iJRASET
International Journal For Research in Applied Science and Engineering Technology



INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 10 **Issue:** V **Month of publication:** May 2022

DOI: https://doi.org/10.22214/ijraset.2022.42181

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 7.538
Volume 10 Issue V May 2022- Available at www.ijraset.com

Studies on Extraction of Chitosan from *Trichoderma Viridae* and Effect of Chitosan Based Edible Coating on Cucumber

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Abstract: ***The Biopolymer chitosan, formed from chitin, is one of the most widespread renewable natural materials on the earth. Chitin is a major component of the cell wall of fungi. In this research extraction of chitosan from the fungi Trichoderma viridae is done by simple deproteinization, Filtration and deacetylation. Total phenol content was measured using Gallic acid as standard. H2O2 scavenging activity of the chitosan was calculated. Furthur antibacterial activity was evaluated against the gram positive bacteria S.aureus and the gram negative bacteria E.coli. Edible coating, for cucumber to extend shelf life, is made from the chitosan using polyethylene glycol and glycerol. Nanocomposite based edible coating solution is synthesised by the addition of AgNO3, for further enhancement of antimicrobial activity. Edible coating to cucumber is done and incubated at room temperature and observed for 7 days. Non-coated samples and chemical coated samples were also maintained. The microbial load was measured by turbidity method on the 2nd and 6th day of coating. Nutrient analysis including Carbohydrate estimation, Protein estimation, Moisture loss estimation was also carried out.***

Keywords: Trichoderma viridae, Chitosan, Edible coating, Cucumber, Shelf life analysis

I. INTRODUCTION

Cucumber (Cucumis sativus L.) is a fruit vegetable that belongs to the Cucumis genus in the Cucurbitaceae family. Cucumber is native to India and is said to have originated in the Himalayan foothills (Jia and Wang 2021). Cucumber is a high-water-content vegetable (about 95 percent) that is rich in potassium, calcium, vitamin A, and vitamin K (Manjunatha and Anurag 2014). According to FAO figures, the global cucumber production in 2012 was 65 million tonnes, grown on 2,109,650 hectares (FAOSTAT, 2013). Cucumber fruits, on the other hand, have a short storage life of fewer than 14 days due to weight and firmness loss, discolouration, and fungal infections (Kahramanoğlu and Usanmaz 2019). According to Hassan (2010), due to postharvest losses, 23.6 to 43.5 percent of fruits and vegetables do not reach consumers. The application of edible coatings is one of the successful ways utilised in postharvest handling procedures to protect postharvest fruit quality (Kahramanoğlu *et al.* 2020). Edible coating controls maturation, development and respiratory rate. They also prevent oxidative browning and decrease microbial growth in fruits and vegetables (S. Kumar & T. Bhatnagar *et al*., 2014). Edible coatings have been made with a variety of biopolymers, with chitosan being a popular polysaccharide (de Oliveira *et al*., 2014; Moustafa *et al*., 2019). Chitosan, which is the linear polymer of 2-amino-2-deoxy- β -D-glucan, is the deacetylated form of chitin (Vaishali *et al*., 2019). Chitin is a kind of polysaccharide found in fungi and some animals. In fungi chitin is found in the cell walls of spores and hyphae. It's linked to glucan microfibrils, which are contained in an amorphous matrix and provide the framework for cell wall morphology (Kikkawa, Y *et al*., 2008). Chitosan possesses antibacterial properties as well as excellent film-shaping capabilities, making it ideal for the creation of edible coatings that have been shown to extend the shelf life of products (Vargas, M., *et al* 2009). Present research work was carried out for the extraction of the biopolymer (chitosan) from *Trichoderma viridae* and to evaluate the effect of chitosan edible coating solution on cucumber to extend shelf life, followed by shelf life analysis and nutritional value also identified.

II. MATERIALS AND METHODS

A. Sub culturing of Trichoderma viridae on MALT agar

The *Trichoderma viridae* strain was collected from CBNR(Centre for Bioscience and Nanoscience Research), Coimbatore, Tamil nadu, India. The obtained fungi was sub-cultured in MALT agar (Himedia, Mumbai, India) after sterilization. The media was prepared by dissolving 45g in 1000ml of distilled water and sterilised under autoclave at 121^0C for 15minutes. Sterilised media was poured to petriplate under aseptic condition and transferred the fungi after solidification. This fungi inoculated plate was incubated at 30^0C for 3-5 days.

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B. Chitosan Production media

The MGYP media (composed of Meat extract-0.4g, Glucose-0.3g, Yeast extract-0.06g, Peptone-0.1g in 20ml distilled water) was prepared and sterilized under autoclave at 121^0C for 15 minutes. When the media is cooled down to room temperature, the fungal growth from the sub-cultured MALT agar plate is cut using a cork borer and transferred to the media. It is then incubated at 30^0C for 3-5 days to obtain mycelial mat.

C. Extraction of Chitosan

Extraction of chitosan involved two steps of Deproteinization and Deacetylation (Madhusudhan, K.N *et al*., 2017). Deproteination of extract was done by using 1N sodium hydroxide at the temperature of 60-70^0C for 3 hours. After deproteination, the filtrate was washed continuously to get neutral pH and the residue was collected. Chitin was deacetylated with 1% Acetic acid and used for further study.

D. Total Phenol

Total phenol content was measured by Folin-Ciocalteu assay method. The 1ml of the extract was mixed with 0.2ml of 10% folin-ciocalteu reagent and 1ml of the 20% Na_2CO_3 solution, the mixture was allowed to mix and incubated in water bath at 45^0C for 45minutes. After incubation the OD value was measured at 765nm under spectrophotometer. Gallic acid was used as a standard to calculate the mg/g of the phenol content.(Jaya Prakash M.A et al.,2019).

E. H_2O_2 Assay

The ability of the sample to scavenge hydrogen peroxide (H2O2) was determined according to the method of Ruch *et al*., (1989). 0.5ml of chitosan extracts was transferred into the tubes followed by the addition 2ml of H_2O_2 solution (20 mM). The contents were mixed well and add 0.9ml of ethanol. The reaction mixture was incubated at room temperature for 10 min, its absorbance was measured at 230 nm. The H2O2 activity was calculated using the following equation:

$$\text{H2O2 scavenging activity percentage} = [(A0-A1)/A0] \times 100$$

where: A0 = Absorbance of control,

A1 = Absorbance of sample.

F. Antibacterial Activity

Antibacterial activity of the sample was identified by using well diffusion method against the bacteria. Mueller hinton agar (39gm in 1000ml) was prepared and swabbed 80µl of the bacterial culture (*E. coli, S. aureus*) using cotton swab and four wells were made with cork borer followed by the sample (chitosan) was added in different concentrations (10µl, 20µl, 30µl, 40µl). Antibiotic disc (amikacin 30mcg) was placed as a positive control, the plate was incubated at 37^0C for 24 hrs. After incubation antibacterial activity of the sample was confirmed based on the zone of inhibition in mm (Jesteena johney *et al*.,2018).

G. Preparation of Edible Coating Solution

Edible coating was done with the protocol of Azevedo *et al*., (2014), with slight modification. To 12ml of chitosan in acetic acid solution, 12ml of polyethylene glycol was added followed by incubation overnight at room temperature. To the above mixture 5ml of 1mM $AgNO_3$ was added and kept under direct sunlight for 30 minutes. This is followed by addition of 1ml of glycerol and stirred using magnetic stirrer for 1 hour. This edible coating solution was incubated at room temperature for 1-2 hrs and used for coating.

H. Coating on Cucumber

Fresh Cucumber was collected from local vegetable market of Coimbatore, Tamil nadu, India, washed thoroughly and allowed to dry. Three sets of cucumber was used. First set of cucumber was coated with chitosan edible nanocomposite coating solution by spraying method.3ml of coating solution was transferred into a sterile spraying bottle sprayed on cucumber for the coating. Second set cucumber was kept as non-coated(control). Third set of cucumber was coated with Chemical(sodium benzoate). They were incubated at room temperature for 7 days and shelf life analysis was carried out.

I. Shelf-life analysis

1) Microbial Load: Microbial load was determined with turbidity with the protocol of Nisha *et al.*, (2016). Nutrient broth was prepared by dissolving 13g in 1000ml of distilled water and sterilised. The media was cooled aseptically and a swab was taken from the sample and transferred to the tube, this was incubated at 37^0C for 24hrs. The microbial load was measured by turbidity method on the 2^{nd} and 6^{th} day of coating, and the cell viability was calculated by measuring the OD at 600nm using the formula; Percentage of cell viability = (Sample OD / Control OD) X100

2) Nutrient Analysis: 1g of sample from chitosan coated, non-coated and chemical coated cucumber were crushed using a mortar and pestle and transferred to a sterile test tube. 5ml of buffer solution was added to each tubes and used for nutrient analysis.

a) Carbohydrate Estimation: 0.5ml of sample was mixed with 2.5ml of the anthrone reagent (2% anthrone prepared in Concentrated H_2SO_4) allowed to stand in boiling water bath for 10 minutes including blank. Cool the sample to room temperature and colour developed was read at 620 nm using spectrophotometer. mg/g of the carbohydrate was calculated with the standard Glucose (David T. Plummer *et al*., 1990).

b) Protein Estimation: Protein content of the sample was identified by Lowry's method. 0.5ml of sample was mixed with 2.5ml of solution C (solution A-2% Na2CO3 and solution B-0.5% CuSO4 in the ratio 50:1) including blank. The contents were mixed well and incubated at room temperature for 10minutes. Add 0.2ml folin-ciocalteu reagent to each test tubes including blank and incubate at room temperature in dark condition for 30 minutes and blue colour is developed. Measure the OD value at 660nm using spectrophotometer. mg/g of the protein was calculated.

c) Moisture LOSS: Weigh about 5g of the sample in a previously dried and weighed petriplate and heated at 100^0C for 1hr. Remove the dish after heating and weigh the final weight (Abdulrasak Musa *et al*., 2013).

Moisture percent = (W1- W2) x 100/ (W1 – W)

Where, W1 = Weight in g of the dish with the material before drying W2 = Weight in g of the dish with the material after drying W = Weight in g of the empty dish.

III. RESULT

A. Subculture of Trichoderma viridae

In the MALT agar plates that is inoculated with *T.viridae* , growth was observed after 5 days of incubation at 30^0C.



Figure 1. Fungal growth on MALT agar

B. Chitosan production media

In the MGYP media mycelial mat growth was observed after 4 days of incubation at 30^0C.

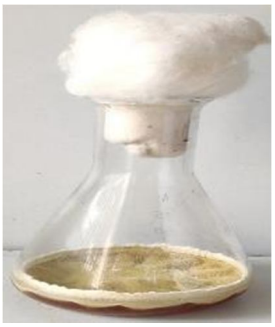


Figure 2. *T.viridae* in MGYP media

C. Extraction of Chitosan

Chitosan was extracted after deproteinization, filtration and deacetylation.



Deproteinization Filtration Deacetylation

Figure 3. Extraction of chitosan

D. Total PHENOL

After incubation, the OD value was measured at 765nm under spectrophotometer. Total phenol content of the chitosan extracted from MGYP media was found to be 0.395mg/g.

E. H_2O_2 Assay

Absorbance was measured at 230 nm.

Sample	OD value
Chitosan from MGYP	0.020
Control	0.186

Table 1. H_2O_2 assay

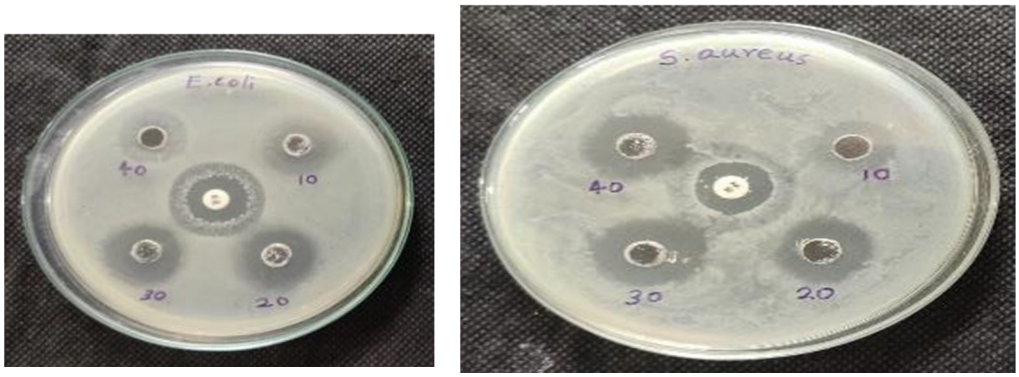
H2O2 scavenging activity percentage was calculated to be 89.24%.

F. Antibacterial activity

Chitosan solution in different cocentrations showed antibacterial activity against *E. coli* and *S. aureus* through well diffusion method.

Sample concentration	*E. coli*	*S. aureus*
10µL	5mm	4mm
20µL	7mm	5mm
30µL	8mm	6mm
40µL	5mm	7mm
AK^{30}	8mm	4mm

Table 2. Antibacterial activity



E. coli S. aureus

Figure 4. Antibacterial activity against *E. coli, S. aureus*

G. Synthesized Edible Coating

Chitosan Nanocomposite based Edible coating solution was synthesized from the chitosan extracted from *T. viridae*

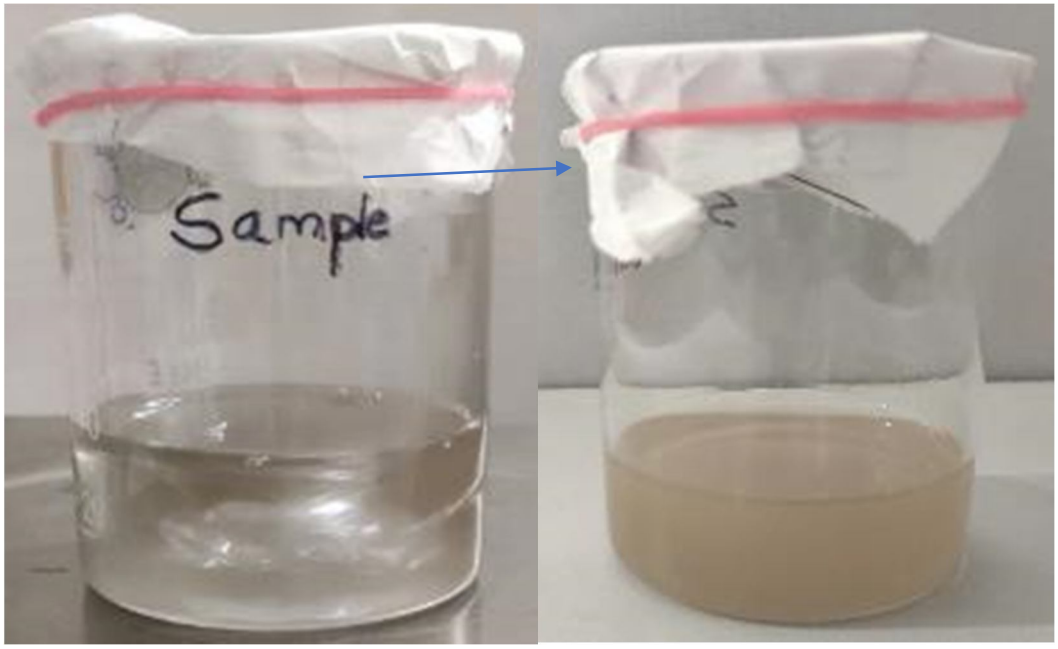


Figure 5.Synthesised composite based edible coating

H. Coating on Cucumber

Cucumber were observed for 7 days. Physical examination showed that Cucumber coated with Chitosan edible coating remained non-spoiled, and more healthy than the Cucumber coated with chemical. While the cucumber that was kept as Control was found to spoiled during the observation period.

Day 0 Day 2



Day 4 Day 6



Fig 6. Appearance of Cucumber coated with Chitosan-nanocomposite based edible coating solution, Control and Chemical coated

I. Shelf-life Analysis

1) Microbial Load: The microbial load was measured by turbidity method on the 2nd and 6th day of coating, and the cell viability was calculated by measuring the OD at 600nm.

Samples	Cucumber on day 2	Cucumber on day 6
Chitosan coated	0.104	0.264
Control	0.771	0.821
Chemical coated	0.172	0.704

Table 3. Microbial load on cucumber

Percentage of cell viability of Chitosan coated Cucumber on Day 2= 13.48%. Percentage of cell viability of Chemical coated Cucumber on Day 2= 22.30%. Percentage of cell viability of Chitosan coated Cucumber on Day 6= 32.15%. Percentage of cell viability of chemical coated Cucumber on Day 6= 85.74%.The results implies that cucumber coated with chitosan edible coating showed significant decrease in microbial population.

2) Nutrient analysis

a) Carbohydrate Estimation

Sample	OD Value
Chitosan coated	0.781
Control	0.672
Chemical coated	0.702

Table 4. Carbohydrate estimation of Cucumber

These absorbance values are compared with the standard curve and the carbohydrate content of the Chitosan coated cucumber was found to be 190mg/g, Non-coated cucumber was found to be 162mg/g, Chemical coated cucumber was found to be 170mg/g.

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b) Protein Estimation

Sample	OD value
Chitosan coated	0.362
Control	0.336
Chemical coated	0.347

Table 5. Protein estimation of Cucumber

These absorbance values are compared with standard curve and the protein content of the Chitosan coated cucumber was found to be 40mg/g, control was found to be 34mg/g, chemical coated cucumber was found to be 38mg/g.

c) Moisture Loss

Sample	Moisture loss in percentage
Chitosan coated	77.74
Control	88.52
Chemical coated	79.62

Table 6. Moisture loss estimation of Cucumber

Fruits and vegetables are rich in moisture content and moisture loss is related to diffusion resistance. The moisture loss percentage of chitosan coated cucumber was found to be 77.74% , control was found to be 88.52%, chemical coated cucmber was found to be 79.62%.

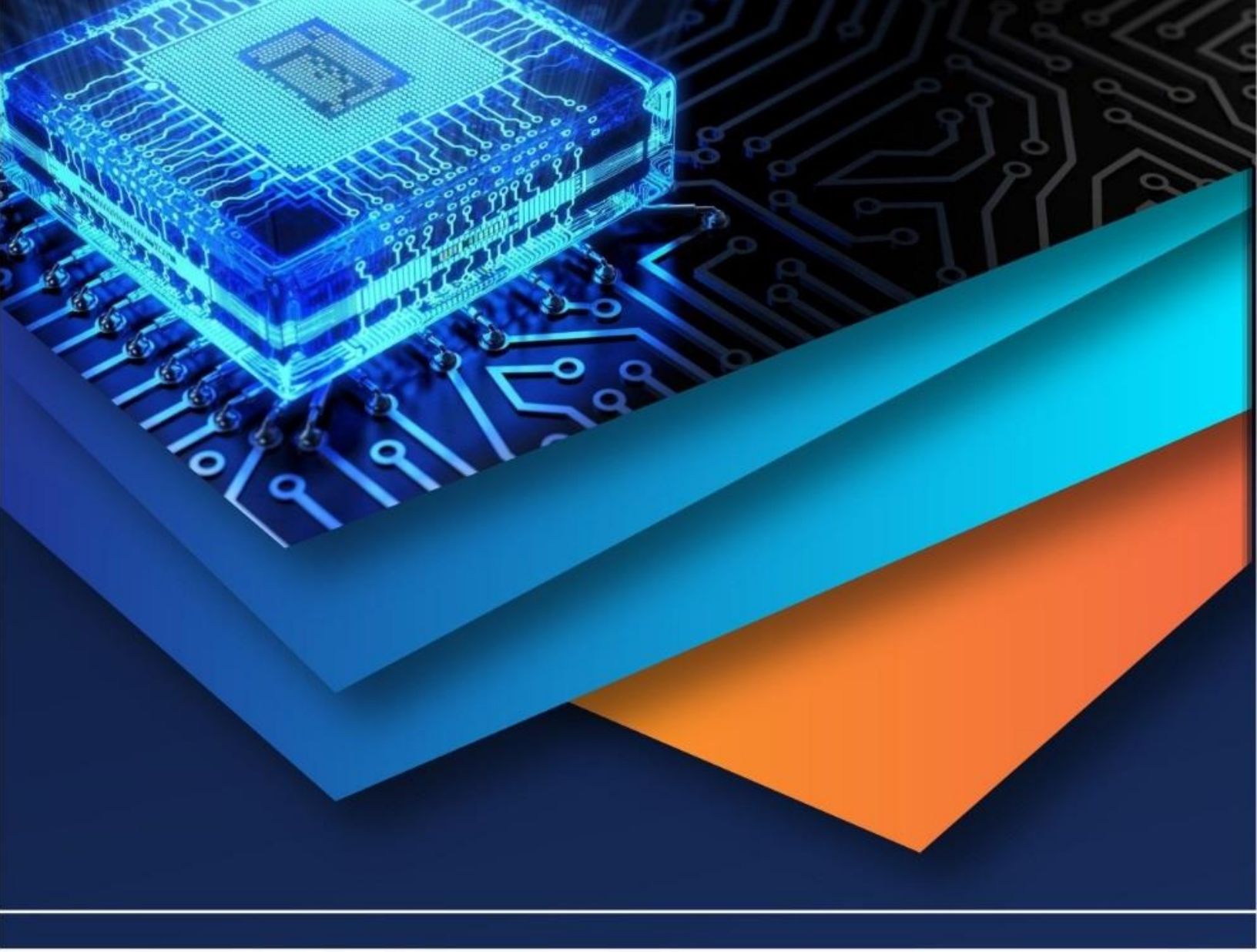
IV. DISCUSSION

The quality of fresh fruits is generally judged by their appearance and freshness at the time of purchase (Kader, 2002). Ripening, senescence, and mechanical damage not only reduce fruit quality, but they also make fresh produce more vulnerable to microbial infections that cause deterioration. Fresh food is preserved using chemical treatments using synthetic or natural pesticides, which are very effective at inhibiting microbial activity and reducing losses. However, because of potentially toxic by-products and residues, this approach may have significant consequences for human and environmental health. For that reason, there is a growing public demand to reduce the use of these chemicals (Shimshoni, J.A *et al*., 2019).Consumers all throughout the globe demand for the natural coating instead of chemical coating for protection of fruit quality (Galed *et al*., 2004). Kardas *et al.* (2012) revealed that chitosan and its derivatives have a wide range of uses in the food business, including food preservation, shelf-life extension, the production of biodegradable films, and food packaging. To effectively extend the shelf life of postharvest fruit and vegetable, chitosan-based coating as a relatively convenient and safe measure, is more and more concerned in food industry in these recent years (Jianglian and Shaoying , 2013). The US Food and Drug Administration has certified chitosan as a generally regarded as safe food additive, dietary fiber (hypocholesterolemic effect), and functional component for consumers (Vidanarachchi *et al*., 2011; Gutiérrez 2017). Chitosan has been shown to be an effective natural antimicrobial agent based on the electrostatic mechanism, as well as a control of respiration rate, weight loss, and water loss, without affecting the taste, odour, and palatability of skinned and fresh cut fruits and vegetables in a number of studies (Duan et al. 2019). Although there are many publications on obtaining chitin and chitosan from fungal sources, very less work was carried out on extraction from *T.viridae*, which was done in this research.

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