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Study on The Microbial Whole Cell Catalytic Preparation of Quercetin

Yuanyuan Zang

College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, China

Abstract

Microorganisms were domesticated from tartary buckwheat using Schatz culture medium, gradually increasing the proportion of quercetin until it became the sole carbon source. The isolated microorganisms were purified, and the strain with the highest rutin-degrading enzyme (RDE) activity in liquid Schatz medium with quercetin as the sole carbon source was obtained. One bacterial strain and one fungal strain were isolated and domesticated, and the RDE conversion rate of the fungal strain was found to be 53.92%, higher than the bacterial strain (2.43%). The fungal strain was then subjected to ecological and biochemical identification, and preliminary identification as a strain of *Aspergillus* was made through colony observation and microscopic observation. After DNA extraction and PCR amplification, a single band with a molecular size of approximately 600 bp was obtained through 0.8% agarose gel electrophoresis. The obtained sequence was compared with the ITS rDNA sequences recorded in NCBI, and a phylogenetic tree was constructed for strain identification, confirming it as a strain of *Aspergillus*. It was then prepared as a whole-cell catalyst, and the optimal reaction system was obtained by optimizing the hydrolysis conditions: the addition amount of the whole-cell catalyst was 6 g/L, the reaction time was 36 hours, and the percentage of natural deep eutectic solvents (NADESs) was 40%. Three verification experiments were conducted under the optimal conditions, and the quercetin degradation rate was found to be 369.543 $\mu g \cdot min^{-1} \cdot L$.

Keywords

Natural deep eutectic solvents; Quercetin; Rutin-degrading enzyme; Microbial identification.

1. INTRODUCTION

Flavonoids are a class of important natural active compounds that have various medicinal values, including anti-proliferative effects on several cancer cells, anti-inflammatory, anti-allergic, antioxidant effects, and preventive effects on atherosclerosis [1-6], In the contemporary food industry, the technology of using microbial systems as necessary catalysts for industrial production has become increasingly mature, Manzanares et al. isolated and identified two glucosidases from Aspergillus niger [7], while Wang Jiali et al. screened and isolated a strain of excellent black Aspergillus from Shanxi aged vinegar koji [8]. Compared to the extraction and preparation of enzymes from cells, the use of microbial whole-cell catalysis method is environmentally friendly, inexpensive, readily available, and has high activity and selectivity [1]. Currently, there have been studies on the microbial preparation of flavonoids, such as the use of Aspergillus oryzae multienzyme system to degrade rutin [9]. The RDE, which catalyzes the hydrolysis of rutin to quercetin, has been proven to be obtained from Penicillium powder blue [10] and black Aspergillus [11], and used for fermentation preparation of quercetin.

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The main advantage of using whole-cell catalysis is that the cells provide a natural environment for the enzymes, preventing conformational changes in proteins that can lead to loss of activity in unconventional culture media. It also allows for the efficient regeneration of cofactors through multi-step reactions under mild operating conditions and environmentally friendly conditions [12]. It enables the production of compounds with high regional and stereo selectivity [13]. It has been successfully applied in the production of 1,4-butanediol from erythritol [14], preliminary studies have also been conducted on the use of whole cell catalysis for the hydrolysis of rutin [15]. Recently, scientists have found that combining unconventional solvents can improve the solubility of reaction substrates [16]. However, the strong toxicity and high cost of ionic liquids are important obstacles to their further practical application [17]. To overcome these problems, in the past decade, deep eutectic solvents (DES) have emerged as a new generation of ionic solvents. DES has customizable properties, low preparation cost, low toxicity, and sufficient biodegradability. In recent studies, the compatibility of DES with purified enzyme preparations has been demonstrated for different categories of enzymes, and its application in whole-cell catalysts has also been proven, such as wild-type Bacillus [18] or Acetobacter [19] species, Saccharomyces cerevisiae [16,20], and recombinant Escherichia coli [21].

In this experiment, we isolated and screened a strain of mold with RDE activity from tartary buckwheat. Enzyme activity assay and microbial identification confirmed it as a strain of Aspergillus. Subsequently, we used this strain for whole-cell catalysis and combined it with the results of previous studies [22] to determine the suitable NADESs system (80% ChGly-water co-solvent) for RDE activity. By optimizing the catalytic system and conditions, we obtained the optimal reaction system and measured the efficiency of whole-cell catalysis under the optimal reaction system.

2. MATERIALS AND METHODS

2.1. Chemical and biological materials

The tartary buckwheat was purchased from Henan. Huaimi was purchased from a local pharmacy in Nanjing. Rutin standard product was obtained from Beijing Bailingwei Technology Co., Ltd. Bacterial liquid PCR amplification primers were synthesized by General Biosystems (Anhui) Co., Ltd. DL 2000 DNA Marker purchased from Shanghai Solebao Biotechnology Co., Ltd

2.2. Preparation of culture medium

Domestication medium: A certain amount of rutin (0, 2, 4, 6, 8, and 10 g) is added to the liquid Charcot culture medium to modify it, while reducing the amount of sugar used. The total amount of sucrose and rutin is maintained at 10 g (50 mL as an example).

Separation medium: 1 L of solid Charcot culture medium

Identification medium: In liquid Chaxhlet medium, modified with rutin instead of sucrose, prepare 50 mL.

2.3. Source and domestication of bacterial strains

Bacterial suspension: Weigh 10g of moldy bitter nourishing powder, add 200 mL of distilled water to dissolve. Filter the obtained solution for future use.

Inoculation: Take 200 μL suspension and add it to sterile culture medium for sterile room operation.

Domestication cultivation: Place the culture bottle in a shaker at a temperature of 28 ℃ and a speed of 200 rpm for 5 days. Inoculate 200 μL of cultured seed solution into media with different rutin contents, repeat the operation, and finally stabilize the growth of the strain.

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202401_10(1).0016

2.4. Strain isolation and purification

Referring to the morphological identification method of common toxin producing fungi in food microbiology testing GB4789.16-2016, the strains obtained through domestication were separated and purified using the plate streak separation method. Incubate the plate at 28 ° C until the strain grows.

Select a single strain, number it, and then perform line separation. Repeat the operation until a single strain is selected.

2.5. Determination of strain vitality

Take 200 μL of each bacterial solution and inoculate them into the identification medium. Cultivate at 28 °C and 200 rpm for 3 days. Collect bacterial solution and centrifuge at 8000 xg for 5 minutes. Take the supernatant and measure UV absorption at 360 nm. Calculate the amount of rutin degradation and compare the activity of the strains.

2.6. Strain Identification

Morphological observation: The suspected fungal colonies isolated by the spot planting method were inoculated into Chaxhlet medium and cultured at 28 °C for 5 days, during this period, observe the color, texture, edge roughness, transparency, etc. of the colonies.

Microscopic observation: Drop a small drop of lactobacillus carbonate cotton blue staining solution onto a glass slide, take a small amount of mycelium and rinse it in a 50% ethanol solution. Then rinse the soaked mycelium with distilled water, immerse it in the staining solution, and cover it with a cover glass. Observe under 10x, 20x, 40x, and 100x optical microscopes respectively, and finally observe and record under a 100x oil microscope.

Refer to the Fungal Identification Handbook [23] for preliminary identification of fungal species.

Molecular Biology Identification: The purified bacterial strains obtained through domestication were cultured on glass paper plates at 28 °C for 3 days. The cultured fungal tissues were taken and ground into fine powder in a mortar with liquid nitrogen. DNA was extracted using the SPARKeasy Fungus DNA kit.

PCR amplification of ITS rDNA target fragments:

a) The primer sequence is:

Upstream primer ITS1: 5 '- TCCGTAGGTGAACCTGCGGG-3'

Downstream primer ITS2: 5 '- TCCTCGCTTATTGATGC-3'

b) PCR system

Sterilized ultrapure water: 19 μL

2x Tap Master Mix: 25 μ L Primer 1:2 μL

Primer 2:2 μL

Substrate template: 2 μL DNA sample solution

c) PCR reaction

96 ° C pre-denaturation: 3 minutes

96 ° C (denaturation): 45 s
55 ° C (annealing): 45 s
72 ° C (extension): 90 s
} 35 cycles
72 ° C (extension): 8 minutes

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202401_10(1).0016

0.8% agarose gel electrophoresis: 0.24 g of agarose powder was placed in a conical flask. Add 30 mL of l x TAE buffer, boil it, wait for it to cool down, add 1.5 μL of ethidium bromide, mix well, and pour it into a rubber plate. After the completion of gel production, place the gel into the electrophoresis tank, add l x TAE buffer until it reaches the gel surface, and sequentially point the sample into the gel hole. Connect the power supply (130 V, 30 min). After gel removal, ultraviolet imaging was performed in the gel imaging system.

Gene sequencing and phylogenetic tree construction: Send PCR products to General Biology for strain identification. Compare the ITS rDNA sequence of isolated fungi with the ITS rDNA sequence recorded by Blast in NCBI, and select strains with homology higher than 95% to construct a phylogenetic tree.

2.7. Whole cell catalysis

Preparation of whole cell catalysts: After 48 hours of liquid cultivation, 5000 xg of the isolated bacterial strains were taken and centrifuged for 15 minutes to obtain mycelium. The mycelium was washed three times with distilled water to obtain a precipitate and stored in a refrigerator at 4 °C.

Whole cell catalytic hydrolysis of rutin: 20 μL of whole cell catalyst was added to 1 mL of PBS (pH=7), and 5 mg of rutin was added to culture at 30 °C and 180 rpm for 24 hours. The supernatant was obtained by centrifugation. The content of rutin before and after the reaction was determined by HPLC according to the method established in the laboratory [22]. The degradation amount of rutin was calculated, and a buffer solution was used instead of the enzyme solution in control one. No substrate was added to control two.

The unit of activity (U) of RDE crude extract is defined as the amount of enzyme required to degrade unit mass (μg) of rutin per minute at 31 °C.

Single factor experiments for optimizing reaction conditions: Optimization of whole cell catalyst addition amount: The substrate amount remains unchanged, and the whole cell addition amounts are 20, 40, 60, 80 and 100 μL, respectively. The degradation amount of rutin is measured.

Optimization of reaction temperature: Cultivate at 25, 32, 37, 40, and 45 °C, with other conditions unchanged, and measure the degradation amount of rutin.

Optimization of reaction time: Keeping the cultivation conditions unchanged, samples were taken at 0, 12, 24, 36, and 48 hours to determine the degradation amount of rutin.

Speed optimization: Shake and cultivate at 40, 80, 120, 160, and 200 rpm to determine the degradation of rutin.

Optimization of NADES content: Adjust the amount of NADES (ChCl: Gly=1:1) added to make the NADES content 0%, 20%, 40%, 60%, and 80%, respectively, and determine the degradation amount of rutin.

Orthogonal design experiment optimization of reaction conditions: Based on the results of the single factor experiment, three factors including the amount of whole cell catalyst added, reaction time, and NADES content were selected to design an orthogonal experiment, and the degradation rate was used as the test indicator to optimize the immobilization conditions. The experimental design is shown in Table 1.

Table 1. The factors and levels for optimizing the reaction conditions

	The amount of whole cell catalyst added (μL)	reaction time (h)	NADESs content (%)
1	20	12	20
2	40	24	40
3	60	36	60

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202401_10(1).0016

3. RESULTS AND DISCUSSION

3.1. The results of the strain domestication

One strain of mold and one strain of bacteria were domesticated and named F1 and B1, respectively.

According to the definition of enzyme activity unit (U) mentioned above: the amount of enzyme required to degrade unit mass (μg) of rutin per minute at 37 °C, the conversion rates of Fl and Bl catalytic systems were calculated to be 53.92% and 2.43%, respectively. Select the strain Fl with high catalytic activity as the subsequent experimental research object.

Colony identification: As shown in Figure 1 A, strain Fl is circular on the culture medium, with flat colonies and cotton like hyphae, white in color; Microscopic observation, as shown in Figure 1 B, shows that the mycelium has a septum, branches, and produces long cylindrical segmented spores.

According to the Fungal Identification Handbook [23] and GB 4789.16-2016, the preliminary identification of this strain is Fusarium. The characteristics of Fusarium culture are that the aerial hyphae grow vigorously on potato glucose agar or Chaffel medium, and the large conidia have various forms, such as sickle, linear, and spindle

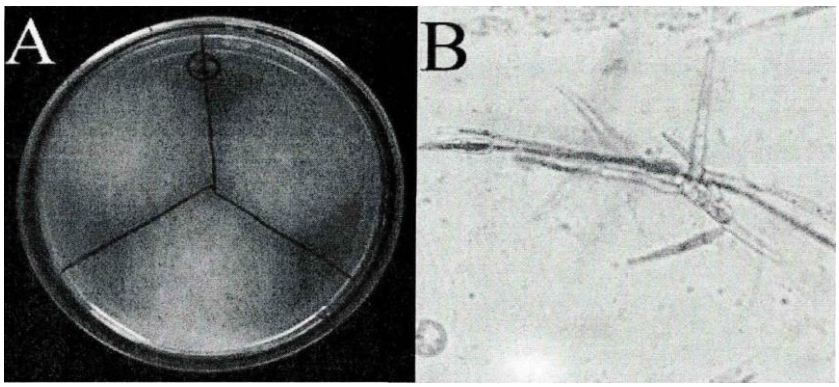

Figure 1. Morphological identification of strains Fl (Figure 1 A Observation of colony morphology; Figure 1 B Microscopic observation)

3.2. Molecular Biology Identification

After PCR amplification of strain F1, it was verified by 0.8% agarose gel electrophoresis. According to Figure 2 below, the fragment size was about 600 pb and the single band of amplification product could be used for subsequent gene sequencing.

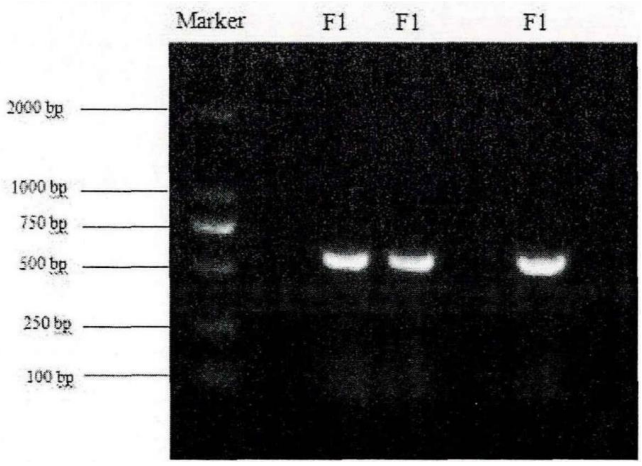

Figure 2. Electrophoresis verification of strains Fl

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202401_10(1).0016

After sequencing by General Biology, the ITS rDNA sequences of the isolated fungi were compared with the ITS rDNA sequences already included in NCBI through Blast, and strains with homologous similarity of over 95% were selected to construct a phylogenetic tree. As shown in Figure 3, F1 and Fusarium can be obtained SPP has strong similarity in genetic relationships, and F1 is identified as Fusarium based on colony morphology observation.

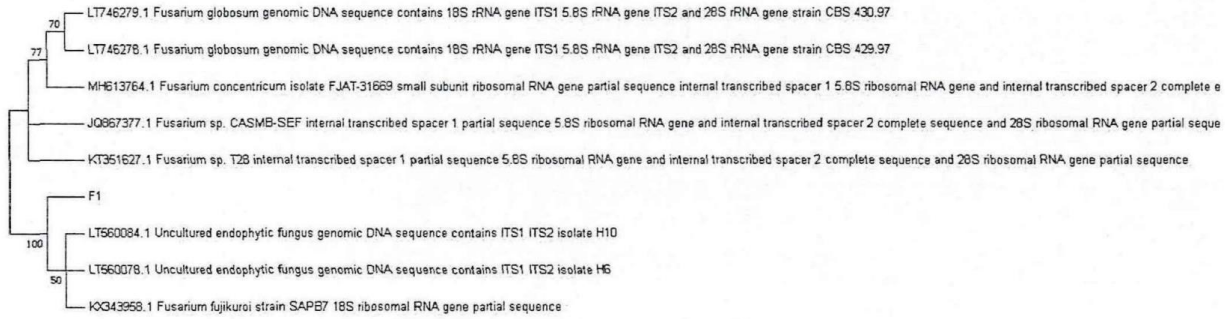

Figure 3. Phylogenetic tree of Pl strain constructed based on ITS rDNA gene sequence

3.3. Whole cell catalysis and its optimization

Single factor test: The single factor experimental results of the effects of bacterial addition, culture temperature, time, rotation speed, and NADES content in the whole cell catalytic reaction on the catalytic rate are shown in Figure 4:

From Figure 4 A, it can be seen that the degradation rate increases with the increase of bacterial addition, reaching its highest point at 40 L. However, as the bacterial addition continues to increase, the degradation rate actually decreases. This is because the binding amount between the enzyme and substrate reaches saturation, and excessive enzyme causes diffusion restriction, which actually inhibits the catalytic reaction.

As shown in Figure 4 B, the degradation rate first increases and then decreases with the increase of cultivation temperature, reaching its maximum value at around 30 °C, which is the optimal temperature for RDE catalytic reaction in the bacterial body.

The effect of cultivation time on degradation rate is shown in Figure 4 C. The degradation rate reaches its maximum value at 12 hours, and then the reaction tends to stabilize. The degradation rate decreases.

The effect of rotational speed on degradation rate is shown in Figure 4 D. Rotational speed affects the degree of enzyme substrate binding during the reaction process. The degradation rate decreases at low rotational speed, reaching its highest value at 160 rpm. Subsequently, due to shear force, enzyme activity decreases [24].

The addition of NADES to the reaction system can enhance the permeability of bacterial cell walls, promote the exchange of enzymes and substrates inside and outside the cell, and increase the degradation rate. From Figure 4 E, it can be seen that the degradation rate reaches its maximum value when the addition amount is 40%. Subsequently, an increase in NADES content leads to an increase in system viscosity and a decrease in degradation rate.

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202401_10(1).0016

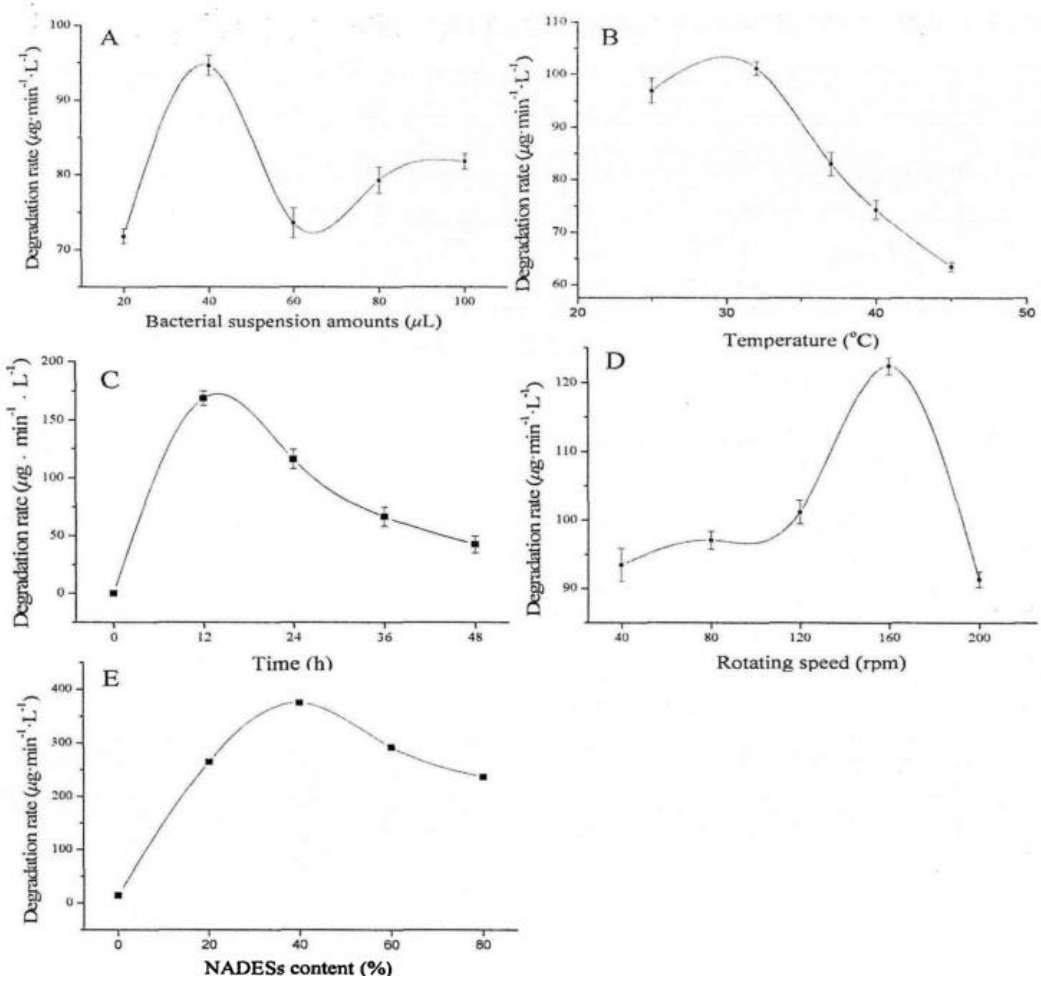

Figure 4. Single factor experiment on the effect of bacterial suspension amounts (A), temperature (B), time (C), rotating speed(D)and NADESs content(E)on the efficiency of whole cell catalytic

Orthogonal design experiment optimization of reaction conditions: Further optimization will be carried out based on three factors: bacterial volume, reaction time, and the percentage of NADES in the reaction system, selected through single factor experiments

Table 2. L9 (3^4) orthogonal test design and results of optimization of whole-cell catalytic conditions [a]

Experiment number	Bacterial quantity(μL)	Reaction time (h)	percentage of NADESs (%)	degradation rate($\mu g \cdot min^{-1} \cdot L^{-1}$)
1	20	12	20	162.68
2	20	24	40	64.35
3	20	36	60	212.08
4	40	12	40	142.10
5	40	24	60	0.00
6	40	36	20	206.08
7	60	12	60	305.04
8	60	24	20	0.00
9	60	36	40	380.17
$\overline{K}_1$	146.37	203.28	122.92	--
$\overline{K}_2$	116.06	21.45	195.54	--
$\overline{K}_3$	228.40	266.11	172.37	--
R_j	112.34	244.66	72.62	--

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The impact of various factors on the experimental results can be obtained from the Rj value. According to Table 2, $R_{j2}>R_{j1}>R_{j3}$ indicates that the reaction time has the greatest impact on the degradation of rutin, followed by the amount of bacterial addition. The percentage of NADES has the smallest impact on the catalytic yield.

According to the K value, the optimal combination of levels for each factor is selected as the optimal reaction conditions, which are: bacterial addition of 60 μL, reaction time of 36 hours, and NADES percentage of 40%. Three validation experiments were conducted under optimal conditions, and the degradation rate of rutin was found to be 369.54 $\mu g \cdot min^{-1} \cdot L$.

4. CONCLUSIONS

In this study, a strain of mold with high RDE activity was identified through domestication and screening from moldy tartary buckwheat. Through colony morphology observation, microscopic observation, and molecular biology identification, it was identified as a *Fusarium* strain.

Subsequently, the bacteria were prepared as a whole cell catalyst, and combined with the experiments in the previous two chapters, whole cell catalysis was carried out in the NADES system. The optimal hydrolysis conditions were optimized to obtain the optimal reaction system as follows: the amount of whole cell catalyst added was 6 μL, the reaction time was 36 hours, and the percentage of NADES was 40%. Three validation experiments were conducted under optimal conditions, and the degradation rate of rutin was found to be 369.54 μg·min-1·L.

The technology of using microbial engineering to produce important reagents such as catalysts required for industry has become increasingly mature, and microorganisms have gradually become one of the main sources of important reagents in modern food and drug industry. In this study, the experiment uses laboratory screened strains for whole cell catalytic preparation of quercetin, which is more environmentally friendly compared to chemical methods and does not require extraction and purification of enzyme preparations compared to traditional catalyst hydrolysis. The operation is simple, providing a reference for the efficient and environmentally friendly preparation of other important products in modern food and drug industry.

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