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Neurotoxic Acrylamide Mitigation By L-Asparaginase Enzyme Extracted From Actinomycetes Species.

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Abstract

Acrylamide is a chemical that is generated in starchy foods, coffee, and bread, etc during high-temperature cooking processes, such as frying, roasting, and baking and is a major food safety concern. Acrylamide toxicity targets the nervous and reproductive systems, causing symptoms like muscle weakness and numbness therefore there is a need to solve this problem by biological method. Actinomycetes are an excellent source of L-asparaginase, an enzyme which specifically catalyses the breakdown of amino acid L-asparagine to aspartic acid and ammonia. If starchy food products are not treated with L-asparginase enzyme then at high temperature L-asparagine present in food converts to acrylamide. Hence, water and soil samples were obtained from Uran, Sai, and Panvel regions and Actinomycetes were isolated onto Starch Casein Agar plates. Biochemical analyses revealed negative results for TSI, nitrate reduction, and citrate but positive for catalase and starch hydrolysis. Noteworthy enzymatic activity at 2.66µg/ml/min was observed. Optimization was performed with various parameters. Production and purification of L-asparaginase by submerged fermentation, followed by ammonium precipitation, dialysis, and subsequent application to pretreat potatoes. After incubation and frying, high-performance liquid chromatography analysis exhibited a reduction in acrylamide levels. The study's meticulous approach, from isolation to application, addresses critical food safety issues, marking a significant contribution.

Keywords: Actinomycetes, L-asparaginase, Acrylamide, HPLC, food safety.

INTRODUCTION

Food is a source of nutrition and basic requirement for living beings to survive but some deep fried, roasted coffee beans and baked food form unhealthy substances as a byproduct such as acrylamide. The Swedish Scientist in the year 2002 accidently discovered the presence of acrylamide in fried and baked food. Acrylamide is a by-product of the Maillard reaction, a complex chemical reaction that occurs among asparagine an amino acid and reducing sugars, predominantly formed in starchy foods subjected to high temperatures and low moisture content. The Maillard reaction leads to the browning and development of characteristic flavours in these foods but, unfortunately, also results in the unintended production of

acrylamide. Acrylamide, a chemical known for its potential carcinogenic, neurotoxic, and genotoxic properties, raised concerns about the safety of commonly consumed food items.
Researchers and food scientists have been exploring various strategies to mitigate its formation, including modifying processing conditions, altering ingredient compositions, and employing enzymatic treatments. Understanding the underlying mechanisms of acrylamide formation is crucial for developing effective strategies to minimize its presence in food products and mitigate potential health risks associated with its consumption. (Pal Murugan Muthaiah et al 2018)
Actinomycetes, soil microorganisms, are a rich source of therapeutically important products. Comprising about 80 genera, these bacteria thrive in terrestrial soils as saprophytes, water, and plant colonizers, displaying marked chemical and morphological diversity. Gram-positive actinomycetes, with a high guanine cytosine content exceeding 55% in their DNA, are recognized for producing secondary metabolites, antibiotics, and bioactive compounds that impact microbial growth. Actinomycetes are also a valuable source of enzymes like L-asparaginase, catalysing the hydrolysis of L-asparagine into aspartic acid and ammonia,
L-asparaginase has been extensively studied for potential applications in the pharmaceutical and food industries, showcasing its versatility and importance in various fields. The treatment of starchy foods with L-asparaginase has been demonstrated to reduce the synthesis of acrylamide significantly, particularly before subjecting the food to high-temperature frying. The key mechanism involves the transformation of asparagine into aspartic acid and ammonium by L-asparaginase prior to baking or frying. This transformation prevents the involvement of asparagine in the Maillard reaction, a process responsible for the browning, crust formation, and toasted flavour in baked or fried foods. As a result, the production of acrylamide in the final food product is significantly reduced. Importantly, the degradation of acrylamide by L-asparaginase does not compromise the taste and appearance of the final product. (Naveed Munir et al 2019).

MATERIALS AND METHODS

1. Enrichment and isolation of actinomycetes:

Samples of soil were collected from various areas around Navi Mumbai, at the depth of 10-12 cm in zip lock bags. Which were treated in a hot air oven to reduce the unwanted bacterial load and serially diluted in sterile distilled water. Samples were then inoculated onto sterile starch casein agar plates and incubated for 4-7 days at 28°C. Primary screening of actinomycetes was done by picking colonies that showed dry and powdery appearance and purified these colonies by four quadrant method onto sterile starch casein agar plates, further these purified cultures were screened by microscopic characterization by using slide culture technique.

2. Qualitative and Quantitative analysis:

Each of the actinomycetes isolate were spot inoculated on asparagine dextrose salt agar plates (ADS) [pH7.0, indicator- phenol red]. Then, plates were incubated at room temperature for 7-8 days. Isolates having the potential of producing L- asparaginase enzyme showed visible pink coloration zones around them.

L-asparaginase activity was determined by measuring the amount of ammonia released by Nesslerization. 1.5 ml of 0.04M L-asparagine was prepared in 0.05M Tris HCl buffer as substrate (pH-8.6). The reaction was started by adding 0.5 ml of crude enzyme and 1.5 ml substrate to make up the volume to 2 ml. The tubes were then incubated at room temperature for 30 minutes. After incubation the reaction was stopped by adding 1% TCA and the precipitated protein was removed. 0.5 ml of supernatant extracted was then mixed with the 2 ml of distilled water. The liberated ammonia in the supernatant was determined by adding 1 ml of Nessler's reagent and enzyme activity was determined using standard graph of nesslerization.

$$\textit{Enzyme activity} = \frac{concentration\ of\ enzyme}{O.D\ of\ crude\ enzyme \times 30\ min}$$

3. Biochemical & Molecular Characterization of isolates.

Characterization by biochemical analysis such as Indole test, Methyl red test, Voges Proskauer, Citrate , TSI, starch hydrolysis, gelatine hydrolysis, casein hydrolysis, catalase, urease were performed.
16s rRNA sequencing was used to validate the strain of Actinomycetes.

4. Optimization, Production and Purification:

Different temperature and pH conditions was set up to carry out optimization. Various types of carbon and nitrogen sources were provided to carry out optimization. Optimization of actinomycetes for various growth

parameters like pH(9 & 5), Temperature (50°C & 25°C), Agitation (0 &150), Incubation in day (2 & 5 days), Inoculum volume/ml (0.1 & 0.2), carbon source and nitrogen source. Dextrose (0.12g & 0.04g), Asparagine (0.2g & 0.8g), Yeast extract (2g & 0.2g), Tryptone (0.2g & 0.8g), K_2HPO_4 (0.12g & 0.04g), NaCl (0.02g & 0.04g).

The isolate 65 showing higher production of L-asparaginase was selected for submerged fermentation for production of enzyme. 1 ml of these samples were inoculated into a specialized medium designed from optimization results. Later, these flasks were incubated at room temperature for 3-4 days on a rotary shaking incubator at 250 rpm. The fermented broth was then subjected to centrifugation at 5000 rpm for 20 minutes at 4°C in cooling centrifuge, to remove cellular mass and extract the enzyme using Tris HCl buffer which was further purified using ammonium sulphate precipitation at 80% saturation. The precipitate was collected by centrifugation at 4°C, 10000 rpm for 30 min. This precipitate was dissolved in 50mM of tris HCl buffer. Dialysis was performed for further purification. Purified Enzyme was used for reducing formation of acrylamide in fried foods.

5. Application on potato slice.

Fried Potato Products

L-asparaginase can effectively inhibit acrylamide formation in fried potato products because of its catalytic hydrolysis of L-asparagine to aspartic acid. Thermostable L-asparaginase has potential application in fried potato products. The procedures used to treat potato slices with the same enzyme concentration 2.664 µg/ml/min blanching in enzyme solution at 40°C for 15 min. The dried potato slices were fried at 175°C for 10 min.

Extraction of Acrylamide

A sample of snack food, homogenised and finely ground, weighing 10.0 g, was defatted twice by adding 50 mL of dichloromethane and 5 mL of ethanol, then 50 mL of dichloromethane alone. The mixture was then vigorously shaken in a shaker for two hours at room temperature. Centrifuging the mixture for 20 minutes at 15 ºC at 3500 rpm was done. After discarding the supernatant, the precipitate was dried at 40ºC in a vacuum oven. 20 millilitres of acetone was added to the defatted sample in order to extract Acrylamide. After about 20 minutes at 40°C in an Ultrasonic water bath, the samples were centrifuged for 25 minutes at 15 ºC at 3000 rpm. A filter paper (What man filter paper No. 1) was used to filter the solvents. The filtrate was dried by evaporating it in a flash evaporator. To dissolve the residue, 2 millilitres of HPLC grade water: acetonitrile (40:60) mobile phase was added and vigorously shaken. Using Solid Phase Extraction tubes (ODS, 500 mg, and 3 mL capacity) to purify the aqueous solution, a 20 µl injection loop was used to inject it into the column. (Muthugan Murugan et al.)

Results and Discussions:-

1. Enrichment and isolation of Actinomycetes from different soil and water samples:

25 different samples collected from various regions of Navi Mumbai, out of which 86 isolates were obtained, these isolated were selected according to their initial appearance showing powdery colonies, gram positive filamentous form of bacteria were further analysed using the slide culture method. The slides were observed under the microscope for aerial, hyphae, mycelium and spore out of 86 isolates 77 showed positive results for actinomycetes.

Figure 1 Sample collection

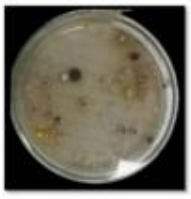


Figure 2 Isolation on starch casein agar

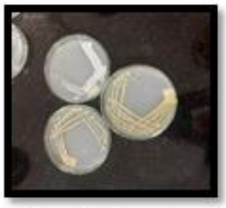


Figure 3 Purification of isolates by four quadrant method

Figure 4 Microscopic observation of slide culture

2. Screening of L- Asparaginase by Qualitative and Quantitative method:

While screening by qualitative method out of 77 isolates 57 isolates showed production of L-asparaginase enzyme. Isolates 65, 23, 86, and 27 showed maximum pink zones around the colonies, producing higher amounts of L-asparaginase enzyme. Hence, they were selected for submerged production of L-asparaginase. The L-asparaginase catalyse L-asparagine to L-aspartic acid and ammonia, and the latter reacts with Nessler's reagent to produce an orange-coloured product. The isolate 65 showed maximum absorbance at 430 nm, its enzyme activity was found to be 2.664 µg/ml/min.

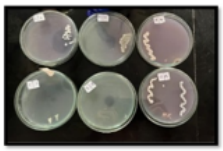
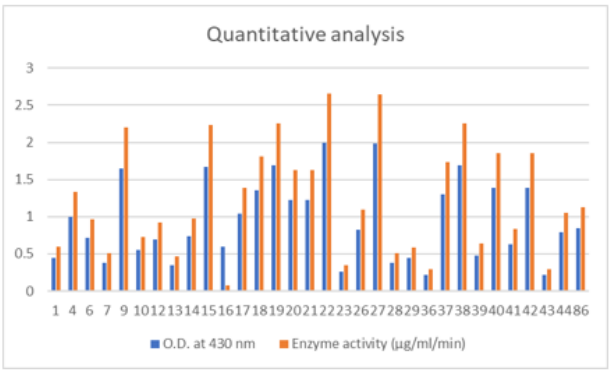


Figure 5 Qualitative analysis

Figure 6 Quantitative analysis



3. Biochemical & Molecular Characterization of isolates

3.1. Biochemical analysis

Biochemical tests performed showed the following results of Isolate no. 65 Citrate negative, Indole positive, TSI negative, Catalase positive, Methyl Red positive, Voges Proskauer positive.

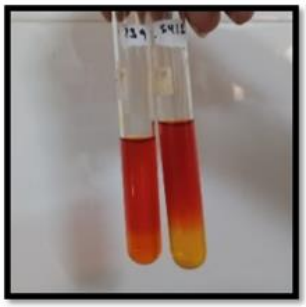


Figure 7 Indole test

Figure 8 Starch hydrolysis

Figure 9 Tryptone

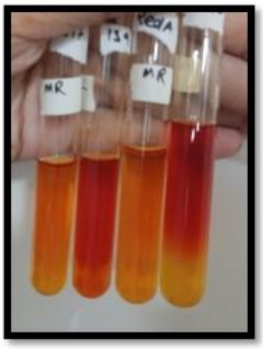


Figure 10 Methyl red

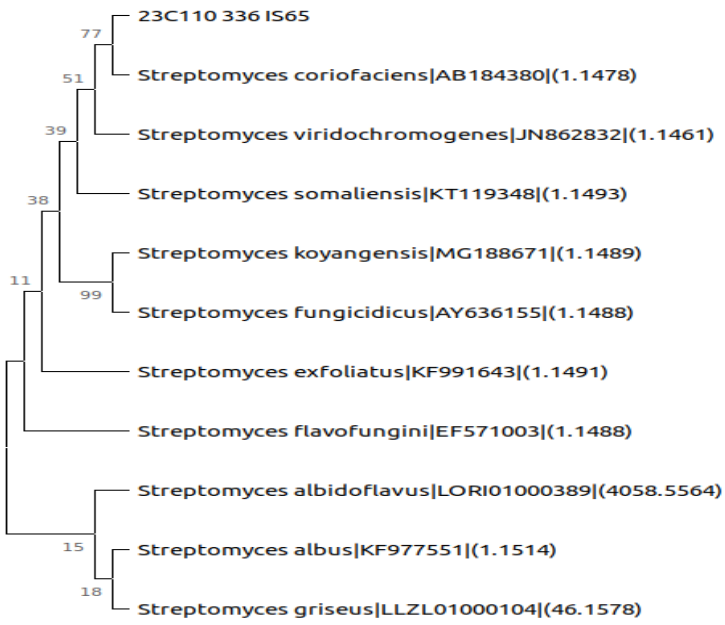
Figure 11 Voges Proskauer

Figure 12 Urease

3.2. 16S rRNA sequencing of potent isolate no 65.

MBS110: From Isolate to Identity

	qseqid	sseqid	pident	length	mismatch	gapopen	qstart	qend	sstart
1	23C110_336_IS65	Streptomyces_griseus|LLZL01000104|(46.1578)	99.796	1473	2	1	1	1473	10
2	23C110_336_IS65	Streptomyces_albidoflavus|LORI01000389|(4058.5564)	99.796	1473	2	1	1	1473	1
3	23C110_336_IS65	Streptomyces_flavofungini|EF571003|(1.1488)	99.796	1473	2	1	1	1473	1
4	23C110_336_IS65	Streptomyces_coriofaciens|AB184380|(1.1478)	99.793	1451	3	0	1	1451	1
5	23C110_336_IS65	Streptomyces_viridochromogenes|JN862832|(1.1461)	99.793	1448	3	0	1	1448	1
6	23C110_336_IS65	Streptomyces_exfoliatus|KF991643|(1.1491)	99.729	1475	2	2	1	1474	1
7	23C110_336_IS65	Streptomyces_somaliensis|KT119348|(1.1493)	99.729	1474	3	1	1	1474	1
8	23C110_336_IS65	Streptomyces_albus|KF977551|(1.1514)	99.661	1473	3	2	1	1473	1
9	23C110_336_IS65	Streptomyces_fungicidicus|AY636155|(1.1488)	99.457	1473	7	1	1	1473	1
10	23C110_336_IS65	Streptomyces_koyangensis|MG188671|(1.1489)	99.456	1471	7	1	1	1471	1



4. Optimisation, Production and Purification.

Optimisation of different growth conditions such as pH(9 & 5), Temperature (50℃ & 25℃), Agitation (0 &150), Incubation in day (2 & 5 days), Inoculum volume/ml (0.1 & 0.2), Dextrose (0.12g & 0.04g), Asparagine (0.2g & 0.8g), Yeast extract (2g & 0.2g), Tryptone (0.2g & 0.8g), K_2HPO_4 (0.12g & 0.04g), NaCl (0.02g & 0.04g), required for the potent isolate 65 and 27 was performed by Placket Burman design and the following result were obtained showing best growth at condition pH 9, temperature 25℃, agitation at 150rpm, inoculum volume 0.2ml, Dextrose 0.12g, Asparagine 0.8g , Yeast extract 2g , Tryptone 0.2g, K_2HPO_4 0.04g , NaCl 0.02g where the optical density obtained was 2 at 430 nm and enzyme activity 2.66 µg/ml/min. The isolate 65 showing higher production of L-asparaginase was selected for submerged fermentation for production of enzyme. 1 ml of this sample was inoculated into asparagine dextrose salt medium. Later, these flasks were incubated at room temperature for 3-4 days on a rotary shaking incubator at 250 rpm. The fermented broth was then subjected to centrifugation at 5000 rpm for 20 minutes at 4°C in cooling centrifuge, to remove cellular mass and extract the enzyme using Tris HCl buffer which was further purified using ammonium sulphate precipitation at 80% saturation.

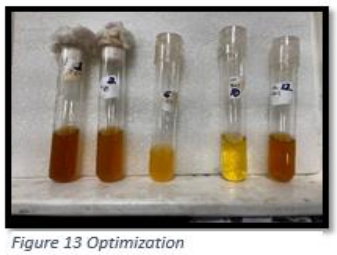


Figure 13 Optimization

Figure 14 Ammonium precipitation

Figure 15 Dialysis assembly

5. Application

5.1.Activity of L-asparaginase on Acrylamide

The purified enzyme showing activity 2.66µg/ml/min was dipped in a potato slice of 10 grams named Test and another potato slice that was not treated named Control. The Test was then incubated at 40℃ for 15 min followed by both fried at 175℃ for 5 min. Then followed by extraction of acrylamide from the fried potato. This extracted acryl amide was detected by HPLC showed 26% reduction.

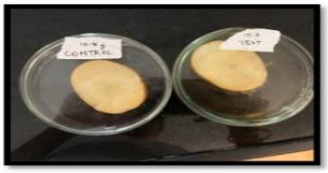
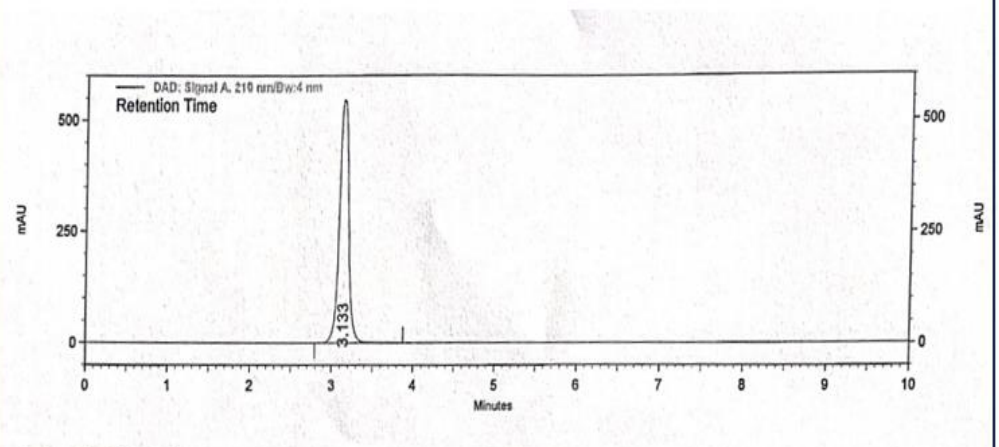


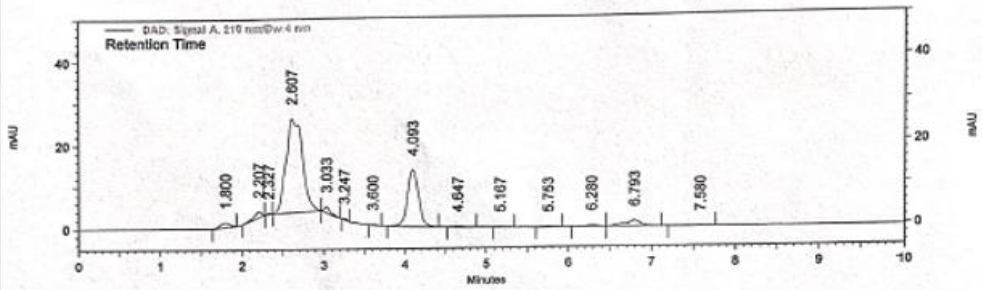
Figure 16 Application on potato slices



DAD: Signal A, 210 nm/Bw:4 nm Results

Peak No.	Name	Retention Time	Area	Area Percent
1	Acrlamide	3.133	45629532	100.000
Totals			45629532	100.000

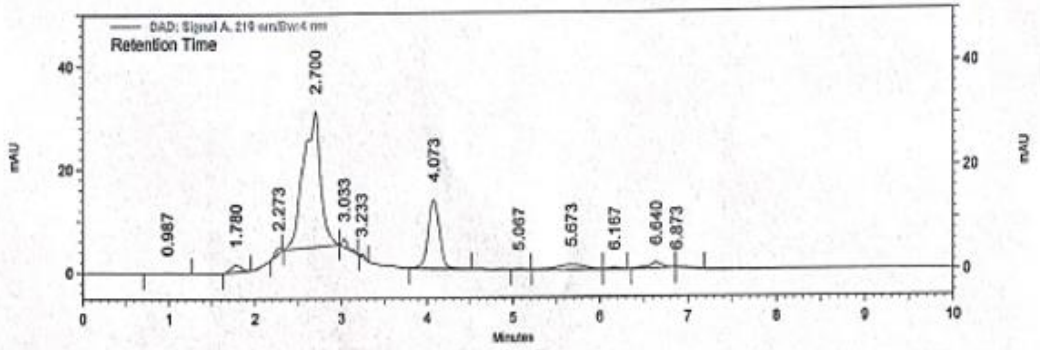
Figure 17 Standard Acrylamide peak



DAD: Signal A, 210 nm/Bw:4 nm Results

Peak No.	Name	Retention Time	Area	Area Percent
1		1.800	88979	1.708
2		2.207	89864	1.725
3		2.327	6830	0.131
4		2.607	3315419	63.625
5	Acrlamide	3.033	82272	1.579
6		3.247	12833	0.246
7		3.600	6109	0.117
8		4.093	1354564	25.995
9		4.647	12835	0.246
10		5.167	6478	0.124
11		5.753	13029	0.250
12		6.280	31325	0.601
13		6.793	182153	3.496
14		7.580	8165	0.157
Totals			5210855	100.000

Figure 18 A2 Control without Enzyme treatment



DAD: Signal A, 210 nm/Bw:4 nm
Results

Peak No.	Name	Retention Time	Area	Area Percent
1		0.987	8124	0.143
2		1.780	109887	1.939
3		2.273	47370	0.836
4		2.700	3802286	67.088
5	Acrlamide	3.033	74687	1.318
6		3.233	17201	0.303
7		4.073	1256677	22.173
8		5.067	10900	0.192
9		5.673	199674	3.523
10		6.167	19279	0.340
11		6.640	115117	2.031
12		6.873	6391	0.113
Totals			5667593	100.000

Figure 19 A1 Test with enzyme treatment

CONCLUSION

Acrylamide is a chemical that is generated in starchy foods, coffee, and bread, etc during high-temperature cooking processes, such as frying, roasting, and baking and is major food safety concern. Acrylamide toxicity targets the nervous and reproductive systems, causing symptoms like muscle weakness and numbness therefore there is a need to solve this problem by biological method. Actinomycetes are excellent source of L-asparaginase. L-asparaginase specifically catalysis the breakdown of amino acid L-asparagine to aspartic acid and ammonia if not treated at high temperature L-asparagine converts to acrylamide. Hence, samples were obtained from Uran, Sai, and Panvel and isolated onto Stach Casein Agar. Biochemical analyses revealed negative results for TSI, nitrate reduction, and citrate but positive for catalase and starch hydrolysis. Noteworthy enzymatic activity at 2.66µg/ml/min was observed. Optimization was performed with various parameters such as pH(9 & 5), Temperature (50°C &25°C), Agitation (0&150), Incubation in days (2 & 5 days), Inoculum volume/ml (0.1&0.2), Dextrose (0.12g & 0.04g), Asparagine (0.2g & 0.8g), Yeast extract (2g & 0.2g), Tryptone (0.2g & 0.8g), K_2HPO_4 (0.12g & 0.04g), NaCl (0.02g & 0.04g), required for the potent isolate 65 and 27 was performed by Placket Burman design and the following result showed optimum growth at growth condition no.8 given in table. Production and purification of L-asparaginase by submerged fermentation in ADS broth, followed by ammonium precipitation at 80% saturation, dialysis was performed for further purification, and subsequent application to pretreat potatoes each of wt.10g namely test and control test treated with L-asparaginase control untreated. After incubation and frying, high-performance liquid chromatography analysis exhibited a reduction in acrylamide levels. The study's meticulous approach, from isolation to application, addresses critical food safety issues, marking a significant contribution.

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