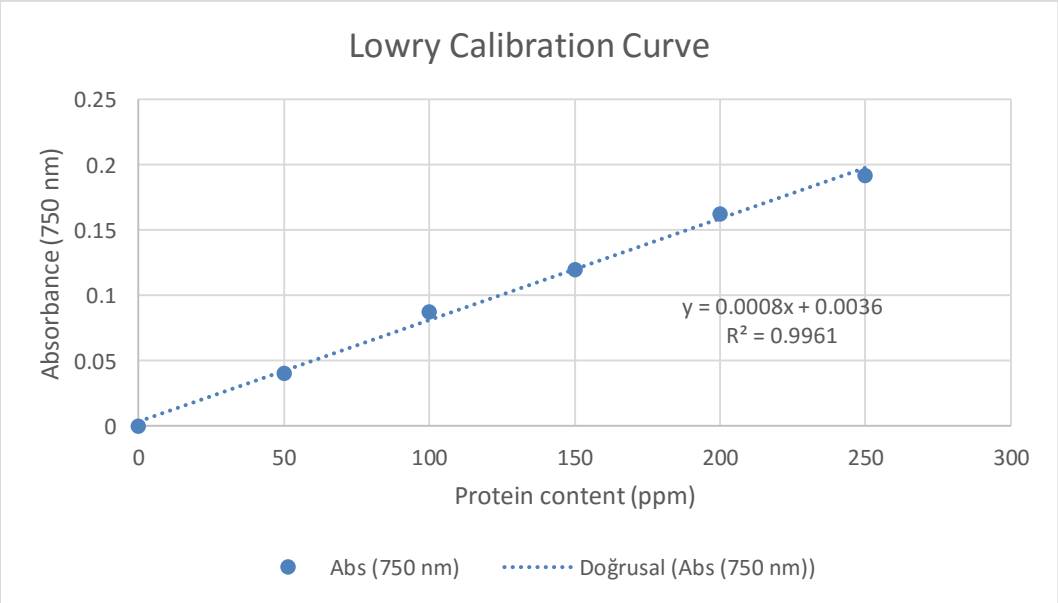

Figure A. 1 Lowry Calibration Curve

APPENDIX B

AAS

Materials:

Certified 1000 ppm AAS standards (Fe, Zn, Cu)

Nitric acid (2% v/v)

Nitric acid (65% v/v)

Sulfuric acid (85% v/v)

Method:

A. Preparation of Unknown: The sample was digested by dissolving 0.10 g in a mixture of 25 mL of concentrated nitric acid and 10 mL of concentrated sulfuric acid in a beaker. Once dissolved, the mixture was added slowly to ~50 mL of distilled water in a 100 mL volumetric flask and diluted to the mark with distilled water.

B. Preparation of Standard Curves:

Table B. 1 Standard Curve Concentrations of Iron

Stock Solution (100 ppm)	0	62.5	125	187.5	250
Distilled Water (μL)	5000	4937.5	4500	4812.5	4750
Fe Concentration (ppm)	0	1.25	2.50	3.75	5.00

Table B. 2 Standard Curve Concentrations of Zinc

Stock Solution (10 ppm)	0	100	200	300	400
Distilled Water (μL)	5000	4900	4800	4700	4600
Zn Concentration (ppm)	0	0.2	0.4	0.6	0.8

Table B. 3 Standard Curve Concentrations of Copper

Stock Solution (100 ppm)	0	25	50	75	100
Distilled Water (µL)	5000	4975	4950	4925	4900
Cu Concentration (ppm)	0	0.5	1.0	1.5	2.0

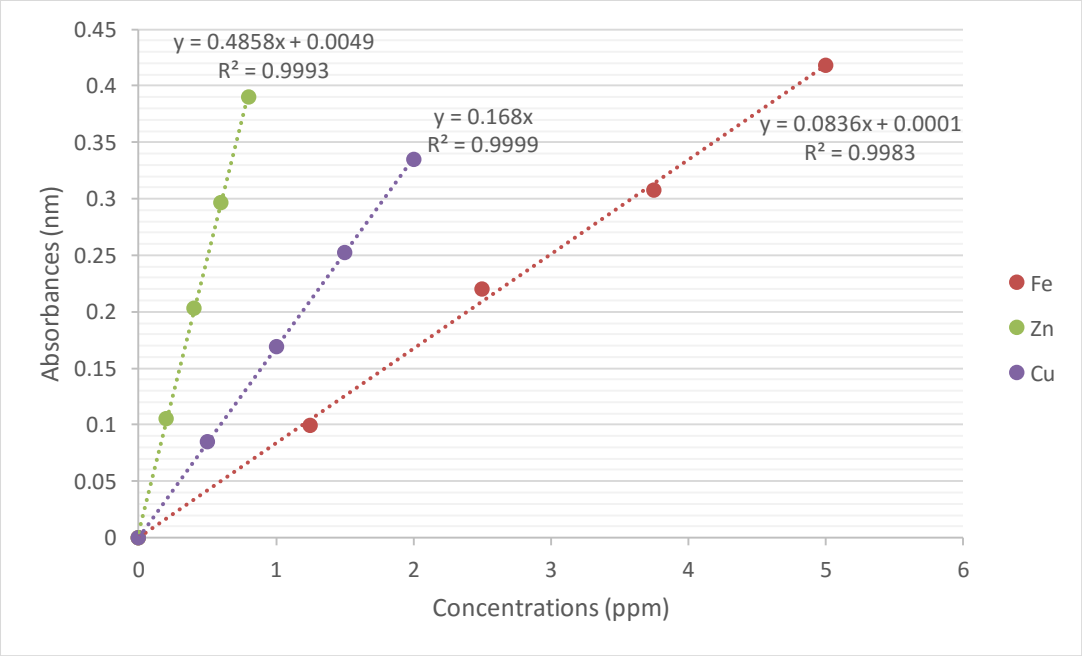

Figure B. 1 Calibration Curves of Iron, Zinc and Copper

APPENDIX C

Calibration Curves for HPLC Analysis

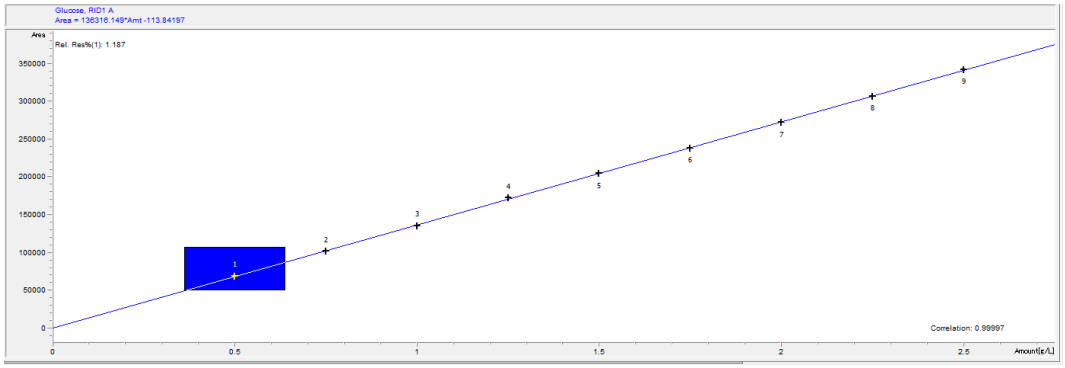

Figure C. 1 Calibration Curve of Glucose for HPLC Analysis

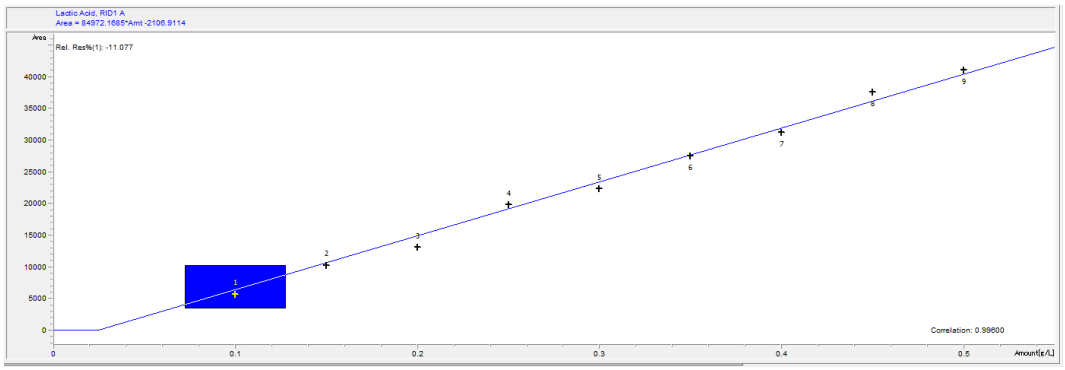

Figure C. 2 Calibration Curve of LA for HPLC Analysis

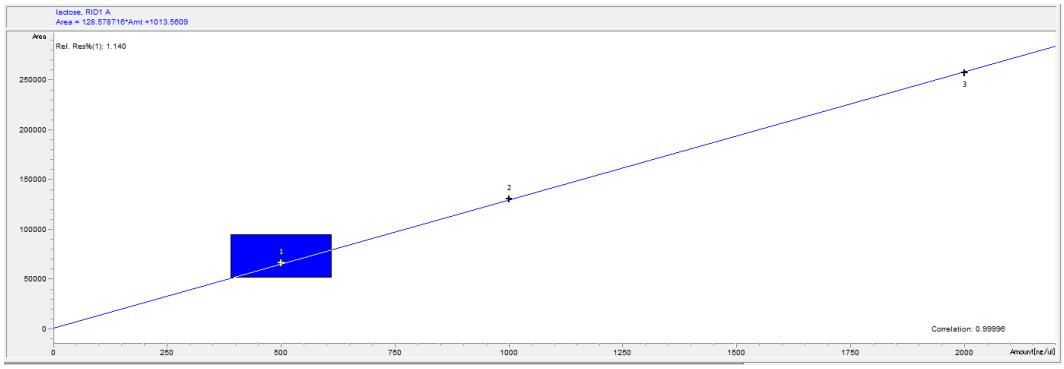

Figure C. 3 Calibration Curve of Lactose for HPLC Analysis

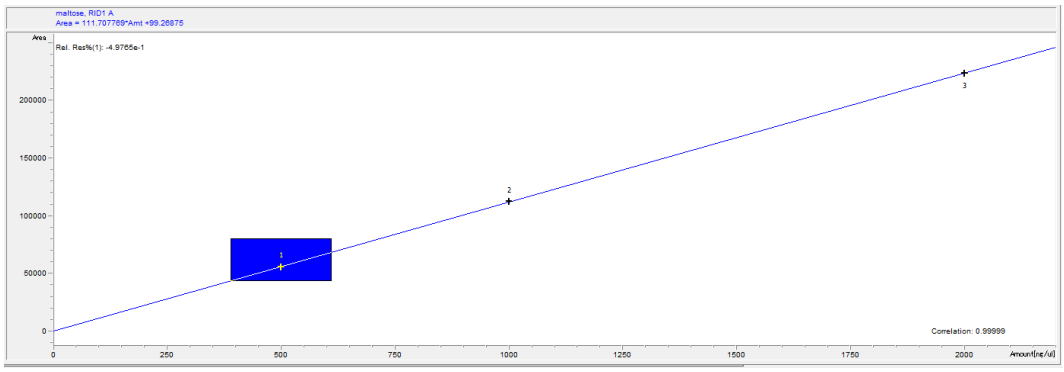

Figure C. 4 Calibration Curve of Maltose for HPLC Analysis