Antibacterial activity and biotechnological application of Endophytic Bacteria isolated from seaweed

ABSTRACT:

Seaweedaremulticellular,photosyntheticorganisms.Seaweed aretypeofalgaemainlygrows in ocean which is also known as sea vegetable. Seaweeds come in various colors like green, red, brown, and black, and they are known for their high nutritional value. Many seaweed species are edibleandofferarangeofculinarypossibilities.Someevenhavecommercialimportance,usedin various products like sushi, nori sheets, and even cosmetics and fertilizers.Seaweeds are often consideredsuperfoodsduetotheirhighnutritionalvalue,richinvitaminsandmineralslikeiodine, calcium, magnesium, and iron. Endophytic bacteria are significant contributors to the plant microbiome and are essential for the survival, development, and growth of the plant.Hormones, nutrients, secondary metabolites, and other tiny molecules are among theSubstances secreted by endophytic bacteria that have a substantial impact on plant growth and Development. Auxins, cytokinin, and gibberellins, for instance, are phytohormones produced by some endophytic bacteria that can encourage cell division and elongation, resulting in greater plant development. Polyhydroxybutyrate (PHB) is a biodegradable and bio-based thermoplastic polyester.PHB is synthesized as an intracellular storage compound by various microorganisms, including certain bacterialike*Cupriavidusnecator,Ralstoniaeutropha,Bacillusmegaterium,*andsomearchaea.It isstoredintheformofdensegranuleswithinthecells.Proteasesplayavitalrole incatalyzingthe breakdownofpeptidebonds in proteins.This enzymaticfunctionis crucialnotonlyfor digestion inthe human bodybut alsoin biotechnologicaland industrial applications.Inindustrieslikefood processing,pharmaceuticals,andlaundrydetergents,proteaseactivityisharnessedtobreakdown proteins.

KEYWORDS: Polyhydroxybutyrate (PHB),Seaweed, secondary metabolites,thermoplastic polyester

INTRODUCTIONS

Seaweeds, also known as sea vegetables, are indeed fascinating marine plants. They thrive in shallowwatersandalongrockyshorelines,formingdistinctzonesbasedonwaterdepth.Kelpisa notable example, oftenfound inwatersup to50 metersdeep.Theseplantsserveasa crucial food source for marine life. Seaweeds anchor themselves to the seafloor through holdfasts, but unlike typical plants, they don't extract nutrients through their roots. Instead, they absorb nutrients directlyfromthesurroundingwater.Seaweedscomeinvariouscolorslikegreen,red,brown,and black, and they are known for their high nutritional value. Many seaweed species are edible and offer a range of culinary possibilities. Some even have commercial importance, used in various productslikesushi,norisheets,andevencosmeticsandfertilizers.Theirversatilityandnutritional benefitsmakethemavaluableresourcebothintheoceanecosystemandforhuman consumption.

Seaweeds are indeed fascinating and diverse organisms found in coastal areas around the world. Theyplayasignificantroleinmarineecosystemsandhavevarioususesforbothhumansandother marine life. Red algae, scientifically known as Rhodophyta, are particularly noteworthy due to their vibrant red hue, which is the result of pigments like chlorophyll A, phycoerythrin, and phycocyanin. These red algae are not only edible and important for human consumption but also serveasavitalsourceof nutritionfor many marinecreatures.Theycontributeto thefood webby providingsustenancetofishesandshellfish.Seaweeds,asagroupofmulticellular,photosynthetic organisms, are integral to marine ecosystems and have diverse applications, from food and medicinetoindustrialpurposes.Theyarecategorizedinto threemaingroupsbasedoncoloration: red,green,andbrownseaweeds.Eachgrouphasitsuniquecharacteristicsand uses.Seaweedsare oftenconsideredsuperfoods duetotheirhighnutritionalvalue,richinvitamins andminerals like iodine, calcium, magnesium, and iron.

Endophyticbacteriaarefascinatingmicroorganismsthathaveasignificantimpactonplantgrowth andhealth.Theysecreteawiderangeofsubstances,includinghormones,nutrients,andsecondary

metabolites,whichplayimportant roles indevelopmentof plant. Phytohormones like auxins, cytokines,andgibberellinsproducedbycertainendophyticbacteriacanstimulatecelldivisionand elongation in plants, leading to enhanced growth. Nitrogen fixation is another crucial service provided by some endophytic bacteria, making nitrogen more readily available to the host plant, which is essential for its growth and development.

The research into the antibacterial activity of endophytic microorganisms has indeed advanced significantly in recent years, and various methods have been employed to study their potential in combatingbacterialinfections.Here'sabriefoverviewofthemethodsmentioned:AgarDiffusion Assay:Thismethodinvolvesembeddingthetestbacteriainanagarmediumandthenapplyingan endophytic bacterial extract or a purified compound onto the medium. The appearance of a clear zone around the application site indicates the presence of antibacterial compounds, as the compounds inhibit bacterial growth. Minimum Inhibitory Concentration (MIC) Assay: In this assay, the target bacteria are exposed to a range of concentrations of the endophytic bacterial extract or purified compounds. The goal is to determine the lowest concentration that effectively inhibits bacterial growth.This provides a quantitative measure of the antibacterial potency of the tested substances.Time-KillAssay:This assay monitors the growth of the test bacteria over time in the presence of the endophytic bacterial extract or compounds. It helps in understanding the kinetics of antibacterial activity, including the rate and extent of bacterial inhibition.

Polyhydroxybutyrate (PHB) is indeed a fascinating biodegradable thermoplastic polyester with a rangeof promising applications. Herearesomekey points aboutPHB:MicrobialSynthesis :PHB issynthesized asan intracellularstoragecompound byvariousmicroorganisms,including certain bacterialike*Cupriavidusnecator,Ralstoniaeutropha,Bacillusmegaterium,*andsomearchaea.It is stored in the form of dense granules within the cells.Biodegradability: One of the primary advantages of PHB is its biodegradability. Unlike traditional petroleum-based plastics, PHB can be broken down by natural processes, making it a more environmentally friendly option.Biocompatibility:PHB is biocompatible, meaning it is well-tolerated by living organisms. This property makes it suitable for various applications in medicine and biomedicine.PHB finds applications in multiple fields such asAgriculture, medicine and biomedical.

Proteasesplayavitalroleincatalyzingthebreakdownofpeptidebondsinproteins,leadingtothe formationofsmallerpeptidechainsoraminoacids.Thisenzymaticfunctioniscrucialnotonlyfor

digestionin thehumanbodybutalsoinbiotechnologicalandindustrialapplications.Inindustries like food processing, pharmaceuticals, and laundry detergents, protease activity is harnessed to break down proteins. For example, in laundry detergents, protease activity is essential for effectively removing stains containing protein-based substances like blood or food. This demonstrates the versatility of proteases in improving product performance and cleanliness in various sectors.

AIM

Thestudytheantibacterialactivityandbiotechnologicalapplication Endophytic Bacteria isolated from seaweed

OBJECTIVES

- Collectandidentifyseaweedspecimensfromthecoastal regionofTamilNadu.
- Isolateandcharacterizeendophyticbacteriaresidingwithintheseaweed.
- EvaluatetheantibacterialpotentialofendophyticbacteriaagainstUTIpathogens.
- ProduceandcharacterizePolyhydroxybutyrate(PHB)usingendophyticbacteria.
- Screenendophyticbacteriaforenzymaticactivity.

MATERIALSANDMETHODS

COLLECTION OF SAMPLES

Seaweed samples were gathered from Sathyabama University in Tamil Nadu. Initially, the seaweeds were observed and selected based on variations in their morphology and color. After selection, the collected seaweed samples were carefully placed in Polythene zip-lock sample covers.They were then promptly transported to a laboratory and rinsed gently with sterile water. To eliminate excess moisture from the seaweed samples, they were gently pressed with sterile tissue paper. Further sterilization of the seaweed samples was performed to remove external microorganisms, facilitating the isolation of endophytic bacteria.

BACTERIALISOLATIONANDIDENTIFICATION

The process involves isolating endophytic bacteria from seaweed. The selected segment of thallus are immersed in 95%ethanol for 30 sec and 4% sodium hypochlorite solution for 3 mins . Rinse the thallus in sterile distilled water three times and dry the surface area under appropriate condition . After drying cut the thallus segment approximately of 0.5cm and place it in the petri

plate containing tryptic soy agar medium(TSA) supplemented with ketoconazole(100mg/L)tosuppressthefungalgrowth.Itincludessurfacesterilizationofseaweed segments, followed by their placement on tryptic soy agar medium supplemented with ketoconazole to suppress fungal growth. After incubation at 37°C for 24 hours, endophytic bacterial colonies are observed and transferred to fresh TSAplates to obtain pure colonies. This method is commonly used for studying endophytic bacteria associated with seaweed.

MORPHOLOGICALCHARACTERISTICS

The colony characteristics like size, shape, color, elevation, texture, margin, and opacity when studying bacterial isolates. All actinobacterial morphotypes were grown in LB brothand incubated at 28±2 °C at 120 rpm.Additionally, observing the growth cycle of individual isolates usingalightmicroscopeandperformingGramstainingevery24hoursforupto10daysprovides valuable information about their growth patterns and Gram stain reactions. This comprehensive approach helps in characterizing and understanding the isolated endophytic bacteria.

MICROSCOPICEXAMINATION

GRAM STAINING:

Aloopful of culture was smeared on the sterile glass slide and air dried. The smear was subsequentlyfloodedwithcrystalviolet,leftfor1minute,washed,andthen floodedwithGram's iodineforanotherminute.Afterthis,decolorizingagents(90%alcohol)wereintroduced,followed by a thorough wash. Finally, the counterstain safranin was applied for 20 seconds, followed by washing. The slide was then examined under a microscope, and the results were recorded.

BACTERIALCONFIRMATIONTEST

VITEK®MSPRIMEisavaluabletoolforbacterialconfirmationtests,offeringautomated identification with a diverse and reliable database for rapid and certain microbial identification.

ANTIBACTERIALANALYSIS

Preparationofbrothcultures:

Two UTI pathogens (Escherichia coli, Staphylococcus aureus was inoculated in Nutrientbroth andincubatedat37˚cfor24hours,itwasusedinantibacterialanalysis.

38 grams of MHA (Muller Hinton Agar) was dissolved in1000 ml of distilled water and then autoclaved for 15 minutes at 121˚c. Once the medium was about 45˚-50˚c, it was poured into Sterile petri dishes. Then it was allowed to set completely

ANTIBACTERIALACTIVITY

Inthisexperiment,youswabbedanovernightbrothcultureontoMHAplates, created wells, added spice extracts, and used Streptomycin as a positive control.After incubation, you measured thediameter of thezoneof inhibition, and theresult is 4.8 mm.

PHBPRODUCTION

Materials:

TheEndophyticBacterialculturewasisolatedandinoculated intothecultureto produce the phb.

Cultureconditions:

AmineralsaltmediumusedforPHA(Polyhydroxyalkanoate)production.The composition of the medium includes:

2.5g/LK2HPO4

2.5g/LKH2PO4

5g/Lglucose(addedseparately) 2

g/L yeast extract

0.2g/LMgSO4.7H2O

0.01g/LFeSO4.7MnSO4

0.007g/LH2O7H2O

ThepHofthemediumwasadjustedto7.5,andsterilizationofthe mediumwithoutglucose was performed for 20 minutes at 121°C. Glucose was sterilized separately and then added to the medium. This process is likely for the cultivation of microorganisms to produce PHA.

Sudan Black B staining:

TheSudanBlackBstainingmethodiscommonlyusedtodetectthepresence of

polyhydroxybutyrate (PHB) granules in bacterial cells. Here's a summary of the process:

Preparation of Sudan Black B Solution:

Mix0.3mgofsolidSudanBlackBdyewith100mLof70%ethanoltopreparethestaining solution.

SmearPreparation:

Smearsofthepositivebacteriaarepreparedandheat-fixedontoglass slides.

StainingwithSudanBlackB:

ThepreparedsmearsarestainedwiththeSudanBlackBsolutionfor15minutes.Thisstaining step helps to visualize the PHB granules.

WashwithXylene:

Afterstaining, thesmearsarewashedwithxylene.Thisstepislikelyperformedto remove excess stain and enhance contrast.

Counter-StainingwithSafranin:

The smears are counter-stained with Safranin (5% in distilled water) for 1 minute. Safranin is a red stain and is used to provide contrastto theblue-black PHB granules stained by Sudan Black B.

MicroscopicObservation:

Thestainedsmearsare thenobservedunderamicroscopewithimmersionoilata magnification of 100×. Cells containing blue-black cytoplasmic granules are considered PHB producers.

Preservation:

PHB-producing cells are preserved in 2% glycerol, likely for further analysis or long-term storage.Stainedslideswereobservedtodeterminethecellularcharacteristicsoftheisolates at the microscope with immersion oil at 100X.

Extraction:

BacterialCulture: Bacterialcellsaregrownandincubatedfor48 hours.

Harvesting:Afterincubation, thebacterialcellsareharvestedbycentrifugationat5,000rpm for 10 minutes. This separates the cells from the culture medium.

Drying:Theharvestedbacterialcellsaredriedtodetermine theCellDryWeight(CDW),which serves as an index of biomass.

PHAandLipidExtraction: Thedriedcellsaretreatedwithboilingchloroformtodissolveboth

thePHAandlipids presentinthecells.

Filtration:Thechloroform-treatedsolutionisthenfilteredusingWhatmanNo.1filterpaperto remove the remaining cell mass. This step isolates the dissolved PHAand lipids.

PrecipitationofPHA: Methanolisaddedtothefiltrate,whichselectivelyprecipitatesthe PHA from the solution.

Centrifugation:Thesolutioncontaining theprecipitatedPHAissubjectedtocentrifugation at 12,000 rpm for 10 minutes. This step separates the PHAfrom the rest of the solution.

Drying:TheisolatedPHAisair-driedtoremove anyremaining solvent.

WeighingandStorage: ThedriedPHAisweighedtodetermineitsyield andisthenstored for further studies or applications.

FTIRAnalysis:

FTIR (Fourier Transform Infrared) spectroscopy is a common technique used to analyzethechemicalcompositionofsubstancesbymeasuringtheirinfraredabsorption spectra

- Chloroformwas usedas amediumforyoursample.
- ThesamplewasappliedasasmearoveranNaCl(sodiumchloride)block,whichisoften used as a sample holder for FTIR analysis.
- ThePerkinElmerRX1FTIRspectrophotometerwasusedfortheanalysis.
- TheFTIRspectrumofthesamplewascollectedin thewavelengthrangeof450-4000 cm^-1.

ENZYMATICACTIVITY

AMYLOLYTICACTIVITY

Thestarchhydrolysistestisacommonmethodtoassessabacterialisolate'sability to produceamylase,anenzyme thatbreaks downstarch.Herearethekeysteps ofthe procedure you described:

PreparationofStarch HydrolysisAgar:Starchhydrolysisagar,whichisaselective medium containingstarch, ispreparedandsterilizedby autoclavingat121°Cand15lbpressurefor15 minutes to ensure it's free from contaminants.

PouringtheMedium: Aftersterilization,theagarmediumiscarefullypouredintopetriplatesin a laminar flow hood to maintain sterility.

CoolingtoRoomTemperature:Thepetriplateswiththeagarmediumareallowedtocoolto room temperature within the laminar flow hood to prevent contamination.

Inoculation:Sterileinoculationloopsorswabsareusedtotransferbacterialisolatesonto the surface of the agar in the petri plates.

Incubation:Theinoculatedpetriplatesarethen incubatedat37°Cfor24hoursto allow bacterial growth and potential amylase production.

IodineTest:Afterincubation,aniodinesolutionisdirectlyaddedtothebacterialcolonies onthe agar surface. The iodine solution will turn blue-black in the presence of starch, but if amylase produced and has hydrolyzed the starch, there will be a clear zone around the bacterial colonies where the iodine solution remains yellow-brown

PROTEOLYTICACTIVITY

Onbloodagarmedia,,theactivityofproteolyticbacteriawasqualitativelytested.Clear zones around the colon show that the bacteria are able to integrate protein.

RESULT AND DISCUSSION

IDENTIFICATIONOFSEAWEED:

Theidentifiedseaweed istheSargassumspecies.They have branchedthallus with hollow berrylike floats (pneumatocysts). The fronds are generally small and have serrated edges Sexual reproduction takes place in most species but the pelagic species Reproduce by fragmentation. The largest members can reach several meters in length.

Figure 5 : Antibacterial activity of isolated endophytic bacteria against *Staphylococcus aureus*

S.NO	ORGANISMS	ZONE OF INHIBITION (mm)			
1.	*E.coli*	20µl	50µl	75µl	Positive control (streptomycin)
		9mm	13mm	17mm	14mm
2.	*Staphylococcus aureus*	10mm	12mm	15mm	15mm

Table 2 : Antibacterial assay of isolated endophytic bacteria against *E.coli* and *Staphylococcus aureus*

PHBPRODUCTION

SUDAN BLACKB STAINING:

Results from the initial Sudan Black B staining on test isolate cultured in petri dishes showedthatcolonies'colorsalteredtoadarkgreenish-bluehuewhenthedyewaspouredintothe dishes. This symptom is taken to indicate that test isolates have PHB accumulations.

PHBPOWDERBioplasticsaremoreimportance nowadays duetotheirbiocompatibleandbiodegradable naturewhichisanalternativeforpetroleumbasedplastics.Bacillus cereus is the bacterium that was isolated from the seaweed andwas foundtoproducePHB upto 900 mg of its dry cell weight.

FTIRRESULT:

ThephbextractionpowderwasanalyzedbyFouriertransforminfrared(FTIR).ThePhb powder functional group was observed by ftir peaks. The alkanes (C-H) group were Located in 2924.09. They show alkanes consist of single-bonded carbon and hydrogen atoms. In 1743.65 peaksshowstheketones(C=O)groupwerelocatedandcarbonylgroupsareComposedofacarbon atom double-bonded to an oxygen atom. Alkenes groups were located in 1643.35. In alkene, carbon carbon double bond (C=C) is the functional group. Nitro Compounds also have been presented. NO2 is strongly present in the extract polymer. In 1442.75 the aromatic compounds (C=C)wereobserved.Thefunctionalgroupthatcontainsonlycarbon andhydrogen isan aromatic ringwhichisasix-carbonringwithalternatingdoublebonds.Alkyl&ArylHalidesgroupsalso presented.Whenamixtureof alkylhalideand arylhalideis treatedwithsodiumindry ether alkyl arene is formed.

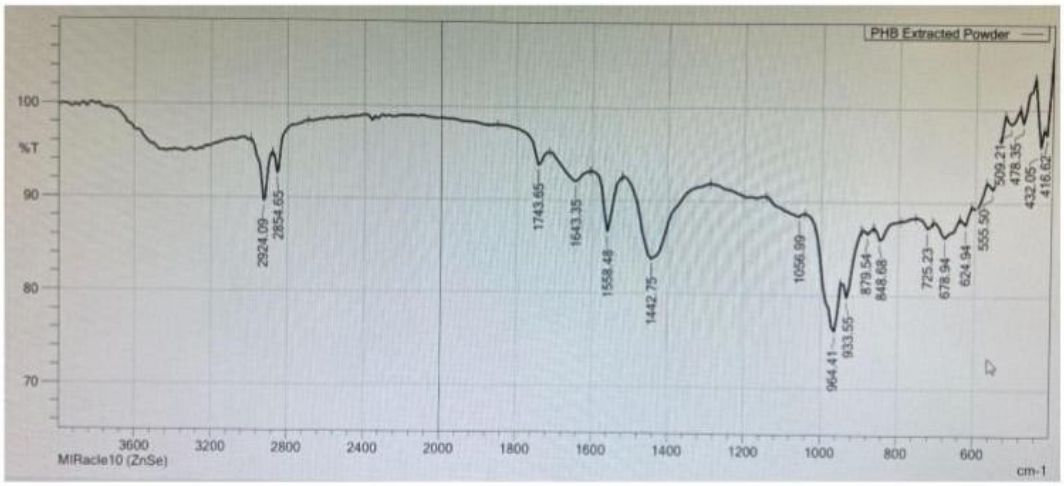

Figure 8 : FTIR report of PHB powder

AMYLOLYTICACTIVITY:

Theisolatedbacteriashowedprominentgrowthonstarchhydrolyzing,azoneof Inhibition was observed after the addition of iodine.

Figure 9: Amylolytic activity of isolated endophytic bacteria on Starch agar

PROTEOLYTICACTIVITY:

The isolate bacteria showed prominent growth on blood agar by the formation of zone of clearance after incubation

Figure 10 : Proteolytic activity of isolated endophytic bacteria on blood agar

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