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One-Pot Consolidated Bioprocessing of Waste Potatoes to L-Malic Acid by a Thermotolerant and Metabolically Versatile *Aspergillus terreus* BD Isolate

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Abstract

This study explores a metabolically versatile and thermotolerant *Aspergillus terreus* BD isolate, which exhibited selective itaconic acid (IA) and L-malic acid (L-MA) production under distinct fermentation conditions at 45°C. An acidic fermentation medium (pH 3.4) with an inorganic nitrogen source favoured predominant IA synthesis. Contrastingly, organic nitrogen supplementation in a near-neutral medium (pH 6.0) diverted the metabolic flux towards L-MA. Relative gene expression analysis supported the differential IA and L-MA production under selective media conditions, showing upregulation of key genes in their respective biosynthetic pathways. Furthermore, the isolate was employed for the valorization of waste potato biomass (WPB), which is a major food waste worldwide. Fermentation of the enzymatic WPB hydrolysate at 45°C generated a substantial L-MA titer (37 g/L) without requiring $CaCO_3$ supplementation. Operating at elevated temperatures reduces cooling demands, minimizes contamination risks, and improves compatibility with saccharification conditions. Eliminating $CaCO_3$ minimizes chemical inputs and neutralization sludge outputs, thereby lowering the environmental burden and downstream complications. Harnessing these significant attributes, the isolate was integrated into a one-pot bioprocess employing simultaneous and semi-simultaneous saccharification and fermentation approaches. Process optimization, including solid loading, pH, inoculum size, pre-hydrolysis time, and enzyme dosage, yielded 60 g L-MA per Kg WPB. Finally, extraction using *n*-butanol led to the recovery of 58% L-MA. Overall, these findings present *A. terreus* BD, integrated into an efficient one-pot bioprocess, as a promising platform for the sustainable and economically viable production and recovery of L-MA from starch-rich agro-industrial waste.

Keywords: L-Malic acid; Potato waste fermentation; Consolidated bioprocessing; Simultaneous saccharification and fermentation; Downstream processing

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1. Introduction

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The demand for bio-based alternatives to petrochemical products and processes is growing, driven by the scarcity of fossil resources and emerging environmental concerns associated with the oil-based petrochemical refineries.[1–3] In this context, biorefineries producing value-added chemicals through microbial fermentation of renewable feedstocks hold promise towards a sustainable future.[2,4,5] *Aspergillus terreus* is a filamentous fungus with significant biotechnological importance.[6] It bears a natural ability to synthesize high-value compounds, particularly lovastatin, a cholesterol-lowering drug, and itaconic acid (IA), a bio-based platform chemical [7,8]. IA is a building-block chemical that could serve as a green alternative for petrochemicals, like acrylic and methacrylic acid, used in the polymer industry.[9,10] Owing to its meticulous synthesis potential, *A. terreus* fermentation has been utilised primary route for industrial IA production for decades.[11] Besides, the production of other organic acids like fumaric acid, gluconic acid, acetic acid, citric acid, malic acid, etc., has also been reported from *A. terreus* fermentation, although in smaller quantities.[12,13]

Malic acid (MA) or 2-hydroxybutanedioic acid ($C_4H_6O_5$) is a value-added chemical with high industrial relevance [14]. It is a widely used additive in cosmetics, pharmaceuticals, food, and beverage industries.[15] Currently, a well-established petrochemical route is employed for its commercial synthesis, generating 80000-10000 tonnes annually worldwide.[16] This method produces a racemic DL-malic acid (DL-MA), which further requires the resolution of enantiomers for industrial utility.[17] The microbial synthesis provides a sustainable alternative that also offers the benefit of enantiospecificity, generating the L-malic acid (L-MA) as the sole isomer.[17] However, fermentation methods are relatively expensive and bear low commercial viability in comparison to conventional manufacturing processes.[14] Effective transition from petrochemical to biobased synthesis necessitates research exploring strategies to reduce the production costs, which could involve the use of waste feedstocks, optimizing integrated fermentation processes, employing high-yielding microbial sources, and reducing downstream processing costs.[15,17,18]

Raw materials and feedstocks account for 40-50% of the manufacturing cost of biochemicals.[16] Utilizing a fermentation medium derived from waste feedstocks presents a viable solution to reduce production costs while

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also offering an effective approach for waste management.[15,16] Among various waste streams available, starchy residues are promising feedstocks as they have high glucose content, which could be easily extracted under mild conditions with minimal pre-treatment and detoxification steps.[19] Potato (*Solanum tuberosum*) is the fourth most cultivated food crop in the world. Potato tubers are either consumed fresh in households/restaurants or industrially processed into products like fries, chips, or starch. They are typically composed of 75-80% water, 15-20% starch, 1-1.5% fibres, 1.5-2% protein, and less than 1% fat and minerals.[20] Huge amounts of potato-derived waste (peels, pulp, scraps, food waste, etc.) are domestically or industrially generated throughout the globe.[21,22] Additionally, a large portion (20-30%) of the total potato produced is discarded across the supply chain due to cosmetic defects or damage caused by diseases, pests, adverse climate, mishandling practices, and improper storage facilities.[23] India is the second-largest producer of potatoes, generating around 50-60 metric tons of potatoes annually, which also leads to the generation of substantial quantities of potato waste.[24] This abundantly available starchy waste remains underutilized but holds great potential to be valorized into biochemicals through fermentation.[23]

Integrated bioprocesses like simultaneous and semi-simultaneous saccharification and fermentation (SSF and semi-SSF) offer a streamlined approach ideal for industrial bioproduction.[25,26] The SSF and semi-SSF processes involve synergistic action of enzymatic breakdown and microbial fermentation in a single pot, which improves bioconversion efficiency by minimizing product inhibition and intermediate loss.[27] These one-pot approaches enable cost-effective manufacturing by reducing energy, labor, raw material, and equipment requirements. Additionally, the simplified workflow in a single vessel saves time and reduces the risk of contamination while minimizing processing steps.[28,29] The key bottleneck in the application of this bioconversion approach is the reduced metabolic efficiency of conventional microbial sources at high temperatures, suitable for enzymatic breakdown.[30] However, with systematic selection of feedstock, enzyme, and microbe, a viable one-pot SSF or semi-SSF bioprocess could be strategized, offering a convenient and scalable approach for industrial biosynthesis from waste feedstocks.

A. terreus BD is a thermotolerant isolate which have been explored for its IA production potential at high temperature in our previous study.[19] The thermotolerant nature of the strain could offer significant benefits for

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biorefinery applications. High temperature fermentation reduces culture viscosity, enhances product solubility, and reduces the risk of contamination by other mesophilic microorganisms. Interestingly, the BD isolate also produced a considerable amount of L-MA in synthetic medium. This metabolic versatility exhibited by the strain was further investigated in the current study, specifically in the context of L-MA production. The conditions for the selective production of IA and L-MA were identified and investigated by examining the expression levels of associated biosynthetic genes and metabolite profiling in pure glucose medium. For a cost-effective bioprocess, the isolate was exploited to valorise waste potato biomass (WPB) through a separate hydrolysis and fermentation (SHF) approach. Further, the isolate was implemented in a one-pot bioprocess involving SSF and semi-SSF approaches for streamlined bioconversion of WPB. Crucial process parameters were optimized to improve bioconversion efficiency, and the produced L-MA was recovered from the fermentation pot via solvent extraction. Harnessing the interesting attributes of the BD strain, including metabolic versatility, thermotolerance, high yield, and robustness under industrially relevant conditions, the present study aims to develop a sustainable, cost-effective, and streamlined approach for L-MA production from starchy agro-industrial waste.

2. Experimental

2.1 Materials

All the salt components were purchased from Fischer Scientific. Glucose, yeast extract, and peptone were purchased from Himedia Laboratories. L-Malic acid and itaconic acid, with ≥99% purity, were procured from Sigma-Aldrich. 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. n-Butanol used for downstream processing was of HPLC grade and was purchased from Fischer Scientific. Damaged/defective 'Chipsona' potatoes were procured from the local market in New Delhi, India.

2.2 Microorganism and culture conditions for IA and L-MA production

The thermotolerant *A. terreus* BD strain used in this study was isolated from soil samples collected at multiple sites across the Dhaka region of Bangladesh.[19] The strain was preserved in 50% glycerol stocks at -80°C and was maintained on malt extract agar at 30°C. The spores were harvested from the plates after 5 days of

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cultivation using a saline solution (0.9% w/v NaCl, 0.05% Tween 20), and the number of spores was determined using a Neubauer chamber. The primary culture medium contained 30 g/L glucose and 5 g/L yeast extract, with a starting pH of 2.5 for IA and 5.5 for L-MA production. The medium was inoculated with 10^6 spores/mL and incubated at 45°C with an agitation speed of 150 rpm for 48h.

The IA production medium was composed of 150 g/L glucose, 1 g/L KH_2PO_4, 3 g/L NH_4NO_3, 1 g/L $MgSO_4.7H_2O$, 5 g/L $CaCl_2.2H_2O$, 1.67 mg/L $FeCl_3.6H_2O$, 8 mg/L $ZnSO_4.7H_2O$, and 15 mg/L $CuSO_4.5H_2O$[31]. The L-MA production medium was composed of 150 g/L glucose, 6 g/L peptone, 0.15 g/L KH_2PO_4, 0.15 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.005 g/L $FeSO_4 \cdot 7H_2O$ and 0.005 g/L NaCl.[32] The fermentation was carried out in 250 mL flasks with a working volume of 50 mL. The initial pH of the glucose solution was adjusted to 3.4 for IA production and 6.0 for L-MA production. The glucose medium was autoclaved, and the respective salt components were filter sterilized (0.2 µm nylon filter) into the solution. Both the media were inoculated with 5% v/v of BD from primary culture and incubated at 45°C with a shaking speed of 200 rpm.

2.3 Relative expression analysis of the key metabolic pathway genes

The relative expression levels of the *A. terreus*' IA biosynthetic gene, including cis-aconitate decarboxylase (*cad* - ATEG_09971), putative L-MA production genes, malate dehydrogenase (*mdh* - ATEG_05653), malate synthase (*mls* - ATEG_07126), and fumarase (*fum* - ATEG_07205), were measured through real-time RT-PCR. The total RNA was isolated using liquid nitrogen and the RNeasy mini kit (Qiagen, USA) from the fungal biomass samples harvested on the 3rd, 6th, and 9th day of fermentation in the respective IA and MA production media. Their purity and concentrations were checked by a NanoDrop2000 spectrophotometer (Thermo Scientific, USA) and 1% agarose gel electrophoresis. The DNase-treated RNA was converted to complementary DNA (cDNA) using the iScript™(BioRad) cDNA synthesis kit as per the manufacturer's instructions. The gene-specific oligonucleotide primers were designed and ordered (**Supplementary Table S1**). The Internal Transcriber Spacer (ITS) gene was used as the reference gene for performing the Real-time RT-PCR using the synthesized cDNA as a template, the primers, and SYBR Green Master Mix (BioRad). The relative normalised expression was analyzed using the CFX Maestro software (CFX96 Touch Real-Time PCR detection system, Bio-Rad).

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2.4 Separate hydrolysis and fermentation (SHF) of WPB

The damaged potatoes were grated/finely chopped and enzymatically hydrolysed to release glucose through amyloglucosidase (AMG) treatment as described previously.[33] Briefly, 160 g WPB (25% solid, 75% moisture) was mixed with 40 mL of 0.01 M citrate buffer (pH 4.5) to make up a final solid loading of 20% w/v. The slurry was autoclaved, and 10 U/g AMG was added to it, followed by incubation at 50°C with 200 rpm for 48 h. After saccharification, the unhydrolyzed residues were removed by centrifugation (10000 rpm X 15 min) and filtration, and glucose concentration was estimated in the supernatant obtained. The resultant enzymatic hydrolysate from waste potatoes (EHWP) was concentrated to adjust the glucose concentration to 150 g/L and divided into 50 mL portions, which were adjusted to different pH values (3.0-6.0). This EHWP medium was filter sterilised (0.2 µm nylon) into autoclaved flasks (250 mL) and inoculated with BD isolate (5% v/v). The metabolic behaviour of the strain was investigated across a range of media pH by culturing it at 45°C with a shaking speed of 200 rpm.

2.5 One-pot (SSF and semi-SSF) bioconversion of WPB to L-MA

Initially, the SSF approach was applied for the bioconversion of WBP to L-MA. Different amounts of WPB were taken in 250 mL flasks, and 0.01 M citrate buffer (pH 4.5, unless otherwise stated) was added to adjust the solid loading to 5-25% w/v, making up the final volume to 50 mL, followed by autoclaving. The BD inoculum (5% v/v) and AMG (5 U/g) were then simultaneously added to the WPB slurry. The flasks were incubated at 45°C and 200 rpm. Critical parameters like pH, inoculum size, and shaking speed were optimized using a one-variable-at-a-time (OVAT) approach with the best yielding WPB loading. The pH of the medium was optimized by using citrate buffer with different pH values (3.0-6.0).

Following SSF, a sequential one-pot semi-SSF approach was employed for WPB bioconversion in a single vessel. Optimal SSF conditions were used, with an additional initial hydrolysis period prior to fermentation. The AMG was added to the autoclaved WPB slurry, which was incubated at 45°C for a defined pre-hydrolysis time (0-48 h). After this phase, the BD inoculum was added to initiate fermentation concurrently with ongoing

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saccharification. Subsequently, the optimal enzyme dosage (0.1-10 U/g) was determined based on the most efficient pre-hydrolysis time.

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2.6 Downstream processing of L-malic acid

The downstream processing of the produced L-MA was conducted using a previously developed solvent-extraction method[33]. After completion of fermentation, the pH of the fermentation broth was reduced to 3.0 with HCl. 50 mL of *n*-butanol was added to the flask and kept for shaking at 200 rpm for 3 h. The flask was allowed to stand to allow complete phase separation, after which the upper solvent layer was manually collected. The extraction process was performed twice, and the collected organic layers were pooled. The pooled solvent was treated with activated charcoal (2% w/v) for the removal of pigments. Finally, the clear solvent was evaporated using a rotary evaporator (IKA, Breisgau, Germany), leaving a dry precipitate, which was then dissolved in Milli-Q water to analyze yield and purity. The solvent was recovered from the rotary evaporator after condensation and was successfully reused in the next batch of extraction.

2.7 Analytical methods

To analyze the residual glucose and produce IA and L-MA in the culture, samples (1 mL) were withdrawn periodically from the fermentation broth (12/24 h). To minimize errors and ensure the representativeness of the aliquots, shake-flask cultures were thoroughly mixed by shaking immediately before and during sampling to achieve the highest possible degree of homogeneity. The samples were centrifuged at 10000 rpm for 10 min, and the supernatant was used for HPLC analysis. A Perkin Elmer HPLC system equipped with a Bio-Rad HPX-87H Aminex ion exclusion column and a refractive index detector (RID) was used for organic acid and glucose quantification. The 5 mM H_2SO_4 with a flow rate of 0.6 mL/min was used as the mobile phase. The fungal growth was monitored in terms of dry cell weight (DCW) obtained by oven drying the precipitated biomass for 2 h.

2.8. Statistical analysis

All the experiments in the study were performed in triplicate, and the results are reported as mean ± standard deviation (SD) of triplicate measurements (n = 3). Statistical analyses among groups were performed using one-

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way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test for pairwise comparisons of data values. Differences among groups were declared statistically significant if $p < 0.05$.

3. Results and discussion

3.1 Selective IA and L-MA production by *A. terreus BD*

Aspergillus terreus strains are widely studied for IA biosynthesis through fermentation of simple and complex feedstocks [34,35]. In our previous study, a novel *A. terreus* BD strain was isolated, identified, and characterized for IA production ability.[19] It demonstrated remarkable thermotolerance as it efficiently grew and maintained its metabolic capability at temperatures as high as 45°C. In the current study, the strain was pre-cultured in yeast extract and glucose medium, followed by cultivation in synthetic medium with pH 3.4, containing 150 g/L glucose and a salt composition specific for IA production.[19] This system generated 34.5 g/L IA over 13 days of fermentation (**Supplementary Figure S1**). At this stage, 20 g/L glucose was retained in the medium with a final DCW of 10.8 g/L and a pH dropping to 1.3. No further growth or production occurred, likely due to the inhibitory effect of the highly acidic medium pH. Additionally, during the course of fermentation, the isolate also accumulated a considerable amount of L-MA, reaching a maximum titer of 7.5 g/L by 192 h. With the pH dropping to 2.0, no further production of L-MA was observed beyond this, suggesting that acidic conditions inhibit L-MA synthesis.

To further investigate the L-MA production potential of BD, it was cultivated in a glucose medium with a composition previously used for L-MA production in species such as *A. oryzae* and *A. niger*.[32,36]. This medium contained a complex nitrogen source, peptone, and had an initial pH of 6.0, favorable for L-MA synthesis. Under these conditions, the isolate demonstrated a preferential shift towards L-MA production (**Figure 1**). Previous studies with *A. oryzae* have shown that the use of complex nitrogen sources like yeast extract and peptone resulted in better fungal growth and L-MA productivity as compared to inorganic nitrogen sources like $(NH_4)_2SO_4$.[32,37]. Consistent with these findings, the presence of peptone, a rich source of amino acids, peptides, vitamins, and minerals, boosted the growth and metabolism of BD and supported L-MA production. It resulted in complete consumption of glucose over 12 days of fermentation and a substantially higher DCW of 18.5 g/L in comparison

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to the IA production medium containing NH_4NO_3 as nitrogen source. The rate of glucose consumption and fungal growth was high during the initial phase of fermentation and gradually declined after 144 h as the medium became acidic with L-MA accumulation. Initially, the L-MA concentration increased sharply to 21.5 g/L till 144 h, reaching a maximum productivity of 0.145 g/L/h. Further, the increment was gradual, ultimately reaching a final titer of 32.3 g/L L-MA, corresponding to a yield and productivity of 0.215 g/g and 0.112 g/L/h, respectively. Notably, the IA synthesis was initiated around 120 h when the medium attained a lower pH (<4.5) suitable for IA production, and a final titer of 3.8 g/L was observed.

Studies have implicated that $CaCO_3$ supplementation in the medium is crucial for L-MA production as it regulates media pH, supplies CO_2 into the biosynthetic pathway, and reduces product inhibition by precipitating L-MA [18,38]. Previous reports have shown that $CaCO_3$ concentrations as high as 120 g/L are required for effective production.[32,33,38] Under similar media composition, added with 100 g/L $CaCO_3$ *A. oryzae* NRRL3485 produced 30.3 g/L L-MA from 50 g/L glucose.[32] In another study, an engineered *A. niger* strain produced a titer of 55.5 g/L from 100 g/L glucose in a medium containing 6 g/L peptone and 80 g/L $CaCO_3$.[36] Geyer et al. showed that medium containing 120 g/L $CaCO_3$ produced 400% more L-MA in comparison to that with 20 g/L $CaCO_3$ using *A. oryzae* NRRL3488.[38] However, the use of such high amounts of $CaCO_3$ not only adds to the cost but also poses operational challenges like high culture viscosity-hindering mass transfer, clogging/fouling of the equipment, and complicated downstream processing.[16,38,39] Interestingly, a high yield of L-MA was achieved from BD without $CaCO_3$ supplementation. This is likely due to a sufficient supply of endogenous CO_2 from central metabolism and efficient recycling to sustain pyruvate carboxylation. The absence of $CaCO_3$ in the medium provides advantages for downstream processing and industrial scalability of the process, making BD a promising cell factory for L-MA synthesis.

The use of complex nitrogen sources, such as peptone, on MA production is well documented. The positive effect of peptone towards MA biosynthesis can be explained by several interconnected biochemical and regulatory mechanisms [32,36,37]. Peptone provides the necessary amino acids, peptides, vitamins, and mineral nutrients that support metabolic flux toward MA synthesis. A study reported a 36% improvement in L-MA titer from *A. oryzae* using 6.0 g/L peptone as the nitrogen source, compared with using ammonium sulfate.[32] RNA

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sequencing and qPCR analysis showed that the pyruvate carboxylase gene is significantly upregulated when *A. oryzae* is grown on peptone rather than on ammonium sulfate, thereby supplying oxaloacetate in the rTCA pathway. It also supplies essential trace metals (Mn^{2+}, Mg^{2+}, Zn^{2+}) that act as cofactors for pyruvate carboxylase, maximizing malate flux and L-MA secretion. Additionally, peptone directly replenishes TCA intermediates via deamination of its amino acids, bypassing the energy-costly ammonium assimilation pathway. Catabolism of the amino acids through the TCA/rTCA cycle generates additional NADH, which supports higher malate flux by providing reducing equivalents for malate dehydrogenase and related steps[9] In contrast, peptone supplementation is detrimental for IA production. IA biosynthesis typically requires a nitrogen-limited condition and is strongly inhibited by the presence of metal ions, particularly Mn^{2+}, which are abundant in peptone and known to inhibit *cis*-aconitate decarboxylase enzyme [40]. This explains the low yield of IA in the L-MA production medium (Figure 1). These findings highlight the metabolic versatility of the BD strain, which exhibits selective IA and L-MA production depending upon media composition and pH. This position is an interesting microbial chassis for the sustainable synthesis of two industrially significant organic acids.

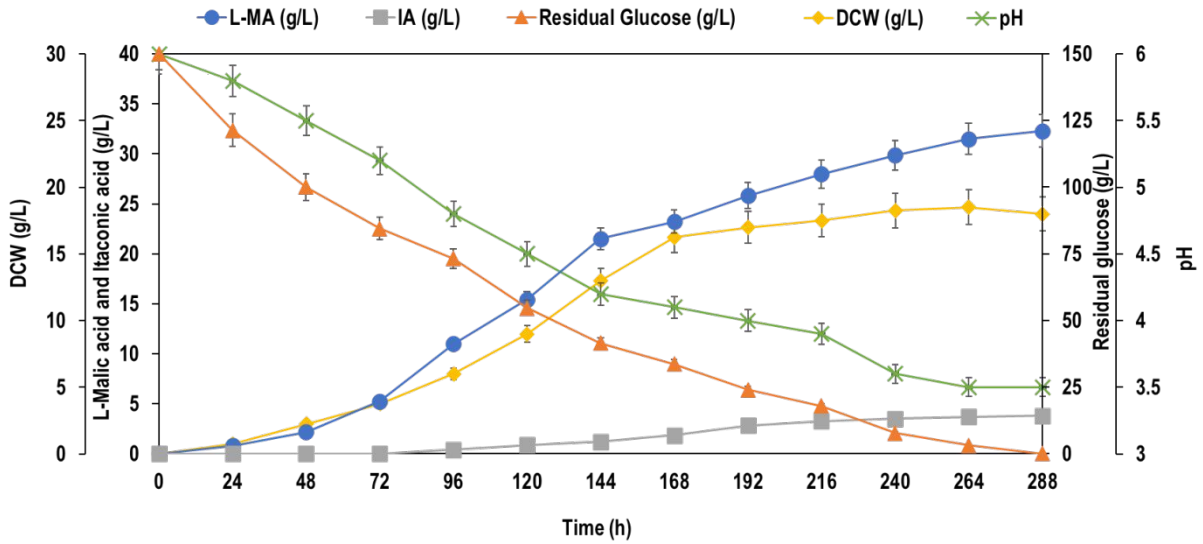

Figure 1: Shake flask cultivation of *A. terreus* BD cultivated on L-MA production medium showing produced L-MA (g/L), IA (g/L), residual glucose (g/L), DCW (g/L), and pH. Symbols: circle (L-MA), square (IA), triangle (residual glucose), rhombus (DCW), cross (pH).

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3.2 Relative expression of key pathway genes involved in IA and L-MA production

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The IA production pathway has been well-characterised in *A. terreus*, which involves *cad* as the critical gene responsible for decarboxylation of *cis*-aconitic acid to form IA.[11,41] In contrast, L-MA biosynthesis has never been studied in this species. However, studies based on the other microorganisms have shown three possible MA production pathways, namely, the reductive TCA (rTCA), oxidative TCA, and the glyoxylate cycle.[42] In these pathways, the key genes are *mdh*, *fum*, and *mls*, which catalyze the formation of L-malate from oxaloacetate in the rTCA pathway, from fumarate in the oxidative TCA pathway, and from glyoxylate in the glyoxylate shunt, respectively.[17] **Figure 2** demonstrates the putative metabolic pathways for IA and L-MA synthesis in fungal cells.

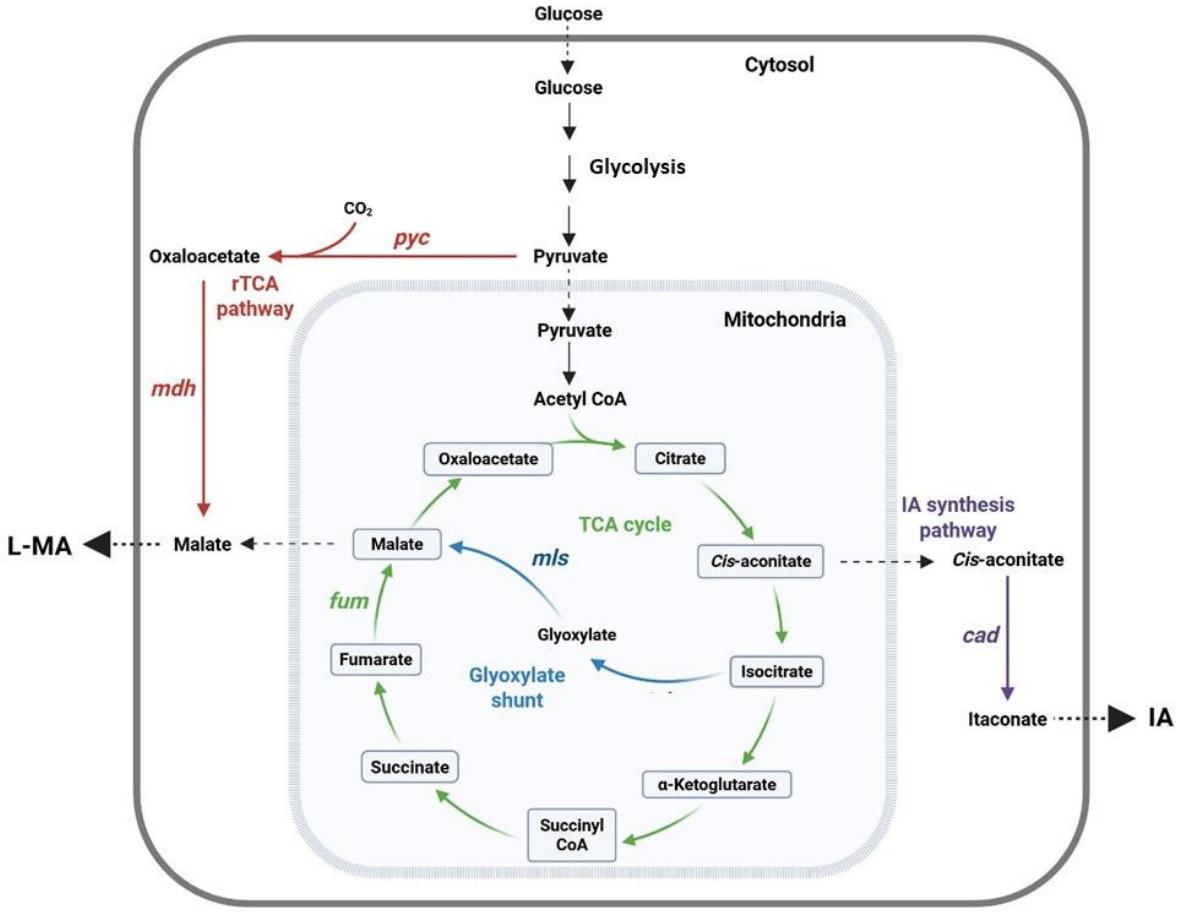

Figure 2: Metabolic pathways for IA and L-MA biosynthesis and their key genes. L-MA synthesis: Oxidative TCA pathway (green) – Fumarase (*fum*); Reductive TCA pathway (red) - malate dehydrogenase (*mdh*), pyruvate carboxylase (*pyc*); Glyoxylate shunt (blue) – malate synthase (*mls*). IA synthesis pathway (purple) – *cis*-aconitate decarboxylase (*cad*).

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Given the peculiar metabolic functionality of the isolate, it was interesting to study the relative expression profiles of these key IA and L-MA production genes in BD strain when cultured on respective production media conditions. The expression of *cad* was feeble when the isolate was cultivated in the MA-specific media having higher pH and peptone, which was well correlated with the low titres of IA (3.8 g/L). In contrast, the *cad* expression in IA production medium was around 11 times higher (up to 6th day) and subsequently decreased towards the end of fermentation **(Figure 3A)**. This justified the high accumulation of IA when the isolate was cultivated with a low pH (3.4) medium with specific salt components **(Supplementary Figure S1)**. A recent study demonstrated that acidic conditions are vital for IA production since the histidine residues present in the active site of the CAD enzyme need to be protonated for effective binding of the substrate, *cis*-aconitate, for its decarboxylation into IA.[43] In line with these findings, the current study also demonstrates a strong influence of medium pH on the IA productivity of the strain. Additionally, studies have shown that nitrogen-limited conditions are more suitable for IA production, as they direct the carbon flux towards the secondary metabolism, whereas a rich nitrogen source like peptone negatively affects the IA productivity.[44]

The MA production genes (*mdh*, *fum*, *mls*) exhibited a relatively higher expression on MA-specific medium as compared to IA medium (**Figure 3**). The gene expression profiles supported the production profile of L-MA with different media conditions. Previous studies on other MA-producing species like *A. oryzae* and A. *flavus* have shown rTCA as the chief production pathway.[15] Similarly, in the current study, the expression levels of *mdh* dominated other key genes of the MA's metabolic machinery, indicating rTCA to be the major pathway in the *A. terreus* isolate as well. Overall relative expression trend of *mdh*>*fum*>*mls* was observed. The results ensured the discrete metabolic versatility in the *A. terreus* BD isolate at the transcriptional levels, where the *cad* gene of the TCA cycle directs the production of IA, and the *mdh* gene of the reductive TCA cycle directs the production of MA when the isolate is grown in the production media for IA and L-MA, respectively. Moreover, a relatively higher expression of the *fum* gene in MA medium suggests that TCA-derived MA might also be contributing to the overall titres, although the *fum* expression was lower than that of *mdh*. When the IA biosynthetic pathway

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was suppressed in MA medium, the upregulated *fum* might have shifted carbon flux towards MA, which would have been transported out of the mitochondria.

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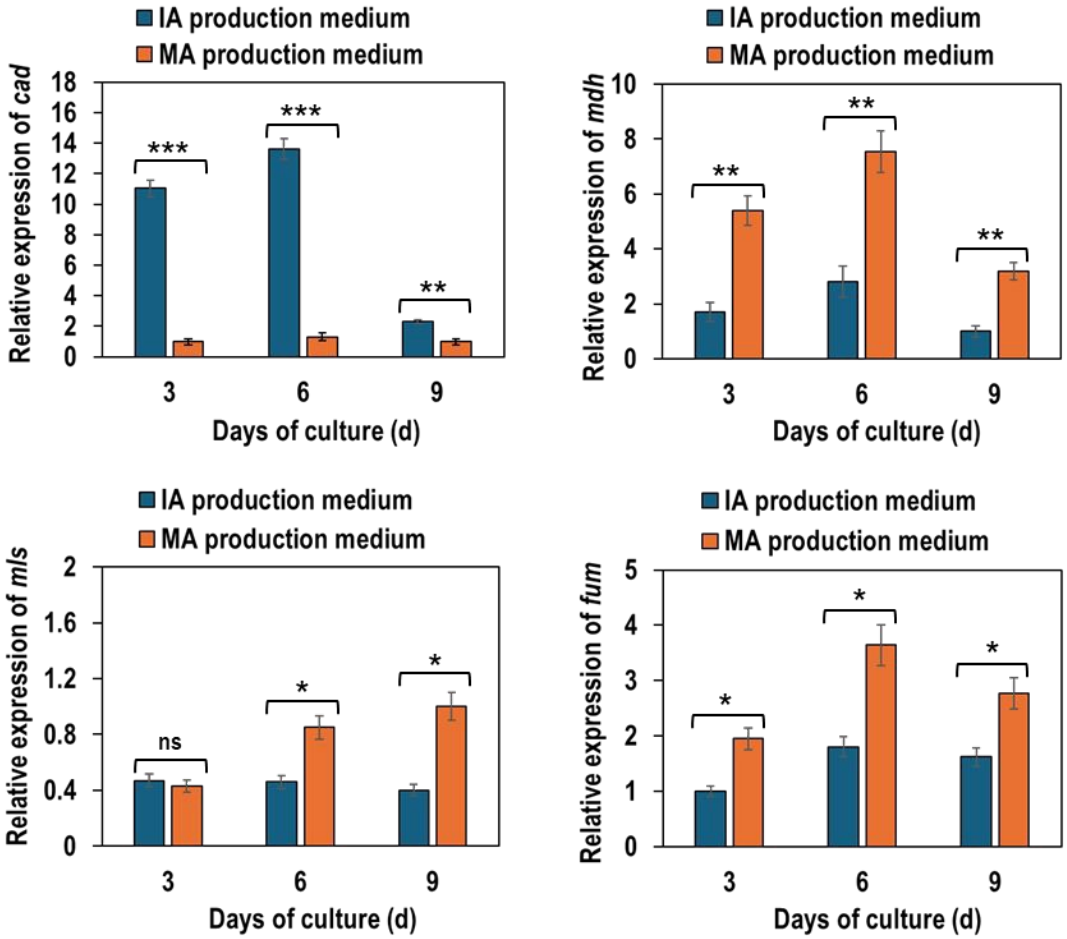

Figure 3: Relative normalized expression of key biosynthetic pathway genes during cultivation of *A. terreus* BD on synthetic IA and L-MA production media. IA production gene: (a) *cis*-aconitate decarboxylase (c*ad*); and L-MA production genes (b) malate dehydrogenase (*mdh*); (c) malate synthase (*mls*); and (d) fumarase (*fum*). Data represent mean ± SD (n=3) of three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test for pairwise comparisons between the relative expression of each gene in the IA and MA production medium. (*p< 0.05, **p < 0.01, ***p < 0.001, ns p ≥ 0.05).

The gene expression studies suggested that in the MA production medium, rTCA and TCA cycles with upregulated *mdh* and *fum* genes contribute to the production of MA as a major product. Whereas in the IA-specific medium, the IA biosynthetic pathway with upregulated *cad* is functional, which directed the metabolic

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flux towards IA. The presence of complex nitrogen source peptone containing amino acids, peptides, and minerals, not only increased fungal growth but also had a positive influence on MA production pathways in the *A. terreus* BD strain, leading to higher L-MA titre. A near-neutral pH of 6.0 and specific salt composition in the MA medium also supported L-MA production over IA.

3.3 Separate hydrolysis and fermentation (SHF) of WPB

The potato waste, generated from farms, markets, industries, and households, usually ends up in landfills or is used as low-value animal feed. However, being a rich source of starch, it holds promise as a fermentation feedstock, adding value to this discarded waste.[20,21] This is evident from numerous studies employing potato hydrolysate for the production of biofuels and biochemicals.[21,45–47] For instance, Smerelli et al. showed high-yield production of L-lactic acid from enzymatic hydrolysate.[48] In the current study, damaged/discarded potatoes (WPB), unsuitable for consumption, were used to generate a glucose-rich fermentation medium (EHWP) through enzymatic hydrolysis. The WPB was composed of 25% solid and 75% moisture content, and was rich in starch, accounting for 18.5% of the total weight. The autoclaved WPB slurry (20% w/v solid loading) was saccharified using a multifunctional AMG (10 U/g), which has α-D-(1-4), the α-D-(1-6), and the α-D-(1-3) glycosidase activities. This resulted in a glucose release of 140.8 g/L with >85% saccharification efficiency. Several studies have used two-step approaches for starch hydrolysis involving liquefaction and saccharification using multiple enzymes under different conditions.[49] For instance, a previous study involving dried powder of damaged potatoes, also known as “cull potatoes”, achieved a saccharification efficiency of 83.2% using the two-step enzyme approach[23]. Conversely, in this study, comparable saccharification efficiency was achieved with a single enzyme while avoiding the drying, grinding, gelatinization, and liquefaction steps, which saved associated energy and enzyme costs. This was followed by centrifugation, filtration, concentration, pH adjustment, and sterilization of the hydrolysate (EHWP) prior to fermentation.

Given the versatile capabilities of the strain overproducing specific metabolites under different culture conditions, it was interesting to explore its metabolic behaviour in a cost-effective, waste-derived medium. In this context, the EHWP medium was used to cultivate the BD isolate, and L-MA/IA production was monitored across a range of initial media pH (**Table 1**). Notably, the isolate exhibited enhanced growth and metabolic functionality in EHWP

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medium as compared to synthetic medium. This is possibly due to the inherent richness of proteins and minerals in potatoes. As a result, complete consumption of glucose took place within 240 h of fermentation. Under acidic conditions, the strain yielded IA as the major product. A maximum IA titer of 21.5 g/L was obtained from EHWP medium with an initial pH of 3.5. Subsequently, the IA productivity declined with an increase in initial pH and became minimalized (<5 g/L) at pH ≥5.0. In our previous study on BD, 41 g/L IA was achieved from the enzymatic hydrolysate of starch-rich synthetic food waste at pH 3.4 in a bioreactor culture.[19] However, the composition of biomass critically influences the metabolite profile of the isolate, which was seen in this study from potato waste.

Table 1: Influence of initial pH of EHWP medium on production of L-MA and IA. The data have been represented as Mean ± Standard deviation of triplicate measurements.

	L-Malic acid			Itaconic acid		
pH	**Titer (g/L)**	**Yield (g/g)**	**Productivity (g/L/h)**	**Titer (g/L)**	**Yield (g/g)**	**Productivity (g/L/h)**
3.0	7.1 ± 0.45	0.047 ± 0.003	0.030 ± 0.002	16 ± 1.05	0.107 ± 0.007	0.067 ± 0.004
3.5	10.5 ± 0.74	0.070 ± 0.005	0.044 ± 0.003	21.5 ± 1.40	0.143 ± 0.010	0.090 ± 0.008
4.0	13.9 ± 0.9	0.093 ± 0.006	0.058 ± 0.004	20.1 ± 1.30	0.134 ± 0.009	0.084 ± 0.006
4.5	25.21 ± 1.7	0.168 ± 0.012	0.105 ± 0.007	10.9 ± 0.75	0.073 ± 0.005	0.045 ± 0.003
5.0	32.2 ± 2.2	0.215 ± 0.015	0.134 ± 0.009	4.9 ± 0.34	0.033 ± 0.002	0.020 ± 0.002
5.5	33.8 ± 2.3	0.225 ± 0.016	0.141 ± 0.01	4.1 ± 0.28	0.027 ± 0.002	0.017 ± 0.001
6.0	36.9 ± 2.5	0.246 ± 0.018	0.154 ± .011	3.1 ± 0.21	0.021 ± 0.002	0.013 ± 0.001

It was observed that cultivation of BD in EHWP medium favored L-MA synthesis. Even at the lowest tested pH of 3.0, a considerably high amount of L-MA (7.1 g/L) was co-produced with 16 g/L IA. This resulted in a mixed metabolite profile at low pH, which could pose downstream processing challenges. However, the L-MA production efficiency improved significantly with pH increase, showing a five-fold increment in titre from pH 3.0 to 6.0. At near-neutral pH (5.0-6.0), the metabolic flux shifted predominantly towards L-MA synthesis, leading to substantially high accumulation of L-MA (>32 g/L) as compared to IA (<5g/L), making the separation of the major product easy. This aligns with the previous findings on pH dependence of microbial L-MA synthesis.[18,50] For

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instance, in our previous study involving *A. wentii*, it was observed that media pH substantially influenced L-MA production, which was diminished under acidic conditions and maximized at a near-neutral pH of 6.0.[33] Multiple reports have implicated the same in other species like *A. oryzae, A. niger*, *Ustilago trichophora*, etc., where a pH regulation around 5.0-6.0 contributed to maximum L-MA production.[37,51,52] In *A. terreus*, IA biosynthesis is embedded in the secondary-metabolism network and controlled by the global regulator *laeA* together with cluster-associated transcription factors, *regA*, which activate the IA gene cluster under IA-producing conditions[53,54]. In contrast, L-MA formation is more closely linked to primary carbon metabolism and rTCA flux; although L-MA-specific regulators have not yet been identified in *A. terreus*, studies in other *Aspergillus* spp. indicate that stress-responsive Msn2/4-like factors and pH-responsive regulators can remodel expression of TCA/rTCA enzymes and organic-acid transporters, thereby favouring malate secretion under L-MA-promoting conditions.[32]

3.4 L-MA production from WPB hydrolysate via SHF

The existing literature is mostly focused on L-MA production from synthetic fermentation medium containing pure carbon sources (e.g., acetate, glucose, glycerol, etc.). Only a few reports exist based on waste-derived medium, which could enable low-cost L-MA production, suggesting a need for further research exploring more feedstocks and robust microbial sources. Our previous study employing *A. wentii* as a novel L-MA producer achieved titer values of 20.9 g/L and 33 g/L from acidic and enzymatic hydrolysate of cassava peel waste[33]. Corn straw hydrolysate, obtained from steam-explosion pre-treatment and enzymatic digestion, has also been reported for L-MA production using a *Rhizopus delemar* mutant, which generated 120 g/L L-MA.[55] Another study used beechwood for L-MA biosynthesis by *A. oryzae* DSM 1863. After organosolv pretreatment, two fractions were obtained, including cellulose-rich fibers, which were subsequently hydrolyzed, and supernatant rich in hemicellulose, which was used for fermentation after solvent evaporation. The L-MA titer of 37.9 g/L was obtained from the cellulosic hydrolysate, while the hemicellulosic fraction yielded 5.8 g/L L-MA.[56] The current study presents the new *A. terreus* isolate as a promising platform for efficient and inexpensive L-MA synthesis using starchy agro-industrial waste.

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Figure 4 illustrates the fermentation profile of BD cultivated in EHWP medium adjusted to an initial pH of 6.0, that resulted in maximized production of L-MA. Similar to synthetic medium, the growth rate was higher during the initial phases of fermentation. The strain grew more efficiently in EHWP medium, leading to a maximum DCW of 22.7 g/L, while occurring in a dispersed morphological state (**Supplementary Figure S2**). After complete consumption of glucose in 10 days, a final L-MA titer of 36.9 g/L was achieved. The corresponding yield (0.246 g/g) and productivity (0.154 g/L/h) surpassed those obtained from synthetic medium. The strain started secreting feeble amounts of IA after 120 h, reaching a final concentration of 3.1 g/L. Additionally, the EHWP medium showed considerable buffering capacity, which is evident by a final pH of 4.3 despite high-level of acid accumulation. These findings indicate that the BD isolate could be efficiently employed for the valorisation of waste potatoes, favouring the production of L-MA, with a conversion efficiency of 36.4 g L-MA per Kg WBP, from the hydrolysate under high temperature conditions suitable for industrial applicability. However, the SHF involves multiple intermediary steps (filtration, concentration, sterilisation, etc.) between saccharification and fermentation, taking place in separate bioreactors/flasks under different conditions, which adds to operational cost and makes SHF a complex and time-consuming process.

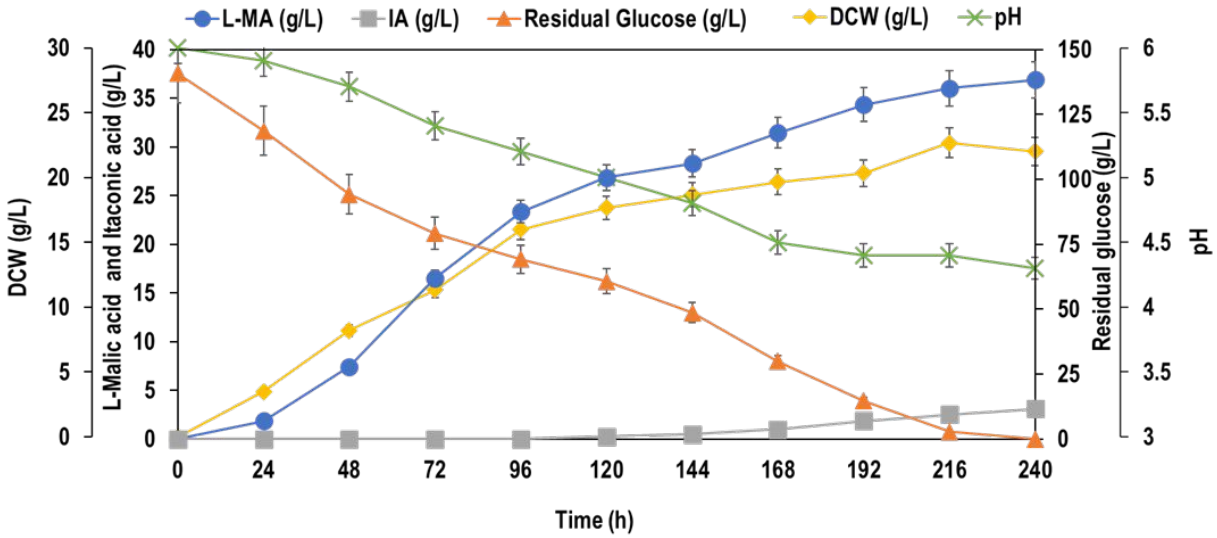

Figure 4. Time course fermentation profiles for glucose consumption, pH, biomass, and product (L-MA & IA) formation by *A. terreus* BD cultivated on EHWP medium adjusted to an initial pH of 6.0. Symbols: circle (L-MA), square (IA), triangle (residual glucose), rhombus (DCW), cross (pH).

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3.5 Simultaneous saccharification and fermentation (SSF) of WPB

While studies have mostly employed SHF bioprocess for organic acid production due to its compatibility with most microbes, the SSF presents a more viable approach for industrial bioconversion by being a one-step process taking place in a single vessel.[25] Moreover, starchy residues are ideal feedstock in an SSF system due to their amorphous and homogenous nature, with low formation of inhibitory compounds.[57] This was shown in a previous report, where SSF of starch-rich bread waste resulted in high yields of lactic acid (250 mg/g) using *Lactobacillus* sp.[58] Several reports have used this approach for bioethanol production from *Saccharomyces cerevisiae.*[25,28] A study conducted SSF of potato waste co-culture of *A. niger* and *S. cerevisiae,* which resulted in a bioethanol yield of 0.41 g/g starch.[59] However, this approach is less common in filamentous fungi due to oxygen transfer limitation and the inability of mesophilic fungal systems to work under high temperature conditions.[60]

The potential of *A. terreus* BD to produce L-MA from potato hydrolysate, without $CaCO_3$, at high temperature conditions close to the temperature optima of AMG makes it an ideal candidate for an SSF bioprocess. Initially, the solid loading of WPB was optimized by taking varying amounts of WPB in citrate buffer (pH 4.5). The slurry was prepared by considering the dry matter (25% w/w) and water (75% w/v) content of WPB for adjusting the solid loading (5-25% w/v), and citrate buffer was suitably added to make up the final working volume of 50 mL in 250 mL flasks (**Supplementary Table S2**). The autoclaved slurry was simultaneously inoculated with BD and AMG to carry out SSF at 45°C. The strain grew around the potato starch granules while they were being degraded by the enzyme. The autoclaving process not only sterilized the medium but also contributed to the initial gelatinization and degradation of starch granules in the potato biomass, making them more accessible to the enzyme. **Supplementary Figure S2** depicts SEM micrographs of unfermented potato-starch granules and the morphology of BD in the SSF culture.

The time profile of L-MA and glucose obtained from the SSF with varying solid loading amounts of WPB is demonstrated in **Figure 5A**. In the initial 24 h, the glucose level sharply increased as enzymatic saccharification

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took place, which was subsequently consumed by the isolate for growth and L-MA production over the course of fermentation. The maximum yields obtained from 5, 10, 150, 20, and 25% solid loading were 39.2, 34.8, 35, 37.5, and 29 g L-MA per Kg WPB, respectively, when nearly all the glucose was consumed in the medium. An increase in the WBP amount enhanced the product accumulation, and the highest L-MA titre of 30 g/L was obtained with the solid loading of 20% w/v, within 192 h of cultivation. At higher solid loading (25% w/v), the saccharification efficacy, glucose assimilation rate, and L-MA productivity were substantially compromised. This could be attributed to limited enzymatic accessibility to starch granules and reduced mass transfer to cells in a denser slurry.

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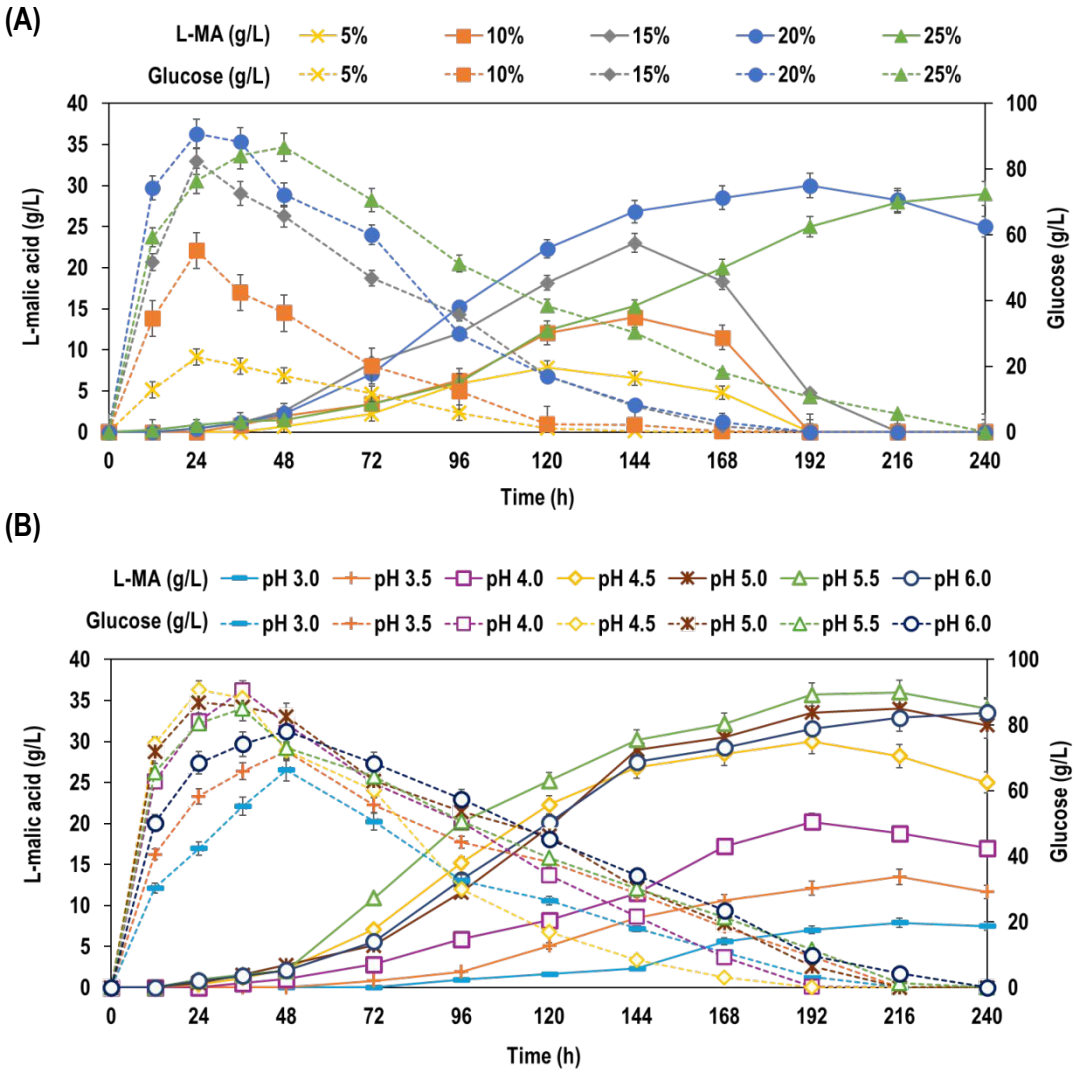

Figure 5. Time course profiles of glucose (dotted line) and L-MA (solid line) produced in the one-pot SSF culture of WPB. Effect of (A) solid loading (5-25% w/v), and (B) initial pH (3.0-6.0). Symbols (A): cross (5%w/v), square (10%w/v), rhombus (15%w/v), circle (20%w/v), and triangle (25%w/v). (B): minus (pH 3.0), plus (pH 3.5), empty square (pH 4.0), empty rhombus (pH 4.5), asterisk (pH 5.0), empty triangle (pH 5.5), and empty circle (pH 6.0).

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Following optimization of solid loading (20% w/v), the next steps involved optimization of physicochemical parameters, including inoculum size, shaking speed, and pH. These factors are crucial in determining fermentation kinetics and overall process efficiency. The optimum shaking speed of 200 rpm and inoculum size of 2.5% v/v allowed balanced growth and substrate conversion, maximizing L-MA yield (**Supplementary Figure S3A, S3B**). Given that the pH optimum for AMG was 4.5, and L-MA productivity was best at pH 6.0 in SHF, it was imperative to investigate the influence of pH on the fermentation profile of the SSF process (**Figure 5B**). Acidic conditions (pH <4) resulted in minimized enzymatic activity as well as L-MA productivity. As the pH increased, the L-MA production efficiency gradually improved, reaching a maximum titer of 36 g/L at pH 5.5, with a production rate of 0.176 g/L/h. This corresponded to an elevated yield of 45 $g_{L\text{-}MA}/Kg_{WPB}$. Notably, despite low enzymatic activity at higher pH (5.0-6.0), the L-MA yield remained substantial. The final optimized process yielded 47.5 g L-MA per Kg WPB. This suggests that the metabolic functionality of the isolate is the predominant factor deciding the conversion efficiency of WPB in the SSF process, even when the glucose release was slower. These findings indicate that BD could be efficiently incorporated into an SSF process for L-MA production using WPB.

3.6 Semi-simultaneous saccharification and fermentation of WPB

The semi-SSF bioprocess is a sequential one-pot strategy that incorporates a short pre-hydrolysis stage, involving partial biomass breakdown, prior to inoculation. This hybrid approach integrates the positive aspects of both the SHF (high initial glucose supply) and SSF (minimized product inhibition) processes, potentially enhancing the bioconversion efficiency with minimum cost and fewer processing steps.[26,30] Studies have demonstrated improved yield of bioethanol from a semi-SSF system as compared to standard SHF and SSF processes.[26,30,61] This technique has been particularly useful with high biomass loadings.[26,62] Schmatcz showed that a 24 h pre-hydrolysis of delignified sugarcane bagasse resulted in 87% conversion of cellulose, yielding 15.2 g/L ethanol.[61] In a study on cashew apple bagasse, better bioethanol conversion efficiency was achieved in a semi-SSF with 24 h of pre-hydrolysis (84.6%) compared to 12 h (72.4%), while the SSF process resulted in

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65.5% conversion efficiency.[30] This suggests that the efficiency of a semi-SSF process largely depends on the duration of the initial hydrolysis step.

Aiming to improve the efficiency of WPB bioconversion into L-MA, the semi-SSF approach was employed. In this context, the optimized SSF conditions (solid loading: 20% w/v; shaking speed 200 rpm; inoculum size: 2.5% v/v; pH: 5.5) were tested with varying pre-hydrolysis time (0-48 h), and resultant glucose and L-MA levels were monitored (**Figure 6A**). The theoretical maximum glucose concentration achievable with complete saccharification of 20% (w/v) WPB solids was calculated to be 205 g/L. Partial hydrolysis of WBP with 5 U/g AMG for 12, 24, 36, and 48 h resulted in glucose concentrations of 60.1, 95.4, 115.4, and 125.2 g/L, respectively, at the onset of fermentation. During the first 24 h of fermentation, the glucose levels continued to rise as the hydrolysis proceeded, while simultaneously being consumed by the growing fungus. Higher initial glucose levels boosted fungal metabolism, leading to an enhanced rate of assimilation and production. The maximum L-MA titre of 45.5 g/L was obtained with a pre-hydrolysis of 24 h. This corresponded to a 20% improvement in yield (56.9 $g_{L\text{-}MA}/Kg_{WPB}$) in comparison to the SSF (47.5 $g_{L\text{-}MA}/Kg_{WPB}$) without a pre-hydrolysis step. The L-MA began to degrade beyond the complete consumption of glucose, which occurred within 8 days of fermentation. Interestingly, extending the pre-hydrolysis step beyond 24 h did not improve L-MA production; instead, a slight decrease in titer was observed. High glucose levels appear to downregulate the metabolic pathways responsible for L-MA synthesis. Additionally, enzyme activity may also be suppressed due to excessive product accumulation, which occurs without simultaneous microbial consumption. Hence, maintaining an optimal balance between sugar generation and fungal uptake is crucial for ensuring maximum conversion efficiency.

Despite the use of waste substrates, the cost of the enzyme for the saccharification process substantially drives up the production cost[63]. Therefore, minimizing enzyme usage is crucial to ensure an economically feasible bioprocess. To address this, the influence of enzyme dosage was evaluated in the semi-SSF conditions (**Figure 6B**). The availability of free glucose at the onset of fermentation depended on the enzyme loading, ranging from 35.8 g/L at 0.1 U/g to 120.5 g/L at 10 U/g, with a 24-hour pre-hydrolysis. The glucose supply remained steady during the initial stages of fermentation as saccharification continued concurrently. The L-MA production significantly improved with an increase in enzyme loading up to 2 U/g, reaching a maximum titer of 48 g/L with

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a yield of 60 $g_{L\text{-}MA}/Kg_{WPB}$. However, at higher enzyme dosage (5 and 10 U/g), the L-MA production was slightly reduced, possibly due to substrate inhibition. These findings confirm that in the sequential one-pot semi-SSF system, the enzyme efficacy is greatly enhanced as compared to the complete SSF mode. This could be attributed to catabolic synergy between the enzyme and the microorganism, and to reduced glucose-mediated inhibition of the enzyme/*A. terreus*, pre-hydrolysis boost, and continued availability of the enzyme for complete breakdown of biomass. These combinatorial effects elevate the saccharification dynamics and bioconversion efficiency, even at lower enzyme dosage. The optimized semi-SSF process achieved a balanced carbon flux by effectively synchronizing glucose supply with microbial uptake, ultimately resulting in a high L-MA yield.

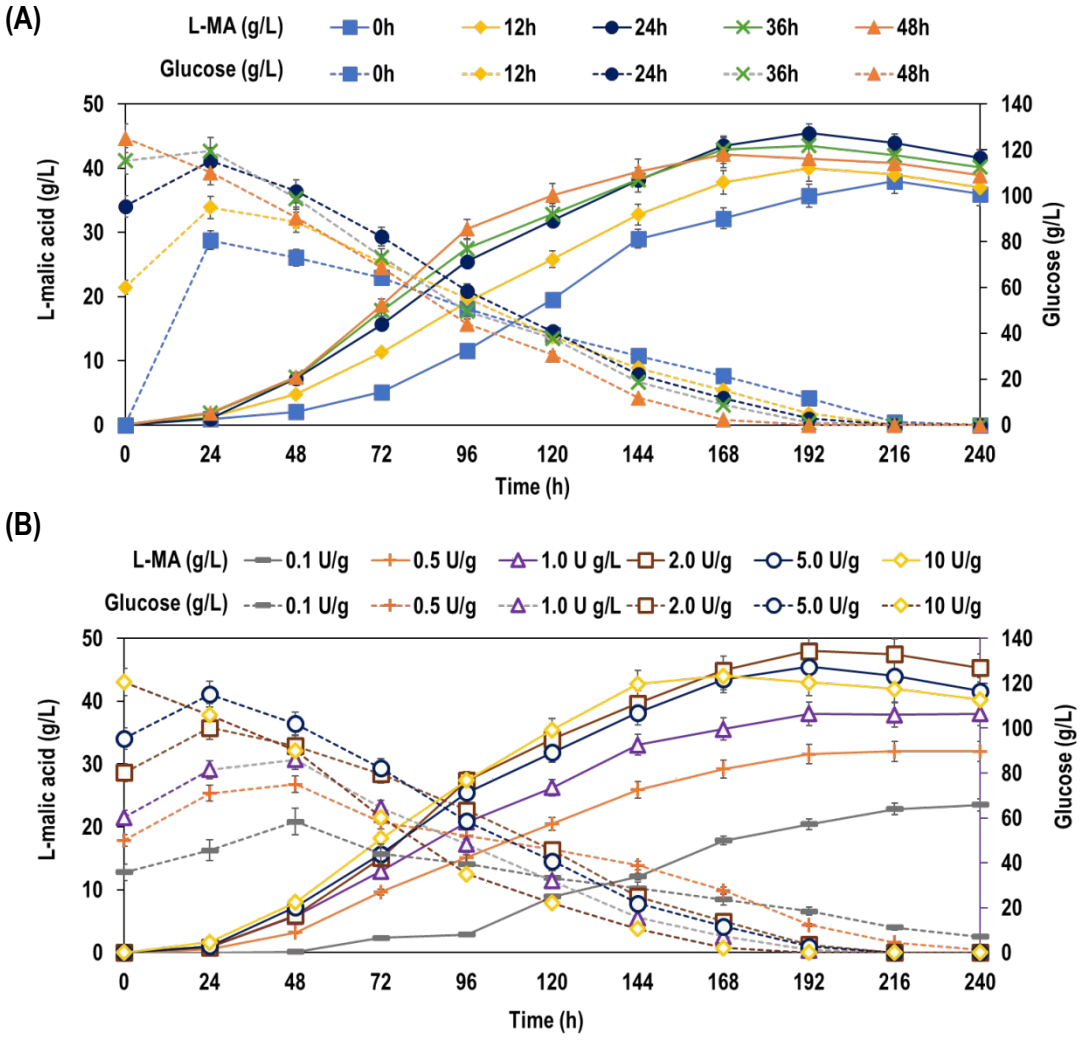

Figure 6. Time course profiles of glucose (dotted line) and L-MA (solid line) produced in the one-pot semi-SSF culture of WPB. Impact of (A) pre-hydrolysis time (0-48 h), and (B) enzyme dosage (0.1-10 U/g). Symbols (A): square (0h), rhombus (12h), circle (24 h). cross (36 h), triangle (48h) (B): minus (0.1 U/g), plus (0.5 U/g), empty triangle (1.0 U/g), empty square (2.0 U/g), empty circle (5.0 U/g), and empty rhombus (10 U/g).

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Previous studies on L-MA production from crude feedstocks have either employed by-products of the fuel industry (crude glycerol, thin stillage) or hydrolysates of agricultural residues (**Table 2**). SSF-based L-MA synthesis has remained unexplored due to the temperature incompatibility of standard L-MA producers. A single report on poly(β-L-malate) production from *Aureobasidium pullulans* showed SSF of liquefied corn starch, which produced 49 g/L in a 10 L fermenter.[64] In the current study, owing to its peculiar attributes of producing L-MA at high temperatures without $CaCO_3$ supplementation, the BD isolate demonstrated a strong potential towards SSF or semi-SSF based L-MA production from an agro-industrial waste through a one-pot bioprocess. The shake flasks experiments establish a proof-of-concept one-pot (SSF/semi-SSF) process for L-MA biosynthesis from WPB, a configuration that, to our knowledge, has not yet been reported in the literature. The flask-scale set-up was used to demonstrate process feasibility and to identify key process parameters influencing the productivity of the *A. terreus* BD under one-pot conditions using WPB. Future studies will focus on translating this one-pot bioconversion process to controlled, aerated bioreactors, where oxygen transfer, mixing, and heat removal can be quantitatively characterised and optimised. Furthermore, integrated techno-economic analysis and life-cycle assessment will be conducted to quantitatively evaluate sustainability metrics and validate the industrial feasibility of the proposed process.

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Table 2: Summary of L-MA production from waste-derived feedstocks

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Microbial source	Carbon source	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
A. niger ATCC 9142	Thin stillage	17	0.8	0.09	[65]
A. niger ATCC 10577		19	0.8	0.10	
Zygosaccharomyces rouxii	Crude glycerol	72.1	-	0.15	[66]
A. niger PJR1	Crude Glycerol	77.4	1.28	0.4	[67]
U. trichophora TZ1	Crude glycerol	196	0.82	0.39	[68]
A. oryzae DSM 23081	Pyrolytic aqueous condensate	9.77	0.23	0.041	[69]
A. oryzae DSM 23081	Beechwood cellulose hydrolysate - SHF	37.9	0.97	0.23	[56]
	Beechwood hemicellulose fraction - SHF	5.8	-	0.03	
	M. giganteus cellulose hydrolysate - SHF	30.8	0.32	0.18	
Rhizopus. delemar HF-121	Corn straw hydrolysate - SHF	120.5	0.96	2.01	[55]
Engineered *T. fusca* muC	Cellulose	62.7	0.62	0.51	[70]
	Corn stover	21.7	0.43	0.18	
Acetobacter tropicalis H1	Corn hull hydrolysate - SHF	77.1	0.77	0.64	[71]
A. wentii MTCC 1901	Cassava peel waste hydrolysate – SHF	33.1	0.34	0.14	33
A. terreus BD	Potato waste hydrolysate – SHF	36.9	0.24	0.15	This study
	Potato waste – SSF (one-pot)	38	-	0.20	
	Potato waste – SemiSSF (one-pot)	48	-	0.25	

3.7 Downstream processing of L-MA from the fermentation broth

Downstream processing is one of the key bottlenecks in microbial L-MA production, contributing to around 30-40% of the manufacturing cost.[72] Fewer studies exist in the literature that deal with the downstream processing of L-MA. The reactive extraction is gaining popularity as a high efficiency (>90%) technique, which could be

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coupled with the ongoing fermentation (*in-situ*) process.[73,74] However, this approach involves complexation of L-MA with a tertiary/quaternary amine, followed by extraction in a solvent, where recovery and reuse of each component (L-MA, amine, solvent) is complicated and expensive. Crystallization is another approach reported for L-MA recovery; however, recovery from a waste-derived medium, containing different components, is challenging. A study showed the recovery of 28% L-MA accumulated from beech wood hydrolysate using crystallization.[75] The recovery of L-MA from fermentation broth is particularly challenging due to its high water solubility (363 g/L) and the use of $CaCO_3$ in the fermentation in previous studies.[17] The precipitated Ca-malate needs to be treated with H_2SO_4 to recover the L-MA, which leads to the formation of solid gypsum ($CaSO_4$), posing challenges in its disposal and environmental impact. In our previous study, a simple one-step downstream processing method was developed, which involved *n*-butanol extraction. The formation Fof gypsum was eliminated by using HCl, in place of H_2SO_4, to solubilize the precipitated Ca-malate and $CaCO_3$. However, a large volume of HCl was required, which diluted the culture and made it acidic. Consequently, a moderately low recovery (31%) of L-MA was achieved.[33]

In the current study, the isolate could produce L-MA without $CaCO_3$ supplementation, which eliminated the need for acid solubilisation, potentially allowing better recovery. The pH of the fermentation broth was reduced to 3.0 with HCl prior to extraction, ensuring that the pH remained below pKa_1 (3.4) and that the majority of L-MA was present in the protonated form. An equal volume of *n*-butanol was added to the fermentation "pot" and allowed to mix thoroughly (200 rpm, 3h) for partitioning of the L-MA into the organic layer. The process was repeated after manual separation of the solvent. The pooled solvent acquired a brown colour due to coextracted potato and fungal pigments and yielded a coloured precipitate upon evaporation. To address this, the solvent was treated with activated charcoal (2% w/v), which adsorbed the pigments and generated a colourless extract, leading to a white precipitate upon evaporation. Finally, the downstream strategy resulted in the recovery of 58% L-MA with 75% purity from the one-pot culture, and 65% recovery with 80% purity from the SHF culture. Due to the polar nature of MA, liquid-liquid extraction typically requires polar organic solvents, which are inherently partially miscible with water.[33] Among these, *n*-butanol offers a favorable balance as its water solubility (73 g/L at 25 °C) is lower than that of many alternative polar solvents, while still maintaining sufficient affinity with L-

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MA.[76]. In the current process, phase separation is maintained due to the limited mutual solubility between *n*-butanol and the aqueous phase, enabling recovery of a butanol-rich organic layer. Furthermore, the effective solubility of *n*-butanol in fermentation broth is expected to be lower than in pure water due to salting-out effects arising from dissolved salts and metabolites, which enhances phase separation, and solvent loss remains relatively limited (<7%). The solvent recovered after evaporation and condensation was effectively recycled into the subsequent batch of solvent extraction. This method enabled direct recovery from the same "one-pot system" at the end of fermentation without complex processing in multiple compartments. It also does not involve extra chemicals or reactions, and allows solvent reusability. Thus, this process presents a sustainable and cost-effective approach towards high-yield recovery of L-MA produced from the WPB-based one-pot bioprocess suitable for industrial applicability. Additional downstream optimization strategies, such as salt-induced phase separation (salting-out extraction) and fine-tuning of aqueous phase pH adjustment, may be implemented in future studies to further improve solvent recovery and extraction efficiency, as demonstrated in our previous work on itaconic acid.[77]

4. Conclusion

This study highlights the exceptional metabolic versatility and thermotolerance of a fungal isolate, *A. terreus* BD, establishing its potential as a robust microbial platform for biotechnological applications. The strain demonstrated condition-specific production of IA and L-MA, regulated by differential gene expression under varying pH and nitrogen conditions. Importantly, its ability to produce high levels of L-MA at elevated temperatures (45°C) without the need for $CaCO_3$, from the enzymatic hydrolysate of potato waste (EHWP), underscores its industrial relevance. With such interesting attributes, the isolate was implemented into a one-pot bioprocess, which provided a streamlined, scalable, and cost-effective approach with enhanced L-MA productivity, achieving an impressive yield of 60 g L-MA per kg of waste potato biomass (WPB). Further, the solvent-extraction-based downstream approach allowed efficient L-MA recovery (58%) directly from the fermentation "pot" with minimum processing steps. Overall, this research is a comprehensive study of the peculiar thermotolerant *A. terreus* BD isolate, dealing with its gene expression profiles, defining synthetic media

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conditions for L-MA and IA production, and channelizing its potential to develop a more industrially relevant and sustainable route for L-MA production using a starch-rich agro-industrial waste. This work lays a strong foundation for future applications of *A. terreus* BD in circular bioeconomy frameworks aimed at waste valorization and industrial synthesis of biochemicals. Future work will apply multivariate design-of-experiments approaches, such as response surface methodology, to quantify interaction effects among key parameters and refine the one-pot process conditions. Further work will focus on bioreactor-scale studies with controlled aeration and mixing to quantify oxygen-transfer limitations and validate the scalability of the one-pot L-MA process beyond shake-flask conditions. Additionally, metabolic engineering strategies would be applied to *A. terreus* BD to further improve organic acid production, leveraging its unique metabolic capabilities.

Author Contributions

Deeksha Gopaliya: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Nitin Srivastava:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Vinod Kumar:** Conceptualization, Supervision, Writing – review & editing. **Sunil Kumar Khare:** Conceptualisation, Supervision, Resources, Writing – review & editing.

Conflicts of 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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The data supporting this article have been included as part of the Supplementary Information.

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