

Characterization and Optimization of L-Malic Acid Production by Some Clinical Isolates of *Aureobasidium pullulans*



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Citation Jafarian-Haris T, Tavakkoli AR, Najafzadeh MJ, Danesh A. Characterization and Optimization of L-Malic Acid Production by Some Clinical Isolates of *Aureobasidium pullulans*. Research in Molecular Medicine. 2020; 8(4):209-214. <https://doi.org/10.32598/rmm.8.4.1173.1>

doi <https://doi.org/10.32598/rmm.8.4.1173.1>



Article Type:
Research Paper

Article info:
Received: 30 Aug 2020
Revised: 28 Sep 2020
Accepted: 26 Oct 2020

Keywords:
Poly L-malic acid, L-Malic acid, *Aureobasidium pullulans*, Fungal natural products

ABSTRACT

Background: Poly-L-malic acid (PLMA) comprises aliphatic polyester polymers with broad applications in pharmaceutical industries. The fungal microorganisms are among the best natural sources recruited to supply L-malic acid (MA) as a precursor of PLMA. In this study, we investigated MA production ability of 7 clinical isolated of the fungus *Aureobasidium pullulans*.

Materials and Methods: Seven clinical isolates of *A. pullulans* acquired from Westerdijk Fungal Biodiversity Institute were studied, and the isolate with the highest total MA production was selected for the optimization process. We tried to optimize the output by applying different concentrations of CaCO₃ in fungus medium (1.5%, 3%, and 6%) and various incubation temperatures (27°C, 32°C, and 37°C) during 3, 7, and 14 days.

Results: Intra-strains variation was significantly strong ($P < 0.0001$), and the highest production of MA was carried out by the isolate *A. pullulans* var. *melanigenum* dH 21931, UTHSC 06-456. The amount of MA produced by this strain was significantly higher in medium with 3% CaCO₃ compared with other concentrations of CaCO₃ and after 7 days incubation than the other fermentation times ($P < 0.05$). Although MA production was higher at 27°C, the differences between the investigated various temperatures were not significant ($P > 0.05$).

Conclusion: Overall, we obtained the highest MA production in Sabouraud dextrose agar (SDA) medium with 3% CaCO₃ at 27°C after 7 days of incubation. Our study indicated that the fermentation period and CaCO₃ concentration significantly alter MA production in *A. pullulans* var. *melanigenum*.

1. Introduction

P

oly L-malic acid (PLMA) consists of repetitive L-malic acid (MA) units, a four-carbon dicarboxylic acid unit polymers that are widely used as the second-generation

of biomaterials for drug delivery systems [1]. Depending on the connection of their MA units, these polymers have three forms of poly- α , poly- β , and poly- α,β , each of which could be synthesized chemically or obtained from living organisms. The β -type has the broadest applications in drug-carrier complexes because of its versatile pen-

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dant α -carboxyl groups, which provide sites for chemical modifications to target the delivery of drugs [2]. PLMA could also be easily ionized in water mediums and provide highly soluble poly-carboxylates [3]. In this condition, it can conjugate with cationic compounds such as anticancer drug doxorubicin during a stable ionic reaction. Additionally, these carboxylic polymers are suitable candidates for pH-sensitive drug delivery systems [4].

At present, the main production method of MA as a precursor of PLMA is the hydration of fumaric acid or maleic acid under high pressure and temperature or fumarate biotransformation by fumarase enzyme. However, these processes require the malic anhydride-derived from petroleum- or fumaric acid and relatively expensive catalysts and therefore are limited due to economic issues [5-7]. Therefore, recent research is focused on developing microbial sources for MA production by using renewable raw materials. Several different microorganisms are studied for this purpose. Some of them such as *Zygosaccharomyces rouxii*, *Brevibacterium flavum*, *Saccharomyces cerevisiae*, and different *Aspergillus spp.* as well as some metabolically engineered *Escherichia coli* can produce MA from glucose or other carbohydrates [9-14].

A. pullulans is a melanized yeast-like saprophytic fungus, which is widely found in the environment and can cause rare infections such as fungemia and phaeohyphomycosis in immunodeficient and critically-ill patients [15, 16]. This fungus is currently applied for commercial production of a food additive namely pullulan which is a polymer of maltotriose. It is also used to bio-remediate some environmental pollutions, such as oil spillage pollutions [17]. Some studies reported different antifungal and anti-bacterial metabolites from this fungus [18, 19]. Moreover, some other research studies have shown that *A. pullulans* can encode some valuable enzymes such as alkaline protease [20] and endo-1,4- β -xy lanase [21].

Additionally, several studies have indicated that *A. pullulans* could be considered a good potential source for PLMA supply [22-24]. The question is if opportunistic pathogenic isolates of *A. pullulans* can produce MA and what nutritional elements can alter their MA production. In this study, we investigated the ability of some clinical isolates of *Aureobasidium* for MA production. We tried to optimize production and increase efficiency by altering some environmental and nutritional factors.

2. Methods

Chemicals

Sabouraud dextrose agar (SDA) medium, glucose, CaCO_3 , KH_2PO_4 , KCl, NaNO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and sulfuric acid were

purchased from Merck, Germany. L-malic acid assay kit (Manual Format) was obtained from Megazyme, Ireland.

Fungal samples preparation

At first, a collection of 40 fungal isolates of *Aureobasidium spp.* were received from Prof G. Sybren de Hoog collection in Westerdijk Fungal Biodiversity Institute, the Netherlands (presented by dH codes). Each of the 40 types of fungi was inoculated into two tubes containing the SDA medium. The samples were then incubated at 25°C for 4 days, and among them, only 7 isolates were recovered (Table 1). Next, each of these 7 isolates was inoculated to three 250-mL Erlenmeyer flasks (in addition to three ones without any inoculation for the negative standard), containing a liquid medium consisting of glucose (12 g), CaCO_3 (3 g), NaNO_3 (0.2 g), KH_2PO_4 (0.01 g), KCl (0.05 g) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02 g) in 100 mL distilled water. CaCO_3 solution was prepared and autoclaved separately due to the possible reaction with other medium components in high temperatures. All flasks were sterilized by autoclaving at 121°C and incubated in a shaker incubator for 7 days at 27°C. The fermented mediums were then transferred to Falcon tubes and centrifuged (8000 rpm for 30 minutes), and the resulted supernatants of each isolate were transferred equally to test tubes. The tubes were treated with the same volume of sulfuric acid (2 M) and incubated at 100°C for 12 hours to hydrolyze PLMA into MA. The prepared test samples were examined by L-malic acid assay kit based on their UV absorbance at 340 nm. The final concentration of MA in fermented mediums was calculated and reported as g/L.

Optimization procedure

Based on the previous step's results, the isolate dH 21931, UTHSC 06-456, which produces the highest amount of MA, was selected for the optimization process (Figure 1). First, the selected isolate was fermented as per the previously-described method except for the difference in medium CaCO_3 concentration, which was adjusted as different states of 1.5%, 3%, and 6% to find in which condition the highest production of MA occurs. Next, the best CaCO_3 concentration was fixed, and the flasks were incubated at different temperatures (27°C, 32°C, or 37°C) for different periods (3 days, 7 days, or 14 days). Each of the specific planned conditions of fermentation (including temperature, time, and CaCO_3 concentration) was carried out in three separate flasks, and reported values were the average of their results.

Statistical analysis

GraphPad Prism software v. 8.3.0 for Windows (GraphPad Software, La Jolla California USA) was used for statistical

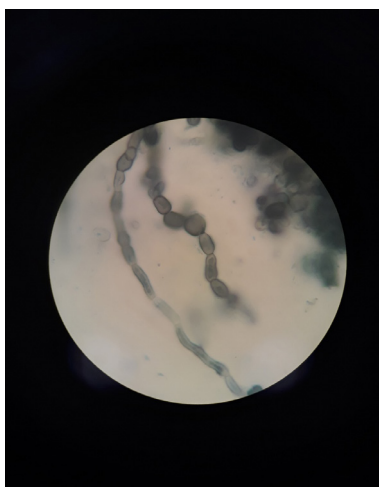


Figure 1. Melanized conidiophores (right) and short hypha (left) of *A. pullulans* var. *melanigenum* dH 21931, UTHSC 06-456 on Sabouraud dextrose agar medium×1000

analysis. The results were presented as Mean±SD. The Shapiro-Wilk pretest was used to confirm the normal statistical distribution in each group. One-way or 2-way ANOVA and Tukey-Kramer posttest were used to compare the different groups. P<0.05 was considered statistically significant.

3. Results

Screening of the isolates

As shown in Figure 2, the highest MA production was obtained by the isolate dH 21931, UTHSC 06-456. The intra-isolate variations were strongly significant (P<0.0001), as the highest strain production was 8-fold more than the lowest one.

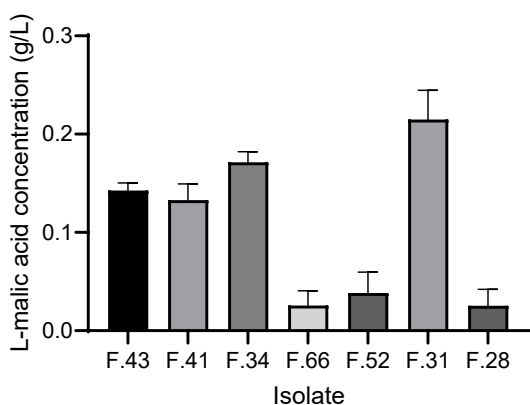


Figure 2. MA concentration in fermented medium of seven selected fungal isolates, after 7 days of incubation at 27°C. The results are shown as Mean±SD (n=3).

Optimization results

Figure 3 shows the average amount of MA produced by isolate dH 21931, UTHSC 06-456 in different concentrations of CaCO₃. The results showed that the highest amount of MA production happened with 3% CaCO₃ to the culture medium, and subsequently 6% and 1.5%. Statistical analysis with 1-way ANOVA showed a significant difference between these three concentrations in the production of MA (P=0.0413). Tukey-Kramer posttest indicated that in multiple comparisons, the significant difference is only observed in the case of 1.5% vs. 3% concentrations.

Table 2 presents the amount of MA produced by dH 21931, UTHSC 06-456 isolate on 3, 7, and 14 days after inoculation, at different temperatures (27°C, 32°C or 37°C). As indicated in Figure 4, the best output was achieved after 7 days at 27°C. Statistical analysis by 2-way ANOVA test showed no significant difference between distinct incubation temperatures (P>0.05). Still, the fermentation period had a significant effect on MA amount in general (P=0.0288). The Tukey-Kramer posttest indicated that in multiple comparisons, a significant difference is observed in the case of 3 days vs. 7 days (P=0.0226). This correlation was stronger in the case of 27°C temperature, and 7 days yield was significantly higher than 3 days (P=0.0115).

4. Discussion

Fungal microorganisms are among the notable sources of green production of MA [25, 26]. Considering some of the previous studies, which had highlighted the effect of incubation temperature and CaCO₃ concentration in MA production by fungal microorganisms such as *Zygosaccharomyces*

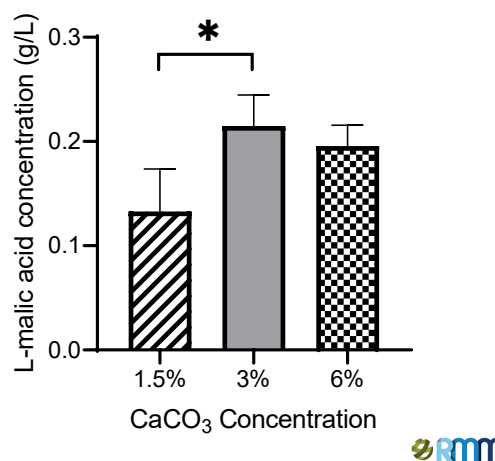


Figure 3. MA concentration in dH 21931, UTHSC 06-456 isolate medium with different concentrations of CaCO₃ at 27°C after 7 days of incubation. Results are shown as Mean±SD (n=3). * Indicates P<0.05 and different significance.

Table 1. Characteristics of seven investigated isolates

No.	Identification	Code	Source	Country
F.43	<i>A. pullulans</i> var. <i>pullulans</i>	dH 21943, UTHSC 07-2037	Cerebrospinal fluid	The USA
F.41	<i>A. pullulans</i> var. <i>melanigenum</i>	dH 21941, UTHSC 07-1927	Bronchial wash	The USA
F.34	<i>A. pullulans</i> var. <i>melanigenum</i>	dH 21934, UTHSC 07-22	Ethmoid sinus	The USA
F.66	<i>A. pullulans</i> var. <i>melanigenum</i>	dH 21966, UTHSC 10-1003	Bronchial wash	The USA
F.52	<i>A. pullulans</i> var. <i>pullulans</i>	dH 21952, UTHSC 08-1356	Toe nail	The USA
F.31	<i>A. pullulans</i> var. <i>melanigenum</i>	dH 21931, UTHSC 06-456	Forehead	The USA
F.28	<i>A. pullulans</i> var. <i>melanigenum</i>	dH 21928, UTHSC 03-3450	Bone marrow	The USA



rouxii and *Schizopyllum commune* [26], we decided to investigate these factors effects in the case of *A. pullulans* var. *melanigenum*.

CaCO₃ addition into the fermentation medium is performed for two major purposes: to provide CO₂ for the reductive Tricarboxylic Acid Cycle (TCA) pathway, where pyruvate is converted to oxaloacetate and then malate, and buffer the pH at ~6.0 [27]. It has been well documented that the con-

centration of CaCO₃ in the medium is contributed to PLMA production by *A. pullulans*, but the reports are controversial. Zhang et al. (2011), for example, reported 4% CaCO₃ as the optimum CaCO₃ concentration for PLMA production by *A. pullulans* [10], while another research reported that a medium containing 6.5% of CaCO₃ is ideal [28]. On the other hand, Cao et al. has obtained the highest PLMA production by adding 1.5% CaCO₃ to *A. pullulans* medium [29]. The present study proved a significant correlation between

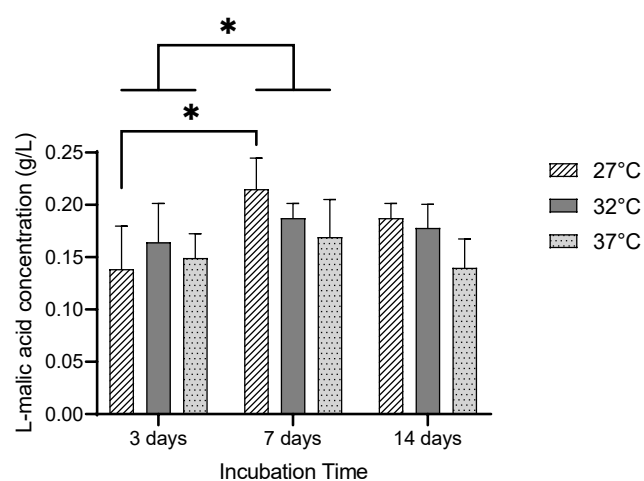


Figure 4. MA concertation in dH 21931, UTHSC 06-456 isolate medium with different fermentation temperatures and times. The results are presented as Mean±SD (n=3). *Indicates P<0.05 and different significance.

Table 2. MA concertation in dH 21931, UTHSC 06-456 isolate medium with different fermentation temperatures and times

Incubation Time	27°C	32°C	37°C
3 days	0.138±0.041 g/L	0.164±0.037 g/L	0.149±0.023 g/L
7 days	0.215±0.029 g/L	0.187±0.014 g/L	0.169±0.035 g/L
14 days	0.187±0.014 g/L	0.178±0.022 g/L	0.140±0.027 g/L



CaCO₃ concentration and MA production by *A. pullulans*; although, we achieved the highest MA production (0.21±0.02 g/L) by adding 3% CaCO₃ in *A. pullulans* fermentation medium. The observed differences in the CaCO₃ concentration effect may be contributed to intra-strains variations; however, further investigations are needed.

Besides, our findings represented that MA concentration was highest after 7 days of fermentation and then decreased to the 14th day. We measured total MA in our samples by treating them with sulfuric acid (2 M) before assay by kit, which hydrolyzes the present PLMA into MA [26]. It is shown that in fungal cells, the production of PLMA as a major form of MA is associated with cell growth in the early exponential growth phase [27, 30], so the end of exponential growth in the second week of fermentation could be the cause of the observed MA level decrease.

5. Conclusion

MA production ability significantly differs between clinical isolates of *A. pullulans*. By studying the role of medium CaCO₃ concentration and incubation time and temperature on MA production on the isolate dH 21931, UTHSC 06-456 of *A. pullulans* var. *melanigenum*, we found that the output is significantly affected by medium CaCO₃ concentration and fermentation period. Overall, the highest amount of MA production was obtained after 7 days of incubation at 27°C, with a medium contained 3% CaCO₃. The limitations of our study include the low scale of fermentation and missing some other potentially useful factors on MA biosynthesis yield, such as pH and trace elements of the medium. Besides, we propose to perform additional studies on other *Aureobasidium spp.* and genetic studies on the MA biosynthesis pathway.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them.

Funding

This work was supported by Mashhad University of Medical Sciences (Grant No.: 921255).

Authors' contribution's

Conceptualization and Supervision: Abolghasem Danesh; Methodology: Mohammad Javad Najafzadeh, Abolghasem Danesh; Investigation, Writing – original draft, and Writing – review & editing: All authors; Data collection: Taha Jafarian-Haris; Data analysis: Taha Jafarian-Haris, Alireza Tavakkoli; Funding acquisition and Resources: Mohammad Javad Najafzadeh, Abolghasem Danesh.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

This work was supported by Mashhad University of Medical Sciences (Grant No.: 921255).

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