Indian Journal of Applied Microbiology
ISSN (Online): 2454-289X, ISSN (Print): 2249-8400
Copyright © 2022 IJAM, Chennai, India
https://doi.org/10.46798/ijam.2021.v24i01.3

Volume 24 Number 1
April - June 2022, pp. 21-31

Estimation of Antimicrobial Efficacy of Bio-enzyme Extracts

Prerna S Kapadi*[1], Vedita Hegde Desai[2], Shailaja Mallya[2], Yogita Sardessai[3]

[1]Research Student, Department of Pharmacology, Goa College of Pharmacy

[2]Assistant Professor, Department of Pharmacology, Goa College of Pharmacy

[3]Head of Department of Microbiology, Goa College of Pharmacy

Abstract

Background: Bioenzymes are organic products of fermentation of fruits/vegetables waste which have shown promising antimicrobial activity against a wide range of pathogenic microorganisms. Following fermentation, the antibacterial properties of these Bioenzymes are enhanced multi-fold as organic substances after decomposing,yield bioactive compounds or phytochemicals. **Aim:** To evaluate the antimicrobial potency of Bioenzymes prepared using *Emblica officinalis* (Amla) fruit, *Cucumis sativus* (Cucumber) peel and Hibiscus *Rosa-sinensis* (Hibiscus) petal and a mixture of all three. **Method:** 4 Types of Bioenzymes were prepared, 3 of them prepared using Amla, Hibiscus, Cucumber individually while the fourth one having equal parts of all three plant parts. These 4 types of Bioenzymes were prepared in two sets namely Set I (fermented for 12 weeks) and Set II (fermented for 25 days). All of these eight test samples were subjected to antimicrobial susceptibility testing, using the Agar well diffusion method against 5 Bacterial strains. **Result:** It was observed that Bioenzymes from both these sets were capable of successfully producing large zone of inhibition. Bioenzymes from Set I were also analysed to determine their Minimum Inhibitory Concentration against *S. aureus* and it was observed that Amla Bioenzyme Set I and Mixture Bioenzyme Set I was found to be at 50% concentration. **Conclusion:** The Bioenzymes have shown the potential to act as promising antimicrobial agents against various pathogenic bacteria.

*Author for Correspondence. E-mail: kapdiprer97@gmail.com

This work is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 4.0 International License.

Keywords: Antimicrobials, Anti-oxidants, Bacteria, Pollution, Fruit peels, Fermentation, Minimum Inhibitory Concentration, Pathogenic bacteria, Bioenzymes

Introduction

Bioenzymes are a complex mix of various bioactive components such as proteins, vitamins, minerals, salts etc. produced as a by-product of fermentation of vegetable or fruit scrapes by the microbial flora found in nature. This method is so simple and cost effect that it makes use of simple ingredients like Jaggery which isnot only the carbon source but it is also packed with probiotics like *Lactiplantibacillus plantarum* [1] which aid innatural fermentation avoiding the use of any additional fermentation starters. This method was actually brought into light in the 1970s when Dr. Rosukon Poompanvong, a university student of agriculture in Thailand was working with chemically treated crops, but exposure to these chemicals compromised her health hence her search for an eco-friendly, non-toxic alternative led to the creation of Bioenzymes.[2]

Literature shows that Bioenzymes have been found as an effective alternate to Sodium Hypochlorite against *E. faecalis* in the root canal procedure as the latter is known to be cytotoxic.[3] Another study conducted on the biological treatment of Dairy waste-water showed that Bioenzyme were excellent at controlling parameters like alkalinity, pH, COD (Chemical oxygen demand), TDS (Total dissolved solids), TS (Total solids), TSS (Total suspended solids), oil & grease and even BOD (Biological oxygen demand) hence proving as an affordable and effective method for wastewater treatment.[4]

Due to the excessive, inappropriate & indiscriminate use of antibiotics have increased the risk of antimicrobial resistance rendering the currently available synthetic medications ineffective. Alternatively, bacteria may develop resistance to medicinal components of plant origin if only one active ingredient with a specific target is involved. Bioenzyme being a blend of complex Phyto-components can act by inducing intrinsic antibacterial activity by acting on multiple metabolic processes or by interrupting the synthesis of vital components and at the same time pose low chances of side effects which are otherwise seen in synthetic antibiotics.

The reason for choosing these particular plant parts is because these plants have been extensively investigated for their use as antimicrobials [5,6,7,8] but the extraction methods used to isolate the therapeutics components in these plants involves the use of organic solvents and heat which can either destroy these essential medicinal properties or cause toxicity to humans by leaving behind residue.

The rationale of this study was to check if Bioenzymes prepared using *Emblica officinalis* (Amla) fruit, *Cucumis sativus* (Cucumber) peel and Hibiscus *Rosa-sinensis* (Hibiscus) petal & a mixture of all three were effective as antibacterial agents against various strains of Gram-positive and Gram-negative bacteria. And secondly since the original method involves fermentation of about 12 weeks there was an alternate method using *Saccharomyces cerevisiae* commonly known as Baker’s Yeast to speed up the process under 25 days.

Materials

The plants used in this study—*Phyllanthus emblica* (Amla), *Hibiscus rosa-sinensis* (Hibiscus) and *Cucumis sativus* (Cucumber) were identified and authenticated at the Dhempe College of Arts & Science, Miramar, Panaji Goa.

The microbial strains used as test organisms were procured from the Department of Microbiology, Goa Medical College, Bambolim Goa. In total 5 strains were used which included: *Staphylococcus aureus*, ATCC 25923, *Enterococcus faecalis*, ATCC 29212, *Escherichia coli*, ATCC 25922, *Pseudomonas aeruginosa*, ATCC 27853, *Klebsiella pneumoniae.*

Methods

A. Preparations of Bioenzymes

This study was performed in the Department of Microbiology, Goa College of Pharmacy, Panaji-Goa. In total four types of Bioenzymes (in sets of two) were prepared for this study. Three of which were prepared solely from one type of fruit, peel or petal.

Table No.1: Preparation of Bioenzyme by two different methods:

Set I Bioenzyme (No yeast)	**Set II Bioenzyme (Yeast present)**
3 parts test material + 5 parts of DW + 1 part of Jaggery	3 parts test material + 5 parts of DW + 1 part of Jaggery + 1 tsp of Baker's yeast
Fruits Amla (A)	Fruits Amla (A')
Petals of Hibiscus (B)	Petals of Hibiscus (B')
Peels of Cucumber (C)	Peels of Cucumber (C')
Equal parts of A + B + C= (D)	Equal parts of A' + B' + C'=(D')
Fermentation period: 12 weeks	Fermentation period: 25days

DW: Distilled water

The bottles were opened frequently to air out the gases produced as a result of fermentation and placed in a cool dry place away from sunlight. After the completion of fermentation period each Bioenzyme was strained using a mesh strainer and filtered using a whattman filter paper which was ready to be used for furthertests. [3,4,9]

B. Organoleptic Evaluation

The colour & odour were recorded individually through sensory and visual perceptions. [11]

C. Preliminary Phyto-chemical screening

Saponins

Foam test: 5ml of test sample was shaken vigorously with 20ml water. The presence of saponins is indicated by formation of persistent foam. [11]

Tannins

Ferric chloride test: add few drops of 5% Ferric chloride solution to 2ml test sample. Blue colour indicates the presence of hydrolysable tannins, brown colour indicates the presence of pseudo-tannins while green colour is given by gallo-tannins. [11]

Triterpenoids

Salkowski test: shake 5ml of test sample with 2ml chloroform. To this chloroform layer, add 3ml conc. Sulphuric acid slowly to the sides of the test-tubes. The presence of triterpenoid is indicated by the formation of yellow colour in the lower layer. [11]

Fig No: 1a: Amla

Fig No: 1b: Hibiscus

Fig No: 1c: Cucumber

Fig No: 1d: Jaggery

Fig No: 1e: Gram-Positive &Gram-Negativebacterial cultures

Fig No: 1d: Bioenzymes Set I & Set II post 12 weeks & 25 days fermentation respectively

Phenolic compounds

Lead acetate test: 10% Lead acetate is added to 2ml of the test sample. Presence of phenol is indicated by the formation of white precipitate. [11]

D. Preparation of culture suspensions

The working station was swabbed clean with Dettol and the Bunsen burners were turned on to create an aseptic environment. The cultures were removed from the refrigerator 15 mins prior to their use to stabilise at room temperature. Using a sterile nichrome loop, few specs of the culture were inoculated into sterile test-tubes containing saline to obtain a bacterial suspension of 0.5 McFarland. [11]

E. Anti-Microbial assay

Agar-Well diffusion assay

Required quantity of media was prepared as per the instructions on the media bottle and then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After autoclaving the sterilised growth media was allowed to cool to about 45-50°C. Once cooled about 50ml of the media was poured into a sterile glass beaker and to that 100µL of bacterial suspension was added. This was then mixed and poured into sterile Petri plates which were kept un-disturbed for about an hour. Once these plates were set, using a clean sterile cork borer 4 wells were punched into the plates. Immediately, using a sterile tip 200µL of test solution was pipetted into these wells using a micro-pipette. The solution in the wells were allowed to diffuse for 30mins and then wrapped and kept in the incubator for 24 hours. This process was carried out for every single microbial strain and for both the sets of bioenzymes. Zone of inhibition reading was taken post 24hrs.[9,10]

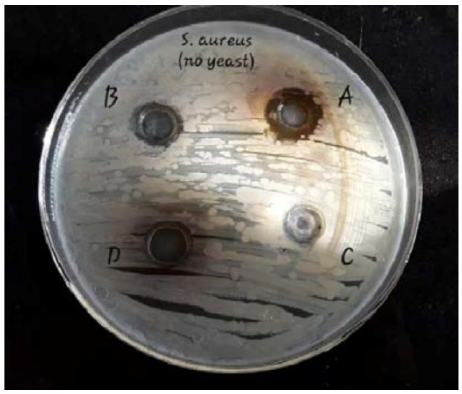

Fig No 02

Fig No 03

Fig No 04

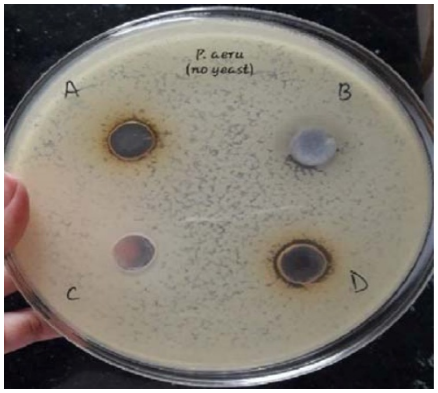

Fig No 05

Fig No 06

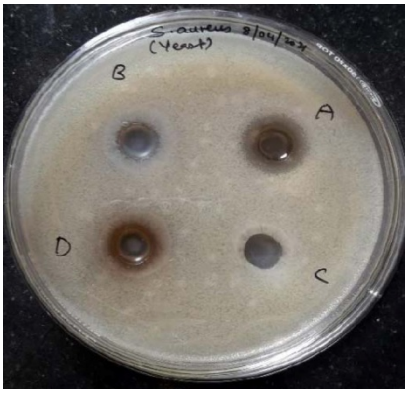

Fig No 07

Fig No 08

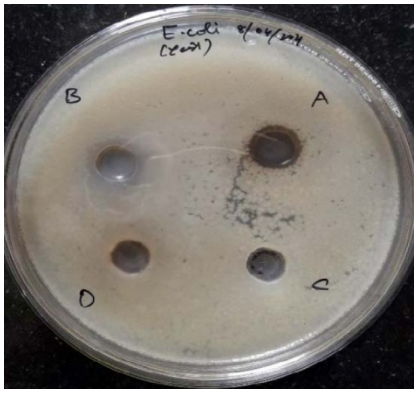

Fig No 09

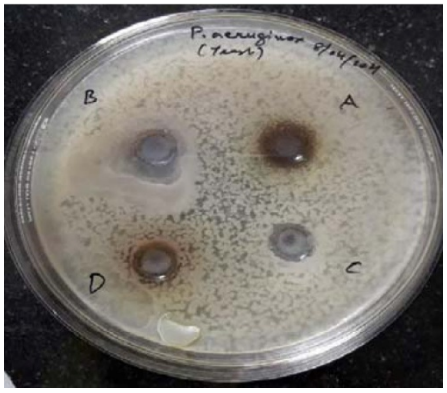

Fig No 10

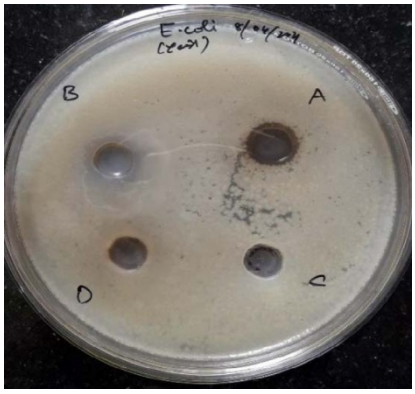

Fig No 11

Fig No 02, 03, 04, 05 & 06:plates of Zone of Inhibition of 4 BioenzymesA, B, C, D from Set I on *S. aureus, E. faecalis, E. coli, P. aeruginosa and K. pneumoniae* respectively

Fig No 07, 08, 09, 10 & 11:plates of Zone of Inhibition of 4 BioenzymeA', B', C', D' Set II on *S. aureus, E. faecalis, E. coli, P. aeruginosa and K. pneumoniae*respectively

Table No 2: Organoleptic properties & Preliminary Phyto-chemical analysis

Test Sample	Colour	Odour	Test for Tannins	Test for Triterpenoids	Test for phenolic compounds	Test for saponins
SET I						
Amla (A)	Yellow	Strong alcoholic	+	-	+	+
Hibiscus (B)	Dark Red	Mild acidic-alcoholic	+	-	+	+
Cucumber (C)	Buffy white	Mild alcoholic	-	-	+	+
Mixture (D)	Orange	Mild alcoholic	+	-	+	+
Set II						
Amla (A')	Yellow	Strong alcoholic	+	-	+	+
Hibiscus (B')	Dark red	Alcoholic	+	-	+	+
Cucumber (C')	Pale Yellow	Alcoholic	-	-	+	+
Mixture (D')	Orange	Mild alcoholic	+	-	+	+

Table No 3: Agar Well-diffusion Assay:

Test Cultures	Bioenzyme Set I				Bioenzyme Set II			
	A	B	C	D	A'	B'	C'	D'
Staphylococcus aureus	24mm	17mm	0mm	19mm	25mm	19mm	0mm	23mm
Enterococcus faecalis	19mm	18mm	17mm	21mm	23mm	19mm	18mm	22mm
Escherichia coli	19cm	18mm	19mm	20mm	20mm	17mm	0mm	0mm
Pseudomonas aeruginosa	20mm	18mm	0mm	20mm	22mm	20mm	0mm	19mm
Klebsiella pneumoniae	19mm	20mm	21mm	20mm	24mm	0mm	18mm	25mm

Bioenzyme Set I: A: Amla, B: Hibiscus, C: Cucumber, D: Mixed;

Bioenzyme Set II: A': Amla, B': Hibiscus, C': Cucumber, D': Mixed

Discussion

In this study, we used 3 different plant parts namely amla fruit, hibiscus petals, cucumber peels and equal parts of all three, to prepare four types of bioenzymes. These were prepared in two sets I and II and fermented over two different time periods along with addition of yeast in the latter set. The main aim of this study was to prepare naturally generated alcohol based anti-microbials which would not only replace harsh chemicals but also provide cost effective, environment friendly ways of reducing biodegradable waste.

From the organoleptic evaluation it was found that the bioenzymes ranged from mild acidic to strong alcoholic smell in both the sets which indicates that indeed alcohols and acids were produced during the fermentation process. Similarly, the preliminary phytochemical tests confirmed the presence of Phenolic compounds and Saponins in both the Bioenzyme sets while tannins were only found in Amla, Hibiscus and Mixture Bioenzymes of both the sets. Test for Triterpenoids was shown negative by all these bioenzymes. Phytoconstituents like Tannins, Saponins and Phenolic Compounds are known to interact with enzymes and proteins of the bacterial cell membrane causing its disruption further causing cell death due to a greater influx of protons or inhibition of synthesis of vital components required for the survival and growth of the bacteria, hence presence of these in the Bioenzymes indicate that these bioenzymes have anti-microbial property

To confirm the anti-microbial activity of these Bioenzymes they were tested by the Agar well diffusion method and to know the minimum inhibitory concentration the Broth dilution method was used.

A comparison was made between Set I (No Yeast) (Fig No: 02, 03, 04, 05 & 06) and Set II (Yeast present) (Fig No: 07, 08, 09, 10 & 11) which were kept for fermentation for 12 weeks and 25 days respectively and the observations are as follows:

Against *S. aureus*: Amla, Hibiscus and Mixture bioenzymes from Set II (Fig No 07) showed a higher zone of inhibition than that shown by the same bioenzymes from Set I (Fig No 02). While cucumber bioenzyme from both sets did not show any activity.

Against *E. faecalis*: all four bioenzymes from Set II (Fig No 08) were leading with respect to their anti-bacterial activity as compared to the same Bioenzymes from Set I (Fig No 03). Amongst the eight samples tested Amla Bioenzyme Set II (Fig No 08) showed the highest zone of Inhibition against *E. faecalis*.

Against *E. coli*: Here all four Bioenzymes from Set I (Fig No 04) produced a zone of inhibition against *E. coli* while only Amla and Hibiscus Bioenzyme from Set II (Fig No 09) showed their activity against it. Amla Bioenzyme from Set II (Fig No 09) produced a higher Zone as compared to Amla Bioenzyme Set I (Fig No 04).

Against *P. aeruginosa*: Amla and Hibiscus Bioenzymes from Set II (Fig No 10) were leading in their activity as compared to the same Bioenzymes from Set I (Fig No 05), while Mixture Bioenzyme Set I (Fig No 05) showed a higher zone of inhibition as compared to the same

preparation from Set II (Fig No 10). On the other hand, Cucumber Bioenzyme from both the Sets didn't produce any zones against *P. aeruginosa* indicating no activity against it.

Against *K. pneumoniae*: Amla and Mixture Bioenzymes from Set II (Fig No 11) and Hibiscus and Cucumber from Set I (Fig No 06) showed largest zones amongst the ones produced by other samples tested. While Hibiscus Bioenzyme from Set II (Fig No 11) was the only one that did not produce any zones indicating no activity against *K. pneumoniae*.

This shows the potential of Bioenzymes to diffuse from their respective reservoir wells through the agar medium, and get adsorbed onto the cells of the surrounding culture, leading to the obstruction of metabolism processes of the bacteria.This further inhibits cellular division within the area of the zone. Some Bioenzymes were not able to produce effective results against certain bacterial strains namely Hibiscus Bioenzyme Set II against *K. pneumoniae*, Cucumber Bioenzyme Set II and Mixture Bioenzyme Set II against *E. coli*. This could be because these specific Bioenzymes require a longer fermentation time for their anti-bacterial effects to mature or enhance in order to be effective against pathogens like *K. pneumoniae* and *E. coli* respectively, as these same test samples that were fermented for 3 months period were able to produce effective results in these test strains.

Conclusion

The findings of this study suggest that Bioenzymes may possess the potential to act as anti-bacterial agents as the test samples were able to produce promising results individually and also synergistically when used as a mixture. The idea of using yeast (as in set II) was an alternative to speed up the fermentation process but availability of yeast cannot be always guaranteed. Hence the original method (as in set I) involving only Plant parts + jaggery + Water even though has a longer fermentation period, was proven to be economical and functional without the need of additional fermentation starters. This process also omits the conventional methods of extraction like use of organic solvents which otherwise pose as health hazards and environmental pollutants and most of the times too expensive and in other cases can cause loss of phytoconstituents due to heat or high temperatures. However further studies are required for an in-depth screening to determine the major components that contribute to the antimicrobial activity shown by these Bioenzymes and also to obtain an account of their safety and efficacy against different pathogenic strains. Even though these Bioenzymes were found to be effective against many pathogenic strains that colonise different parts of the body, their use in these areas still need a thorough investigation in order to understand its safety and effectivity in reaching the deep ends in order to completely eradicate the infection.

Acknowledgements

I would like to extend my deepest gratitude towards my guides Mrs. Vedita Hegde Desaiand Mrs. Shailaja Mallyafor encouraging and supporting me throughout the process of this study. I am also very thankful towards Dr. Yogita Sardessai, for providing me guidance and advice and allowing me to work on this project in the Microbiology Lab. A humble thanks to Dr. Savio Rodrigues,

Head and Professor, Department of Microbiology and Dr. S.M. Bandekar, Dean, Goa Medical College for giving us the approval to procure microbial culture strains from their Microbiology department. I am extremely thankful to Mr. Ashish V. Prabhugaonkar, Assistant Professor, Department of Botany & Mrs. Manjiri M. Barve, Head and Professor, Department of Botany, Dhempe College of Arts and Science for the timely assistance in the identification and authentication of my plant species. I am also very grateful towards Dr. Madhusudan P. Joshi, Head and Professor, Department of Pharmacology & Dr. Gopal Krishna Rao, Principal, Goa College of Pharmacy for giving me an opportunity to carry-out this project and providing access to resources used in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Surve S, Shinde DB, Kulkarni R.2022 Feb 4;“Isolation, characterization and comparative genomics of potentially probiotic *Lactiplantibacillus plantarum* strains from Indian foods”. *Sci Rep.* 12(1):pg1-6.
2. Outstanding farmer from Thailand honoured at World Food Day celebration [Internet]. [cited 2021 May 15]. Available from http://www.fao.org/asiapacific/news/detail-events/en/c/47368/
3. Mavani HAK, Tew IM, Wong L, Yew HZ, Mahyuddin A, Ghazali RA, et al. 2020,“Antimicrobial efficacy of fruit peels eco-enzyme against *Enterococcus faecalis*: An in vitro study”. *Int J Environ Res Public Health*.17(14):1–12.
4. Srimathi N, Niranjana T.2020,“Biological Treatment of Dairy Wastewater using Bio Enzyme from Citrus Fruit Peels”. *Int J Recent Technol En.,* (9):pg 2277–3878.
5. Vaou N, Stavropoulou E, Voidarou C, Tsigalou C, Bezirtzoglou E. 2021 Sep 27“Towards advances in medicinal plant antimicrobial activity: A review study on challenges and future perspectives”. *Microorganisms*.,9(10):pg 2041
6. Hasan MR, Islam MN, Islam MR.2016 “Phytochemistry, pharmacological activities and traditional uses of Emblica officinalis: A review”. *Int Curr Pharm J.,5(2)*:pg 14–21.
7. Ruban P, Gajalakshmi K.2012,“In vitro antibacterial activity of Hibiscus rosa-sinensis flower extract against human pathogens”. *Asian Pac J Trop Biomed, [cited 2021 May 18*];2(5):pg 399–403.
8. Mukherjee PK, Nema NK, Maity N, Sarkar B, 2013“Phytochemical and therapeutic potential of cucumber”. Fitoterapia, 84(1):pg 227–36.
9. Saramanda G, Kaparapu J.2017“Antimicrobial Activity of Fermented Citrus Fruit Peel Extract”. *J Eng Res Appl*.,7(117):pg 2248–962225.
10. Balouiri M, Sadiki M, Ibnsouda SK.2016,“Methods for in vitro evaluating antimicrobial activity: A review”. *J Pharm Anal*.,6(2):pg 71–9.
11. Gokhale, Kokate, Purohit, 2009. A Text Book of Pharmacognosy. Nirali Prakashan