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Article

PRODUCTION OF KOJIC ACID FROM LEMON (*CITRUS LIMON*) PEEL USING *ASPERGILLUS NIGER* (PQ585363) AND *ASPERGILLUS FLAVUS* (PQ585407) IN SUBMERGED FERMENTATION

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ABSTRACT: Kojic acid, valued for its antioxidant and antimicrobial properties, is typically produced using expensive substrates. This study explores the potential of lemon peels, a citrus industry by-product, as a sustainable substrate for Kojic acid production. Lemon peels were analyzed for their proximate composition, revealing moisture (9.12%), protein (11.46%), fat (2.79%), and carbohydrate content (55.61%). Fungal isolates were obtained through natural fermentation and characterized using both morphological and molecular methods, with *Aspergillus niger* (PQ585363) and *Aspergillus flavus* (PQ585407) identified as producers of kojic acid. Screening for kojic acid production was carried out using a 1% ferric chloride solution. Results showed that *A. niger* and *A. flavus* tested positive. Submerged fermentation of lemon peels using single and co-cultures of the fungal isolates produced Kojic acid. The co-culture exhibited higher Kojic acid concentrations (7.50 g/l) compared to a single culture of *A. niger* (6.99 g/l). Submerged fermentation conditions were optimized using Response Surface Methodology (RSM) by varying key parameters, including pH, incubation period, glucose concentration, yeast extract, and substrate levels. The optimal fermentation conditions were determined to be 150 g/L glucose, 6 g/L yeast extract, a 96-hour incubation period, and pH 5.0. Under these conditions, co-cultures produced 7.80 g/L kojic acid, while single cultures produced 7.40 g/L. Kojic acid was extracted using ethyl acetate, and its purity was confirmed through FTIR spectroscopy, showing a purity level of 97.00%. This study demonstrates the potential of lemon peels as a viable and sustainable substrate for kojic acid production through submerged fermentation using *Aspergillus* species.

1. INTRODCTION

Kojic acid, also known as 5-hydroxy-2-hydroxymethylgamma-pyrone (KA), is a significant secondary metabolite synthesized by various microorganisms, including *Aspergillus oryzae*, *A. flavus*, *A. tamarii*, *Penicillium* species, and specific bacteria such as *Bacillus subtilis*, *Pseudomonas fluorescens* and *Streptomyces griseus* [1]. Kojic acid is a versatile compound with properties of weak acids and is classified as a multifunctional, reactive Gamma-Pyrone [2]. Its reactivity is predominantly observed at all positions on a ring, and numerous valuable industrial products can be derived from it, such as; metal chelates, ether, pyrimidine, pyridones, azodies, mannich base etc [3]. It has versatile applications across chemistry, health, food and cosmetics, acting as an antibacterial, antifungal, anti-melanosis agent, and a chelating agent [4]. Kojic acid is an inhibitor of bacterial, fungal growth and viral multiplication. It also inhibits the catecholase activity of tyrosinase, making it useful in the cosmetics industry for skin lightening and as an organic chelation and decolourizing agent [5].

Citrus limon commonly known as lemon are versatile nutritious fruit, widely used in cooking, health, cosmetics, medical and household applications. The global lemon industry generates massive amounts of lemon peel, primarily as a by-product of

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juice and oil production. Despite being considered waste, lemon peel is rich in valuable bioactive compounds like essential oils, pectin, flavonoids, and citric acid [6], making it a valuable resource for various industries. The disposal of lemon peel waste poses significant environmental concerns, mirroring those of other citrus wastes. Conventional methods like landfilling and incineration are harmful, releasing greenhouse gases, toxic compounds, and leachate that pollute the environment. To address this, leveraging biotechnology to convert lemon peel waste into valuable products, such as Kojic acid, and various chemicals and materials, offers a sustainable solution. This approach aligns with global initiatives to reduce waste and foster a circular economy [7,8].

Chib *et al.* [9] successfully produced Kojic acid using a newly discovered fungal strain, *A. sojae* SSC-3, isolated from rice husk. Meanwhile, Zohri *et al.* [10] developed an innovative approach to optimize Kojic acid production, utilizing cane molasses as a cost-effective substrate. This breakthrough makes the production process more sustainable and economically viable.

The agro-processing industry, especially in food processing and beverage production, produces escalating amounts of waste, resulting in substantial environmental concerns [11]. To mitigate this issue, researchers have been investigating the potential of agro-waste, such as sweet potato peels [12] and cane molasses [10], for valorization. However, there is a notable knowledge gap in Nigeria regarding the sustainable, domestic production of Kojic acid using lemon peel. The present study is motivated by the need to address environmental challenges arising from increasing waste in agro-processing industries by focusing on the isolation, identification, screening, utilization of single and co culture of Kojic acid-producing fungi isolated from lemon peel waste and optimizing different parameters to maximize yield.

2. Materials and Methods

2.1. Collection and Preparation of Samples

Fresh lemon peels were sourced from Kulende market, Ilorin, Kwara State. The peels were cleaned thoroughly to remove impurities and oven-dried at 55°C for 6 hours to prevent nutrient loss. After drying, the lemon peels were pulverized into fine particles using blender (BAJAJ BRAVO DLX) and stored in an airtight container at room temperature until further use.

2.2. Proximate Analysis of Lemon Peel

The proximate compositions were obtained using standard analytical methods. The proximate analysis carried out include moisture content, ash content, crude fiber content, crude lipid content, crude fiber content and carbohydrate content [13].

2.3. Preparation of Media

Potato dextrose agar was prepared according to manufacturer's instructions, sterilized in an autoclave for 15 minutes at 121°C and supplemented with 250 mg/l of chloramphenicol to suppress bacterial growth [14].

2.4. Isolation of Naturally Occurring Fungi

Ten grams of lemon peel were added to 90ml of sterilized distilled water in a flask. The mixture was incubated at 28±2 ^{0}C for 7 days, allowing natural fermentation to occur. Following fermentation, serial dilutions were performed on the fermentation broth until dilution of 10^{-3}. One ml of the diluted sample was introduced into each Petri dish, followed by the addition of 15-20 ml of potato dextrose agar (PDA). The plates were mixed well to disperse the suspension in the medium. Triplicate plates were prepared for each sample and incubated at 28±2°C for 8 days. The resulting colonies were counted and calculated as colony-forming units (CFU) per 1 g of lemon peel [15].

2.5. Screening for Kojic Acid Production and Preparation of the Inocula

One percent (1%) ferric chloride containing 0.1M of hydrochloric acid (HCl) was added to already prepared potato dextrose agar (PDA) in a conical flask. It was poured in a plate and allowed to solidify. Fungal innocula from a five days old culture was then inoculated into it, and was incubated at 28±2°C for 5 days and zone of clearance was measured by using a ruler to measure the diameter of the red-colored zone [16]. The inoculum was prepared by growing the fungus on PDA for 5 days and their spores were harvested and suspended into 50ml of sterile water and counted using hemocytometer. The spore suspension concentration was adjusted to be 2×10^6 spores per ml for the single culture while the co-culture was adjusted to be 1×10^6 each [17].

2.6. Identification of Fungal Isolates using Phenotypic and Molecular Identification

The fungal morphology was studied macroscopically by observing the colony features (color, shape, size, hyphae) and microscopically by a compound microscope using a lactophenol cotton blue stained slide mounted with a small portion of the mycelium and stored on PDA agar slants in the refrigerator for further use. Molecular identification and characterization of *Aspergillus* species was carried out using extraction of DNA, PCR amplification and blasting using primer sequences 5'–3'(F-GCATCGATGAAGAACGCAGC

and R-TCCTCCGCTTATTAGATATGC). The phylogenetic tree construction was a gold standard in characterizing our sequences to their respective genotypes. Analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software, ver. 11 http://megasoftware.net/. The latter was carried out by uploading the aligned sequence on MEGA 11, the phylogenetic tree window was utilized, and the appropriate type of sequence and the best DNA substitution model were selected and then run to construct a tree of ancestral genomic relatedness using the Neighbor-joining tree method of the phylogeny [14].

2.7. Kojic Acid Fermentation and Assay of Kojic Acid Production from Lemon Peels as Carbon Sources

Lemon peels were added to the basal fermentation medium as described by Manan and Webb [18] in amount equivalent to 100 g/l of carbon and the following components: 10g of mycological peptone, 0.05 of magnesium sulphate-7-hydrate and 1g of potassium phosphate dibasic anhydrous. Both single and co-cultures underwent incubation at 28°C ± 2°C for a duration of 10 days. The concentration of Kojic acid produced was quantified in the culture supernatant using a colorimetric assay, which employed ferric chloride (FeCl3) reagent, as described by Bentley [19].

2.8 Optimizing Culture Conditions for Kojic Acid Production from Lemon Peels using Response Surface Methodology (RSM)

The effects of various culture conditions, including substrate concentration, nitrogen source (yeast extract), and incubation period, was examined on Kojic acid biosynthesis in the fermentation medium, following the methodologies outlined by Prabhu *et al.* [20].

2.9 Effect of Ethyl Acetate Volume Ratio on Kojic Acid Extraction Yield

The method described by Zhao *et al.* [21] was used in order to determine the effect of different amounts of ethyl acetate on the yield of Kojic acid extraction. Exactly 300 mL of fermentation broth was added to ethyl acetate for extraction according to the fermentation broth: ethyl acetate (volume ratio) = 1:0.8, 1:1.0, 1:1.2, 1:1.4.

2.10 Fourier Transform Infrared Spectroscopy (FTIR)

The dried Kojic acid sample obtained from the extraction process was analyzed using an FTIR spectrometer. Approximately 1 mg of the sample was mixed with 100 mg of potassium bromide (KBr) and pressed into a transparent pellet under high pressure. The FTIR spectra were recorded in the range of 500–4000 cm^{-1} at a resolution of 4 cm^{-1} [22].

2.11 Statistical Analysis

Data obtained was analyzed using Statistical Package for Social Sciences (SPSS) version 25. All the work were done in triplicates and values were the mean (%) +/- standard deviation. The data obtained was subjected to analysis of variance (ANOVA), and Duncan's multiple range tests were used to determine the significance level of means.

3. Results

The proximate analysis of the lemon peel sample is shown in Table 1. All measurements were done in duplicates and values presented in percentage.

Table 1. Proximate Composition of the Prepared Lemon Peels

Sample Parameter (%)	Result (%)
Moisture	9.12±0.03
Crude Fiber	14.17±0.01
Crude Protein	11.46±0.01
Fat	2.79±0.01
Ash Content	6.85±0.07
Carbohydrate	55.61±0.04

Values are means of triplicate readings ± SD

Table 2. Screening Reaction for Kojic Acid Production by Fungal isolates

Fungal Isolates	Screening Reaction	Zone of Red Clearance(mm)
Isolate A	+	8.50
Isolate B	+	6.20

The screening reaction yielded positive results for fungal isolate A and B as represented in the Table 2.

Table 3: Microscopic and Macroscopic Characteristics of the Isolates

Characteristics	Isolate A	Isolate B
Hyphae color	Dark Brown	Yellow-green
Hyphal septation	Septate	Septate
Hyphae shape	irregular	wavy
Colony color before Sporulation	White	Pale Yellow
Color of Spore	Dark Brown	Yellow-green
Reverse View	Black	Dark orange
Days before Sporulation	2 Days	3 Days
Type of Spore	Conidia	Conidia
Conidia Head	Radiate	Radiate
Conidiophore	Roughened	Smooth
Stipe of Conidiophore	Double Walled	Single Walled
Conidia	Double Walled	Single Walled
Hyphae	Aseptate	Septate
Mycelia	Spreading	Spreading
Probable Organism	*Aspergillus niger*	*Aspergillus flavus*

The fungal isolates from the samples were identified phenotypically to be *A. niger* (isolate A) and *A. flavus* (isolate B) as shown in Table 3.

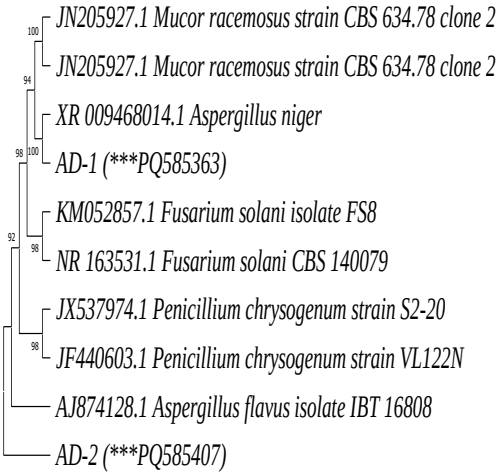

Figure 1. Phylogenetic tree of Kojic acid producing fungi isolates and related sequences obtained from NCBI. Numbers in parenthesis represent GenBank accession numbers

Based on morphological and molecular identification, isolate A was given the name *A. niger* and sequence was deposited in the GenBank under accession number PQ585363 and strain AD-1 and isolate B was given the name *A. flavus* and sequence was deposited in the GenBank under accession number PQ585407 and strain AD-2 as shown in **Figure 1**.

The results, presented in the **Figure 2**, show the mean Kojic acid concentration (mg/L) produced by the single culture of *A. niger* (PQ585363) and the co-culture of *A. niger* (PQ585363)*/A. flavus* (PQ5853407) at different time intervals, obtained from the Kojic acid fermentation of lemon peels.

Table 4 and 5 show the yield of kojic acid as affected by different variables using the fungal isolates**.**

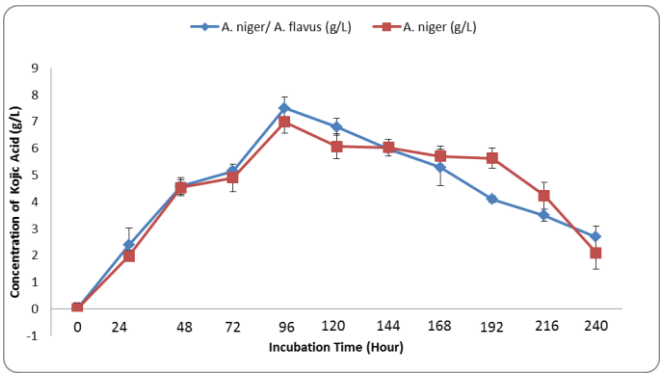

Figure 2. Fermentation by Fungal Isolates for Kojic Acid Production Using Lemon Peel as Carbon Source.

Table 4: The Yield of Kojic Acid as Affected by Process ariables with *Aspergillus niger* (PQ585363)

SD	Run	A: Glucose	B: Yeast Extract	C: Incubation Period	D: Substrate Concentration	E: pH	R1 (Kojic Acid Yield)
		g/l	g/l	Hours	g/l		g/l
30	30	150	6	96	150	5.0	7.4
29	10	150	6	96	150	5.0	7.4
28	1	150	6	96	150	5.0	7.4
27	25	150	6	96	150	5.0	7.4
26	29	150	6	96	150	5.0	7.4
25	18	150	6	96	150	5.0	7.4
24	4	150	6	96	250	5.0	5.5
23	12	150	6	96	50	5.0	4.2
22	26	150	6	144	150	5.5	6.2
21	7	150	6	48	150	5.0	5.5
20	23	150	14	96	150	5.0	7.4
19	19	150	2	96	150	5.0	7.4
18	5	250	6	96	150	5.0	7.4
17	6	50	6	96	150	5.0	7.4
16	2	200	10	120	200	5.0	7.0
15	9	100	10	120	200	5.0	7.0
14	8	200	2	120	200	5.0	7.0
13	14	100	2	120	200	5.0	7.0
12	27	200	10	72	200	6.0	6.3
11	21	100	10	72	200	6.0	6.3
10	28	200	2	72	200	6.0	6.3
9	13	100	2	72	200	6.0	6.3
8	24	200	10	120	100	5.5	7.0
7	15	100	10	120	100	5.5	7.0
6	20	200	2	120	100	5.5	7.0
5	11	100	2	120	100	5.5	7.0
4	22	200	10	72	100	6.0	6.3
3	16	100	10	72	100	6.0	6.3
2	17	200	2	72	100	6.0	6.3
1	3	100	2	72	100	6.0	6.3

Table 5: The Yield of Kojic Acid as Affected by Process Variables with *Aspergillus niger* (PQ585363)/ *Aspergillus flavus* (PQ585407)

SD	Run	A: Glucose	B: Yeast Extract	C: Incubation Period	D: Substrate Concentration	E:pH	R1 (Kojic Acid Yield
		(g/l)	(g/l)	Hours	(g/l)		(g/l)
30	30	150	6	96	150	5.0	7.8
29	10	150	6	96	150	5.0	7.8
28	1	150	6	96	150	5.0	7.8
27	25	150	6	96	150	5.0	7.8
26	29	150	6	96	150	5.0	7.8
25	18	150	6	96	150	5.0	7.8
24	4	150	6	96	250	5.0	5.8
23	12	150	6	96	50	5.0	4.5
22	26	150	6	144	150	5.5	6.5
21	7	150	6	48	150	5.0	5.7
20	23	150	14	96	150	5.0	7.8
19	19	150	2	96	150	5.0	7.8
18	5	250	6	96	150	5.0	7.8
17	6	50	6	96	150	5.0	7.8
16	2	200	10	120	200	5.0	7.3
15	9	100	10	120	200	5.0	7.3
14	8	200	2	120	200	5.0	7.3
13	14	100	2	120	200	5.0	7.3
12	27	200	10	72	200	6.0	6.6
11	21	100	10	72	200	6.0	6.6
10	28	200	2	72	200	6.0	6.6
9	13	100	2	72	200	6.0	6.6
8	24	200	10	120	100	5.5	7.3
7	15	100	10	120	100	5.5	7.3
6	20	200	2	120	100	5.5	7.3
5	11	100	2	120	100	5.5	7.3
4	22	200	10	72	100	6.0	6.6
3	16	100	10	72	100	6.0	6.6
2	17	200	2	72	100	6.0	6.6
1	3	100	2	72	100	6.0	6.6

Data in the Table 6 show the optimal fermentation conditions for maximizing kojic acid yield using the fungal isolates. Both the single culture of *A. niger* and the co-cultures of *A. niger* and *A. flavus* had the highest yield of kojic acid with 150 g/L of glucose, 6 g/L of yeast extract. 150 g/L of substrate concentration, 5.0 pH at 96 hours of fermentation yielding 7.4 and 7.8 g/L respectively.

Table 6. Optimal Fermentation Conditions for Maximizing Kojic Acid Yield Using the Fungal Isolates.

Culture Type	Glucose (g/L)	Yeast Extract (g/L)	Incubation Period (Hours)	Substrate Concentration (g/L)	pH	Kojic Acid Yield (g/L)
A. niger	150	6	96	150	5.0	7.4±0.12
A. niger/ *A. flavus*	150	6	96	150	5.0	7.8±0.20
p-value						0.014

p-value is less than 0.05, suggesting a statistically significant difference in Kojic Acid Yield between *A. niger* and *A. niger*/ *A. flavus* at $\alpha = 0.05$

The final product mass, purity, and extraction rate as shown in the Table 7, indicate that the efficiency of Kojic acid extraction increased with higher ratios of ethyl acetate.

Table 7. Kojic acid Extraction Efficiency Using Different Ethyl Acetate Volume Ratio

Fermentation	Ratio of fermentation broth to ethyl acetate			
	1:0.8	1:1.0	1:1.2	1:1.4
Final product mass	Emulsified	2.15	2.58	3.05
Purity (%)	_____	94.80	96.50	97.00
Extraction rate (%)	_____	17.30	20.55	23.05

FTIR spectrum analysis of the sample showed the presence of various active functional groups, which had been indicated by the peaks which presents the presence of Alkane and Alkyl group C-H stretch (at 3253.59 and 3179.41 cm^{-1}) of crystals. Samples also showed the presence of alkenes at 1629.86 cm^{-1} (Cyclic-c=c). Furthermore theses samples showed the presence of alcoholic compound (O-H stretch) at 3600 cm^{-1} and ethers, esters and carboxylic acids (C-O) 1245.23 cm^{-1} at wavelength as shown in the Table 8 and as illustrated in the FTIR graph in **Figure 3**.

Table 8. Functional Groups in Kojic Acid as Revealed by FTIR

Wavelength	Functional Group	Names	Commercial Kojic acid	Produced Kojic acid
3600 cm^{-1}	O-H	Hydroxyl Group	Present	Present
1629.86 cm^{-1}	C=C	Alkene and Aromatic Compound	Present	Present
1245.23 cm^{-1}	C-O	Ethers, Esters and Carboxylic Acids	Present	Present
3253.59 and 3179.41cm^{-1}		Alkane and Alkyl Groups	Present	Present

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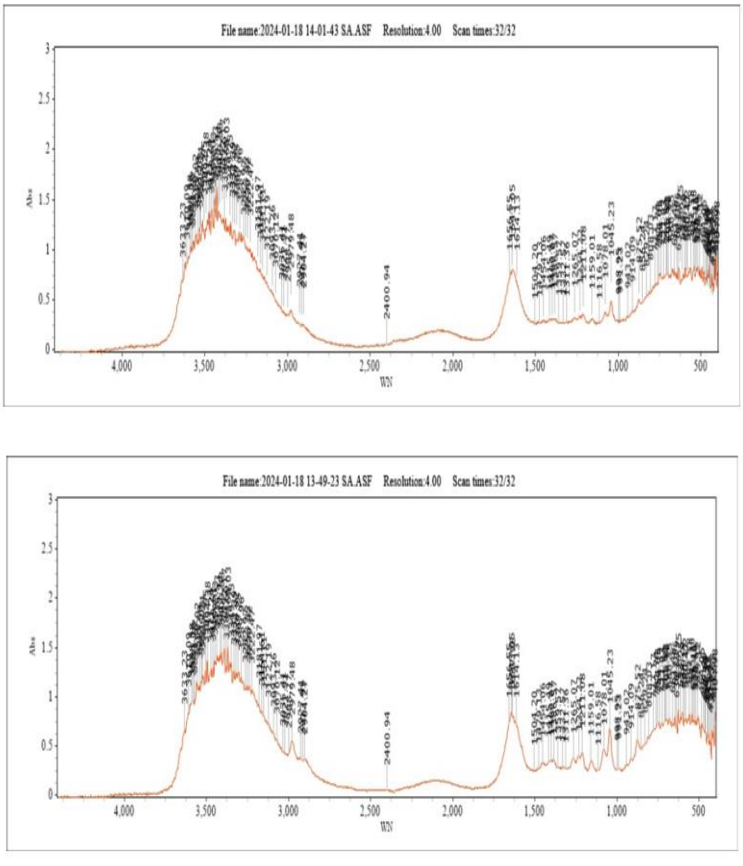

Figure 3. FTIR characterization of Kojic Acid: (A) the produced and (B) the commercial

4. Discussions

Lemon peel is rich in carbohydrate and moderate protein, which makes it suitable for the production of Kojic acid using fungi because high amount of sugar is required to synthesize Kojic acid. This finding align with the report of Rasmey and Abdel-Kareem [15]. Furthermore, the moderate protein content suggests that lemon peel can contribute beneficial nitrogen sources during fermentation, enhancing the nutritional profile of the final product through essential microbial growth and metabolism. Permatananda and Pandit [23], suggest that a protein content of 10-15% is optimal for supporting the metabolic activities of fermentative fungi, ultimately leading to improved yields of fermentation products.

This study successfully isolated fungi that produced Kojic acid from lemon peel, consistent with previous findings by Senanayake *et al.* [24] and Badar *et al.* [2]. The isolated fungal species were identified as prominent producers of Kojic acid, commonly found in natural substrates. Morphological characteristics, such as conidia and colony features, aligned with established fungal taxonomies [25]. Molecular identification confirmed the presence of these fungal species.

The *Aspergillus* species exhibited strong positivity for Kojic acid production was indicated by the red zone of clearance. This significant finding confirms the ability of these fungi to produce Kojic acid, a valuable compound in the food and pharmaceutical industries. These results are consistent with previous research by Mohamed *et al.* [26], who also successfully produced Kojic acid using these fungal species.

This study's results affirm the promising potential of single and co-cultures of the isolated *Aspergillus* species for kojic acid production, showcasing remarkable production capacities. These findings are in agreement with the research conducted by Kamal-Eldin *et al.* [4].

The co-cultures of *Aspergillus* species yielded a significantly higher concentration of kojic acid compared to single cultures, highlighting the benefits of combined fungal interactions. This finding is consistent with previous studies by Yamada *et al.* [27] and Saraphanchotiwitthaya and Sripalakit [28], which demonstrated the efficiency of co-cultures in producing kojic acid, particularly when optimized for specific substrates. These results suggest that synergistic interactions between microbial species can enhance kojic acid production [4]. The optimal conditions identified in this study are comparable to those reported by Mahmoud *et al.* [29]. Furthermore, the extraction and purity results revealed that increasing the solvent ratio improved the efficiency of kojic acid extraction. This finding is in agreement with studies by Xinhe *et al.* [21], which demonstrated the significant impact of solvent ratios on extraction efficiency.

The functional group analysis revealed a striking similarity between the commercial Kojic acid and the Kojic acid produced in this study, indicating that the *Aspergillus* species can synthesize Kojic acid that is chemically identical to its commercially available, synthetically produced counterpart. This is also in line with the study Prantika *et al.* [30]. The yield of Kojic acid obtained in this study is comparable to that reported in previous studies utilizing solid-state fermentation for Kojic acid production. For instance, Patel *et al.* [31] achieved a yield of 4.5g/kg using *A. flavus*, and Costa *et al.* [32] reported a yield of 6.1g/kg using *Penicillium* spp.

In comparison to the production of Kojic acid by the fungi isolated from this study, *Penicillium aurantiogriseum*, *Emericella nidulan* and *A. oryzae* has been reported to produce 2.07±0.19, 2.36±0.7 and 9.05±0.09 respectively using submerged fermentation [33].

5. Conclusion

This study demonstrated the feasibility of using lemon peels as a sustainable substrate for Kojic acid production through fermentation by locally isolated *Aspergillus* species, aligning with circular economy and eco-friendly practices. The optimized conditions, achieved through response surface methodology, significantly enhanced Kojic acid yield while minimizing costs and environmental impact. Utilizing local microbial strains eliminated the need for imported strains and chemicals, promoting indigenous production processes.

Its recommended that there should scaling up of production to industrial levels through pilot-scale studies and exploring alternative substrates like other citrus wastes, millet husk, sweet potato peels, and cassava waste for kojic acid production. The utilization of advanced optimization techniques, such as artificial neural networks (ANNs) or genetic algorithms (GA) would also enhance the production of kojic acid.

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