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Integrated fermentative production and downstream processing of L-malic acid by *Aspergillus wentii* using cassava peel waste

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Abstract

L-malic acid (L-MA) is an industrially significant chemical with enormous potential. The fungal cell factories could be exploited to harvest it on large scales. In our study, *Aspergillus wentii* strain (MTCC 1901^T) was explored for L-MA production. Initially, the L-MA production was carried out using glucose with optimization of parameters influencing product accumulation (pH and CaCO₃). The fermentation resulted in L-MA titer of 37.9 g/L with 0.39 g/g yield. Then, cassava peel waste (CPW) was used for L-MA production by separate hydrolysis and fermentation. Optimized acidic and enzymatic hydrolysis resulted in glucose

release of 0.53 and 0.66 g/g CPW, respectively. The strain accumulated 20.9 g/L and 33.1 g/L L-MA with corresponding yields of 0.27 g/g and 0.34 g/g during batch cultivation using acid and enzyme hydrolysate, respectively. Finally, the produced L-MA was separated using an inexpensive solvent extraction method. Among various solvents used, n-butanol exhibited maximum L-MA extraction efficiency (31%).

Keywords: L-malic acid, Separate hydrolysis and fermentation, Starchy waste fermentation Downstream processing, Cassava peel waste

1. Introduction

Malic acid (MA) or 2-hydroxybutanedioic acid ($C_4H_6O_5$) is a commercially important chemical with a large variety of applications in food & beverage, cosmetics, agricultural, polymer, pharmaceutical, and textile industries (Iyyappan et al., 2019; Schmitt et al., 2022). It is a promising building block for the synthesis of homo- and heteropolymers owing to its dicarboxylic structure. With an estimated global market of 80,000–100,000 tonnes per year, most of the MA is currently produced through fossil-based chemical routes, which result in a racemic mixture of L- and D-MA (Ding and Ye, 2023; Kövilein et al., 2020). The racemic DL-MA is used chiefly in beverages, foods, personal care products, and cleaning supplies; however, enantiopure malic acid is often preferred for specialized purposes in the pharmaceutical or polymer industry (Wei et al., 2021). For example, the use of racemic DL-MA for polymer synthesis provides an amorphous product, while enantiopure precursors produce polymers with enhanced crystallinity and greater melting temperatures (Schmitt et al., 2022). Resolution of the chemically produced racemate is expensive and consequently appears unsuitable for mass production (Kövilein et al., 2020).

In recent years, a search for alternative bio-based resources has been prompted as fossil fuels are non-renewable in nature and result in excessive carbon emissions, which ultimately contribute to global warming. In this context, microbial cell factories naturally producing chemical building blocks have emerged as a viable platform to reduce reliance on fossil fuels (Gopaliya et al., 2021; Nitzsche et al., 2021). MA is an intermediate of microbial metabolism, and some fungi that can overproduce and secrete MA in their extracellular environment could be employed for industrial production (Dai et al., 2018; Schmitt et al., 2022; Wu et al., 2022). The biochemical reactions are highly specific and provide the benefit of enantio-specificity, and in the case of MA, producing the L-isomer solely through metabolic processes (Ding and Ye, 2023). However, microbial MA has not been able to compete sufficiently with its petrochemical counterpart in economic terms. A major portion of the bio-based MA production cost is drawn from the expenses of raw materials and the tedious product extraction processes (Kövilein et al., 2020; Wei et al.,

2021). In 2004, the US Department of Energy prepared a list of platform chemicals obtainable from biomass where MA was at the top position, and later in 2009, it was dropped when the list was revised (Bozell and Petersen, 2010). The domain of fermentation technology for MA synthesis employing biomass substrates is less explored and requires substantial research to replace its conventional manufacturing technique (Dai et al., 2018; Wu et al., 2022).

Designing and developing a cost-effective bioprocess utilizing waste resources followed by inexpensive downstream processing techniques for L-MA production and recovery is the need of the hour and a significant area of research. In the quest for exploring alternative carbon sources for L-MA production, most studies have been limited to crude glycerol which showed substantial results (Bharathiraja et al., 2022; Selvakumari et al., 2021). Some reports demonstrated the use of renewable raw materials like acetate and xylose in their pure form (Kang et al., 2022; Kövilein et al., 2022). Only a few research have focussed on the use of readily available agroindustrial substrates to achieve low-cost L-MA production with waste valorization (Ding and Ye, 2023; Wu et al., 2022). For instance, a recent study employed pyrolytic aqueous condensate (a novel and unused waste stream) obtained from fast pyrolysis of lignocellulosic biomass, for L-MA production (Kubisch and Ochsenreither, 2022). Moreover, a couple of studies have been conducted to investigate L-MA production from enzymatic hydrolysates of lignocellulosic biomass (Dörsam et al., 2017; Li et al., 2014). In the global scenario, agroindustrial wastes are abundantly available feedstocks which could be efficiently bioconverted into industrially important chemicals like L-MA following a cost-effective and circular bioeconomic approach.

Cassava (*Manihot esculenta*) is the third most important carbohydrate-rich food crop in the tropics, including Africa, Asia, and Latin America (Chennakrishnan and Thenmozhi, 2020). Cassava tubers have cyanogenic glycosides and are categorized as bitter or sweet depending upon their relative levels. While sweet cassava can be used as food after sufficient soaking and cooking, bitter cassava finds its utility only in starch production industries (Flores et al., 2020). A huge amount of waste is produced from the cassava industries, which needs to be managed due to its high content of toxic cyanogen, which restricts its usage in animal feed. Besides, random disposal in landfills could consequence disorders in animals and accumulation in the food web (Ekop et al., 2019). A potential application of this carbohydrate-rich trash could be its utilization as a fermentation substrate to produce industrially significant value-added chemicals (Nizy and Kannan, 2022).

In this study, an *Aspergillus wentii* strain MTCC 1901^T was explored for L-malic acid (L-MA) production, which has never been reported for this species. The strain was initially examined for L-MA production by utilizing glucose as a carbon source. The perusal of the literature reveals that pH and CaCO₃ concentration are the influencing parameters for the fermentative production of L-MA; hence, these critical parameters were optimized for maximizing L-MA productivity. Further, cassava peel waste (CPW) was used as an inexpensive feedstock for L-MA production via separate hydrolysis and fermentation (SHF). Saccharification of CPW was conducted employing acidic and enzymatic treatments, and the resultant glucose-rich hydrolysates were used for fermentative L-MA production. Finally, a simple and inexpensive liquid-liquid extraction technique was used for the extraction of L-MA from the fermentation broth. Thus, the present study demonstrates an integrated approach for the production and recovery of L-MA from CPW.

2. Material and methods

2.1. Materials

All the media components, including glucose and salts, were purchased from Fischer Scientific. Cassava tubers were obtained from the INA market in New Delhi, India. The peels were separated from the tubers and were ground to make fine powder after drying in an oven at 80°C for 3 h. The amyloglucosidase (AMG) (260 U/mL) from *Aspergillus niger* with α -D-(1-4), the α -D-(1-6), and the α -D-(1-3) glycosidase activities was procured from Sigma-Aldrich. HPLC-grade solvents were used for downstream processing and were purchased from Sigma-Aldrich.

2.2. Microorganism and inoculum preparation

Aspergillus wentii MTCC 1901^T was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India, and was preserved in 50% glycerol stocks at -80°C. The strain was maintained on malt extract agar plates and slants at 30°C. The spores were harvested from the plates after 7 days of cultivation using 0.9% w/v NaCl solution with 0.05% Tween 20. The concentration of spores in the suspension was determined using a Neubauer chamber.

2.3. Fermentation for L-malic acid production and process optimization

Fermentation with *A. wentii* was carried out using the method adopted by Kövilein et al. (2021) with slight modifications in terms of inoculum size and glucose concentration. Further, the conditions of pH and CaCO₃ concentration were varied to study their effect on the L-MA yield of the studied organism. The pre-culture media was composed of 40 g/L glucose, 4g/L (NH₄)₂SO₄, 0.75 g/L KH₂PO₄, 0.98 g/L K₂HPO₄, 0.1g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂·2H₂O, 5 mg/L NaCl, and 5 mg/L FeSO₄·7H₂O. The seed culture was inoculated with 10⁶ spores/mL in 250 mL Erlenmeyer flasks with 50 mL medium and incubated for 48 h at 30°C on a rotary shaker with an agitation speed of 150 rpm.

The fermentation media for L-MA production was composed of 100 g/L glucose, 1.2 g/L (NH₄)₂SO₄, 0.1 g/L CaCl₂·2H₂O, 0.1 g/L MgSO₄·7H₂O, 0.17 g/L K₂HPO₄, 0.1 g/L KH₂PO₄, 60 mg/L FeSO₄·7H₂O, and 5 mg/L NaCl. All the fermentation experiments were conducted in 250 mL flasks with a working volume of 50 mL. The effect of the initial pH of the media was studied by adjusting the pH values to 3.0-8.0, and 90 g/L CaCO₃ was added to the media unless stated otherwise before autoclaving. Other salts were filter sterilized and added to the media after autoclaving. The CaCO₃ was added to the media at different levels (0, 30, 60, 90, and 120 g/L) after adjusting the pH to the optimized value. The fermentation media was inoculated with 5% (v/v) inoculum from the 48 h old pre-culture and then incubated at 30°C and 150 rpm.

2.4. Saccharification of cassava peel waste (CPW) via acidic and enzymatic hydrolysis

Powdered CPW was used for L-MA production through a separate hydrolysis and fermentation (SHF) bioprocess. Saccharification of CPW was carried out using two approaches involving acid and enzymatic treatment similar to the method adopted by Narisetty et al. (2022) for the hydrolysis of bread waste. In the first approach involving acid-based saccharification of CPW, we attempted to optimize the HCl concentration and solid loadings to achieve maximum glucose release. A varying amount of HCl (0.5-2.5% v/v) was added to a slurry of 10% (w/v) CPW followed by autoclaving at 121°C for 20 minutes. Subsequently, different solid loadings (5-25% w/v) of CPW were used with the

optimized HCl concentration to obtain a hydrolysate with maximum glucose concentration. In the second approach, CPW was saccharified by treatment with the enzyme amyloglucosidase (AMG). The enzymatic hydrolysis was done at pH 4.5, 50°C, and 200 rpm after autoclaving the CPW slurry (Narisetty et al., 2021). Different enzyme loadings (0.5-20 U/g) and solid loadings (5-25% w/v CPW) were used to optimize the process for maximum saccharification efficiency. Samples were withdrawn at regular time intervals (24, 48, 72 h) and centrifuged at 10,000 rpm for 10 min. The glucose concentration was estimated in the supernatant (filtered by 0.2µm nylon syringe filter) by high-performance liquid chromatography (HPLC).

After saccharification under optimized conditions, suspensions were centrifuged and filtered to remove the unhydrolyzed solid from the slurry using Whatman filter paper no.1. The glucose concentrations in the obtained acidic hydrolysate (AH) and enzymatic hydrolysate (EH) were adjusted to 100 g/L. The hydrolysate was supplemented with other nutrients (salt components) and used as fermentation media for L-MA production under optimized conditions of pH and CaCO₃.

2.5. Downstream processing of L-malic acid from the fermentation broth

The produced L-MA was extracted from the fermentation broth through a simple liquid-liquid extraction method. Various water-immiscible polar organic solvents, namely ethyl acetate, methyl ethyl ketone, methyl isobutyl ketone, n-butanol, sec-butanol, and dichloromethane, were used for the separation of L-MA from the fermentation broth. After the completion of fermentation, the culture broth was treated with concentrated HCl so as to dissolve all the insoluble carbonates and malate salts. Thereafter, an equal volume of solvent was added to the fermentation flask and kept for overnight shaking at 200 rpm. The contents of the flask were transferred into a separating funnel, and it was allowed to stand still for some time. The organic layer with the extracted L-MA was separated and transferred into a round bottom flask for solvent evaporation using IKA rotary evaporator. Thereafter, the remaining precipitate was dissolved in MilliQ water, and the L-MA was estimated with HPLC and characterized through FT-IR and NMR spectroscopy.

2.6. Analytical techniques

Starch was estimated in the CPW using the method described by McCleary et al. (2019). After saccharification of the CPW through acidic and enzymatic treatments, saccharification yield was calculated using the following formula:

Samples (1 mL) were withdrawn from the culture at regular intervals of 24 h, and their pH was determined. The samples were treated with 1 mL of 2 M HCl by heating at 80°C

$$\text{Saccharification yield (\%)} = \frac{\text{Glucose (g) in hydrolysate of 'x' g peel powder}}{\text{Starch content in 'x' g peel powder} \times 1.11} \times 100$$

for 20 min to solubilize the calcium salts (malate/carbonate), followed by centrifugation at 10,000 rpm (Liu et al., 2018). The supernatant was filtered with a 0.2µm nylon filter and used for the estimation of L-MA and residual glucose, while the remaining fungal biomass was dried to determine dry cell weight (DCW).

HMF and furfural were estimated in the hydrolysate using a Perkin Elmer HPLC system equipped with a C-18 column and UV-Diode array detector (UV-DAD) (270nm wavelength). A 15%v/v methanol in water was used as the mobile phase with a flow rate of 0.5 mL/min. Acetic acid, xylose, maltose, glucose, and L-MA were quantified using a

Bio-Rad HPX-87H ion exclusion column, a refractive index detector (RID) (Perkin Elmer Series 200a), and 5mM H₂SO₄ as mobile phase with a flow rate of 0.6 mL/min (Yadav et al., 2021).

The extracted L-MA was characterized by Fourier-transform infrared (FT-IR) spectroscopy to identify the functional groups by pelleting it with KBr, and spectrum (4000–400 cm⁻¹) was recorded on Agilent (Perkin Elmer IR version 10.6.0, USA). For the nuclear magnetic resonance spectroscopy (¹H NMR) studies, the extracted L-MA was dissolved in deuterium oxide (D₂O), and spectra were recorded at 25°C on an NMR spectrometer operating at a resonance frequency of 500 MHz (Bruker 500 MHz, Germany) (Kumar et al., 2020).

3. Results and discussion

3.1. Selection of *Aspergillus wentii* for L-malic acid production

The *Aspergillus* genus has been extensively explored for the abundant production of various organic acids (Iyyappan et al., 2019). The best *Aspergillus* sp. naturally producing L-MA are *A. oryzae*, and *A. flavus* (Iyyappan et al., 2019; Kövilein et al., 2021). Several *Aspergillus* strains viz. *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus wentii* were procured from the culture collection centers. The *A. oryzae*, *A. flavus*, and *A. wentii* strains produced high yields of L-MA, using glucose as the substrate. *A. oryzae* is a widely studied species for L-MA production (Kövilein et al., 2022), and the production of aflatoxins delimits the intensive applications of *A. flavus* despite its high L-MA titers (Dai et al., 2018).

Despite its lower L-MA productivity as compared to the above species, we chose *A. wentii* for further fermentation studies based on two factors. Firstly, the strain was not previously studied for L-MA production. Hence it was worth exploring the strain in this regard. Secondly, the biomass (CPW) utilized for valorization in the current study consists of high amounts of toxic cyanogenic glycosides. Hence a fungal strain derived from a similar environment could be more potent (Florescia et al., 2020). Since the *A. wentii* strain is an unreported fungal isolate derived from soybean, which comprises cyanogenic compounds (Schrenk et al., 2019), it became our choice for the successful production of L-MA from cassava waste. The optimization of the pH of the fermentation media and the CaCO₃ concentration in the media was further performed using *Aspergillus wentii* to achieve higher yields of L-MA.

3.2. Optimization of pH and CaCO₃ concentration for L-malic acid production

The standard media used in this study, as described in the materials and methods section, has already been reported for the most studied producer of L-MA, i.e., *Aspergillus oryzae* (Kövilein et al., 2021). The studies implicate two other factors besides carbon and nitrogen source that delimit the high titers of L-MA. These are media pH and CaCO₃ concentration in the fermentation media (Wu et al., 2022). Hence, the optimization of pH and CaCO₃ concentration was performed.

Fermentation processes are chiefly dependent on the pH of the media. Also, during the course of fermentation, as organic acid accumulates, it reduces the pH and creates stress for fungal growth and metabolism (Iyyappan et al., 2019). The *A. wentii* was grown in the fermentation media with the initial pH value ranging from 3.0–8.0, containing pure glucose as substrate with 90 g/L CaCO₃ as pH regulator in the media. The cultivation under acidic conditions was accompanied by minimal growth of the fungus and low L-MA production (Fig. 1a). At pH 3, 4, and 5, only 3.88, 12.45, and 25.16 g/L L-MA titers were produced with a final fungal biomass concentration of 5.86, 9.12, and 18.97 g/L,

respectively (Table 1). The maximum growth and glucose consumption by the fungus was observed in a slightly acidic to neutral environment (pH 6.0-7.0), with maximum L-MA titer (37.9 g/L) and yield (0.39 g/L) being at pH 6.0 after 10 days of fermentation. The titer value sharply declined to 8.9 g/L on moving towards alkaline conditions (pH 8). Similar behavior has been reported for other MA-producing microorganisms. For instance, a media pH of 5.5 has been reported as optimal for L-MA production in studies conducted using *A. oryzae* (Kövilein et al., 2022). Zambanini et al. (2016) showed that for efficient malate synthesis by *Ustilago trichophora* TZ1, a pH higher than 5.4 is required. In another study, it was observed that *A. niger* which is a major producer of citric acid, exhibits L-MA as the major product when the pH of the fermentation media is 6.0, whereas citric acid production dominates at low pH values (2.0-3.0) (West, 2015). The CaCO_3 in the fermentation media is reported to play a pivotal role in L-MA production by different species (Geyer et al., 2018). Since optimal growth and production were observed in neutral pH conditions, adding CaCO_3 to minimize the acidic stress caused by the synthesized L-MA seems to be a critical factor in the current bioprocess. Hence, fermentation under varying CaCO_3 concentrations was carried out to understand its effect on L-MA production under optimized pH (6.0) conditions. It was observed that the L-MA production by the strain was proportional to CaCO_3 concentration. This pattern was analogous to that of other natural L-MA producers. Studies indicate in most producers, L-MA production majorly takes place through the reductive tricarboxylic acid pathway, wherein pyruvate is carboxylated to oxaloacetate by pyruvate carboxylase, which further gets converted to L-malate by malate dehydrogenase (Wu et al., 2022). Thus, besides functioning as a pH regulator, CaCO_3 also acts as a supplier of CO_2 , which gets channelized to the biosynthetic pathway of L-MA (Dai et al., 2018). This phenomenon has been evident in the current study as well (Table 1). There was negligible L-MA production when no CaCO_3 was added to the media, and it enhanced with the increase in the initial CaCO_3 concentration in the media. The highest L-MA titer (37.9 g/L) was obtained when the culture medium was supplemented with 90 g/L CaCO_3 , beyond which there was no significant change in the productivity (Fig. 1b). Additionally, the synthesized L-MA precipitates in the fermentation broth in the form of calcium malate, which reduces the possibility of product inhibition (Geyer et al., 2018). Moreover, when CaCO_3 concentrations used during fermentation were 0, 30, 60, 90, and 120 g/L, the glucose consumption after 240h was 71.94, 82.68, 88.2, 96.92, and 95.12 g/L, respectively. Similar observations have been made in a study investigating the effect of CaCO_3 on L-MA production by *A. oryzae* NRRL 3488. An increase in CaCO_3 led to an increment in glucose consumption which was only 65% for 20 g/L CaCO_3 but rose to 91 and 100% when 80 and 120 g/L CaCO_3 were used in the media. The L-MA production was 300 and 400% more in the case of 80 and 120 g/L CaCO_3 as compared to 20 g/L CaCO_3 (Geyer et al., 2018). In another study involving *A. oryzae* DSM 1863 as the microbial source and acetate as substrate, maximum acetate consumption and L-MA production (titer: 8.77 g/L; yield: 0.19 g/g) was reported with 90 g/L CaCO_3 (Kövilein et al., 2021). During the fermentation of glycerol (250 g/L) by *U. trichophora* TZ1, the MA titer value reached 196 g/L when 100 g/L CaCO_3 was used in the media (Zambanini et al., 2016). Additionally, calcium plays important role in fungal cell development, stress responses, cell wall growth and remodeling. The calcium responsive modifications in the fungal cell morphology are triggered in the presence of higher amounts of CaCO_3 . This results in the activation of various transcription factors that affects the cell membrane integrity, cell wall organization, and stress tolerance besides regulating the calcium metabolism (de Castro et al., 2019). The better cell membrane stability, cell wall integrity, and stress tolerance could be an effect of higher concentrations of CaCO_3 exposure to the

Aspergillus fungus which enhances its growth and could possibly lead to higher titres of L-MA (Table 1) in the current study. Besides, there have been few reports on the use of some other pH neutralizers like MES buffer, MgCO_3 , K_2CO_3 , NaHCO_3 , etc., but the knowledge gained is limited and has a scope of further exploration (Iyyappan et al., 2018). The above experiments defined the optimized pH and CaCO_3 levels which were used in subsequent experiments.

3.3. Fermentation profile of L-malic acid production in pure glucose media

When fermentation was carried out under optimized conditions (pH 6.0 and 90 g/L CaCO_3), ~97% of glucose was exhausted, leading to fungal growth in the form of 1.5mm pellets with a final DCW of 21.65 g/L and accumulated 37.9 g/L L-MA, which corresponded to a yield of 0.39 g/g with an overall productivity of 0.157 g/L/h. The maximum productivity (0.202 g/L/h) was determined at 144 h. CaCO_3 added to the media maintained the pH above 5.0 for 7 days even after substantial synthesis of L-MA, as shown in Fig. 2. Beyond this point, as the pH dropped with further acidification by L-MA, fungal growth was arrested. This also coincides with the fact that the maximum L-MA production rate was observed in the log phase (5-7 days), where fungal growth was maximum and was successively reduced with acid stress below pH 5.0.

Since L-MA is a key intermediate in the metabolic pathways of most living cells, researchers have explored different organisms for L-MA production in a synthetic media with pure glucose as the sole carbon source (Wu et al., 2022). For instance, among the *Aspergillus* spp., L-MA titer as high as 113 g/L has been obtained from 120 g/L glucose by *A. flavus* ATCC 13679 (Dai et al., 2018). On the other hand, the most studied natural producer, *A. oryzae* DSM 1863, produced 58.16 g/L L-MA from 120 g/L glucose through a 353h shake flask batch fermentation which was equivalent to 0.51 g/g yield and 0.16 g/L/h productivity (Ochsenreither et al., 2014). In a recent study, an L-MA concentration of 178 g/L was reached in a 19-day-long repeated-batch fermentation with 5 cycles using *A. oryzae* DSM 1863, which showed maximum productivity of 0.90 g/L/h. Regular supplementation of CaCO_3 and nitrogen source enhanced the efficiency of the process for a prolonged period (Schmitt et al., 2022). The best productivity reported to date is with *Penicillium viticola* 152, which generated 198 g/L calcium malate in 96h with a yield and productivity of 1.28 g/g and 1.75 g/L/h, respectively. The fermentation was conducted from 140 g/L glucose supplemented with 0.5% corn steep liquor in a 10L bioreactor (Khan et al., 2014). Under similar conditions, excluding the addition of corn steep liquor, *Penicillium sclerotiorum* K302 produced 92 g/L calcium malate, corresponding to 0.88 g/g yield and 1.23 g/L/h (Wang et al., 2013). In another study, yeast *Zygosaccharomyces rouxii* showed production of 74.9 g/L L-MA from 300 g/L glucose in 360h (Selvakumari et al., 2021).

3.4. Acidic and enzymatic saccharification of cassava peel waste

The Cassava industry generates huge amounts of waste, which can be divided into four general categories: peels from initial processing, fibrous remnants from crushing and screening, residues left over after starch has settled, and wastewater effluent (Li et al., 2017). In the current study, hydrolyzed CPW was used for fermentation. Saccharification of CPW was conducted through two approaches which were compared in terms of the level of sugars generated, fermentation inhibitors, and L-MA produced upon fermentation of the resultant hydrolysates. Starch content in the CPW used in this study was 0.69 g/g. The first approach involving treatment with HCl during autoclaving is an inexpensive and rapid method for starch saccharification. Different HCl concentrations (0.5, 1, 1.5, 2, and

2.5% v/v) were used with 10% (w/v) CPW loading. Glucose concentration in the hydrolysate increased from 41.27 to 51.59 g/L with an increase in acid loading from 0.5 to 1%, corresponding to a saccharification yield of 53.57 and 65.66%, respectively. The process efficiency remained unchanged with further increment in acid concentration (Fig. 3a). Thereafter, when different solid loadings (5, 10, 15, 20, 25% w/v) of CPW were used with 1% (v/v) HCl, the maximum glucose concentration (105.5 g/L) was achieved with 20% (w/v) loading as shown in Fig. 3b. The saccharification yield (%) for 5, 10, 15, 20, and 25% (w/v) solid loading was determined to be 72.44, 66.14, 60.52, 68.49, and 50.67%, respectively. Hence, treatment of 20% (w/v) CPW with 1% HCl was used for the preparation of fermentation media for L-MA production. Apart from 105.5 g/L glucose, 15.83 g/L maltose and 13.06 g/L xylose were also present in the hydrolysate. HCl catalyzed hydrolysis at high temperatures led to the accumulation of fermentation inhibitors viz. acetic acid (3.78 g/L), furfural (0.087 g/L), and 5-hydroxymethylfurfural (1.29 g/L) in the final hydrolysate prepared under optimized conditions. The amount of glucose released per gram of dried and powdered cassava peel using this approach was 0.53 g/g. Similarly, in a study comprising cassava peel bioconversion to ethanol, hydrolysis was carried out using 4.5 M H₂SO₄ with 20% solid loading at 80°C (Olayemi et al., 2019). In another study, saccharification of bread waste, a starchy material, with 2% HCl gave glucose yields of 0.45 and 0.38 g/g from solid loading of 15% (w/v) and 20% (w/v), respectively (Narisetty et al., 2022).

In contrast, the second approach involving the treatment of CPW with starch-digesting enzyme AMG is less economical but more efficient than acid hydrolysis. To design a process leading towards the highest glucose production with minimum cost, different enzyme loadings of 0.25, 0.5, 0.75, 1.0, 2.5, 5, 7.5, 10, 15, and 20 U/g (units/CPW) were added to a slurry of 10% (w/v) CPW in distilled water (pH 4.5) and saccharification was done for 72h at 50°C with shaking at 200 rpm. Fig. 3c shows that the release of glucose by CPW increased exponentially with an increase in enzyme loading at lower concentrations of the enzyme (0.25-2.5 U/g). However, at higher enzyme loadings (5-20 U/g), glucose concentration in the hydrolysate was leveled off, and there was no further increment. It was observed that >85% saccharification yield was achieved within 48h when 5U/g of AMG was used. Treatment for more time with a higher concentration of enzyme could eventually add to the overall cost of the process, which could be favorably avoided. When different solid loadings of 5, 10, 15, 20, and 25% w/v CPW were treated with 5 U/g AMG to prepare a glucose-rich hydrolysate, the obtained glucose concentrations were 32.08, 65.76, 99.29, 117.94, and 115.2 g/L, respectively, which is equivalent to saccharification yields of 83.28, 86.66, 85.59, 76.54 and 59.81%, respectively (Fig. 3d). Our process optimized for minimum cost and maximum saccharification efficiency, involving 15% (w/v) CPW and 5 U/g enzyme, led to the production of 0.66 g glucose per g of cassava peel powder.

Enzymatic hydrolysis of starch is a two-step process involving liquefaction and saccharification with different enzymes under different conditions (Gopaliya et al., 2021). Our study included one-step saccharification using a single enzyme (AMG) with α -D-(1-4), the α -D-(1-6), and the α -D-(1-3) glycosidase activities. Autoclaving of the CPW before treatment weakened the glycosidic bonds, which allowed easy digestion into glucose with maximum efficiency using a minimal enzyme. Similarly, Narisetty et al. (2022) conducted a single-enzyme treatment of bread waste which resulted in 95.9% saccharification yield using 0.6m g/g enzyme (Dextrozyme peak) with 20% (w/v) solid loading. In another study, multi-step treatment of cassava peels (10% w/v) sequentially with 3.68% HCl (soaking for 2.57h at 69°C followed by autoclaving), α -amylase (125.30 U/g),

amyloglucosidase (74.06 U/mL), and cellulase (2.34% v/w) generated 0.58 g fermentable sugars per g CPW (Aruwajoye et al., 2020). Literature also shows reports on CPW saccharification using starch-digesting microorganisms like *A. niger* (Adeoye et al., 2015; As et al., 2020).

3.5. Fermentation for L-malic acid production from cassava peel hydrolysates

In previous studies, cassava-derived feedstocks have been employed for the production of various organic acids, viz. lactic acid (Trakarnpaiboon et al., 2017), citric acid (Adeoye et al., 2015), and succinic acid (Thuy et al., 2017), etc. In the current study, the CPW hydrolysates obtained from acid and enzyme treatments were used for L-MA production. After saccharification, 50 mL of the filtered hydrolysates were transferred into 250 mL flasks, pH was adjusted to 6.0 using NaOH, and 90 g/L CaCO₃ was added aseptically. Using the conditions optimized for pure glucose, hydrolysates containing 100 g/L glucose were used for fermentation. The time profile of L-MA production, glucose consumption, fungal dry cell weight (DCW), and pH during the course of fermentation are presented in Fig. 4.

When AH was used as fermentation media, it showed low potency for L-MA production due to the presence of fermentation inhibitors. Additionally, the pH adjustment step would have led to salt accumulation in the media which could have hindered the fermentation process. The presence of additional monomeric sugars (maltose and xylose) in sufficient amounts apart from glucose did not seem to contribute much towards L-MA production. Table 2 shows a comparison of the L-MA production indices between the three-fermentation media. The fungal growth was sluggish during the initial phase of fermentation, rose sharply after 72 h consequently producing L-MA, and got arrested after 168h. L-MA production by the fungus was chiefly observed after 72 h, and a maximum titer value of 20.94 g/L was obtained at 216 h, which corresponded to a yield of 0.27 g/g (L-MA/glucose) and 0.097 g/L/h productivity. The fungus grew in the form of pellets 4mm in diameter, and the highest dry cell weight of 15.6 g/L was attained at 216 h.

The application of EH as fermentation media resulted in an L-MA production profile resembling that of pure glucose. The absence of fermentation inhibitors in the CPW hydrolysate prepared from AMG resulted in better efficacy for L-MA production. A titer value of 33.06 g/L L-MA was obtained after 10 days of fermentation, corresponding to a yield and productivity of 0.34 g/g and 0.14 g/L/h, respectively. Similar to glucose fermentation, the L-MA production rate was maximum (0.18 g/L/h) in the middle of the fermentation period at 144 h. The fungal morphology was in the form of very small pellets (0.5mm) with a final cell dry weight of 19.82 g/L. The results indicate that EH could be considered a less economical but more efficient fermentation media than AH. There have been fewer studies on renewable resources, especially biomass hydrolysates, as substrates for fermentative L-MA production.

Literature shows reports on microbial production of L-MA from waste resources. Most studies dealt with the utilization of glycerol, a major renewable waste from petroleum industries, for L-MA production using various organisms viz. *A. niger*, *A. oryzae*, *Z. rouxii*, *U. trichophora*, etc. (Iyyappan et al., 2018; Selvakumari et al., 2021; West, 2015; Zambanini et al., 2016). On the other hand, studies involving the valorization of lignocellulosic/starchy waste feedstocks into L-MA have been limited. Previously, hydrolysates from beechwood and *Miscanthus giganteus* have been reported as substrates for L-MA biosynthesis using *A. oryzae* DSM 1863. Organosolv-pretreated beechwood and *Miscanthus* biomasses were subjected to enzymatic hydrolysis, and the resultant hydrolysates were used for fermentation, as done in this study. L-MA titers of 37.9 g/L

and 5.8 g/L were respectively obtained from cellulosic hydrolysate and the hemicellulosic fraction of beechwood. While the fermentation of cellulosic hydrolysate of *M. giganteus* generated 30.7 g/L MA with 0.32 g/g yield and 0.18 g/L/h productivity (Dörsam et al., 2017). In another study, corn straw hydrolysate, obtained from steam-explosion pretreatment and enzymatic digestion, was used as a substrate for L-MA production by *Rhizopus delemar*. L-MA concentration of 120 g/L was obtained in 96 h upon fermentation with a mutant strain HF-121 (Li et al., 2014).

As per the results of our study, the amount of L-MA that can be obtained from 1 Kg of CPW via acidic and enzymatic hydrolysis is 121.4 and 218.2 g/Kg, respectively. Previously, whole cassava tubers have been employed production of poly(β -L-malic acid) using *Aureobasidium pullulans* (Liu et al., 2022). To our knowledge, this is the first report on L-MA production from CPW. Cassava peels are starch-rich waste that accounts for 20–30% of the approximately 270 million tonnes of cassava tuber produced annually worldwide (Rogoski et al., 2023). Starchy wastes are promising feedstocks for fermentation owing to their simple structure and the presence of weak glycosidic bonds. CPW has great potential in this regard and could be explored for the production of various bio-based chemicals (Aruwajoye et al., 2020).

The findings of the current study depicts that owing to the absence of fermentation inhibitors (HMF, acetic acid, and furfural) in the enzymatic hydrolysate, better L-MA titer (33.06 g/L) is obtained, as compared to acid hydrolysate. However, acidic hydrolysis, inspite of the presence of inhibitors, showed a considerable amount of fungal growth and corresponding L-MA production (20.94 g/L). This indicates that the inhibitors pose less effect on fermentation in the current study. Moreover, the use of acid hydrolysate is an economic process, hence could be applied for a cost-effective production of L-MA besides its comparatively lower yields than enzymatic hydrolysate driven process. Further investigations could be conducted around detoxification strategies like chromatographic separation, zeolite adsorption, activated charcoal treatment, etc. for the removal of inhibitors from the acid hydrolysate to improve the efficacy of the process for better L-MA yields.

3.6. Downstream processing of L-malic acid from the fermentation broth

The downstream processing method for carboxylic acids accounts for 30–50% of the total production cost (Bekatorou et al., 2016). In the present study, a simple and inexpensive one-step downstream processing technique was used for the extraction of produced L-MA from the fermentation broth. The process involved the partitioning of MA between the aqueous phase of fermentation broth and an immiscible organic phase. Liquid-liquid extraction was carried out using different polar organic solvents, which are immiscible in water and have high volatility (BP <120°C). The potential solvents fitting the criterion were ethyl acetate, n-butanol, sec-butanol, methyl ethyl ketone, methyl isobutyl ketone, and dichloromethane. Since the produced L-MA is present in the form of insoluble calcium salt, direct partitioning from the media is not feasible. Hence, concentrated HCl was added dropwise to the media till all insoluble salt components get solubilized, and calcium malate gets converted to L-MA. The resultant solution was subjected to solvent-based extraction with different solvents by shaking it overnight for partitioning of L-MA into the organic layer followed by evaporation of solvent to obtain pure L-MA in solid form. Among the studied solvents, n-butanol showed the maximum L-MA recovery (31%) followed by sec-butanol (see supplementary material). Since MA has high water solubility, its partitioning behavior is relatively low. The solvent, owing to its low BP, could be easily evaporated using a rotary evaporator and could be reused after condensation making the process more economical. The FT-IR and NMR spectra of the

extracted L-MA showed characteristic bands and signals similar to that of standard L-MA indicating its recovery in pure form (see supplementary material).

Studies on downstream processing techniques developed for recovering L-MA from the fermentation broth are scarce (Wu et al., 2022). Iyyappan et al. (2020) used a reactive extraction method involving a trioctylamine as an extractant and n-octanol as an organic diluent for in-situ extraction of L-MA during fermentation by *A. niger* mutant PJR1. In a similar study, n-decanol was used with trioctylamine (Inyang and Lokhat, 2020), and 93% extraction efficiency was reported. Another approach of downstream processing reported for L-MA recovery is crystallization. Nitzsche et al. (2021) treated the fermentation broth with 70% H_3PO_4 and removed the fungal mycelia and insoluble $Ca_3(PO_4)_2$ through autoclaving and filtration. Crystals of L-MA were obtained from the remaining solution by cooling it to 0°C. This method resulted in a recovery of 29% L-MA with 84% purity. The process used in our study is a preliminary method that allows easy product recovery without involving complex reactions and additional chemicals. Further optimization of pH, solvent volume, and salt addition could enhance the efficiency of the process, imparting a scope for improvement with further research.

4. Conclusion

Unlike chemical synthesis, fermentative routes could generate enantiopure L-MA. The current study depicts an integrated approach for the production and recovery of M-LA by an unreported *A. wentii* via SHF of CPW. The enzymatic approach was discerned to be more efficient than acid-based saccharification resulting in higher saccharification yield (87%) with an absence of inhibitors, and better fungal growth (19.82 g/L) producing high L-MA titer (33.1 g/L). Further, downstream processing resulted in 67.28 g of pure L-MA/Kg CPW, which is comparable to existing studies. Future work will be directed towards optimization and scale-up to improve the efficacy of the bioprocess.

Appendix A. Supplementary data

E-supplementary data of this work can be found in the online version of the paper.

Credit author statement

Deeksha Gopaliya: Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Saniya Zaidi:** Methodology, Validation. **Nitin Srivastava:** Methodology, Writing – original draft. **Bhumika Rani:** Methodology. **Vinod Kumar:** Writing - review & editing. **Sunil Kumar Khare:** Supervision, Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Legends to tables

Table 1: Comparison of malic acid production at different initial pH and CaCO₃ levels. Effect of the critical process parameters viz. pH and CaCO₃ concentration on L-MA titre, yield, productivity, fungal growth (DCW), and glucose consumption.

Table 2: Malic acid production using pure glucose (100 g/L) and sugar-rich hydrolysates (100 g/L glucose) obtained from acidic and enzymatic saccharification of CPW. Effect of fermentation media on L-MA titre, yield, productivity, fungal growth (DCW), and glucose consumption.

Legends to figures

Fig. 1: Time course profiles of L-MA production by *A. wentii* in pure glucose media. (a) Effect of initial pH of the media (3.0-8.0) (b) Effect of CaCO_3 concentration (0-120 g/L). The samples were aliquoted from the fermentation broth at equal time intervals (48 h) and L-MA concentration (in g/L) was estimated.

Fig. 2: Time course profile of glucose consumption, pH, biomass and L-MA production by *A. wentii* from pure glucose (100 g/L). The samples were aliquoted from the fermentation broth at equal time intervals (24 h). Symbols: L-MA (in g/L) (circle), residual glucose (in g/L) (square), DCW (in g/L) (rhombus), and pH (triangle).

Fig. 3: Optimization of saccharification conditions of CPW. Glucose released (g/L) (bar graph) and saccharification yield (%) (line graph) obtained from acidic (a, b) and enzymatic (c, d) saccharification of CPW. (a) Effect of HCl loading (% v/v) (b) Effect of solid loading (% w/v CPW) in saccharification with 1% HCl (c) Effect of enzyme loading (U/g) and treatment time (h) (d) Effect of solid loading (% w/v CPW) in saccharification with 5 U/g amyloglucosidase.

Fig. 4: Time course profile of glucose consumption, pH, biomass and L-MA production from CPW hydrolysate (100 g/L glucose) by *A. wentii* (a) Acidic hydrolysate (b) Enzymatic hydrolysate. The samples were aliquoted from the fermentation broth at equal time intervals (24 h). Symbols: L-MA (circle), residual glucose (square), CDW (rhombus), and pH (triangle).

Tables

Table 1:

		C (g/L) Maximum MA titer	L- [T ₁ Time(h)]	R (g/L) Residual glucose	Y (g/g) Yield (L-MA/ consumed glucose)	P (g/L/h) Overall producti vity at T ₁ time	P _m (g/L/h) Maximum productivity [T ₂ Time(h)]	DC W Dry cell weig ht at T ₁ time (g/L)
pH	3	03.88 [240]		42.69	0.07	0.016	0.016 [192]	5.86

	4	12.45 [240]	29.16	0.17	0.051	0.088 [144]	9.12
	5	25.16 [240]	7.98	0.27	0.104	0.131 [144]	18.9 7
	6	37.90 [240]	3.08	0.39	0.157	0.195 [144]	21.6 5
	7	31.40 [240]	1.95	0.32	0.131	0.146 [192]	21.7 3
	8	08.97 [192]	24.33	0.12	0.047	0.047 [192]	12.3 5
CaC O ₃ conc. (g/L)	0	01.42 [240]	28.06	0.02	0.006	0.006 [192]	14.8 5
	30	16.75 [192]	17.32	0.20	0.069	0.087 [144]	16.4 2
	60	24.81 [192]	11.81	0.28	0.103	0.132 [144]	19.2 4
	90	37.90 [240]	3.08	0.39	0.157	0.195 [144]	21.6 5
	120	36.06 [240]	4.88	0.37	0.150	0.162 [144]	21.9 2

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Table 2:

Fermentation media	C (g/L) Maximum MA titer [T₁ Time (h)]	L- R (g/L) Residual glucose	Y (g/g) Yield (L-MA/ consumed glucose)	P (g/L/h) Overall productivity at T₁ time	P_m (g/L/h) Maximum productivity [T₂ Time(h)]	DC W Dry cell weight at T₁ time (g/L)
Pure glucose	37.90 [240]	96.92	0.39	0.158	0.202 [144]	21.6 5
Acid hydrolysate	20.94 [216]	16.66	0.27	0.097	0.097 [216]	15.6 2
Enzyme hydrolysate	33.06 [240]	2.74	0.34	0.137	0.178 [144]	19.8 2

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797 **Figures**

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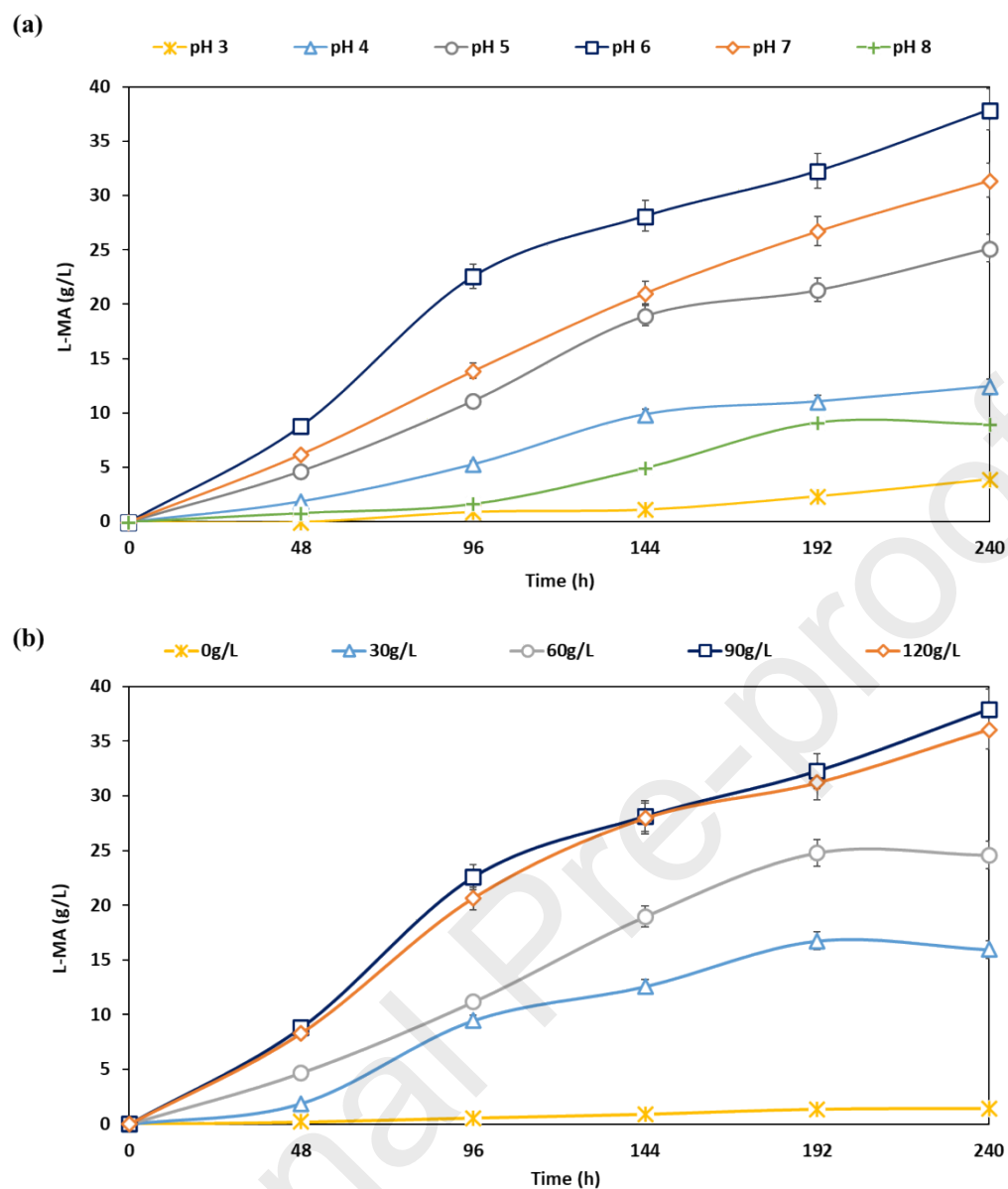


Fig. 1

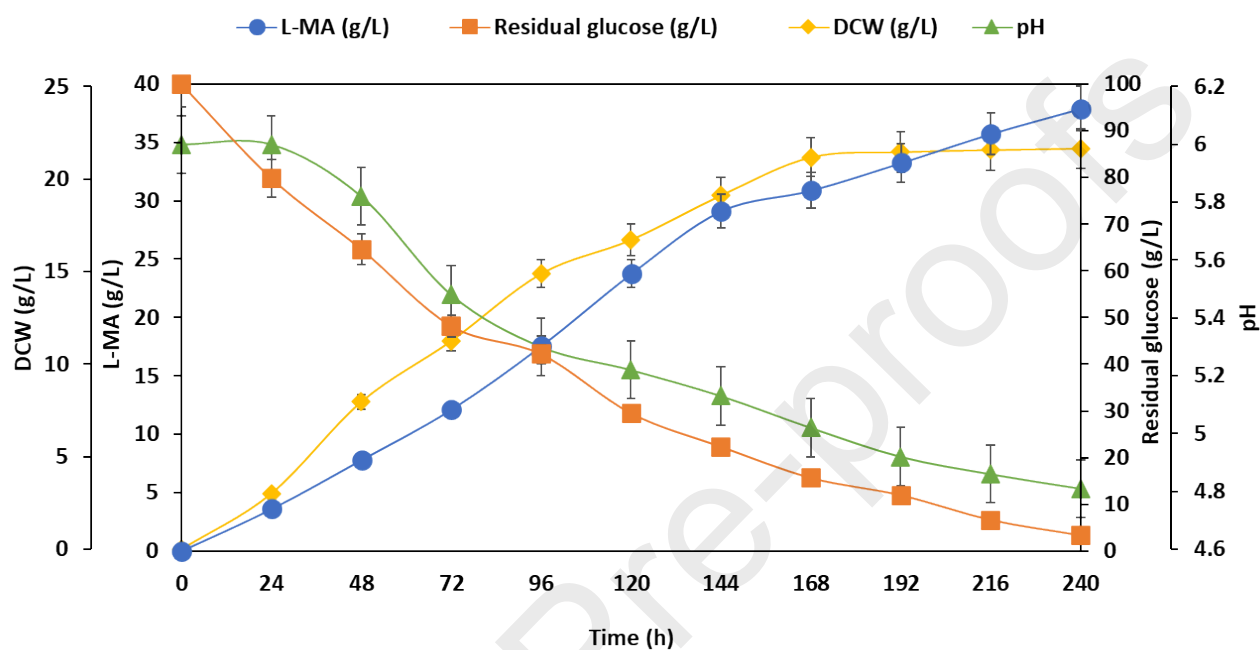


Fig. 2

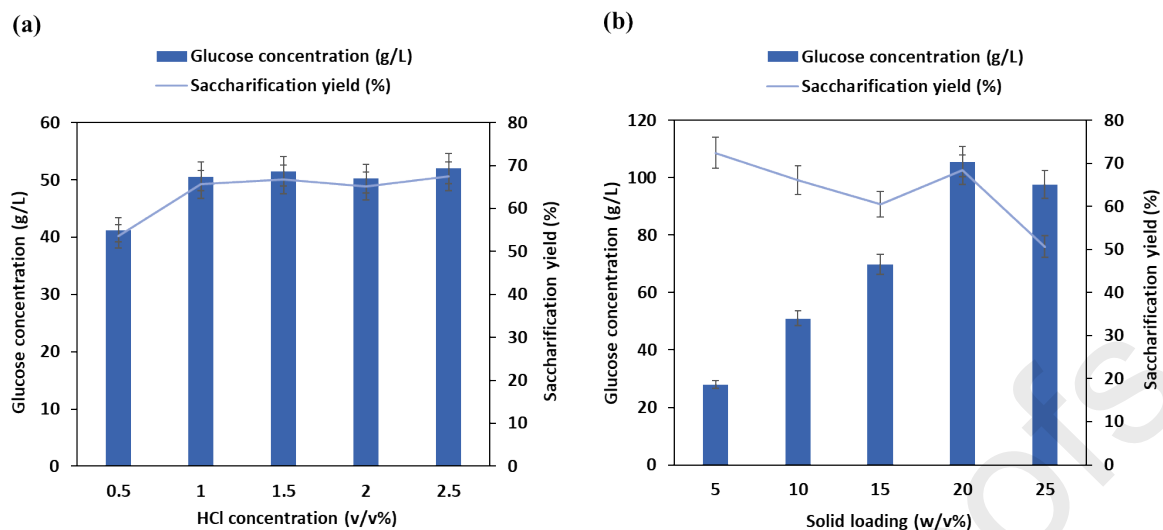
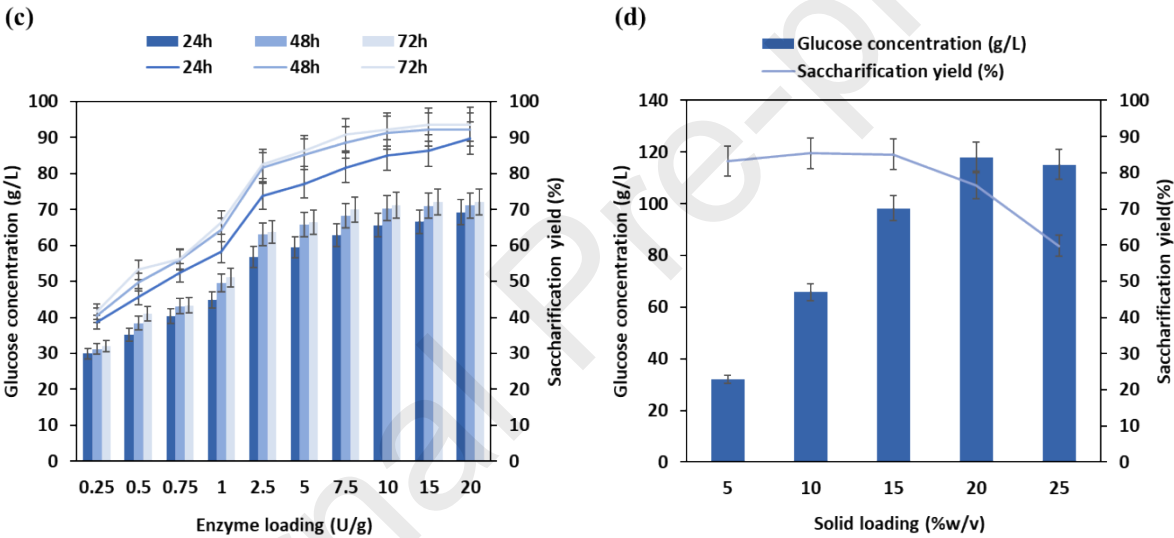


Fig. 3



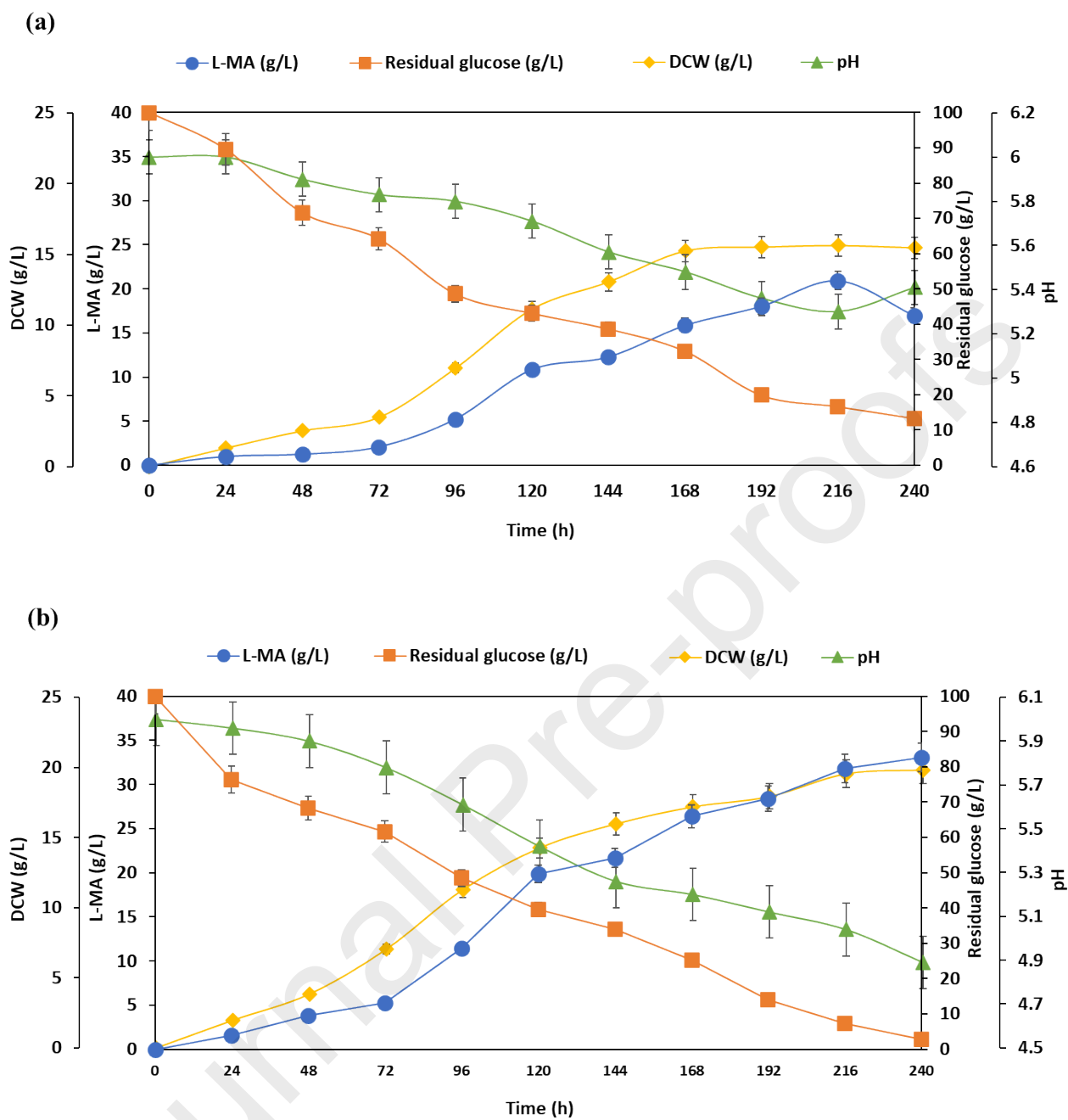
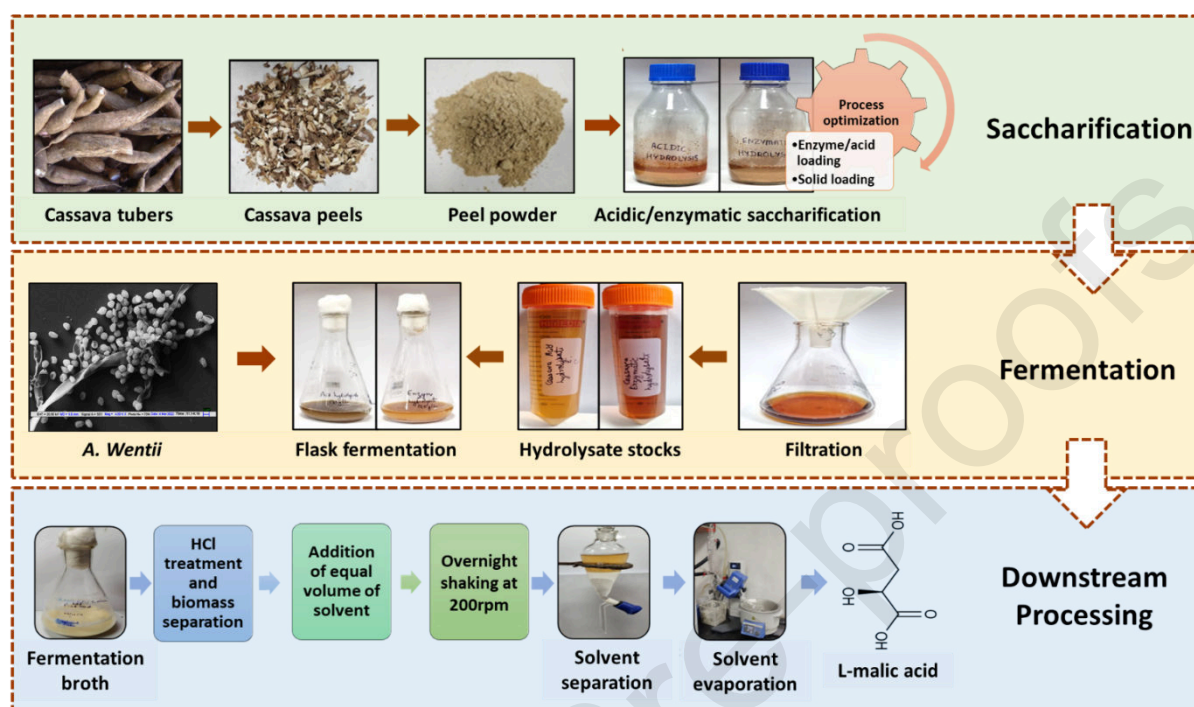


Fig. 4



Graphical Abstract

Highlights

- L-malic acid (L-MA) production by *Aspergillus wentii* using cassava peel waste (CPW).
- A glucose yield of 500-700g/kg CPW was obtained by acid and enzymatic hydrolysis.
- L-MA titer of 37.9 g/L and yield of 0.39 g/g was achieved on pure glucose.
- Acid and enzymatic hydrolysate generated 20.9 g/L and 33.1 g/L L-MA, respectively.
- *n*-Butanol-based solvent extraction method resulted in 31% recovery of L-MA.

Credit author statement

Deeksha Gopaliya: Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Saniya Zaidi:** Methodology, Validation. **Nitin Srivastava:** Methodology, Writing – original draft. **Bhumika Rani:** Methodology. **Vinod Kumar:** Writing - review & editing. **Sunil Kumar Khare:** Supervision, Conceptualization, Writing - review & editing.

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Integrated fermentative production and downstream processing of L-malic acid by *Aspergillus wentii* using cassava peel waste

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