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Optimized Biomass Production of Probiotic Bacterium *Pediococcus acidilactici* TMAB26 Using Pineapple Peel: A Response Surface Methodology Approach

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Abstract

Probiotics, being non-pathogenic, offer distinct health advantages tailored to specific species and demonstrate antioxidant properties. Currently, there is a growing interest among people in probiotics due to their therapeutic and health-promoting benefits. The study aimed for utilization of pineapple peel waste as carbon source for biomass production of probiotic bacterial strain *Pediococcus acidilactici* TMAB26 demonstrating promising avenues for sustainable and efficient production methods. By employing Central Composite Design (CCD) Response Surface Methodology (RSM), the study successfully optimized fermentation conditions, highlighting the significance of pH, tempe rature, and inoculum size in maximizing biomass yield. The pineapple peel waste was used as complete carbon source that replaced half of the other nutrient components of commercial MRS broth. The optimization of medium for biomass production of *Pediococcus acidilactici* TMAB26 was carried out using Central Composite Design (CCD) in 2 L Erlenmeyer flasks. The findings highlight the pivotal role of the physical parameters in enhancing biomass production, with an optimal combination of 2.5% inoculum, pH 6.5, and temperature 35 °C resulting in a significant biomass yield of 2.56g/100ml. Furthermore, the pineapple peel extract exhibited notable total phenolic content of 262 µg/mL and glucose content of 1.83%, indicating its significance as a valuable nutrient source. Moreover, the probiotic strain TMAB26 showed impressive antioxidant potential, as evidenced by its high hydroxyl radical scavenging activity (92.1 ± 4%) with an IC50 of 24.76 µg/ml.

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Introduction

Probiotics, classified as living microorganisms that confer health benefits upon ingestion in specific quantities, play a pivotal role in maintaining host immune system.[1] Among all microorganisms, bacteria are the most widely exploited organisms used as probiotics.[2,3] The cultivation of probiotics, particularly lactic acid bacteria, necessitates an environment conducive to growth and a complex medium comprising various biochemical components.[4] These components typically include carbon and nitrogen sources such as dextrose, yeast extract, peptone, and beef extract, as well as essential minerals like Mg2+ and Mn2+. Additionally, buffering agents like di-sodium-glycerophosphate and sodium acetate are employed to maintain the optimal pH levels required for robust bacterial growth. Researchers and industry professionals are actively investigating for the development of low-cost media and probiotic drinks to replace expensive commercial media components for probiotics cultivation and biomass production.[15]

To enhance competitiveness in the probiotics market, it is imperative to optimize high yield production through the utilization of low-cost, nutrient-rich media.[6] Commercial manufacturing of probiotics typically relies on expensive media such as de Man, Rogosa, and Sharpe (MRS). Consequently, the exploration of agro waste as an alternative medium holds promising results for reducing production costs.[1] Pineapple (*Ananas comosus*), a prominent fruit globally and a leading member of the Bromeliaceae family, stands out as a significant candidate.[7] Pineapple juice ranks third in global preference after orange and apple juices. However, approximately 55-60% of pineapple products are going waste due to rough handling during transportation and storage, leading to the generation of by-products such as stems, leaves, pulp, and peel.[8,9] While unsuitable for direct human consumption, pineapple waste serves as feedstuff for animals. The disposal of unfit waste contributes to environmental pollution. Pineapple peel is rich in soluble sugars (glucose, sucrose, and fructose), and fiber content, where as poor in proteins, minerals, and other nutrients, including Bromelain, a digestive enzyme.[9,10] Carbon serves as primary energy source for biomass production and influences the synthesis of primary and secondary metabolites in microbes.[11] Presently, pineapple waste is increasingly utilized as a substrate for microorganisms to produce organic acids such as lactic acid, citric acid, and acetic acid.[12]

Response Surface Methodology (RSM) is an assembly of efficient statistical and mathematical techniques applied for optimizing fermentation development. RSM is utilized for various fermentation scale-up processes, such as enzymes, metabolites, biomass, and spore production.[13] The advantage of RSM lies in its ability to evaluate numerous parameters and their interactions simultaneously, measuring the individual outcomes of each parameter and observing their potential association.[14] The statistical method, RSM proves useful in reducing the number of experimental trials, thereby decreasing laborious work. It is less time-consuming, and simple calculations, like ANOVA analysis of the obtained data, are required when compared to other methods.[15] The study emphasizes the prime role of indigenous medium for mass production of potential probiotic *Pediococcus acidilactici* TMAB26 bacteria using fruit waste like pineapple peel. The present study focuses on the development of pineapple peel-based indigenous medium for the production of an antioxidant-efficient probiotic *Pediococcus acidilactici* TMAB26.

Materials and Methods

Antioxidant Property of *P. acidilactici* by DPPH Radical Scavenging Assay

Preparation of Cell Free Supernatant

The probiotic bacterium TMAB26 strain sample was prepared following the modified methodology outlined by Xing J.[16] Active culture was centrifuged at 10,000 rpm for 5 minutes, followed by neutralization of the supernatant pH using 1 M NaOH. The resulting cell free filtrate (CFF) was filtered using 0.22-µm membrane filters. Various concentrations (10, 20, 40, 60, 80, 100 µg/mL) of the sample were prepared and added to a 96-well plate. Subsequently, 100 µL of freshly prepared 0.2 mM DPPH solution in methanol was added, and the volume was adjusted to 200 µL with deionized water. The entire mixture was vortexed and then incubated in a dark room for 30 minutes at 37 °C. The scavenging activity of DPPH was assessed by measuring the optical density at 517 nm using a microplate reader (Epoch Biotech) with a blank. Ascorbic acid was used as the standard. Methanol with DPPH served as the control, and methanol without DPPH served as the blank. The scavenging activity of *P. acidilactici* TMAB26 CFF

was quantified in terms of the percentage of DPPH free radicals eliminated and expressed as IC_{50}, representing the concentration of the strain sample at which the absorbance of the DPPH radicals was reduced by 50%. The following formula was employed to calculate the percentage inhibition of DPPH free radical scavenging activity.[16]

DPPH activity (µl/ml) = [(Ao-Ae)/Ao] × 100

where,

Ao- absorbance of the control and Ae – test samples.

Preparation of Pineapple Peel-Based Medium

The pineapple waste were sourced from local juice centers in Hyderabad, Telangana, India. The collected pineapple peel underwent washing under tap water and subsequently sun-dried for 2 days. Following sun-drying, the peel were pulverized into a fine powder using a mixer grinder. Subsequently, 10 g of pineapple peel powder was dispersed in 100 ml distilled water within a conical flask and subjected to boiling for 30 minutes at 90 °C. The resulting boiled extract was then filtered using Whatman filter paper, and the filtrate volume was adjusted to 100 ml with distilled water. A customized culture medium was formulated by substituting glucose with the filtrate obtained from pineapple peel powder, and reducing nutrient concentrations to half of those in the standard MRS broth formulation. The pH of the medium was adjusted to 6.2 ± 0.2 at room temperature. MRS medium served as the reference medium.

Total Phenolic Estimation

The total phenolic content of the pineapple peel extracts was determined using the Folin-Ciocalteu reagent method. Different concentrations of standards and samples were prepared, with the test sample (0.1 mL) mixed with 8.4 mL of water and 0.5 mL of Folin-Ciocalteu's phenolic reagent (1:1 dilution). Following a 5-minute incubation in a dark at room temperature, 1 mL of 20% sodium carbonate solution was added to the mixture. The optical density at 750 nm was measured against a blank using a spectrophotometer. Calibration curves were constructed using various concentrations of Gallic acid (ranging from 20 to 200 µg/mL) to determine the total phenolic content.[17]

Estimation of Glucose in Pineapple Peel Extract by HPLC

Sample preparation and sugar analysis were conducted using modified methodology outlined by Siti Roha.[18] A standard glucose solution was prepared to generate a standard curve for glucose quantification. Glucose was dissolved in distilled water and subsequently filtered through a Millipore 0.45 µm membrane filter. The glucose content in the samples was quantified by comparing the peak areas of sample with the standard obtained from High-Performance Liquid Chromatography (HPLC) of Shimadzu Lab Solutions, Singapore equipped with an amine column (Shim-pack GIST NH2, particle size 5 µm, dimensions 250 × 4.6 mm).

The HPLC analysis was conducted at a controlled temperature of 22 °C. The mobile phase utilized was a mixture of acetonitrile and HPLC-grade water in a 60:40 ratio, chosen for optimal analyte separation. Detection was carried out at a wavelength of 254 nm, suitable for UV-absorbing compounds. The flow rate was set to 1 mL/min, and the injection volume was 20 µL, balancing sensitivity with column capacity. The analysis was completed in 15 minutes. To ensure accuracy and prevent interference, all the solvents were sonicated and degassed prior to use.

Effect of Physical Parameters on the Biomass Production of *Pediococcus acidilactici* Effect of Temperature

The impact of temperature variations on biomass production of probiotic isolate was assessed at different temperatures ranging from 25 °C to 45 °C for over 24 hours. The initial pH of the growth medium composed of pineapple peel was maintained at 6.0 ± 0.5. The dry weight of the obtained biomass of the probiotic isolate was measured using an analytical balance.

Effect of Different pH Conditions

The influence of initial pH on growth was investigated by culturing the probiotic isolate in pineapple peel based fermentation growth medium with varying pH levels ranging from 4.0 to 8.0, adjusted using 0.1 N HCl and 0.1 M NaOH solutions. The flasks were then incubated at 37 °C for 24 hours, and the resulting biomass was collected and dry weight was determined.

Effect of Inoculum Size

Different percent of inoculum ranging from 1% to 45%, were added to the growth medium composed of pineapple peel with an initial pH of 6.0 ± 0.5. The cultures were then incubated at 37 °C for 24 hours to evaluate the effect of inoculum size on biomass production.

Experiment Design

Three independent factors were considered, each with 5 different levels (Table 2), and these factors were screened in a total of 20 experimental runs (Table 1). The lowest and highest settings of each factor were coded as -1 and +1, respectively, while the midpoint was coded as 0. The design was further extended up to a minimum (-α) and maximum (+α) to ensure thorough exploration of the factor space (Table 2). The optimal concentrations of the various factors were determined using statistical optimization technique and by analyzing the response surface plots, enabling the identification of the most favorable conditions.[19]

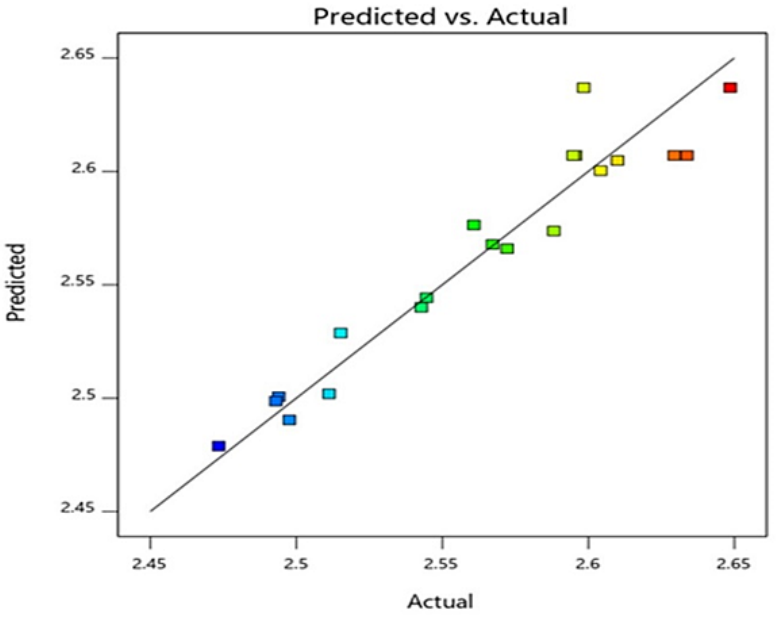

Fig. 1: Plots showing the correlation of actual conversions and values predicted by the model for biomass production of *P. acidilactici* TMAB26

The confirmation experiment depicted in Figure 1 demonstrates strong concordance between the observed response values and the predicted response values. Notably, a linear distribution is evident, indicating a well-fitted model. Furthermore, the straight line displayed in Figure 1 corroborates the fulfillment of the normality assumption.

Based on the experimental outcomes, biomass production was estimated utilizing a polynomial relationship of dependent factors. The primary equation employed to model this relationship is as follows

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \quad x_i \qquad ...(1)$$

Where Y is the predicted response; β0, βi are the constant coefficient, and xi is the coded independent factor

To ascertain the optimal levels and combinations of the selected variables for biomass production of *P. acidilactici* TMAB26, Response Surface Methodology and Central Composite Design were employed. Table 1 presents both the predicted and observed values from 20 experimental runs, while Table 3 illustrates the results of the analysis of variance based on Central Composite Design experiments. The biomass production is predicted by the following equation.

$$Y = +2.10 + 0.081\,A + 0.0020\,B + 0.017\,C + 0.00015AB + 0.00050\,AC - 0.000012\,BC - 0.0097\,A2 - 0.000054\,B2 - 0.000297\,C2$$

Where Y is responsible for Biomass and A, B, C are responsible for pH, inoculum, temperature, respectively.

Table 1: The various experiment compositions of the CCD for independent variables as well as responses

Run. No	Initial pH	Inoculumsize (%)	Temperature (ºC)	Biomass production percentage of grm	
				Actual	Predicted
1	6	2.5	35	2.60	2.61
2	4	1.0	45	2.54	2.54
3	6	2.5	35	2.59	2.61
4	4	4.0	25	2.57	2.57
5	4	1.0	25	2.56	2.58
6	6	2.5	35	2.63	2.61
7	8	1.0	45	2.49	2.50
8	8	4.0	45	2.51	2.50
9	8	4.0	25	2.49	2.50
10	8	1.0	25	2.50	2.49
11	4	4.0	45	2.52	2.53
12	6	2.5	35	2.63	2.61
13	6	2.5	51.8179	2.54	2.54
14	9.36359	2.5	35	2.47	2.48
15	6	-0.226892	35	2.61	2.60
16	6	2.5	18.1821	2.57	2.57
17	6	2.5	35	2.60	2.64
18	6	5.02269	35	2.60	2.60
19	2.63641	2.5	35	2.59	2.57
20	6	2.5	35	2.65	2.64

Table 2: Levels and Ranges of experimental variables.

Factors	Levels of factors				
	-α	-1	0	+1	+α
pH	2.64	4	6	8	9.36
Inoculum (%)	-0.2269	10	25	40	50.23
Temperature (ºC)	18.18	25	35	45	51.82

Data Analysis and Software

The Design Expert Software Version 11.1.2.0 statistical techniques as RSM was used for experiments, construction models and statistical parameters were carried out by analysis of variance.

Results

DPPH Radical Scavenging Activity of *P. acidilactici* TMAB26

The radical scavenging activity of strain TMAB26 cell-free filtrate (CFF) at concentrations of 10, 20,

40, 60, 80, and 100 µg/mL demonstrated hydroxyl radical scavenging percentages of 36 ± 0, 45.9 ± 2.6, 67.3 ± 3, 74.3 ± 2, 79.4 ± 1.5, and 92.1 ± 4%, respectively, with an IC_{50} value of 24.76 µg/mL. The antioxidant activity increased proportionally with the concentration of the sample, as illustrated in Figure 2.

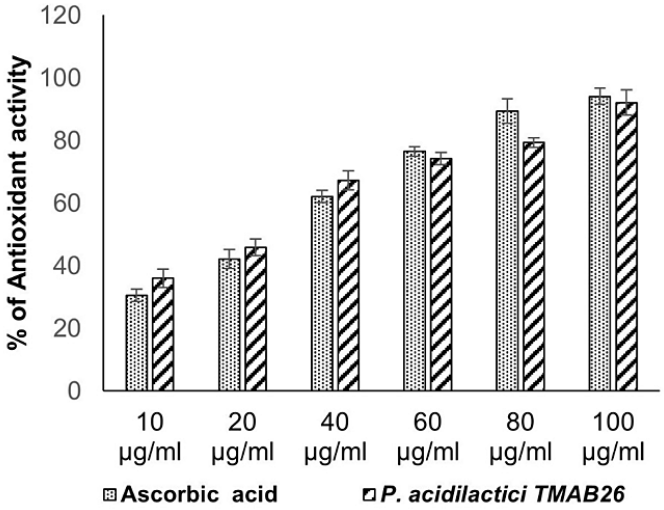

Fig. 2: Antioxidant abilities of *P. acidilactici* TMAB26 using different concentrations (10, 20, 40, 60, 80, 100 µg/ml) of cell-free filtrate and Ascorbic acid was taken as standard. Error bars represent the standard deviation of the mean values obtained from the micro soft word

Phenolic Compounds of Pineapple Peel

Total phenolic compounds were quantified using the Folin-Ciocalteu method, with Gallic acid equivalent in µg/mL serving as the standard curve. The obtained calibration curve for Gallic acid equivalent at different concentrations (µg/mL) facilitated the calculation of total phenolic compounds. The equation of the standard curve was determined as y = 0.0012x + (-0.0233), where y = 0.156, with a correlation coefficient (R^2) of 0.9856. The total phenolic content in pineapple peel extract was measured as 262 µg/mL.

Glucose of Pineapple Peel

The total glucose content of pineapple peel was analyzed on HPLC and determined to be 18.35 mg/mL, while the remaining glucose in the spent pineapple medium was found to be 9.747 mg/mL.

Preparation of Pineapple Peel-Based Medium

The pineapple peel medium was prepared according to the protocol outlined in the Materials and Methods section. Customized culture medium was formulated by replacing glucose with pineapple peel powder filtrate and reducing the nutrient content by half while maintaining the composition of MRS broth. The pH was adjusted to 6.2 ± 0.2 at room temperature. MRS broth served as the standard working growth medium for the probiotic strain TMAB26.

Effect of Response Parameters on the Production of Biomass

The interaction effects of variables on the considered responses were investigated by plotting 3D surface curves, comparing any two independent variables while maintaining another variable at the central (0) level. The results obtained from this study demonstrate a better agreement between the actual response values and the predicted response values as shown in Table 1.

Effect of pH on Biomass Production of *P. acidilactici* TMAB26

The combined effect of initial pH and temperature, as well as pH and inoculum size, on biomass production of *P. acidilactici* showed promising results. Maximum biomass production occurred at pH 6.5; deviations from this pH value resulted in decreased biomass production.

Effect of Temperature on Biomass Production of *P. acidilactici* TMAB26

The interaction of temperature with pH and inoculum size, and temperature with inoculum size, on biomass production of *P. acidilactici* TMAB26 showed promising results. The results reveled that the maximum biomass production occurred at 35 °C; deviations from this temperature led to a decrease in biomass production.

Effect of Inoculum Size on Biomass Production of *P. acidilactici* TMAB26

Furthermore, the combined effect of inoculum size with pH and temperature, and inoculum size with temperature, on biomass production of *P. acidilactici* TMAB26 observed to be maximum biomass production at a 2.5% inoculum size; deviations from this inoculum size resulted in reduced biomass production of strain TMAB26.

Model Fitting

The model fitting was evaluated based on R^2 (coefficient value), P (probability), and F (Fisher value) values obtained through the estimation of ANOVA (Table 3). The R-squared value elucidated the correlation between experimental values and predicted values of factors and their interactions. For biomass production of *P. acidilactici* TMAB26, the obtained R^2 was 0.9166, with adjusted R^2 and predicted R^2 values of 0.8332 and 0.6780, respectively. The Predicted R^2 of 0.6780 showed reasonable agreement with the Adjusted R^2 of 0.8332, with a difference of less than 0.2, indicating the goodness of fit of the model. The Adeq Precision, measuring the signal-to-noise ratio, yielded a ratio of 10.003, which indicates an adequate signal in our experiment. Therefore, this model can effectively navigate the design space.

Furthermore, the Lack of Fit F-value of 0.46 was found to be insignificant, suggesting that the model fit was good due to the lack of significance in the lack of fit. Additionally, the Model P-value being less than 0.05 indicated that the model terms were significant.

Optimized Conditions Obtained by RSM

The optimal fermentation conditions for achieving maximum biomass production of *P. acidilactici* TMAB26 were determined to be a combined inoculum size of 2.5%, a pH of 6.5, and a temperature of 35°C, resulting in a biomass production of 2.561 g/100 mL.

Table 3: Analysis of variance (ANOVA)

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Block	0.0034	1	0.0034			
Model	0.0449	9	0.0050	10.99	0.0007	Significant
A-pH	0.0109	1	0.0109	23.91	0.0009	
B-inoculum	0.0000	1	0.0000	0.0532	0.8228	
C-Temperature	0.0008	1	0.0008	1.78	0.2150	
AB	0.0002	1	0.0002	0.3892	0.5482	
AC	0.0008	1	0.0008	1.80	0.2129	
BC	0.0000	1	0.0000	0.0540	0.8215	
A^2	0.0220	1	0.0220	48.53	< 0.0001	
B^2	0.0021	1	0.0021	4.68	0.0589	
C^2	0.0127	1	0.0127	27.94	0.0005	
Residual	0.0041	9	0.0005			
Lack of Fit	0.0015	5	0.0003	0.4597	0.7918	Not significant
Pure Error	0.0026	4	0.0006			
Cor Total	0.0524	19				

SD. 0.0213	R^2 0.9166
mean 2.56	adjusted R^2 0.8332
C.V. % 0.8311	predicted R^2 0.6780
	Adeq precision 10.0025

Discussion

The bacterium *Pediococcus acidilactici* TMAB26 has demonstrated promising probiotic efficacy and associated health benefits.[20] Numerous recent studies investigated the antioxidant activity of probiotics, few of which utilized probiotics as supplements or directly incorporated them into the diet. Additionally, certain probiotics were shown to enhance antioxidative enzymes or protect cells from damage induced by oxidative stress and carcinogens.[21]

The total phenolic content in pineapple peel extract was estimated to be 262 µg/mL using Spectrophotometric assays.[22] In our study, no significant effect of phenolic content on the biomass production of strain TMAB26 was observed.

The total glucose content in pineapple peel was found to be highest at 1.835%, which aligns with findings by Siti Roha, who reported a glucose content of about 2.18% in pineapple variety N36 peel.[18]

Lactic acid bacteria (LAB) growth and biomass production typically require nutrient-rich media such as MRS and M17. To address cost concerns, we aimed to develop inexpensive, nutrient-rich medium from fruit by-products, like peel. In the present study, we utilized pineapple peel for the biomass production of probiotic strain TMAB26, capitalizing on its abundance of sugars and nutrients generated during pineapple processing.

Previous research has demonstrated the utilization of pineapple waste for the production of lactic acid, citric acid, and vinegar.[23]

In our study, we investigated the influence of physical parameters like pH, temperature, and inoculum size on the biomass production of probiotic strain TMAB26. Biomass production varied across temperatures ranging from 25 to 45 °C, with optimal growth observed at 35 °C. Similarly, the inoculum size ranged from 1 to 5%, with maximum biomass obtained at 2.5%. The effect of pH spanned from 4 to 8, with maximum biomass production observed at pH 6.5.

Ultimately, the optimal fermentation conditions for achieving higher biomass production of *P. acidilactici* TMAB26 were determined to be a combined inoculum size of 2.5%, a pH of 6.5, and a temperature of 35°C, resulting in a biomass production of 2.561 g/100 ml.[24]

Conclusion

The present study successfully formulated a native medium to cultivate *Pediococcus acidilactici* TMAB26 biomass, utilizing pineapple peel as a substrate. Through Response Surface Methodology, key parameters like inoculum size, pH, and temperature were fine-tuned for optimal growth. The optimal conditions for maximum biomass production of probiotic strain TMAB26 using pineapple peel based medium were determined to be 2.5% inoculum required to inoculate the medium with pH 6.5, and a incubation temperature of 35 °C maintained for 24 hours.

These results highlight the diverse health advantages and therapeutic possibilities of probiotics sourced from natural origins. They also open avenues for further investigation and harnessing of agricultural by-products like pineapple peel for valuable microbial biomass production. The tailored medium and optimized conditions present a sustainable and economical strategy for large-scale manufacturing of *P. acidilactici* TMAB26 biomass, with potential applications across various sectors including food, pharmaceuticals, and biotechnology.

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Conflict of Interest

Authors have no conflict of interest.

Data Availability Statement
This statement does not apply to this article.

Ethics Statement
This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement
This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration
This research does not involve any clinical trials.

Authors' Contribution
- **Anuradha Barigela:** Participated in framing of the idea of the performed work.
- **Bhima Bhukya:** Conceived the idea, designed the concept, monitored all the experiments.

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