ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 8, Issue 4, 2015

ISSN - 0974-2441

Research Article

ENDOPHYTIC BACTERIA: OPTIMIZATON OF ISOLATION PROCEDURE FROM VARIOUS MEDICINAL PLANTS AND THEIR PRELIMINARY CHARACTERIZATION

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Received: 02 May 2015, Revised and Accepted: 18 May 2015

ABSTRACT

Objective: The aim of this study was to the isolate the endophytic bacteria, optimize its isolation procedure and preliminary characterize the isolated endophytes.

Methods: Ethanol, sodium hypochlorite, and mercuric chloride at various concentrations and duration were employed to optimize the surface sterilization for the isolation of endophytes from *Catharanthus roseus*, *Ocimum sanctum, Mentha arvensis,* and *Stevia rebaudiana*. Microscopic and biochemical characterization of isolates were carried out.

Results: A total of 35 endophytic bacteria have been isolated from four medicinal plants. Combination of 2% sodium hypochlorite, 70% ethanol, and 0.1% mercuric chloride was found effective for the surface sterilization of *C. roseus* and *O. sanctum*. In case of *M. arvensis* and *S. rebaudiana*, 70% ethanol and 2% sodium hypochlorite was found suitable for the surface sterilization. In preliminary screening 18 isolates were found Gram-positive cocci, 11 were Gram-negative bacilli, 6 were found to be Gram-positive bacilli, 18 isolates showed positive results for endospore staining, 24 gave positive results for catalase test, 13 gave positive results for oxidase test, and 15 isolates were found to be motile.

Conclusion: Ethanol, sodium hypochlorite, and mercuric chloride were found effective decontaminating agents in optimum condition. In the preliminary screening diverse colony, different shapes, color, margins, and textures were observed.

Keywords: *Catharanthus roseus, Ocimum sanctum, Mentha arvensis, Stevia rebaudiana*, Surface sterilization, Endospore, Catalase, Oxidase.

INTRODUCTION

Plants are one of the most vital sources of medicines. Currently, large numbers of drugs in use are derived from plants. Medicinal plants are the chief source of secondary metabolites used as drugs and essential oils of therapeutic importance. The important advantages of medicinal plants for therapeutic uses in various ailments are their safety and also being inexpensive, effective, and their easy accessibility. These advantages of the medicinal plants forced the traditional medical experts for extensively used in their day to day practice. Endophytic bacteria living in plant tissues deprived of doing substantive harm or gain benefit other than residency [1]. The term endophyte (Gr. *endon*, within; *phyton*, plant) was first coined in 1866 by De Bary [2]. An endophyte can be defined as a microorganism such as fungi or bacteria that expends either the complete or part of its lifecycle within the healthy tissues of a living plant, typically causing no symptoms of disease [3,4]. Now we are familiar with the fact that endophytic microorganisms that reside in the tissues of living plants are promising, less explored and useful sources of novel natural products for exploitation in agriculture, medicine, and industry. The importance of endophytes had been demonstrated over a long period as a source of pharmaceutical bioactive compounds, as many of endophytes were exposed to produce novel bioactive metabolites such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive drugs, and many related compounds. Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, etc. Importantly, secondary metabolites produced by endophytes provide a variety of fitness enhancements and exert several beneficial effects on host plants, such as stimulation of plant growth [5], nitrogen fixation [6,7], and induce resistance to drought, herbivorous, parasitism [8,9], etc. Endophytes are generally isolated for following good reasons such as for their characterization, for studying population dynamics and diversity, use of microbial inoculants to improve plant growth and plant health, and as sources of novel biologically active secondary metabolites [10,11]. Endophytes enter the plant tissue chiefly through the root zone; on the other hand, above ground portions of plants, may also be used for entry, such as cotyledons, stems, and flowers [12]. Bacteria get entrance in the tissues via germinating radicles [13], secondary roots [14], stomata [15], or from the damage site [16]. Endophytes inside a plant may either remain confined at the point of entry or reside within the cells [17], the intercellular spaces [18] or the vascular system [19] and can also spread throughout the plant [20]. Endophytic bacteria are found in stems, leaves, roots, seeds, fruits, ovules, tubers, and as well inside legume nodules [5,21]. In general, bacterial populations are in decreasing order from roots, stems and leaves. In most plants, generally roots have greater numbers of endophytes compared with aboveground tissues [22]. Bacterial endophytes have been isolated from leaves, stems, roots, flowers, seeds, and fruits of various plant species [12]. The rationale behind this research work was to ensure the purity of endophytes during isolation and purification process by optimization of isolation and purification steps. The isolation procedure is an important and critical step while working with endophytic bacteria and fungi. It must be sensitive enough to recover endophytic microorganisms, but at the same time it should be strong enough to eliminate epiphytes from the plant surface. In this report, result of optimization of isolation of endophytes from four different medicinal plants whose potential as medicinal plants are well known is presented. This will be the first report on the optimization of isolation and purification procedures to get bacterial endophytes from medicinal plants.

Catharanthus roseus is an important medicinal plant, belongs to the family Apocynaceae and is a vital source of alkaloids, which are obtainable from all parts of the plant. *C. roseus* can be called as an important drug because different parts are used in the treatments of various diseases such as cancer, diabetes, menstrual regulators, hypertension, and antigalactagogue. It is used as medicinal plants in different countries such as Australia, Brazil, China, Cook Island,

Dominica, Europe, England, French Guiana, France, India, Jamaica, Kenya, Mexico, Mozambique, North Vietnam, Philippines, Pakistan, Peru, South Africa, South Vietnam, Taiwan, Thailand, USA, Venda, Vietnam, West Indies, etc. [23]. Widely used alkaloids such as the anticancerous drugs vinblastine and vincristine, as well as the antihypertension compounds such as ajmalicine and serpentine have been isolated from different parts of *C. roseus* [24]. Catharanthine, tabersonine, and lochnericine are indole alkaloids also found in *C. roseus* [25].

Ocimum sanctum belongs to family Lamiaceae is a widely grown in India has high medicinal value. Tulsi leaves contain bright yellow volatile oil, reported possessing anti-bacterial properties and also acts as insecticides. Their leaves are traditionally used for getting relief from the common cold, bronchitis, cough, and digestive problems [26]. *Ocimum* (Tulsi) plants are considered as sacred plants in India and known for its antimicrobial, antipyretic, immunomodulatory, anti-inflammatory, antistress, antiasthmatic, hypotensive, hypoglycemic, and analgesic activities [27,28]. It is also reported that *O. sanctum* exhibited antibacterial activity against *Vibrio cholera, Escherichia coli*, *Staphylococcus aureus, Klebisella* sp., and *Proteus* sp. Maximum zone of inhibition was noticed against *S. aureus* [29].

Mentha arvensis belongs to family Lamiaceae, and is one of the most widespread essential oil crops. They are widely distributed and cultivated in the temperate and sub temperate regions of the world [30]. *M. arvensis* possess considerable chemical diversity in the essential oil composition and are considered industrial crops as the aerial parts of *Mentha* species produces a number of commercially valuable essential oils containing a complex mixture of monoterpenoids which are generally employed in pharmaceutical, flavor, food, beverages, cosmetics, and allied industries [31,32]. Menthol, menthone, isomenthone, menthofuran, carvone, linalool, linalyl acetate and piperitenone oxide are the aromatic compounds present in aerial parts of the herb, used in pharmaceutical, food, flavor, cosmetics, beverages, and allied industries. In India, mint species are mainly cultivated in Punjab, Haryana, and Uttar Pradesh, and they had occupied more than 162,800 hectares of agricultural land [33,34].

Stevia rebaudiana is a shrub of the family Asteraceae and it is a member of genus *Stevia* producing sweet steviol glycosides. It was earlier cultivated in Paraguay and Brazil and now it is also cultivated in some region of Canada, Europe, and Asia [35]. Stevia has been successfully cultivated in many areas of the Indian states. The growing demands for natural sweeteners have motivated the farmers in India toward large-scale Stevia cultivation. It is rich in terpenes and flavonoids. The phytochemicals present in *S. rebaudiana* are austroinullin, β-carotene, stevioside, niacin, dulcoside, riboflavin, rebaudi oxides, steviol, and tiamin [36]. Compounds obtain from *Stevia* have important industrial uses as energizers, beverages as well as medicinal uses such as a vasodilator, cardiotonic, anesthetic and anti-inflammatory and to lower uric acid. The natural sweeteners that have been extracted from *Stevia* are the group of diterpene glycosides. The leaves of wild *Stevia* plants contain 9.1% stevioside, 3.8% rebaudioside A, 0.6% rebaudioside C, and 0.3% dulcoside [37]. Extract of *Stevia* has been known to exert beneficial effects on human health, including antihypertensive [38], antihyperglycemic [39], and antihuman rotavirus activities [40]. Sweeteners are also thoughts to influence glucose metabolism [41] and renal function [42].

Studies were conducted to optimize the effectiveness of the decontamination procedures for the isolation of endophytic bacteria from *C. roseus*, *O. sanctum*, *M. arvensis,* and *S. rebaudiana* and to evaluate the microscopic and biochemical properties of the isolate in order to characterize them in different groups.

METHODS

Collection of plant material

For the isolation of endophytic bacteria, healthy leaves, stems, and roots of *C. roseus*, *O. sanctum*, *M. arvensis*, and *S. rebaudiana* were collected from randomly selected healthy wild and cultivated plants from the medicinal garden of Birla Institute of Technology, Mesra, Ranchi. Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose.

Isolation and purification of bacterial endophytes

Surface sterilization is the initial and mandatory step for isolation of endophyte in order to kill all the surface microbes. It is usually accomplished by treatment of plant tissues with oxidant or general sterilizing agent for a period and then by 3-5 times sterile rinse. The most commonly used isolation procedures combine surface sterilization of the plant tissue, plating small sterilized segments onto nutrient agar and also by the maceration of the plant tissue and streaking onto nutrient agar, or vacuum or pressure extraction. Theoretically, the sterilizing agent should kill any microbe on the plant surface without affecting the host tissue and the endophytic microorganisms. Though, this is challenging to achieve because the conditions required to kill the last microbe on the surface may already be lethal for some endophytic microorganisms and in time the agent may penetrate the plant tissue. In general, isolation and purification of endophytic bacteria from plant tissue consist of the following steps:

Pre-treatment

The leaves, stems, and roots of each plant were washed separately under tap water to remove adhering soil particles and the majority of microbial surface epiphytes is a part of pre-treatment.

Surface sterilization

Freshly collected leaves, stem, and roots were washed under slow running tap water for 15 minutes followed by washing in Tween 20 (1 drop in 200 mL sterile distilled water [SDW]) for 1 minute and then were rinsed three times with SDW in the laminar air flow cabinet. Commonly used sterilizing agents are sodium hypochlorite: 1-5% for 2-10 minutes [43], ethanol: 70-95% for 30 seconds - 4 minutes [44], hydrogen peroxide [45] and mercuric chloride 0.05-0.2% for 2-5 minutes [46]. After a proper literature survey, 2% sodium hypochlorite, 70% ethanol and 0.1%, mercuric chloride at different treatment duration and combination was used for the surface sterilization agent for the current study for the process of surface sterilization.

Test for effectiveness of surface sterilization

Only if, complete surface sterilization of the plant tissue is confirmed, then only the isolated microorganisms are said to be endophytes. Validation of the surface sterilization procedure was done by imprinting the surface sterilized plant tissue onto nutrient media [47,48], culturing aliquots of water from the last rinsing onto nutrient media [45], dipping the surface sterilized explants into nutrient broth [13]. They were also incubated as control of the test sample.

Media for isolating endophytic bacteria

The choice of the growth medium is crucial as it directly affects the number and type of endophytic microorganisms that can be isolated from the root tissue. Nutrient agar media were used for the isolation of endophytic bacteria. Since there is no component in nutrient agar which can suppress the growth of endophytic fungi, so the media used for the isolation of endophytic bacteria were supplemented with an antifungal agent, nystatin at a concentration of 30 µg/mL of each to suppress fungal growth.

Isolation, purification, and subculture of endophytic bacteria

After proper drying of surface sterilized plant material, using aseptic procedure the surface of the stems were removed using a sterile scalpel in the laminar air flow cabinet and leaves were cut into pieces and each piece was placed on nutrient agar medium supplemented with antifungal agents. Plates with plant tissues are sealed using parafilm tape and incubated at 28±2°C in order to recover the maximum possible colonies of bacterial endophytes. The observation was made for 48 hrs. After 24 hrs from the bacterial cultures, morphologically different bacterial colonies were selected and are repeatedly streaked in order to achieve bacterial isolates. All selected isolates were subcultured in nutrient agar slants and finally, all the purified endophytes were maintained at 4°C till further used.

Preliminary characterization of endophytic bacteria

Phenotypic characteristics such as microscopic features, gram reaction, endospore staining, motility, catalase, and oxidase activity of all the isolates were determined by using standard procedures.

Statistical analysis

Different sterilizing agents alone and along with their different combination were used to get the optimum condition for the surface sterilization of the four different medicinal plants as given in Table 1. Experiments were repeated thrice and data represents the mean of three experiments. After analyzing statistically the effectiveness of sterilizing agent alone and in combination, variable results were found in four different medicinal plants. These results were subjected to standard error.

RESULTS

Isolation and purification of bacterial endophytes

Surface sterilization

To check the efficiency of sodium hypochlorite, ethanol, and mercuric chloride as effective sterilizing agents and survival percentage of treated explant. And to see the effect on explant treated individually and by the different combination of a chemical disinfectant. Sterilization procedures that were initially selected and used were not found efficient sterilizing agent individually. Therefore, a high percentage of the explants were found contaminated. However, different combination and duration of 70% ethanol, 2% sodium hypochlorite, and 0.1% mercuric chloride were applied to the explant to achieve a satisfactory result. For the surface sterilization and the percentage survival of explants after treatment of *C. roseus* and *O. sanctum,* 2% sodium hypochlorite, 70% ethanol, and 0.1% mercuric chloride were found effective, whereas treating *M. arvensis* and *S. rebaudiana* with mercuric chloride damaged the explant and plant died (Figs. 1 and 2). Therefore, only 2% sodium hypochlorite and 70% ethanol was used for the surface sterilization of *M. arvensis* and *S. rebaudiana.* The results of the Optimization process for the surface sterilization of *M. arvensis* and *S. rebaudiana* is shown in the Figs. 3 and 4.

Table 1: Different duration, combination, and concentration of treatment used for sterilization

Sterilization treatment	Explant	Duration
70% ethanol (A)	Stem, leaves and roots	1 minute
2% sodium hypochlorite (B)	Stem and leaves roots	1 minute 30 seconds 3 minutes
0.1% mercuric chloride (C)	Stem and leaves roots	30 seconds 1 minute
70% ethanol+ 2% sodium hypochlorite (A+B)	Stem and leaves	Ethanol for 1 minute+ sodium hypochlorite for 1 minute 30 seconds
	Roots	Ethanol for 1 minute+ sodium hypochlorite for 3 minutes
2% sodium hypochlorite+ 0.1% mercuric chloride (B+C)	Stem and leaves	Sodium hypochlorite for 1 minute 30 seconds+ mercuric chloride for 30 seconds
	Roots	Sodium hypochlorite for 3 minutes+mercuric chloride for 1 minute
70% ethanol+ 0.1% mercuric chloride (A+C)	Stem and leaves	Ethanol for 1 minute+ mercuric chloride for 30 seconds
	Roots	Ethanol for 1 minute+ mercuric chloride for 1 minute
70% ethanol+ 2% sodium hypochlorite+ 0.1% mercuric chloride (A+B+C)	Stem and leaves	Ethanol for 1 minute+ sodium hypochlorite for 1 minute 30 seconds+ mercuric chloride for 30 seconds
	Roots	Ethanol for 1 minute+ sodium hypochlorite for 3 minutes+Mercuric chloride for 1 minute

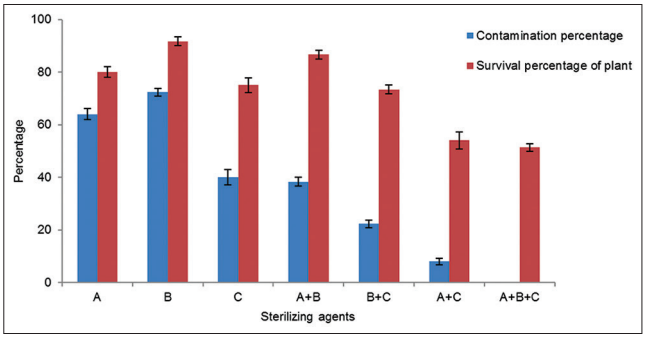

Fig. 1: Optimum condition for the surface sterilization of *Catharanthus roseus*. (Error bars show standard errors)

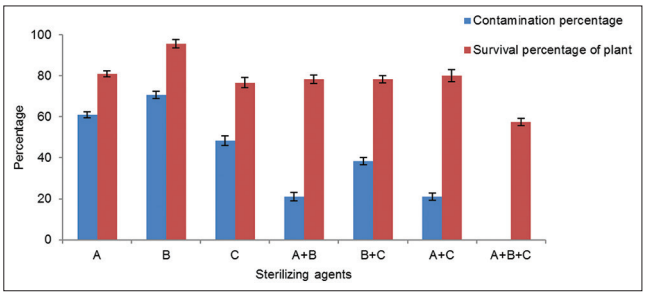

Fig. 2: Optimum condition for the surface sterilization of *Ocimum sanctum*. (Error bars show standard errors)

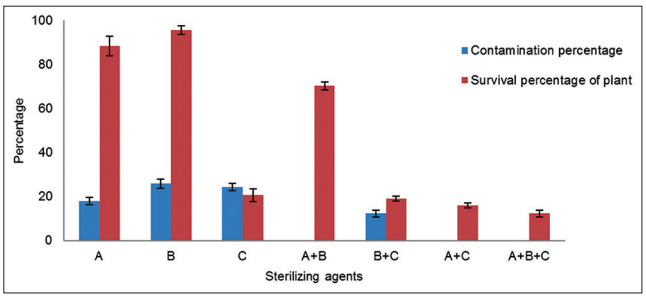

Fig. 3: Optimum condition for the surface sterilization of *Mentha arvensis*. (Error bars show standard errors)

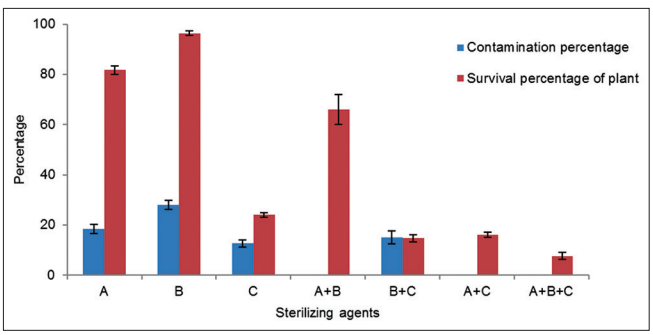

Fig. 4: Optimum condition for the surface sterilization of *Stevia rebaudiana*. (Error bars show standard errors)

Effectiveness of surface sterilization

Three methods of sterility check namely imprinting the surface sterilized plant tissue onto nutrient media, culturing aliquots of water from the last rinsing onto nutrient media and dipping the surface sterilized explants into the nutrient broth gave comparable results and under optimal condition, no microbial growth occurred on the control medium, so the surface sterilization is considered complete and isolated bacteria are considered as endophytes.

Isolation and purification

Nutrient agar media has been used for the isolation of endophytic bacteria and different colonies have been recovered after culture plate. About 35 bacterial endophytes have been isolated in pure form from four medicinal plants used in this study as shown in the Fig. 5. Emergence of endophytic bacteria on the cultured stem and leaves of *C. roseus* and *M. arvensis* are shown in Figs. 6 and 7. Purified culture of endophytic bcateria from Catharnathus roseus and Mentha arvensis is shown in Fig. 8. From the total 35 isolates, 10 isolates were selected for further investigation.

Preliminary characterization of endophytic bacteria

For morphological characterization, isolated endophytic bacteria exhibited the diverse colony, different shapes, color, margins and textures including round to irregular colonies, off white, pink, yellow colonies with regular or wavy margins. Out of 35 isolates, 26 are pigmented and 9 were non-pigmented isolates. Regarding cell shape and gram staining, 18 were Gram-positive cocci, 11 were Gram-negative bacilli, and 6 were found to be Gram-positive bacilli. In total 18 isolates showed positive results for endospores staining, possibly belonging to the genus Bacillus. Endophytic bacteria showed creamy, slimy, soft, and sometimes mucoid, rough, dry and embedded colonies. For biochemical and physiological tests, 24 isolates gave positive results for catalase and 13 showed positive results for oxidase test. The result indicates that they can produce catalase and oxidase enzyme. Likewise, 15 isolates showed positive results for motility tests. The culture characteristics of isolated endophytic bacteria are given in Table 2. Distribution of important features of isolated bacterial endophytes is shown in the Fig. 9.

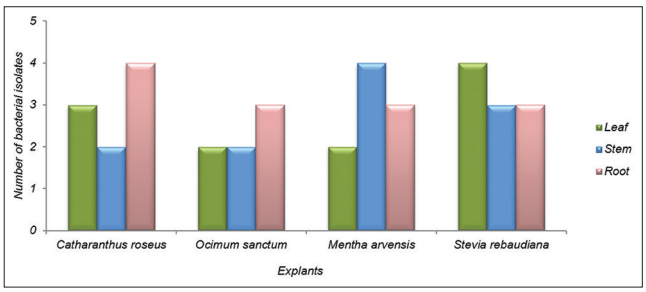

Fig. 5: Number of endophytic bacteria isolates from four medicinal plants

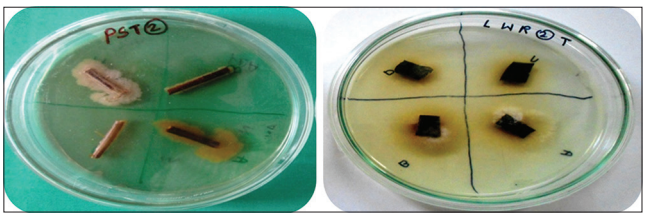

Fig. 6: Growth of endophytic bacteria on the stem and leaves of *Catharnathus roseus*

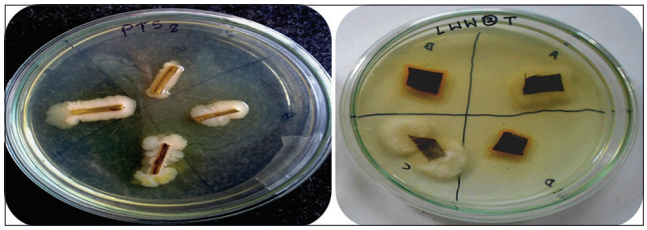

Fig. 7: Growth of endophytic bacteria on the stem and leaves of *Mentha arvensis*

DISCUSSION

These studies demonstrated the occurrence and diversity of culturable endophytes. In India, only countable number of the reports showed the diversity of endophytic bacteria and fungi from the medicinal plants. There is no report on endophytic bacteria from Jharkhand. This work may be the first report on endophytic bacteria from *C. roseus*, *O. sanctum*, *M. arvensis,* and *S. rebaudiana* form Jharkhand state. There are reports on the isolation of fungal endophytes from *C. roseus*, *O. sanctum*, *M. arvensis,* and *S. rebaudiana* [49], but there are only few reports on the isolation of endophytic bacteria [50,51]. In general, endophytic bacteria occur as lower population density than rhizospheric bacteria or bacterial pathogen. The surface of plants carries a wide range of microbial contaminants. To avoid this source of infection and for the isolation of endophytes, explants must be thoroughly surface-sterilized before inoculating them onto the nutrient medium. Chemical disinfectant has been employed for surface sterilization of excised plant tissue to remove epiphytes and immersion of the tissue in ethanol, sodium hypochlorite, and mercuric chloride has shown significant success in these studied. In all the four plants, *C. roseus*, *O. sanctum*, *M. arvensis,* and *S. rebaudiana,* contamination percentage was found to be high in individual treatments when compared to the treatment in which different combination of sterilizing agents were used. The contamination percentage and survival percentage of the plant, does not gave satisfying results when the treatment duration or concentration of sterilizing agent is either increased or decreased. The optimum

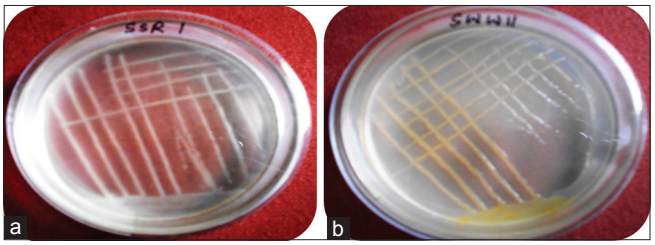

Fig. 8: Purified culture of bacterial endophytes (a) from *Mentha arvensis* and (b) from *Catharanthus roseus*

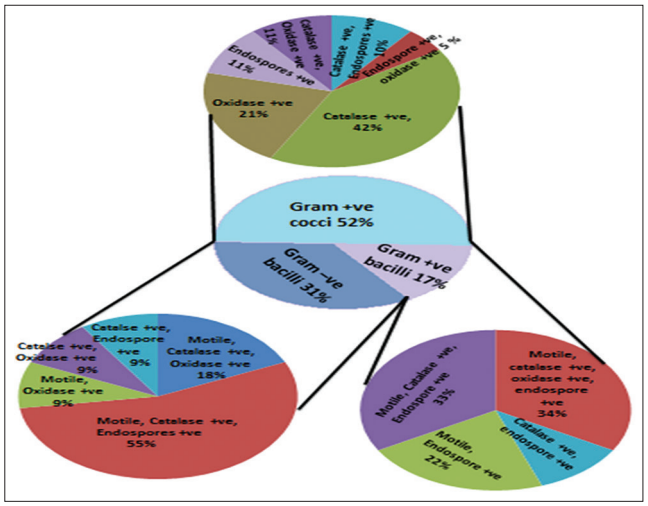

Fig. 9: Distribution of important features of isolated bacterial endophytes

Table 2: Colony features and biochemical characteristics of endophytic bacteria

S. no	Culture	Color	Size	Margin	Consistency	Texture	Gram staining	Size	Motility	Catalase	Oxidase	Spore staining
1	CRL1	Colorless	Large	Irregular	Creamy	Raised	+ve	Cocci	-	+	-	+
2	CRL2	Yellow	Small	Circular	Slimy	Raised	-ve	Bacilli	+	+	+	-
3	CRL3	Regular	Large	Regular	Mucoid	Raised	+ve	Bacilli	+	+	+	+
4	CRS1	Colorless	Small	Irregular	Creamy	Flat	–ve	Bacilli	+	+	+	-
5	CRS2	Colorless	Small	Irregular	Creamy	Raised	+ve	Bacilli	-	+	-	+
6	CRR1	Yellow	Small	Irregular	Slimy	Raised	+ve	Cocci	-	-	+	+
7	CRR2	Colorless	Small	Circular	Creamy	Raised	+ve	Cocci	-	+	-	-
8	CRR3	Colorless	Large	Circular	Creamy	Raised	+ve	Cocci	-	-	+	-
9	CRR4	Yellow	Small	Irregular	Slimy	Flat	-ve	Bacilli	+	+	-	+
10	OSL1	Colorless	Large	Irregular	Creamy	Raised	+ve	Cocci	-	+	-	+
11	OSL2	Orange	Small	Regular	Mucoid	Flat	+ve	Cocci	-	+	-	-
12	OSS1	Colorless	Small	Irregular	Creamy	Raised	+ve	Cocci	-	+	-	-
13	OSS2	Colorless	Small	Irregular	Creamy	Raised	+ve	Cocci	-	-	-	+
14	OSR1	Yellow	Small	Circular	Slimy	Raised	+ve	Bacilli	+	+	+	+
15	OSR2	Colorless	Large	Irregular	Creamy	Raised	+ve	Cocci	-	+	-	-
16	OSR3	Colorless	Small	Irregular	Creamy	Raised	+ve	Cocci	-	+	-	-
17	MAL1	Colorless	Small	Irregular	Creamy	Raised	+ve	Cocci	-	+	-	-
18	MAL2	Orange	Small	Irregular	Mucoid	Flat	-ve	Bacilli	+	-	+	-
19	MAS1	Colorless	Small	Irregular	Creamy	Raised	+ve	Bacilli	+	+	+	+
20	MAS2	Yellow	Small	Regular	Slimy	Flat	-ve	Bacilli	-	+	+	-
21	MAS3	Colorless	Large	Irregular	Creamy	Raised	+ve	Cocci	-	-	-	+
22	MAS4	Colorless	Small	Circular	Creamy	Raised	+ve	Cocci	-	+	-	-
23	MAR1	Colorless	Small	Irregular	Creamy	Flat	-ve	Bacilli	+	+	-	+
24	MAR2	Colorless	Small	Regular	Creamy	Raised	+ve	Cocci	-	+	+	-
25	MAR3	Colorless	Large	Irregular	Creamy	Raised	-ve	Bacilli	+	+	-	+
26	SRL1	Yellow	Small	Irregular	Slimy	Raised	+ve	Cocci	-	-	+	-
27	SRL2	Colorless	Small	Regular	Creamy	Raised	-ve	Bacilli	+	+	-	+
28	SRL3	Colorless	Small	Regular	Creamy	Flat	-ve	Bacilli	+	+	-	+
29	SRL4	Colorless	Small	Regular	Creamy	Raised	+ve	Bacilli	+	-	-	+
30	SRS1	Colorless	Small	Irregular	Creamy	Raised	+ve	Cocci	-	-	+	-
31	SRS2	Colorless	Small	Irregular	Creamy	Raised	+ve	Cocci	-	+	+	-
32	SRS3	Colorless	Small	Circular	Creamy	Raised	+ve	Cocci	-	+	-	-
33	SRR1	Colorless	Small	Regular	Creamy	Raised	-ve	Bacilli	-	+	-	+
34	SRR2	Yellow	Small	Irregular	Mucoid	Flat	+ve	Bacilli	+	+	-	+
35	SRR3	Colorless	Large	Irregular	Creamy	Raised	-ve	Bacilli	+	+	-	+

Codes regarding origin CRL: *Catharanthus roseus* leaf, CRS: *Catharanthus roseus* stem, CRR: *Catharanthus roseus* root, OSL: *Ocimum sanctum* leaf, OSS: *Ocimum sanctum* stem, OSR: *Ocimum sanctum* root, MAL: *Mentha arvensis* leaf, MAS: *Mentha arvensis* stem, MAR: *Mentha arvensis* root, SRL: *Stevia rebaudiana* leaf, SRS: *Stevia rebaudiana* stem, SRR: *Stevia rebaudiana* root and isolate number is indicated as 1, 2, 3 and 4

condition for the surface sterilization of the *C. roseus* and *O. sanctum* was found as treating the stem, leaves, and roots with 70% ethanol for 1 minute followed by treatment of 2% sodium hypochlorite for 1 minute 30 seconds for stem, leaves and 3 minutes for roots, then treating with 0.1% mercuric chloride 30 seconds for stem and leaves and 1 minute for roots. Optimum conditions for the surface sterilization of the *M. arvensis* and *S. rebaudiana* were found by treating the stem, leaves, and roots with 70% ethanol for 1 minute followed by treatment of 2% sodium hypochlorite for 1 minute 30 seconds for stem and leaf and 3 minutes for root explant, respectively. Use of mercuric chloride for the surface sterilization of *M. arvensis* and *S. rebaudiana* was not found effective because mercuric chloride was found to be a good decontaminating agent, but the survival percentage of plant explants decreased. These wide range of biochemical and physiological characteristics of the endophytic bacterial isolates indicated that they are different bacterial species. There appeared significant variation in the types of indigenous bacteria isolated from diverse host plant species. Various factors may explain these differences in the line of explanation given by Kobayashi and Palumbo [12]. These factors are geographical distribution, host specificity, plant age and tissue type. Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans [3,52]. Endophytes can be a promising source of bioactive compounds, and should be continuously isolated, characterized, and investigated for the discovery of lead bioactive compounds which can be employed in agriculture, medicine, and industries [11,51]. Endophytes can be an alternate source of drugs which will help to conserve biodiversity and drug resistance.

CONCLUSION

The contamination percentage of culture explant is higher when 70% ethanol, 2% sodium hypochlorite, and 0.1% mercuric chloride used separately. The contamination percentage of all the cases is lower when mercuric chloride was used for decontamination, but the survival percentage is lower. The combination of 70% ethanol, 2% sodium hypochlorite, and 0.1% mercuric chloride is best for surface sterilization of explant from *C. roseus* and *O. sanctum*. For surface sterilization of *M. arvensis* and *S. rebaudiana*, a combination of 70% ethanol and 2% sodium hypochlorite was found sufficient. In present work around 2/3 of the endophytic bacterial isolates are Gram-positive. Again 2/3 of endophytic bacterial isolates are catalase positive and about half of total isolates are spore forming. Keeping in view the importance of the four selected ethnomedicinal plants, their pharmaceutical applications and biological activity, further study on purification, optimization, and structure elucidation of bioactive compound produced by the best isolates that exhibit wide spectrum activity and the identification of the potential isolate is under is under progress.

ACKNOWLEDGMENT

The authors acknowledged the facility provided from the Department of Bioengineering, Birla Institute of Technology, Mesra, Ranchi, for carrying out research work.

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