



A Comparative Study on the Biodegradation of 17 β -Estradiol by *Candida utilis* CU-2 and *Lactobacillus casei* LC-1

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The release and fate of estrogens have attracted more and more public attention. Biodegradation is an important method for estrogen removal from the environment. However, few comparative studies concentrated on the degradation of 17 β -estradiol (E2) by fungi and bacteria. In this study, the removal efficiencies of E2 by fungi (*Candida utilis* CU-2) and bacteria (*Lactobacillus casei* LC-1) were investigated through influencing factors, kinetics, and biodegradation pathways. The results demonstrated that both *C. utilis* CU-2 and *L. casei* LC-1 have the same degradation efficiency, and they can effectively degrade E2 (10 μ M) with nearly 97% degradation efficiency. However, the biodegradation efficiency of the two strains only reached 20% when E2 was used as a sole carbon source, while it increased to 97% with 1.2 g/L sucrose, glucose, or sodium acetate supply, indicating the occurrence of co-metabolism. In addition, the results indicated that 35°C and 0.6 g/L sucrose favored the degradation. However, the addition of excessive carbon sucrose (10 g/L) significantly inhibited the biodegradation of E2. Besides, the degradation of E2 with ~0–10 g/L sucrose as co-substrate followed the first-order kinetics well. Through intermediate products analysis, 12 degradation products were identified, and they were mainly produced via hydroxylation and methylation, among others, among which C₁₄H₂₂O₄ (m/z:[M + H]⁺ = 255) was detected as the product with the smallest amount of carbon in this study. Based on the detected products and previous studies, five biodegradation pathways were proposed. To our knowledge, there are few reports about the comparisons of E2 removal between fungi and bacteria. Moreover, the results confirmed that the strain CU-2 and the strain LC-1 may have similar degradation characteristics and metabolic mechanisms in the degradation of E2. This study may provide a promising bio-treatment method with low energy consumption for E2 removal from aqueous environments and help in understanding their biodegradation mechanisms.

Keywords: biodegradation, 17 β -estradiol, co-metabolism, degradation products, pathways

INTRODUCTION

Natural estrogens, including 17 β -estradiol (E2), estrone (E1), and estriol (E3), mainly come from human activity and animal excretion (Ying et al., 2002; Zhao et al., 2019). With the growth of population and the demand for livestock products, the release of estrogens to the environment significantly increased in recent years (Wise et al., 2011; Whitman, 2017). Besides, the reports on the exposure of estrogens in the environment increased gradually. Lange et al. (Lange et al., 2002) reported that the annual estrogen contents coming from the excretion of farm animals were ~33–49 tons in the European Union and the US. In addition, estrogens could cause long-term toxicity to organism, especially to the endocrine and reproductive organs (Cevasco et al., 2008). Previous studies reported that E2, as a typical type of estrogens (Li et al., 2019), was of great significance for the growth, maintenance, and contraception of female reproductive tissues (Sacdal et al., 2020), and it could induce a male to produce female characteristics even at very low concentrations (≥ 1 ng/L) (Peiris et al., 2020), showing its great harm to human and animal physiology and behavior (Young et al., 2002; Adeel et al., 2017). In Europe, intersex fish had been found near sewage treatment plants (Jobling et al., 2005). Besides, the phenomenon of intersex was also found in black basses (*Micropterus* spp.) in the US (Hinck et al., 2009). Moreover, Lambert et al. (2015) found that the offspring sex ratios of wild frogs in suburban ponds were affected by E2 exposure. Owing to its chemical stability, it was very hard to completely remove E2 in sewage treatment plants (Ting and Praveena, 2017). Therefore, it is of great significance to develop an effective method for E2 removal.

Until now, various treatment processes, such as physical methods, chemical oxidation, and microbial degradation, have been applied in the removal of E2. Physical methods such as activated carbon adsorption and membrane process have high operation cost and high energy consumption (Snyder et al., 2007). Chemical oxidation methods such as chemical treatment and electrochemistry also have the problem of high energy consumption (Klavarioti et al., 2009). While, microbial degradation technology was considered as a promising and effective method because of its advantages, including no secondary pollution, low energy consumption, high efficiency, low cost, etc (Yu et al., 2013). It was reported that many bacteria (*Rhodococcus* spp., *Sphingomonas* spp., *Pseudomonas* spp., etc.) and some fungi showed a high capacity to degrade E2 and convert it to harmless products (Hyungkeun and Kung-Hui, 2010). Fujii et al. (2002) found that *Novosphingobium* ARI-1 could completely degrade 5 mg/L of E2 in 20 days. Yu et al. (2016) reported that 1 mg/L of E2 could be completely degraded by *Rhodococcus* spp. DS201 with 1% inoculation quantity in 3 days at 30°C. Xiong et al. also claimed that a 90% removal efficiency of 10 mg/L of E2 could be achieved by the strain SJTH1 with the initial OD600 of 0.05 (Xiong et al., 2020). All these above strains could effectively degrade E2, but it was difficult to compare their degradation efficiencies due to different experimental conditions. Furthermore, co-substrate or co-metabolism also had important effects on the degradation of pollutants. The degradation ratio

of ciprofloxacin could be promoted with the presence of glucose and sodium acetate (Xiong et al., 2017). *Stenotrophomonas maltophilia* DT1 exhibited the highest degradation efficiency for tetracycline when glucose was added compared with when glycerol, lactose, or sucrose was added (Leng et al., 2016). Therefore, it can be inferred that the removal efficiency of E2 was significantly related to the species of strains and experimental conditions.

Khattab et al. (2018) investigated the removal of E2 by two kinds of bacteria, namely *Klebsiella* and *Bacillus*, and the degradation efficiencies of the strains of *Klebsiella* and *Bacillus* to E2 were 100 and 91.56%, respectively. Li et al. (2018) also investigated the removal efficiencies of E2 by two kinds of bacteria and found that the E2 removal efficiency by *Acinetobacter* LM1 was higher than that by *Pseudomonas* LY1. Except for the bacterial strains, three kinds of fungi (*Pycnoporus* spp. SYBC-L3, *Trametes versicolor* TV, and *Hymenochaete spreta* PL-1) were investigated, and the degradation ratios of E2 were 78.4, 62.7, and 14.5%, respectively (Liu et al., 2016). Although these studies have enhanced the comparability of E2 degradation efficiency, most of them only focus on single fungi or bacteria, and there are few studies on the comparison of E2 removal by fungi and bacteria. Thus, it is desirable to explore the difference and mechanism of E2 removal by fungi and bacteria.

Aside from these research studies, the degradation products and pathways of E2 have also been concerned. In the degradation process of E2, E1 was identified as the first step in previous studies (Shi et al., 2004). While other E2 conversion products have also been found in recent years, Kurisu et al. (2010) observed a new intermediate metabolite, named 4-hydroxyestradiol, and they affirmed that it was converted from the hydroxylation of the C-4 site on E2 rather than first converting E2 to E1. Besides, the formation of estratetanol (E0) also confirmed the fact that it was formed by the dehydration of the C-17 site on E2 (Nakai et al., 2011). Furthermore, another new degradation product X1 contained lactones in the D ring, which was proposed in a previous study (Yu et al., 2013), and X1 was considered to be further degraded by entering the tricarboxylic acid cycle. The results provided a new idea for the degradation pathway of E2. According to the previous studies, although some biodegradation intermediate metabolites of E2 have been reported, the degradation mechanism of E2 is not clear.

In this study, *Lactobacillus casei* LC-1 and *Candida utilis* CU-2 were selected to be the representative strains of bacteria and fungi for E2 removal. The degradation efficiency and mechanism of E2 by the two strains were explored. Furthermore, the differences in the following aspects were mainly compared: (i) the effects of carbon source substrate, sucrose concentration, and temperature on the biodegradation of E2; (ii) the biodegradation kinetics of E2 under different sucrose concentrations; and (iii) the intermediate products analysis and the degradation pathways of E2. This research may provide a new idea for E2 degradation through the comparative study of fungi and bacteria and elaborate their degradation mechanisms *via* the degradation pathways of E2.

MATERIALS AND METHODS

Chemicals and Materials

17 β -estradiol (purity \geq 98%) was purchased from Beijing InnoChem Science & Technology Co., Ltd. (Beijing, China). The concentration of E2 stock solution was 2 mM. It was prepared by dissolving 54.4 mg of E2 in 150 ml of methanol solution. Sucrose (purity 99%), glucose (purity 99%), and sodium acetate (purity 99%) were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). Methanol (chromatographic grade) and acetonitrile (chromatographic grade) were purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). The Milli-Q ultrapure water (18.2 M Ω) used in this study was provided by Beijing Wahaha Company (Beijing, China).

A mineral medium (MM) containing 1.5 g/L K₂HPO₄, 0.5 g/L KH₂PO₄, 0.2 g/L MgSO₄, and 1.5 g/L NH₄Cl was used for the enrichment of estrogen-degrading strains (Cao et al., 2019). A Luria–Bertani medium (LB) containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl was used for the cultivation of the strain CU-2, and strain LC-1 was cultivated with a minimal salt medium (MSM), which was composed of 10 g/L casein peptone, 10 g/L beef extract, 5 g/L yeast extract, 5 g/L glucose, 5 g/L sodium acetate, 2 g/L diamine citrate, 1 g/L Tween 80, 2 g/L K₂HPO₄, 0.2 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄·H₂O, and 20 g/L CaCO₃. A solid medium was prepared by adding 15 g/L agar to the medium above.

Strain Sources and Cultivation Conditions

The strains were purchased from the China General Microbiological Culture Collection Center (CGMCC), and they were *L. casei* CGMCC 1.29 (LC-1) and *C. utilis* CGMCC 2.2878 (CU-2), respectively.

The strains were activated by the solid medium and cultured at 25°C. After about 2 days of cultivation, colonies were obtained, and then, a single colony was picked and inoculated into liquid medium at 30°C for 36 h to prepare for the next experiment. The strain CU-2 and strain LC-1 were enriched in LB and MSM, respectively.

Morphological Observation of Strains

The activated strains were observed by biochemical methods. The cells cultured for 24 h were treated with Gram staining, and the Gram reaction was observed with an optical microscope (Shanghai Yongheng Optical Instrument Manufacturing Co., Ltd., XSP-63XD, Shanghai, China).

Biodegradation Experiments

The biodegradation experiments were performed with strain CU-2 and strain LC-1 in 150-ml Erlenmeyer flasks containing 75 ml MM. All experiments were performed in a shaker with a speed of 150 rpm, and three parallel experiments were conducted for each group. *C. utilis* CU-2 and *L. casei* LC-1 reached an exponential growth phase in liquid LB medium and liquid MSM for about 24 h, and then, cells were harvested by centrifugation at 4,500 rpm for 5 min under ambient temperature. In this step, the strain was washed twice by sterilized 0.9% NaCl solution (pH = 7.0) and then resuspended in 0.9% NaCl solution, and the concentration of cells in the strain were adjusted to optical density (OD) 600

= 0.8 (Pan et al., 2017). Finally, the treated bacterial solution was added to 75 ml MM (2%, v/v) in a 150-ml Erlenmeyer flask. Based on preliminary experimental results, three influencing factors, namely, carbon substrate (glucose, sucrose, and sodium acetate, all at 1.2 g/L), sucrose concentration (0.05–10 g/L) and temperature (15, 25, 35, and 40°C), were designed to explore their effects on E2 degradation by the two strains. For the earlier experiments, the initial E2 concentration of each group was 10 μ M, and the blank control group without strain was added in all series of experiments except the temperature series. In addition, liquid samples were collected daily for 6 days and filtered through a 0.22- μ m nylon filter before further analysis.

The first-order kinetics (Lu et al., 2019) was used in this study to evaluate the biodegradation rate of E2 under different sucrose concentrations.

Analytical Methods

Bacterial Growth

Cell density was measured by an UV-visible spectrophotometer (Thermo evolution 201, Thermo Technology Co., Ltd., Chachoengsao, Thailand) at an OD of 600 nm to assess bacterial growth (Pan et al., 2017; Moreira et al., 2018).

Quantitative Analysis of E2

To measure E2 concentration, 1.5 ml liquid sample was drawn with a 2-ml syringe and passed through a 0.22- μ m filter to eliminate impurities, and it was finally preserved in brown flasks at 4°C. Stored samples were analyzed by high-performance liquid chromatography (HPLC, Agilent Technologies, Inc., Santa Clara, CA, USA). A column C18 (4.6 \times 75 mm, 5 μ m, Agilent Technologies, Inc., Santa Clara, CA, USA) was operated with the mobile phase consisting of 40% water and 60% acetonitrile at a flow rate of 0.3 ml/min. The total analysis time of each sample was 5 min, and the injection volume was 90 μ l. In addition, the instrument was operated at a column temperature of 35°C, and the detection wavelength was 279 nm.

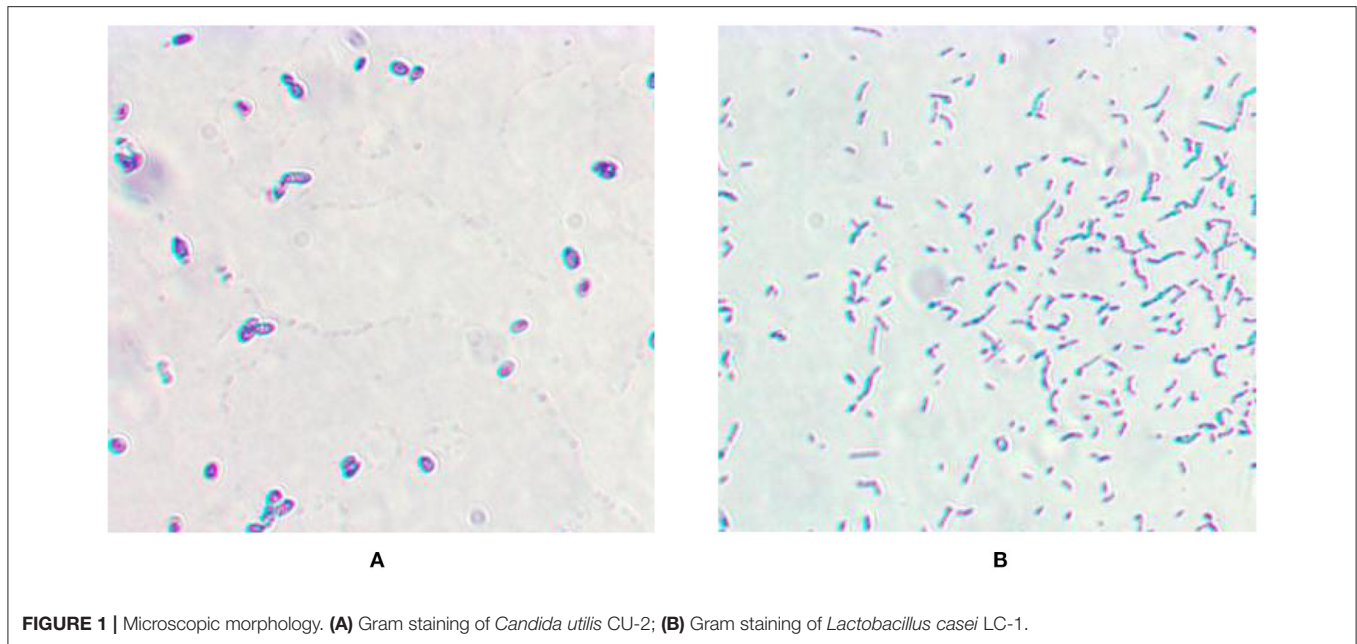
Identification of Biodegradation Products of E2

The detection and identification of the biodegradation products of E2 were carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS) equipped with an electrospray ionization mode (ESI). The ESI was used in a positive ion source (ESI+), and the mass spectrums were operated in a full-scan mode (100–1,500 m/z). The samples were pretreated before determination. Using a solid phase extraction cartridge, 50-ml sample solutions were extracted and eluted with 5 ml of methanol for concentration. Chromatography was operated using a Waters Acquity UPLC BEH C18 (2.1 \times 100 mm, 1.7 μ m), and the flow rate of the mobile phase was 0.3 ml/min. Additional details are shown in **Supplementary Text 1**.

RESULTS AND DISCUSSION

Morphological Characteristics of Strains

As shown in **Figure 1**, both *L. casei* LC-1 and *C. utilis* CU-2 were gram-positive. The strain LC-1 was rod-shaped and the strain CU-2 was elliptical.



Biodegradation of E2 by *C. utilis* CU-2 and *L. casei* LC-1

Effects of Different Carbon Substrates on E2 Biodegradation

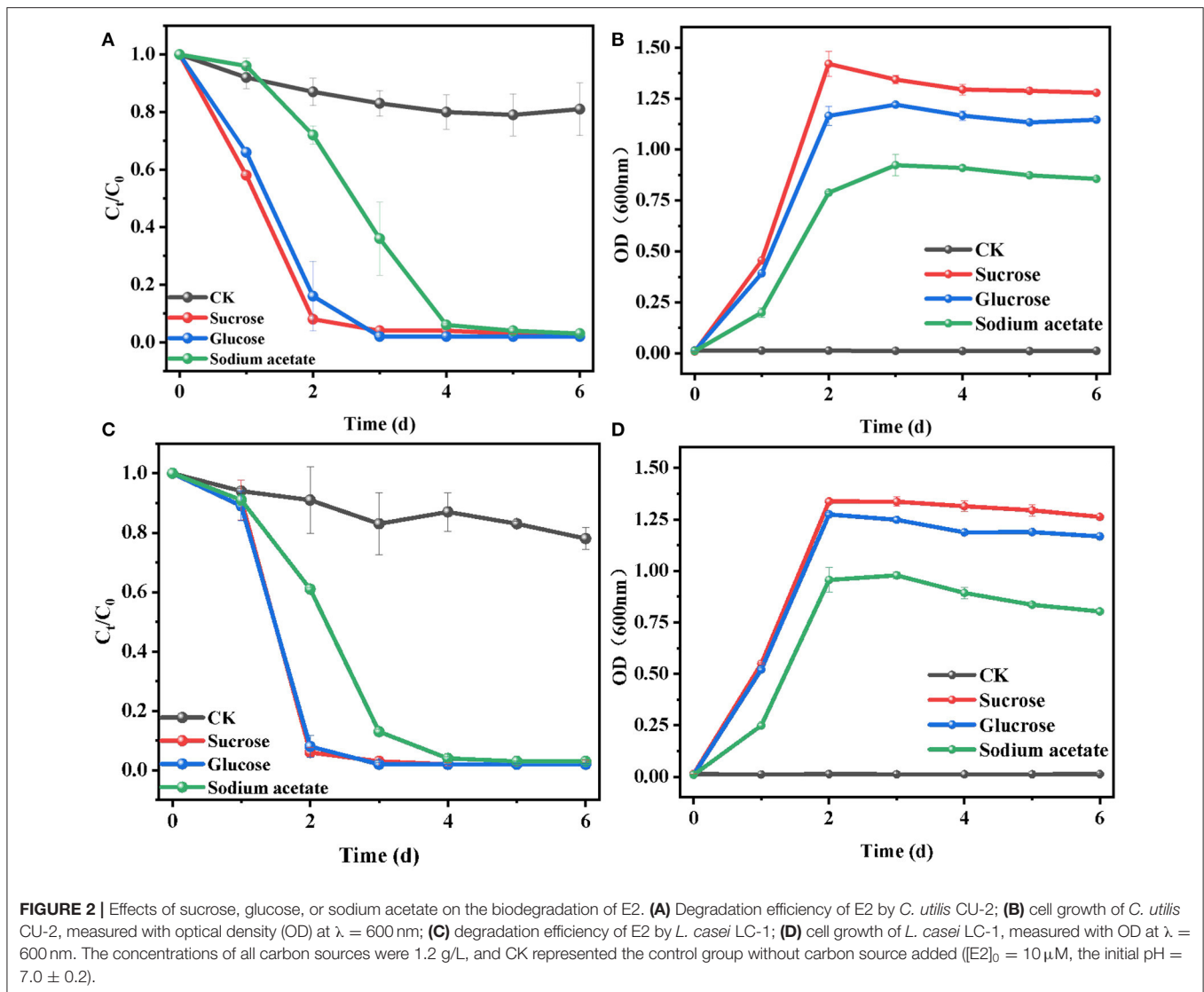
The effects of the addition of substrates on E2 biodegradation by *L. casei* LC-1 and *C. utilis* CU-2 were shown in **Figure 1**, and the experiments were carried out with three single carbon sources, namely, sucrose, glucose, or sodium acetate, with the concentration of 1.2 g/L. The experiments were conducted in MM with 10 μ M of E2. The results showed that all groups with the supply of carbon sources exhibited higher degradation efficiency than the control group (without the addition of carbon sources) (**Figures 2A,C**). Consistent with E2 degradation, the cell growth of the two strains was also more rapid than the control group (**Figures 2B,D**). The addition of carbon sources promoted cell growth and E2 degradation. This phenomenon could be explained by the occurrence of biological activity and co-metabolism (Dawas-Massalha et al., 2014). *C. utilis* CU-2, as shown in **Figure 1A**, could eventually remove about 97% of E2 in 6 days with supplemented substrates, indicating that it was a highly effective strain for E2 degradation. Besides, 92% of E2 could be removed in 2 days by *C. utilis* CU-2 with sucrose addition. However, the removal of E2 under glucose and sodium acetate supplement could only reach 84 and 28% in 2 days. The results indicated that sucrose addition resulted in higher removal efficiency of E2 than the other two carbon sources. This could be explained by the fact that sucrose was more easily utilized by the strain than the other carbon sources (Chen et al., 2013). For *L. casei* LC-1, as shown in **Figures 2C,D**, the degradation efficiencies of E2 were 94, 92, and 39%, respectively, after adding sucrose, glucose, or sodium acetate in 2 days, which were higher than those of *C. utilis* CU-2. This difference in E2 degradation efficiencies may be related to the difference in

the growth of the two strains, that is, the cell concentration of *L. casei* LC-1 was higher than that of *C. utilis* CU-2 in the early stage. This could be accounted to that high biological activity was beneficial to the degradation of E2. While, over time, *C. utilis* CU-2 and *L. casei* LC-1 could basically achieve equal biomass at the later stage in **Figures 2B,D**, and the final removal efficiency of E2 by *L. casei* LC-1 could also reach about 97% with the increase of reaction time under three different carbon sources.

In summary, these results indicated that the addition of carbon substrate (sucrose, glucose, or sodium acetate) could accelerate cell growth and promote E2 removal compared with the control group, which was due to co-metabolism. Moreover, when sucrose was used as carbon source, the cell growth rate and the E2 biodegradation efficiency were faster than that with glucose or sodium acetate as a carbon source. Thus, sucrose was selected as the optimal carbon source and was used for the following experiments.

Effects of Different Sucrose Concentration on E2 Biodegradation

The effect of sucrose concentration (\sim 0.05–10 g/L) on E2 removal was investigated, and the results are shown in **Figure 3**. It can be seen that the biodegradation efficiency of E2 improved with the increase of sucrose concentration (\sim 0.05–1.2 g/L), while it decreased when the sucrose concentration increased to 10 g/L. For cell growth (**Figures 3B,D**), all sucrose added at different concentrations improved the growth of the two strains to different extents. Moreover, cell growth was accelerated with the increase of sucrose concentration (\sim 0.05–10 g/L). To some extent, cell growth may explain the increasing removal efficiency of E2, that is, the enhancement of biological activity in high cell concentration accelerated the biodegradation to a certain



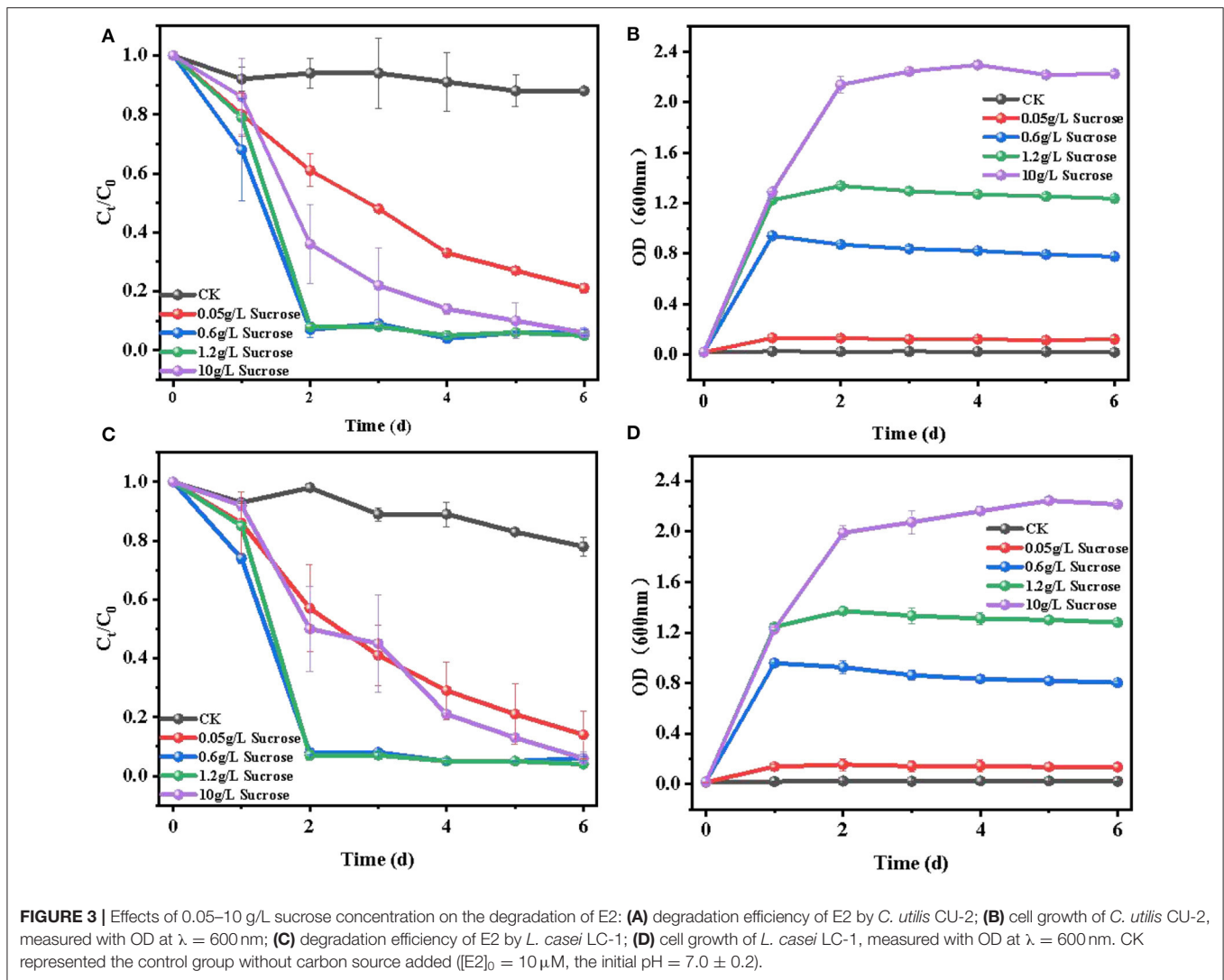
range (Ziagova and Liakopoulou-Kyriakides, 2007). Meanwhile, the degradation of E2 (Figures 3A,C) was slowly removed with the addition of 0.05 g/L sucrose. When the sucrose concentration ranged from 0.6 to 1.2 g/L, over 95% of E2 was rapidly degraded in 2 days. However, when the initial concentration of sucrose was increased to 10 g/L, the removal efficiency of E2 significantly decreased to about 40% in 2 days, and the maximum removal efficiency of E2 appeared until the sixth day. The results suggested that increasing sucrose concentration promoted cell growth, and the suitable concentration of sucrose was beneficial to the degradation of E2. Too high or too low carbon source concentration would affect the carbon-to-nitrogen ratio, leading to weak biodegradation (Dawas-Massalha et al., 2014). Similar phenomena have been reported in the degradation of some other contaminants by microorganisms. Min et al. (2018) reported that 5 g/L succinate supplement had a lower removal efficiency compared with 0.5 g/L succinate in the degradation of 2,6-dibromo-4-nitrophenol by *Cupriavidus* sp. strain CNP-8.

Pan et al. (2017) also proposed that suitable sodium acetate concentration accelerated the biodegradation by *Thermus* sp. in the degradation of ciprofloxacin. Therefore, it is necessary to select the appropriate concentration of carbon source for the removal of pollutants.

In conclusion, there was no significant difference in E2 degradation between *C. utilis* CU-2 and *L. casei* LC-1 at different sucrose concentrations. In addition, the results also revealed that the suitable sucrose concentration could result in a rapid degradation of E2. Simultaneously, considering the degradation efficiency of E2 and cell growth, the optimal concentration of sucrose was 0.6 g/L for further experiments.

Effects of Temperature on E2 Biodegradation

In the biodegradation process, temperature had a great influence on the growth of microorganisms and the activity of some important enzymes. Choosing an optimal culture temperature was a key environmental factor for E2 removal. To investigate the



influence of temperature on E2 biodegradation and the growth of *C. utilis* CU-2 or *L. casei* LC-1, a series of experiments were carried out at 15, 25, 35, and 40°C. The results, as shown in **Figure 3**, demonstrated that E2 degradation was positively influenced by the increase of temperature in the range of 15–35°C, while the biodegradation efficiency of E2 significantly decreased at 40°C. For *C. utilis* CU-2 (**Figures 4A,B**), when the temperature ranged from 15 to 35°C, the biodegradation of E2 and the bacterial growth rate presented an upward trend with the increasing temperature. Furthermore, the fastest degradation efficiency within 1 day and the most rapid cell growth within 12 h was observed at 35°C. Meanwhile, as the temperature was raised to 40°C, about 90% removal efficiency of E2 was not achieved until the fourth day, and the maximum cell concentration was 0.34 (OD600), which was about half of that (OD600 = 0.81) at other temperatures. It was well-known that each microorganism had an optimal temperature range. On the one hand, too high or too low temperatures could reduce the growth and reproduction rates of microorganisms, and it

also reduced the number of effective microorganisms. On the other hand, although denaturation of proteins was not caused at 40°C, the proteins in microbial cells could not maintain structural integrity and catalytic function. Meanwhile, when the temperature was too low, the growth of bacteria was slow and the enzyme activity was also inhibited (Jagdale and Gordon, 1997). Overall, it is generally stated that each microorganism had an appropriate temperature range, and the results also indicated that the optimum temperature of *C. utilis* CU-2 for E2 degradation was 35°C. As shown in **Figures 4C,D**, the optimum temperature of *L. casei* LC-1 was also 35°C. However, unlike *C. utilis* CU-2, *L. casei* LC-1 was more sensitive at 40°C. The cell growth of *L. casei* LC-1 was lower than that of *C. utilis* CU-2 at 40°C. At the same time, the maximum degradation efficiency of E2 by *C. utilis* CU-2 reached 92% within 4 days, while that of *L. casei* LC-1 was only 42%. Overall, *L. casei* LC-1 was similar to *C. utilis* CU-2 in cell growth and E2 biodegradation, and the optimal temperature for E2 biodegradation of the two strains was 35°C.

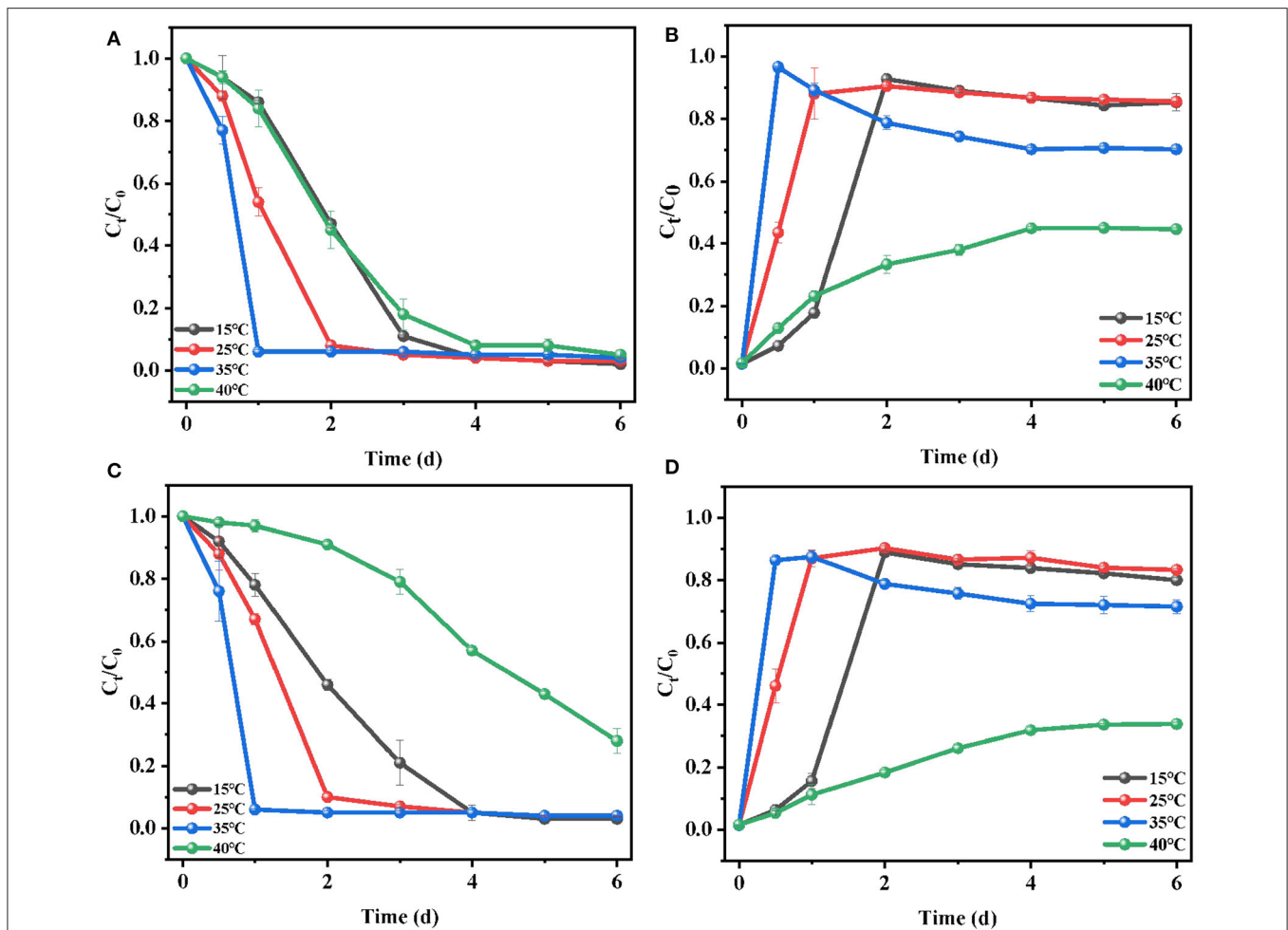


FIGURE 4 | Effects of temperature on the biodegradation of E2: **(A)** degradation efficiency of E2 by *C. utilis* CU-2; **(B)** cell growth of *C. utilis* CU-2, measured with OD at $\lambda = 600$ nm; **(C)** degradation efficiency of E2 by *L. casei* LC-1; **(D)** cell growth of *L. casei* LC-1, measured with OD at $\lambda = 600$ nm. The concentration of sucrose addition was at 0.6 g/L, $[E2]_0 = 10 \mu\text{M}$, the initial pH = 7.0 ± 0.2).

TABLE 1 | First-order kinetic rate constant (k) and half-life ($t_{1/2}$) for E2 degradation by *Candida utilis* CU-2 or *Lactobacillus casei* LC-1.

Sucrose concentration	<i>Candida utilis</i> CU-2			<i>Lactobacillus casei</i> LC-1		
	K (d^{-1})	$t_{1/2}$ (d)	R^2	K (d^{-1})	$t_{1/2}$ (d)	R^2
0 g/L	0.03	4.1	0.77	0.06	3.6	0.85
0.05 g/L	0.27	2	0.99	0.28	2	0.96
0.6 g/L	0.82	0.9	0.98	0.78	0.9	0.93
1.2 g/L	0.65	1.1	0.85	0.64	1.1	0.90
10 g/L	0.49	1.4	0.98	0.37	1.7	0.95

Kinetic Modeling of E2 Biodegradation at Different Sucrose Concentrations

The first-order kinetics was usually used to fit the biodegradation of E2 (10 mM) at different sucrose concentrations. As shown in Table 1, the kinetics of E2 biodegradation at five concentration

sucrose gradients was evaluated. The R^2 values of *C. utilis* CU-2 and *L. casei* LC-1 were ~ 0.77 – 0.99 and 0.85 – 0.96 , respectively, indicating that the removal of E2 at different sucrose concentrations could fit the first-order kinetic models well. During the biodegradation process of E2 by *C. utilis* CU-2, the k -values calculated at different concentrations of sucrose (0–0.6 g/L) were 0.03 d^{-1} , 0.27 d^{-1} , and 0.82 d^{-1} , showing a gradual increasing degradation trend. However, the removal rate constants of 1.2 and 10 g/L of sucrose supplement decreased, which was consistent with the previous statement (Figure 2). Similarly, for *L. casei* LC-1, the removal rate constants at the sucrose concentrations ranging from 0 to 0.6 g/L were 0.06 d^{-1} , 0.28 d^{-1} , and 0.78 d^{-1} , respectively, and the k -value of 10 g/L sucrose decreased to 0.37 d^{-1} , also confirming the above results. That is, when the concentrations of sucrose exceeded a certain range, it would inhibit the degradation of E2. Besides, the maximum rate constants of the two strains were 0.82 d^{-1} and 0.74 d^{-1} , respectively. They all occurred at the condition of 0.6 g/L sucrose addition. Thus, it was

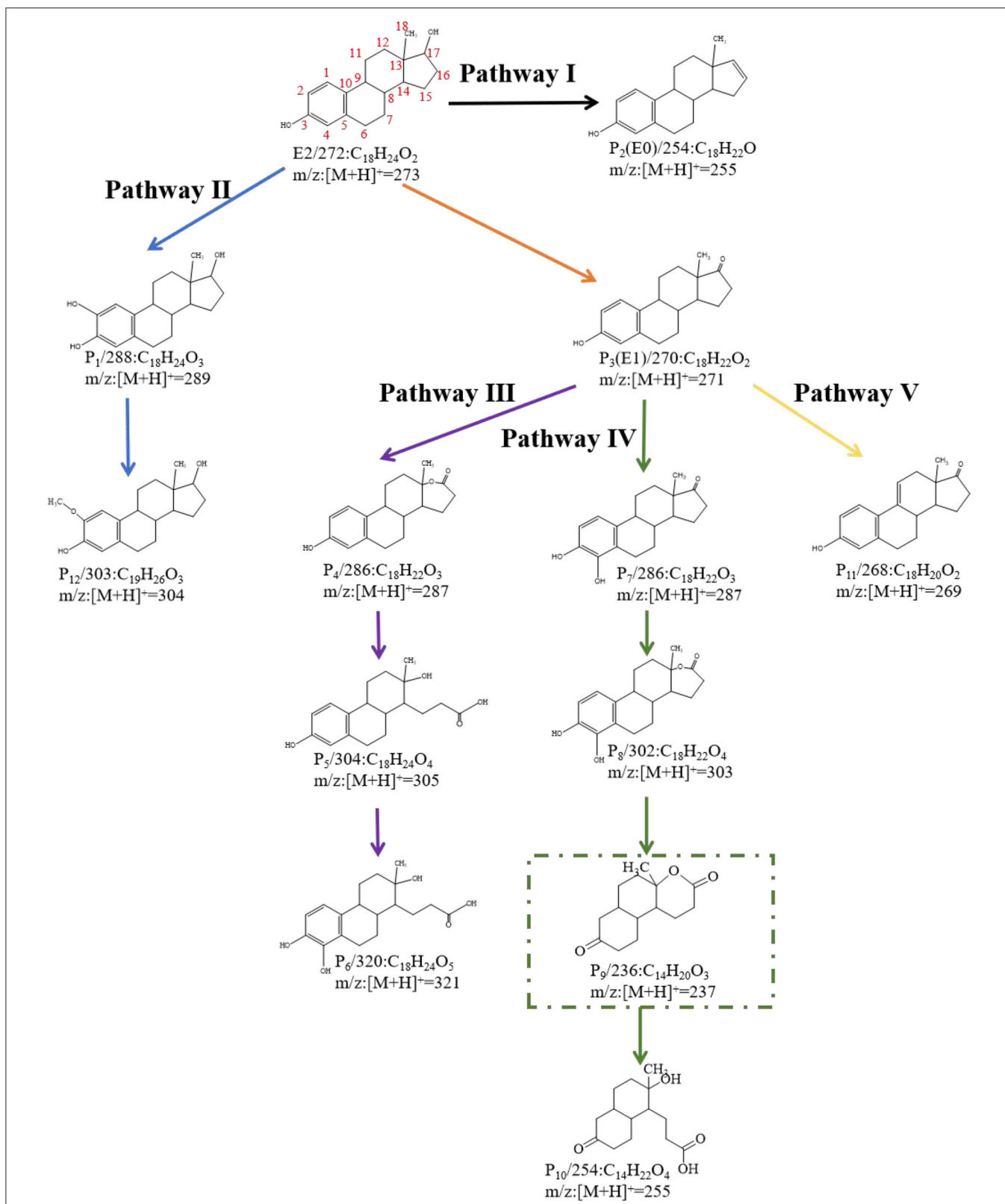


FIGURE 5 | E2 degradation products and predicted biodegradation pathways. The green rectangle indicated the metabolite that was not detected by liquid chromatography tandem mass spectrometry (LC-MS/MS) (the black, blue, purple, green, and yellow lines represent Pathway I, Pathway II, Pathway III, Pathway IV, and Pathway V, respectively).

also reconfirmed that 0.6 g/L was the optimal concentration of sucrose. On the other hand, the *k*-value of sucrose group was higher than that of control group, which reflected that sucrose as co-substrate accelerated cell growth and promoted the biodegradation of E2.

Biodegradation Products and Metabolic Pathways

The degradation intermediates of E2 by *C. utilis* CU-2 and *L. casei* LC-1 were detected by LC-MS/MS, and the degradation pathways were speculated based on accurate MS. In the E2 biodegradation, 12 intermediate products were identified (**Supplementary Table 1**). According to the detected products and the previously reported degradation pathways, five degradation pathways of E2 were proposed. For E2 degradation intermediates, P1 (C₁₈H₂₄O₃, $m/z:[M + H]^+ = 289$), P2 (E0:C₁₈H₂₂O, $m/z:[M + H]^+ = 255$), and P3 (E1:C₁₈H₂₂O, $m/z:[M + H]^+ = 271$) were primary products, and P4 (C₁₈H₂₂O₃, $m/z:[M + H]^+ = 287$)—P12 (C₁₉H₂₆O₃, $m/z:[M + H]^+ = 304$) were secondary products. It was indicated that P4–P11 might be generated from the further degradation of P3. The formation of these products was mainly through hydroxylation, oxidation, and ring cleavage. All intermediate products were detected in the metabolic fluids of *C. utilis* CU-2 and *L. casei* LC-1, indicating that the functions of degrading enzymes produced by *C. utilis* CU-2 and *L. casei* LC-1 may be similar. This needs further analysis and verification.

On the basis of the LC-MS/MS results, the biodegradation pathway of E2 was proposed (**Figure 5**). Specifically, P3, emerging at the time of 16.25 min, contained a mass ion of m/z 271. Its molecular formula was calculated as C₁₈H₂₂O₂, which was determined to be E1 (estrone). In addition, E1 was formed by the dehydrogenation process at the C-17 position of the D ring of E2, and it was suggested that the formation of E1 was caused by 17 β -hydroxysteroid dehydrogenases (Xiong et al., 2020). In previous studies, E1 was generally regarded as the initial step of estrogen degradation (Shi et al., 2004). However, new degradation pathways have been found in recent years, which was not degraded to E1 first. This study also reconfirmed this discovery, and the new pathways were illustrated in pathway I and pathway II. In pathway I, P2 (E0 $m/z:[M + H]^+ = 255$) was formed by dehydration at the C-17 position of the D ring of E2, which was also reported by Nakai et al. (2011). In pathway II, P1 (C₁₈H₂₄O₃, $m/z:[M + H]^+ = 289$) was inferred to be formed by the hydroxylation of E2 (Wang et al., 2019). In addition, the hydroxyl in A ring of P1 was further methylated to form P12 ($m/z:[M + H]^+ = 303$). However, in other three pathways, the products were transformed on the basis of E1. In pathway III, the chemical composition of P4 was calculated to be C₁₈H₂₂O₃, and its structural formula was determined by MS (**Supplementary Table 1**). P4 contained an ether bond, which could be inferred to be formed by the oxidation of E1 saturated ring. This product was also reported in the E2 degradation by microalgae (Wang et al., 2019). In addition, P4 was further

transformed to P5 ($m/z:[M + H]^+ = 305$) by the cleavage of D-ring, and P5 ($m/z:[M + H]^+ = 305$) was further hydroxylated to form P6 ($m/z:[M + H]^+ = 321$) (Wang et al., 2019). In pathway IV, P7 ($m/z:[M + H]^+ = 288$) was identified as 4OH-E1, which was formed by hydroxylation of E1 at C-4 (Yu et al., 2013). Besides, further oxidation between C-13 and C-17 of D-ring on P7 resulted in the formation of degradation intermediate of P8 ($m/z:[M + H]^+ = 303$) (Wang et al., 2019). Another degradation product, P9 ($m/z:[M + H]^+ = 237$), was detected in MS, but its intensity was very low. P9 (C₁₄H₂₀O₃) was further transformed to P10 (C₁₄H₂₂O₄) through ring cleavage. Both of these intermediate products were proposed by Wang et al. (2019). Furthermore, in pathway IV, P10 was the degradation product with the smallest amount of carbon in molecular mass. Some studies had also found that E2 was degraded into small molecules (Ma and Yates, 2018), but such findings were rare. The detection of P10 also indicated the possibility of further biodegradation or even the possibility of E2 being completely degraded into carbon dioxide and water (Yu et al., 2013), but this needs to be confirmed by further research. In pathway V, P11 ($m/z:[M + H]^+ = 237$) was calculated as C₁₈H₂₀O₂, and a double bond was formed by dehydrogenation on the C-ring of E1 (C₁₈H₂₂O₂).

Based on the intermediate degradation products detected by LC-MS/MS, some possible degradation pathways of E2 were proposed. All degradation products were detected in the reaction system of *L. casei* LC-1 and *C. utilis* CU-2. Therefore, it was speculated that *L. casei* LC-1 and *C. utilis* CU-2 may produce the same or similar functional enzymes, while more research and analysis were needed.

CONCLUSIONS

In this paper, the contrastive study of E2 biodegradation by fungi and bacteria was investigated. Taking *C. utilis* CU-2 and *L. casei* LC-1 as functional microorganisms, the environmental impact factors, kinetics, and degradation pathways were analyzed. The results showed that both strains could achieve an optimal degradation efficiency at 35°C with 0.6 g/L sucrose addition. The addition of suitable concentration of sucrose (0.05–0.6 g/L) could significantly promote cell growth and the degradation of E2 with over 97% after reacting for 5 days. However, a high concentration of 10 g/L sucrose as co-substrate would inhibit the biodegradation of E2 compared with the low concentration. *C. utilis* CU-2 or *L. casei* LC-1 could degrade E2 into 12 intermediate products through 5 degradation pathways, among which C₁₄H₂₂O₄ as the minimum-carbon-content degradation product was detected, and other smaller degradation products were not detected due to equipment intensity or low levels. In general, there was no significant difference between *C. utilis* CU-2 and *L. casei* LC-1 in E2 biodegradation characteristics and degradation products, but both of them were E2-efficient degradation strains. It was helpful to understand the degradation mechanism of *C. utilis* CU-2 and *L. casei* LC-1 and provide a new idea for the removal of E2.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

HG: writing-original draft, conceptualization, methodology, data curation, and formal analysis. LZ: conceptualization, formal analysis, writing-review—editing, and supervision. ZD, YL, and LF: validation and investigation. YZ, SW, and YF: methodology and investigation. BL and LY: writing-review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenrg.2021.661850/full#supplementary-material>

The method of LC/MS/MS, basic information, and the secondary MS of E2-degrading products can be found in the **Electronic Supplementary Information**.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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