Lactic acid production from maleic acid pretreated fruit pulp waste using *Lactobacillus delbruckii* and *Lactobacillurhamnosus*

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Abstract

Fruit waste biomass is a renewable substrate whose production is 25-57 million tons (per year). It can contribute to environment problems as the disposal of fruit waste in landfills, leads to greenhouse gas emission, contributing to climate change. It grows pathogens (bacteria fungi and viruses), and causes the spread of diseases and also emit unpleasant odors as it decomposes. Production of important compounds like lactic acid can mitigate the environmental problems caused by fruit waste. The future use of lactic acid produced from fruit waste biomass holds promising potential in various industries like pharmaceutical industry, food and beverage industry, chemical industry and applications like for production of bioplastics (PLA). Lactic acid as a source for biopolymer synthesis from renewable substrates can reduce the dependency on fossil fuels, used as substrate in petrochemical industry. In the current study lactic acid was produced from fruit waste pretreated by organic acids. Organic acid catalyzed pretreatment of fruit waste was carried out using temperature of 121^{0}C and 15 psi pressure. Maleic acid provided highest sugar yield of 15 g/l and the enzymatic hydrolysis with Hemicellulase enzyme was done to enhance the sugar yield to 31.38 g/l. The fermentation of this hydrolysate resulted in lactic acid yields of 6.5 g/l in case of *L. rhamnosus* (MTCC 1423) and 33.5 g/l in case of *L.delbruckii* (MTCC 911).

Introduction

Organic acids are the third - largest category of biological products in the field of food biotechnology [1]. Lactic acid (2-hydroxypropionic acid) occupies a significant position among organic acids as a result of its wide utilization in the food and beverage, cosmetic, pharmaceutical and other industries as a neutralizer [2]. Lactic acid has long been used to ferment and preserve human food. It was discovered by Scheele in 1780 in sour milk [3]. Its molecular formula is (CH3CH(OH)COOH. It is white in its solid state and is miscible with water. It has recently been used as a precursor for ecologically friendly solvents or as a building block for a variety of biodegradable polymers [4]. According to the report Lactic acid Market by Application (Biodegradable Polymers, Food and Beverages, Pharmaceuticals Products), the annual global market for lactic acid in 2020 was estimated to be worth USD 1.1 billion, and it is expected to double by 2025. The market for lactic acid is expected to increase at a compound annual growth rate (CAGR) of 8.0 from 2023 to 2030 from a market size of USD 3.1 billion in 2022. Growing consumer awareness and increased usage of biodegradable plastic packaging in the food application segment are the key reasons for the tremendous growth in the use of lactic acid in biodegradable polymers in recent years [4].

Lactic acid is primarily utilised in the production of polylactic acid (PLA), a compostable thermoplastic created from renewable sources. PLA is a compostable and biodegradable thermoplastic resin made from renewable resources [5]. Due to the declining security of petroleum resources, the importance of polymers from renewable raw materials is expected to increase. The use of biopolymer blends and composites can revolutionize fields such as automotive, pharmaceuticals and packaging. Chemical production of lactic acid is expensive and relies on by-products from other industries derived from fossil fuels. Furthermore, chemical synthesis produces racemic mixtures of lactic acid and for many specific applications only one of the lactic acid isomers is desirable [6]. The production of optically pure D- or L- lactic acid using suitable microorganisms and cheap renewable raw materials with reduced energy consumption has revealed the importance of fermentation technology [7].

Renewable resources like lignocellulosic biomass resources include forestry woody biomass waste, agricultural residue waste, agro industrial waste (fruit waste, animal waste, juice industry waste. Ligno-cellulosic biomass as a renewable resource has recently been exploited as a cheap substitute. The cellulosic, hemicellulosic and lignin content of the raw substrate determines its potential to serve as a raw substrate for the conversion of sugars to lactic acid production, agrowastes are rich in sugars and can be easily assimilated by microorganisms, making them potential substrates [8, 9, 10, 11].

Large amount of waste are generated from the transportation, storage, distribution and consumption of fruits which constitute a major portion of municipal waste. Traditional dumping and landfilling fruit waste causes several environmental problems viz. leachates, unpleasant odor, result of high moisture and organic content as well as low pH. Therefore the development of an appropriate approach for managing fruit waste is of great importance [12, 13].

Maximum sugars are attained after pretreating the potential lignocellulosic waste. The goal of pre-treatment is to reduce the degree of polymerization and crystallinity of cellulose, breakdown hydrogen connections between fibres, and increase the surface area that is available for chemical as well as enzymatic action produced by the miscrobes during the process of fermentation. In order to effectively hydrolyze cellulose and hemicellulose into fermentable sugars, the lignin should be atleast partially eliminated [14, 15]. Several pre-treament methods have been applied in an effort to dissolve the lignocellulosic complex and release sugars for future usage. Sugars are hub for metabolic pathways like EMP pathway followed by lactic acid producing bacteria that tend to produce secondary metabolites viz ethanol, propionic acid acetic acid , lactic acid etc . So it is important to increase the sugar yields for optimum yields of organic acid. In order to optimize the conversion of sugars to lactic acid, further the optical purity and stereo-specificity of lactic acid produced by fermentation depend on the microbial strain and its lactic acid isomer. Due to the use of simple sugars, which enables the creation of a pure product and lowers purifying costs, the main disadvantage of this method on a industrial scale is the significant impact that the raw material has on its economy. Thus, reutilization of agro -wastes is of great interest since, due to legislation and environmental reasons, the industries are being forced to find an alternative use for its residual matter.

Materials and methods

Sample collection:-

In present study, the agrowaste which was used as a renewable substrate was fruit pulp waste, collected from local shop within the University Campus. The composition of the fruit pulp waste comprised of pineapple pulp (20%), orange pulp (50%) and beet root pulp (30%). The sample was air dried at room temperature (280C ± 20C). The waste was mechanically grounded into a fine powder using a grinder, and then passed through of sieve of 1mm which was then subjected to further chemical pre-treatment.

Thermo -chemical pre-treatment:-

Organic acids viz. maleic acid, succinic acid, and acetic acid were used at the concentration of 1%-3% for chemical pre-treatment of fruit pulp waste. The chemically pretreated biomass was subjected to thermal treatment and autoclaved at 121°C for 30 min.

Enzymatic treatment:-

Thermochemically pretreated fruit pulp waste was treated with the commercial enzyme HEMICELULAZA, Z (from Asperigillus niger) from SIGMA Life science at a concentration of 5 units/g at 37°C. The samples were tested for total reducing sugar concentrations at a regular interval of 24 hours.

Fermentation conditions:-

Standard lactic acid producing strains (MTCC 1423 and MTCC 911) were grown anaerobically in MRS media at 37°C under static conditions and the hydrolysates were inoculated with standard lactic acid producing bacterial strains. Samples were collected at the regular interval of 24 hrs for lactic acid estimation. MRS medium (Yeast extract-0.4%; Glucose-2.0% ;Sodium acetate trihydrate-0.5%; polysorbate 80-0.1%; peptone-1.0%; beef extract-1.0%; dipotassium hydrogen phosphate-0.2%; triammonium citrate-0.2%; magnesium sulphate heptahydrate-0.02%; agar-1.0%) was used for growing lactic acid producing strains.

Reducing sugar and lactic acid estimation:-

DNS assay method was adopted for estimation of total reducing sugars obtained from thermochemically and enzymatically pretreated fruit pulp waste. SHF (Separate Hydrolysis and Fermentation) was carried out to determine the lactic acid production.

Reagents used for reducing sugar assay:-

DNS I= DNS (3, 5 – Dinitrosalicylic acid- 1g; Sodium sulphite (Na_2SO_3) - 0.5gm; Phenol- 0.2gm; Sodium hydroxide (NaOH) - 1gm) in 100 ml distilled water.)
DNS II = (Sodium potassium tartarate - 40 gm) in 100 ml of distilled water.

Lactic acid estimation of the fermented broth treated with lactic acid producing standard strains was determined by titrimetric method and formula used for the calculations is given below:

Lactic acid (g/l) = Vol of 0.1 N NaOH X 90.08mol.wt of lactic acid X 100 / 1000

Samples were titrated against 0.1 N NaOH, using 2-3 drops of phenolphathalein as an indicator. Controls and blanks were also taken into consideration.

Results & Discussion

Physical/ mechanical pretreatment:-

Physical pretreatment increases the surface area and also decreases the degree of polymerization and crystallinity by reducing the particle size. It reduces the reaction time and energy consumption by significantly increasing the surface area. The physical methods are environmentally friendly and less toxic.

Figure 1: Physically pretreated fruit pulp biomass

Acid hydrolysis using (one-variable at a time) OVAT

In one-variable at a time (OVAT) approach different organic acids (acetic acid, maleic acid, succinic acid) were used for chemical pretreatment of renewable substrate viz fruit pulp waste. The organic acid hydrolysis was accompanied with thermal pretreatment (121^{0}C at 37^{0}C) at a biomass loading of 10% and acid concentration of 3%. On treatment of fruit pulp waste with organic acid (acetic acid, succinic acid, maleic acid) concentration (3%) and biomass loading of 10%, the maximun reducing sugar yield attained was 15g/l as shown in figure 2 below

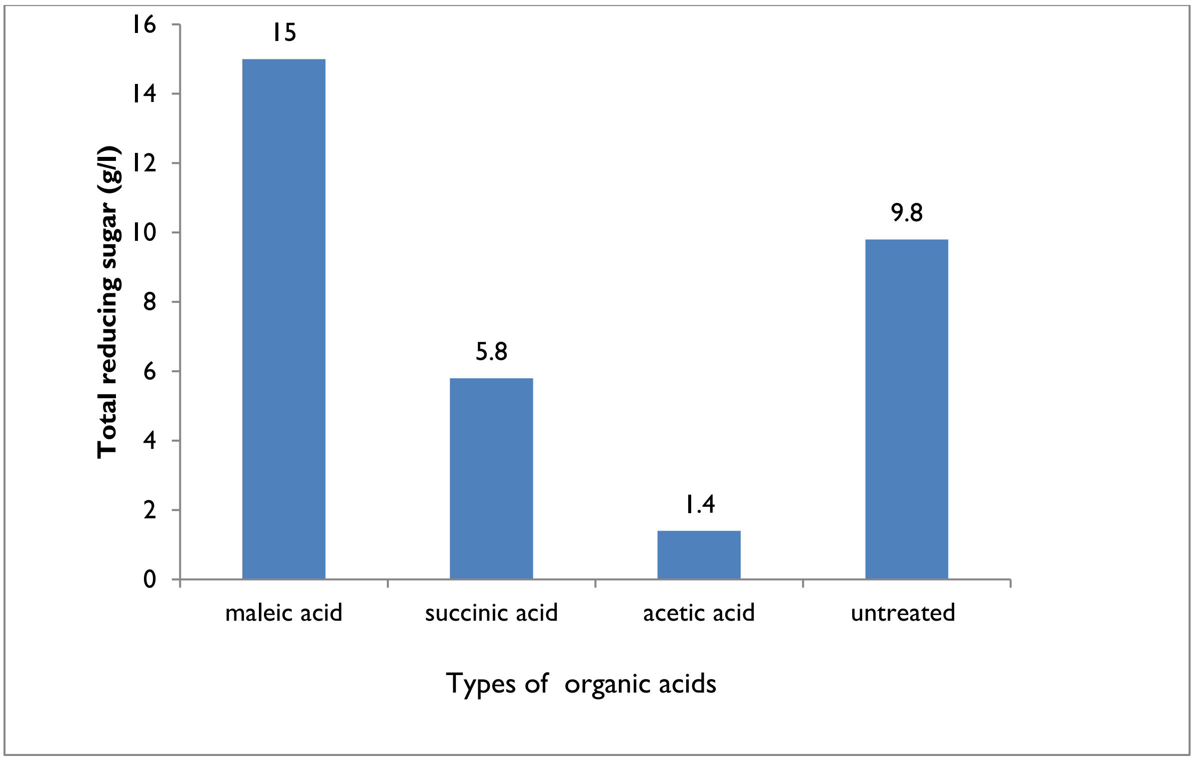

Figure 2: Reducing sugar yields obtained in thermochemical pretreatment of fruit pulp biomass

Enzymatic hydrolysis:

Thermochemical optimization pretreated renewable substrate was subjected to enzymatic hydrolysis for an increase in the yield of reducing sugars. 5U/g concentration of commercial enzyme was added in thermochemically pretreated biomass under optimum conditions of 1% maleic acid concentration and biomass loading of 7.5% . The biomass was treated with hemicellulase enzyme and was incubated at 30°C under shaking conditions at 200 rpm.

Readings were taken after regular intervals of 24 hours. The highest TRS was recorded after every 48 hours with citrate buffer (pH 5.0).

The optimum enzymatic activity of hemicellulase enzyme was ensured at different pH (pH 5.0 was the citrate buffer while pH 6 and pH 7 was the phosphate buffer) conditions with 10 ml of buffer solution. The enzymatic hydrolysis was carried out at 37^{0}C. The samples were analyzed by DNS assay method for determining total reducing sugar (TRS) yields after every 24 hours. The highest TRS was recorded after every 48 hours at pH 5.0 (citrate buffer). The total reducing sugar yield obtained from pretreatment and enzymatic pretreatment was 31.38 g/l.

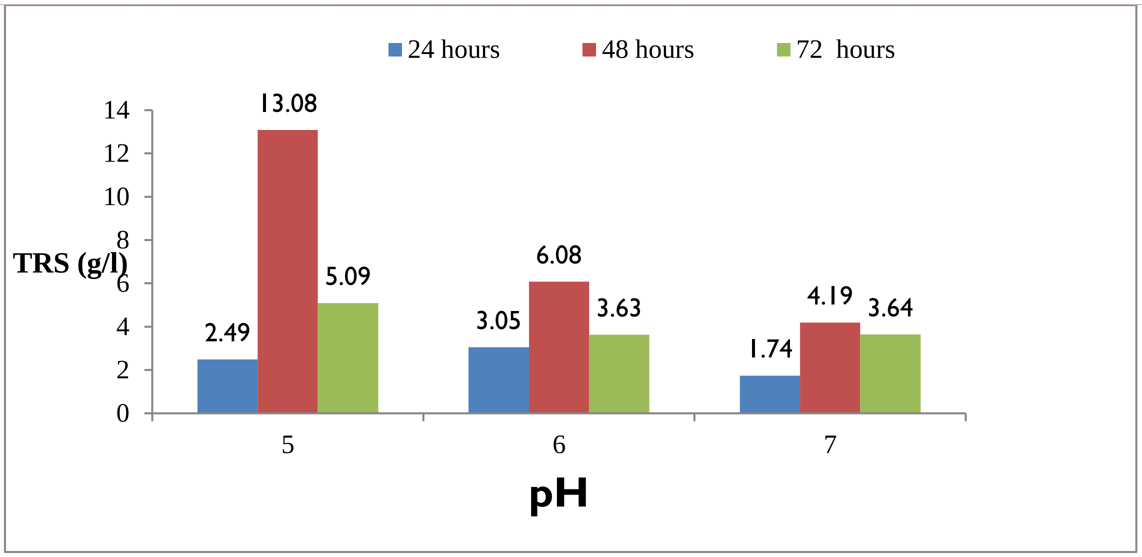

Figure 3: Enzymatic hydrolysis of thermochemically pretreated fruit pulp waste

Production of Lactic acid using standard MTCC bacterial strains:

In order to ferment the TRS obtained from thermo-enzymatically pretreated renewable substrate viz. fruit pulp waste, the microbes used in present study were the standard lactic acid producing strains: *Lactococcus delbruckii (* MTCC 911) and *Lactobacillus rhamnosus* (MTCC 1423) . Fermentative production of lactic acid has certain drawbacks associated with it in case if it is desired to have an enhanced production of the desired compound which includes sensitivity of the microbial strains to extremely high acidic conditions and other by products created during the heterofermentative process. Besides this high sugar yields also have been reported to have a negative feedback for metabolic production of the desired compound.

There have been reports of homo and hetero fermentation processes used by the microbes for production of lactic acid using different renewable substrates as shown in table 3.Thus strains are to be selected should be homofermentative and tend to produce single isomeric form of lactic acid. There are two types of fermentation processes which are generally used for the mass production of lactic acid.

Reports of Simultaneous Saccharification and Fermentation (SSF) as well as Separate Hydrolysis and Fermentation (SHF) have been conducted to obtain optimum yields of desired fermented products from renewable substrates.

Simultaneous Saccharification and Fermentation (SSF):- It was given by Gauss et al in 1976. It is a process used in for the production of lactic acid from lignocellulosic biomass. This process involves employment of fermentative microorganisms in combination with commercial enzyme (hemicellulase) in a fermenter. Sugar accumulation is maximized in this process that favours increased hydrolysis and lactic acid yield.

The principal benefits of performing the SSF are the reduced end product inhibition of the enzymatic hydrolysis and reduced investment cost. The principal drawbacks are the need to find favourable conditions like (temperature and pH) for both the enzymatic hydrolysis and fermentation. The fermenting enzymes and organisms are difficult to recycle.

Separate Hydrolysis and Fermentation (SHF):- enzymatic hydrolysis and fermentation are carried out one after the other using SHF technique. At an ideal temperature for pretreating lignocellulosic biomass is enzymatically saccharified and the optimum conditions for fermentation can be accomplished. Enzymatic hydrolysis and fermentation can be separately optimized. Enzymatic hydrolysis are carried out at an ideal temperature hence less saccharification enzymes are needed here than Simultaneous Saccharification and Fermentation (SSF).

The principal benefit of performing the SHF is its ability to carry hydrolysis and fermentation under different conditions that promote hemicellulase activity and microorganism development. In this instance, the hydrolysis is carried out at a temperature close to 37^0C which is an ideal for hemicellulase performance and fermentation is carried out at 37^0c which is ideal for the microorganism growth. The principal drawback of SHF is that hemicellulase enzyme prolongs the overall process time whereas in other case hemicellulase is hydrolysed concurrently. It also introduces additional challenges and considerations, such as need for enzyme dosage optimization, enzyme stability, and potential product inhibition.

Fermentation results:

Fermentation of thermochemically and enzymatically pre-treated biomass was carried out in SHF mode for lactic acid production. The maximum yield of lactic acid production was observed at 37^{0}C under static conditions for period of three days. The maximum yield of lactic acid was obtained at 48 hrs which was 6.5 g/l in case of *L. rhamnosus* (MTCC 1423) and 33.5 g/l in case of *L. delbruckii* (MTCC 911). Further incubation of the substrate (fruit pulp waste) with the standard strains resulted in decline of lactic acid yields.

References:

[1] Soccol, C.R., Vandenberghe, L.P.S., Rodrigues, C., Medeiros, A.B.P., Larroche, C., Pandey, A. (2008). Production of Organic Acids by Solid-state Fermentation. In: Pandey, A., Soccol, C.R., Larroche, C. (eds) Current Developments in Solid-state Fermentation. Springer, New York, NY. https://doi.org/10.1007/978-0-387-75213-6_10

[2] Y-J Wee; J-N Kim; Ryu H-W. Food Technol. Biotechnol. 2006, 163-172

[3] H. Benninga: A History of Lactic Acid Making, Kluwer Academic Publishers, Dordrecht, Netherlands (1990) pp. 1–61

[4] https://en.m.wikipedia.org/wiki/Lactic_acid

[5] https://www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market

[6] Lipinsky, E.S. and Sinclair, R.G., Chem. Eng. Prog., 82, 26–32, 1986.2. Carothers, H., Dorough, G. L., and Van Natta, F. J., J. Am. Chem. Soc., 54, 761, 1932.

[7] Alves de Oliveira R, Komesu A, Vaz Rossell CE, Maciel Filho R. Challenges and opportunities in lactic acid bioprocess design – From economic to production aspects. Biochem Eng J. 2018;133:219–39. 10.1016/j.bej.2018.03.003

[8] Kylä-Nikkilä K., Hujanen M., Leisola M., Palva A. Metabolic engineering of lactobacillus helveticus CNRZ32 for production of purel-(+)-lactic acid. Appl. Environ. Microbiol. 2000;66:3835–3841.

[9] Reddy G., Altaf M., Naveena B., Venkateshwar M., Kumar E.V. Amylolytic bacterial lactic acid fermentation—a review. Biotechnol. Adv. 2008;26:22–34.

[10] Singh S.K., Ahmed S.U., Pandey A. Metabolic engineering approaches for lactic acid production. Process Biochem. 2006;41:991–1000.

[11] Zheng J., Wittouck S., Salvetti E., Franz C.M.A.P., Harris H.M.B., Mattarelli P., O'Toole P.W., Pot B., Vandamme P., Walter J., Watanabe K., Wuyts S., Felis G.E., Gänzle M.G., Lebeer S. A taxonomic note on the genus Lactobacillus: description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. Int. J. Syst. Evol. Microbiol. 2020;70:2782–2

[12] Schieber, A. Side streams of plant food processing as a source of valuable compounds: Selected examples. Ann. Rev. Food Sci. Technol. 2017, 8, 97–112. Torres-León, C.; Ramírez-Guzman, N.; Londoño-Hernandez, L.; Martinez-Medina, G.A.; Díaz-Herrera, R.; Navarro-Macias, V.; Alvarez-Pérez, O.B.; Picazo, B.; Villarreal-Vázquez, M.; Ascacio-Valdes, J.; et al. Food waste and byproducts: An opportunity to minimize malnutrition and hunger in developing countries. Front. Sustain. Food Systems. 2018, 2, 52.

[13] Torres-León, C.; Ramírez-Guzman, N.; Londoño-Hernandez, L.; Martinez-Medina, G.A.; Díaz-Herrera, R.; Navarro-Macias, V.; Alvarez-Pérez, O.B.; Picazo, B.; Villarreal-Vázquez, M.; Ascacio-Valdes, J.; et al. Food waste and byproducts: An opportunity to minimize malnutrition and hunger in developing countries. Front. Sustain. Food Systems. 2018, 2, 52.

[14]Alvira P., Tomás-Pejó E., Ballesteros M., Negro M.J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour. Technol.

[15] Haldar D., Purkait M.K. A review on the environment-friendly emerging techniques for pretreatment of lignocellulosic biomass: Mechanistic insight and advancements. Chemosphere. 2021;264:128523. doi: 10.1016/j.chemosphere.2020.128523.

[16] Zhang, Z.Y.; Jin, B.; Kelly, J.M. Production of lactic acid from renewable materials by Rhizopus fungi. Biochem. Eng. J.2007,35,251–263.

[17] https://www.marketsandmarkets.com/Market-Reports/polylacticacid-387.html

[18] Ahmed, a. (2020). A review on the lactic acid fermentation from low-cost renewable materials: Recent developments and challenges. *science direct* .

[19] RAHMAN, M. A. (2013). Recent advances in lactic acid production by microbial fermentation processes.

[20] Andrea Komesu, J. A. (2017). Lactic Acid Production to Purification: A Review. BIORESOURCES.

[21] RAHMAN, M. A. (2013). Recent advances in lactic acid production by microbial fermentation processes.

[22] Hamadl, K. (2014). Properties and medical applications of polylactic acid:.

[23] Mohamed Ali Abdel-Rahman a b, Y. T. (2011). Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits. Journal of Biotechnology.

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