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RESEARCH ARTICLE

Production and Characterization of Indole Acetic Acid of Endophytic Bacteria isolated from *Kalanchoe pinnata* (Lam.) in Optimized Media

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Abstract

Objectives: To optimize, quantify and characterize indole acetic acid (IAA) production from endophtic bacteria isolated from the leaves of *Kalanchoe pinnata* (Lam.). **Methods:** The selected endophytic bacteria are identified by biochemical, morphological and 16SrRNA sequencing analysis. Endophytes are screened for IAA using Salkowski reagent and media optimization is carried out for maximum IAA yield and quantification was done with UV Spectrophotometer at 30 and 120 minutes. The mean ± standard error of each sample for three triplicates is calculated from mean value of the samples followed by normal distribution. The characterization of IAA was studied with ethyl acetate extract of endophytes using TLC and HPLC. **Findings:** The selected endophytes are identified as *Bacillus thuringiensis, Bacillus paranthracis, Staphylococcus xylosus* and *Bacillus cereus*. Optimized yeast malt dextrose (YMD) broth containing mannitol, ammonium nitrate, and 0.2% of L-tryptophan at pH 8 and 30°C temperature for 72 h was used to obtain maximum IAA production from these endophytes. *B. paranthracis* produced 82.4733±0.0728µg/ml and 83.5667±0.457µg/ml, *S. xylosus* produced 93.12±0.261µg/ml and 94.7667±0.285µg/ml, *B. cereus* produced 24.4 ±0.113µg/ml and 25.0667 ±0.581µg/ml and *B. thrungiensis* produced 4.1333±0.131µg/ml and 5±0.226µg/ml of IAA at 30 and 120 minutes, respectively. *B. paranthracis* and *S. xylosus* were producing potential IAA with Rf 0.91 and 0.95 respectively compared with standard Rf 0.95. The HPLC analysis of *Bacillus paranthracis* and *Staphylococcus xylosus* were confirmed for the presence of IAA with the retention time of 5.053 and 5.58 minutes respectively compared to the retention time of the standard. **Novelty:** Endophytic bacteria notable in the leaves of *Kalanchoe pinnata* (Lam.) produce indole acetic acid in measurable quantity by UV Spectrometry and HPLC analysis, that can be replace for unstable and expensive synthetic IAA using costly chemicals. This is best pilot scale production of IAA using endophytes in the field of white biotechnology.

Keywords: Endophytes; Kalanchoe pinnata; Indole Acetic Acid; TLC; HPLC

1 Introduction

Plants are storehouse for diverse group of endophytes inside the host tissue including fungi, actinomycetes and bacteria. To consider this, researchers focus on an application study towards the synthesis of biological active phytohormones for development and growth of plants [1]. Several studies proved that endophytic bacteria can synthesis potential IAA, a metabolite of the L-tryptophan which promotes agricultural crops [2,3].

Fresh leaves, fruits and vegetables are the essential habitats for communities of endophytes [4]. During plant-microbial interaction, few microbes enter the plant tissue and lives inside as endophytes. Despite the fact that endophytic microorganism has been often seen in practically all plant tissue [5], their populace fluctuates relying on the host tissue, seasons, the encompassing natural circumstances and developmental stage of plants [6].

Kalanchoe species belongs to Crassulaceae, commonly known as miracle leaf, distributed in tropical and subtropical regions of Asia; mature leaves produce new plantlets since it secretes IAA. In ancient times, this plant species used to treat skin allergies, inflammation, diabetes, wound healing, cardiovascular disorder, ulcer, hypertensive and as antimicrobial agents [7]. The chemical composition of this plants secured wide range of potential ethanomedical applications. Phytochemicals such as alkaloids, flavonoids, phenols, tannins, proteins, sugars, steroids, vitamin, minerals and bufadienolids have been reported [8].

In last two decades, endophytic bacteria have been explored for agricultural application as biofertilizers. Endophytes are liked over other plant development rhizobacteria because of good adaptation and survival against abiotic and biotic stresses. The bacterial endophytes advance the development of host plants directly by means of secreting phytohormones, including indole -3- acetic acid, gibberellins, cytokinins, secondary metabolites, N2 fixation, phosphate solubilization, and indirectly produce siderophore compound as antibiotics [9]. The most significant and dynamic phytohormone is indole-3-acetic acid, controls different natural interaction for plant development [10]. *Agrobacterium fabrum, Agrobacterium tumefaciens, Acinetobacter radioresistant, Bacillus cereus, Bacillus subtilis, Bacillus licheniformis, Brevibacillus brevis, Burkholderia cepacia, Pseudomonas sp, Paenibacillus barengoltzii* and *Lysinibacillus fusiformis* are reported as common endophytic bacteria in various plant species that produce IAA [3,9].

The present study is focused on optimization and characterization of indole acetic acid from endophytic bacteria associated with *Kalanchoe pinnata* (Lam.). In addition, this study explained isolation, characterization of endophytes through biochemical, morphological and molecular studies. Since, the mature leaves of *Kalanchoe pinnata* (Lam.) produce leaflets due to the presence of indole acetic acid, but research on IAA production from endophytic bacteria of this plant was not done as so far. It was shown that endophytic bacteria isolated from this plant possess the mechanism to produce indole acetic acid either direct or indirect mechanism in measurable quantity. This promises to fulfill the agricultural needs for increased growth and yield of agricultural crops.

2 Methodology

2.1 Isolation of IAA Producing Endophytic Bacteria

Fresh leaves of *Kalanchoe pinnata* (Lam.) were ground in 2% saline and serially diluted and plated on LB agar for endophytic isolation. After 24 hrs, morphologically diverse bacterial colonies were selected and maintained at 4°C till further use[(11)].The morphological characters and biochemical tests were followed from standard laboratory manual[(4)]. For authentic conformation, the molecular identification was carried out by 16S rRNA sequencing for isolated endophytes[(11,12)]. These endophytes were further used for production of IAA.

2.2 Confirmation of IAA Production

Salkowski reagent was used to determine the amounts of IAA produced by each isolate[(13,14)]. The isolated endophytes were grown and incubated for 3 days at 30°C in yeast malt dextrose broth (YMD broth, HiMedia, India). The supernatant was collected after centrifugation; to the 1ml of supernatant, added 2ml of Salkowski's reagent and incubated in the dark. The optical density was recorded after 30 and 120 min at 530 nm.

2.3 Optimization of YMD media for IAA production

Optimization of YMD media enhanced with carbon source, nitrogen source, tryptophan concentration, pH, temperature and incubation time was used for improved yield of IAA. The concentration of IAA was assessed using Salkowski method at 30 and 120 minutes;[(12–15)] the values were represented by statistical analysis

2.3.1 Optimization of Carbon Source and Nitrogen Source

Sucrose, glucose, dextrose and mannitol were used as carbon sources and peptone, potassium nitrate, ammonium nitrate and sodium nitrate as nitrogen sources were used in YMD medium for achieving optimum yield of IAA by incubating at 30°C for 72 hours.

2.3.2 Optimization of pH and Temperature

To obtain the optimal IAA production, pH ranges of 6, 7, 8 and 9 and the temperature ranges such as 20°C, 30°C, 40°C and 50°C were used in YMD medium for accomplishing highest yield of IAA by incubating for 72 hours.

2.3.3 Optimization of L-Tryptophan Concentration and Incubation Period

The impact of incubation period and concentration of L-tryptophan on IAA production was tested in endophytic bacteria using YMD broth enhanced with various centralizations of L-Trp (1%, 2%, 3%, 4% and 5%) and followed by incubation period of 24, 48, 72 and 96 hours.

2.4 Extraction and Purification of IAA

The isolated cultures were incubated for 72 hours at 30°C in optimized YMD broth at pH 8. After incubation, broth was centrifuged, the supernatant was mixed with ethyl acetate (1: 2). This mixture was shaked vigorously and stands for 10 minutes to extract IAA.

2.5 Thin Layer Chromatography

Crude ethyl extracts of samples were spotted along with standard IAA (10mg/100ml) on TLC plate bought from Merck Co, made of Silica gel used 8:2 ratios of 1-propanol and water as the mobile phase.

2.6 HPLC Analysis of IAA

The methanolic extract of IAA was additionally affirmed by reverse phase HPLC (Shimadzu Lab Solutions) containing C18 column of 1 ml/ min flow rate. Elution was performed with 60: 40 ratios of HPLC grade water and methanol containing 0.5% acetic acid and estimated by UV/Vis detector at 250 nm.

2.7 Statistical Analysis

The mean ± standard error of each sample for three triplicates is calculated from mean value of the samples followed by normal distribution with a confidence level of 95%. The critical difference value was calculated at a probability of 0.05.

3 Results and Discussion

3.1 Isolation of IAA Producing Endophytic Bacteria

From the leaves of *Kalachoe pinnata* (Lam), 27 bacterial endophytes were isolated. We have selected 4 isolates named as *Bacillus thuringiensis*, *Bacillus paranthracis, Staphylococcus xylosus* and *Bacillus cereus* based on biochemical, morphological characters and 16S rRNA sequencing study for IAA production after the preliminary screening test (Table 1).

3.2 Confirmation of IAA Production

The formation of pink colour indicates the presence of IAA and the reagent acts as a blank. B. *paranthracis, S. xylosus, B. cereus* broth produces pink colour with Salkowski's reagent similar to standard IAA. *B. thrungiensi*s broth and blank does not form pink colour shown in Figure 1. The endophytic isolates of *Bacillus subtilis, Brevibacterium frigoritolerans, Halomonas hydrothermalis* and *K. rosea* form dark pink colour due to production of IAA [16] .

Table 1. Morphological Characters, Molecular identification of Endophytes with Accession number

S. No	Name of Isolates with Gene Bank Accession No	Morphological Characters of Isolates	
		Gram staining	Shape
1	*Bacillus thuringiensis* (OM349623)	Positive	Rod
2	*Bacillus paranthracis* (OK135976)	Positive	Rod
3	*Staphylococcus xylosus* (OM350007)	Positive	Round
4	*Bacillus cereus* (OK135977)	Positive	Rod

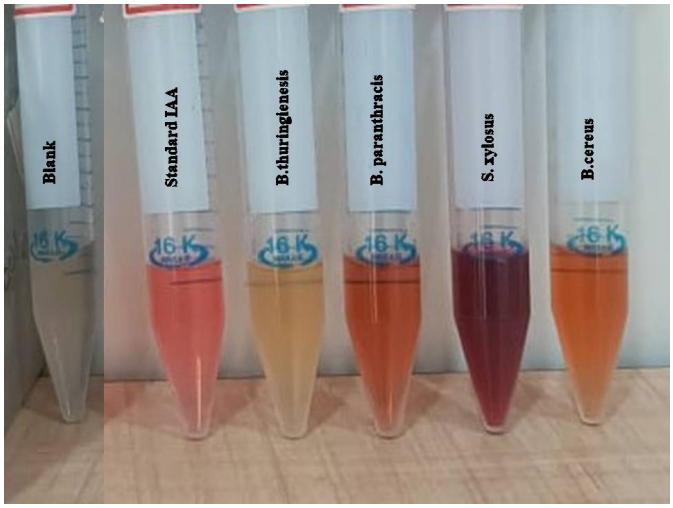

Fig 1. Salkowski's test; Standard IAA, *B. paranthracis, S. xylosus, B. cereus* shown pink colour formation, Blank, *B. thrungiensis* shown no pink colour

3.3 Optimization of YMD Media for IAA Production

3.3.1 Optimization of Carbon Source and Nitrogen Source

Sucrose, glucose, dextrose and mannitol as carbon sources were used in YMD media for maximum yield of IAA. Previous study reported that 1% lactose produced maximum IAA than fructose and sucrose for *Enterobacter cloacae* [17] and mannitol medium

produced maximum yield of IAA for Bacillus sp [14]. In this study, mannitol supplemented YMD produced maximum yield of IAA than glucose, dextrose and sucrose as shown in Table 2 . *B. thrungiensis* produced least amount of IAA such as 7.1 ± 0.113 µg/ml and 5.033 ± 0.065 µg/ml. *B. paranthracis* gave 30.67 ± 1.307 µg/ml and 32.67 ± 0.653 µg/ml, *S. xylosus* produced 41.67 ± 0.653 µg/ml and 42.67 ± 0.653µg/ml, *B. cereus* produced 21.5 ± 0.98 µg/ml and 22.67 ± 0.653 µg/ml at 30 and 120 minutes. *B. paranthracis* and *S. xylosus* produced maximum yield of IAA than *B. thrungiensis* and *B. cereus*.

It was reported that peptone medium produced maximum IAA yield than ammonium nitrate and sodium nitrate for Rhizobium sp [13]. This study stated that ammonium nitrate supplemented YMD produce maximum yield of IAA than peptone, potassium nitrate and sodium nitrate shown in Table 2. *B. thrungiensis* produced 4.67±0.653 µg/ml and 4.23 ± 0.0653µg/ml, *B. paranthracis* gave 25.9 ±0.113µg/ml and 30.73±0.261µg/ml, *S. xylosus* produced 40.67±0.653µg/ml and 43.6±0.113 µg/ml, *B. cereus* produced 19.6±0.599 µg/ml and 25.73±0.261µg/ml maximum yield of IAA at 30 and 120 minutes. Although ammonium nitrate was the best nitrogen source for optimal production of IAA, *B. thrungiensis* and *B. cereus* synthesis minimum IAA concentration compare to *B. paranthracis* and *S. xylosus*.

3.3.2 Optimization of pH and Temperature

An optimum pH and temperature favours the maximum production of phytohormones for endophytic bacteria of different species. It was reported that pH 7 and 8 induces the maximum production of IAA [11,13,14] for various endophytes. In this study, *B. thrungiensis* produced 5.97±0.0653 µg/ml and 6.27±0.0653 µg/ml, *B. paranthracis* gave 24.33±0.653 µg/ml and 32.57±0.523 µg/ml, *S. xylosus* produced 42.3±0.0653 µg/ml and 44.17±0.065 µg/ml, *B. cereus* produced 21.67±0.864 µg/ml and 23.73±0.173 µg/ml at 30 and 120 minutes at pH 8 than pH 6, 7 and 9 shown in Table 2. *B. thrungiensis* produced 3.93±0.131 µg/ml and 4.78±0.0864 µg/ml, *B. paranthracis* gave 26.67±0.653 µg/ml and 32.7±0.236 µg/ml, *S. xylosus* produced 43.03±0.327µg/ml and 43.4±0.299µg/ml, *B. cereus* produced 20.33±0.653µg/ml and 9.78±0.0864µg/ml at 30 and 120 minutes at 30°C shown in Table 2. Previous studies stated that temperature 30°C and 37°C adopted to produce maximum IAA for *Bacillus sp* and *Enterobacter cloacae* [14,17] .

3.3.3 Optimization of L-Tryptophan Concentration and Incubation Period

In this study, 2% tryptophan and 72hrs of incubation time favours the maximum production of IAA shown in Table 2. *B. thrungiensis* produced 5.47±0.285µg/ml and 4.3±0.226µg/ml, *B. paranthracis* produced 35.33±0.753µg/ml and 39.23±0.915µg/ml, *S. xylosus* produced 40.67±0.653µg/ml and 44.67±1.027µg/ml, *B. cereus* produced 22.27±0.879µg/ml and 21.67±1.307µg/ml at 30 and 120 minutes. Previous research reports stated that 2 to 4% tryptophan and 72 and 120 hours used for maximum IAA production by Rhizobium, Bacillus and Pseudomonas sp [2,17,18]. *B. thrungiensis* produced 6.27±0.346µg/ml and 6.13±0.173 µg/ml, *B. paranthracis* gives 35.2 ±0.707µg/ml and 36.33±0.653µg/ml, *S. xylosus* produced 42.13±0.173µg/ml and 43.57±0.581µg/ml, *B. cereus* produced 22.5±0.566 µg/ml and 24.37±0.535µg/ml as maximum IAA at 30 and 120 minutes respectively with 72 hours of incubation.

An optimized YMD media with mannitol, ammonium nitrate, 0.2% L-tryptophan with pH 8 and 30∘C temperature for 72 hours maintained for the maximum yield of IAA for the isolated endophytes. The optimum production of IAA was represented in Figure 2.

3.4 Extraction and Purification

The crude ethyl acetate extract was used for TLC analysis and this crude sample was dissipated in rotatory evaporator at 40°C under vacuum condition, diluted with 2 ml of methanol for HPLC analysis. Most of the studies stated that ethyl acetate was used to extract the sample for analysis [9,10,15] .

3.5 Thin Layer Chromatography

TLC report of *Bacillus paranthracis* and *Staphylococcus xylosus* showed pink colour spot compared to standard IAA at the Rf value with respect to the authentic IAA (0.92). Previous report confirmed the presence of IAA with Rf values ranges from 0.69 to 0.95 [2,3,9,10,18]. It was confirmed that *Bacillus paranthracis* and *Staphylococcus xylosus* producing potential IAA with retention factor 0.91 and 0.95 respectively whereas *Bacillus thuringienesis* and *Bacillus cereus* gave no significant results shown in Figure 3 and Table 3 .

Table 2. Effect of Optimized YMD Media on IAA (μg/ml) production

Parameters	Concentration of IAA in μg/ml at 30 and 120 minutes							
	B. thrungiensis		*B. paranthracis*		*S.xylosus*		*B.cereus*	
	30min	120min	30min	120min	30min	120min	30min	120min
Carbon Source	3.6±0.299	3.6±0.226	21.67±0.65	21.33±0.653	34 ±1.132	35.33±0.653	17±1.132	19.67±0.653
Sucrose Glu-	3.2±0.226	2.166±0.236	22±1.132	12.33±0.653	24.33±0.653	24±1.132	14±1.132	15±1.132
cose Dextrose	4.433±0.131	2.3±0.196	23.5±0.566	25±1.132	35.33±0.653	26.33±0.653	20.33±0.653	21.67±0.653
Mannitol	5.033±0.065	7.1±0.113	30.67±1.307	32.67±0.653	41.67±0.653	42.67±0.653	21.5±0.98	22.67±0.653
Nitrogen Source	1.67±0.653	1.6±0.408	12.67±0.653	21.5±0.113	20.33±0.653	22.33±0.653	8.67±0.653	20.33±0.653
Peptone Potas-	2.47±0.0653	3.53±0.131	13.33±0.653	15.67±0.653	32.67±1.307	35.9±1.764	8.23±0.0653	12.33±0.653
sium nitrate	4.67±0.653	4.23±0.0653	25.9±0.113	30.73±0.261	40.67±0.653	43.6±0.113	19.6±0.599	25.73±0.261
Ammonium nitrate Sodium nitrate	2.67±0.653	1.37±0.131	9.33±0.653	10.33±0.653	18.73±0.728	17.47±0.728	5.2±0.113	6.17±0.173
pH 6 7 8 9	3.17±0.0653	1.77±0.077	14.63±0.623	13.17±0.173	29.4±0.599	27.6±0.493	11.33±0.653	20±1.132
	3.83±0.0653	3.33±0.0653	18.6±0.599	22.63±0.065	29.7±0.588	15.1±0.113	7.33±0.653	9.1±0.113
	5.97±0.0653	6.27±0.0653	24.33±0.653	32.57±0.523	42.3±0.0653	44.17±0.065	21.67±0.864	23.73±0.173
	4.03±0.0653	2.27±0.0653	14.63±0.623	28.1±0.63	35.37±0.236	30.13±0.065	17.33±0.653	10.43±0.131
Temperature	3.47±0.0653	2.07±0.0653	9.27±0.0653	11.5 ±0.113	38.27±0.065	24.6±0.196	18.4±0.113	18.5±0.113
20°C 30°C 40°C	3.93±0.131	4.78±0.0864	26.67±0.653	32.77±0.236	43.03±0.327	43.4±0.299	20.33±0.653	9.78±0.0864
50°C	3.87±0.1312	4.73±0.0653	24.57±0.131	15.57±0.131	39.33±0.653	40.17±0.236	19.67±0.653	17.21±0.125
	2.4±0.0653	2.57 ±0.131	22.67±0.653	20.5 ±0.299	29.67±0.653	11.4 ±0.113	7.03±0.0653	20.1 ±0.113
L-Tryptophan	2.5 ±0.226	2.53±0.236 5.47	28.17±0.327	29.23±0.285	31.07±1.246	33.43±1.561	14.73±1.246	16.93±0.942
1% 2% 3% 4%	4.3 ±0.226	±0.285 4.27	35.33±0.753	39.23±0.915	40.67±0.653	44.67±1.027	22.27±0.879	21.67±1.307
	4.23 ±0.285	±0.346 4.27	33.13±0.261	33.93±0.942	41.3 ±0.898	42.07±1.051	20.8 ±0.588	21.57±0.581
	4.03±0.0653	±0.131	30.33±0.653	31.53±0.857	38.33±0.653	38.27±1.156	17.9 ±0.408	19.17±0.327
Incubation Time	2.7 ±0.299	2.57±0.261	11.5 ±0.297	11.77±0.173	15.5 ±0.299	16.37±0.457	6.47±0.428	7.1±0.113
24hrs 48hrs 72hrs	4.23 ±0.285	5.53±0.535	22.73±0.173	23.33±0.131	27.37±0.719	28.37±0.131	13.47±0.51 22.5±0.5667201.3361.045	
96hrs	6.27±0.346	6.13±0.173 3.43	35.2±0.707	36.33±0.653	42.13±0.173	43.57±0.581		24.37±0.535
	3.27±0.131	±0.236	17.97±1.637	18.5 ±0.566	34.53±0.457	32.97±1.076		16.67±1.116
Optimized YMD broth	4.133±0.131	5 ±0.226	82.46±0.072	83.56±0.457	93.12±0.261	94.77±0.285	24.4 ±0.113	25.067±0.581

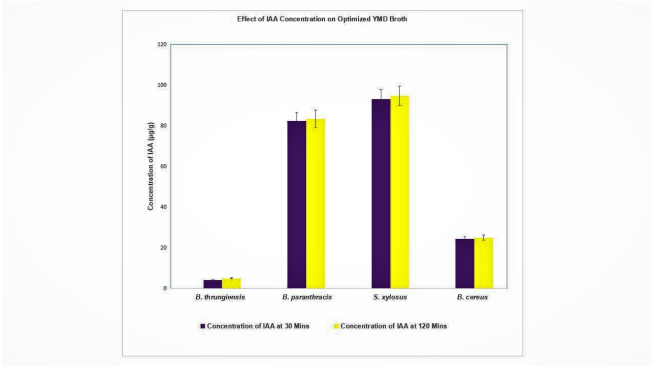

Fig 2. Effect of Optimized YMD Media on IAA (μg/ml) production

Table 3. TLC and HPLC value of IAA

Sample Name	Rf value	HPLC value (μg/ml)
Standard IAA	0.92	100
B. thrungiensis	-	-
B. paranthracis	0.91	48.63
S.xylosus	0.95	57.45
B.cereus	-	-

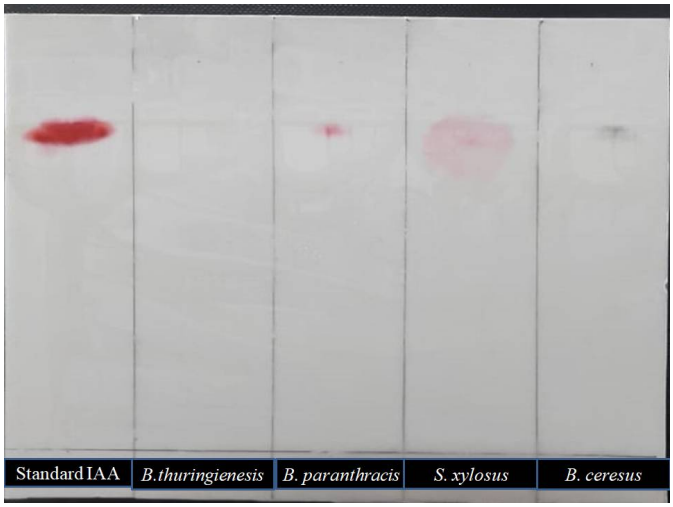

Fig 3. Thin Layer Chromatography Report for IAA

3.6 HPLC Analysis of IAA

Research reports stated that, HPLC retention time peak shown from 5 to 7 minutes for endophytes[18,19]. Here, the standard IAA of HPLC analysis formed retention time peak at 5.33 minutes. The peak of *Bacillus paranthracis* and *staphylococcus xylosus* were confirmed the presence of IAA with retention time of 5.053 and 5.58 minutes compared to standard shown in Table 3 and Figure 4.

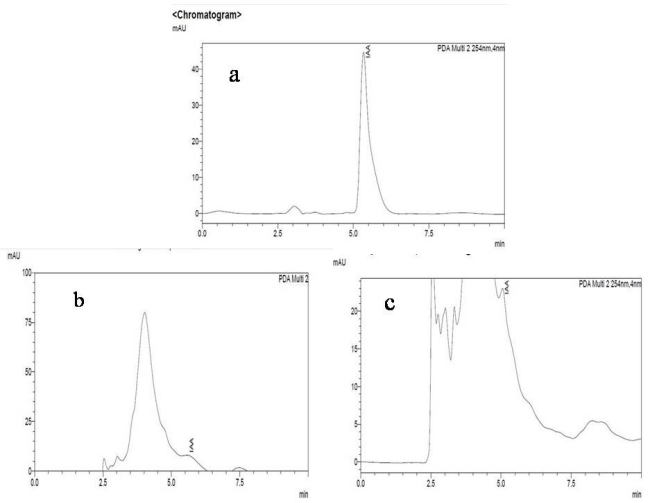

Fig 4. HPLC analysis; a) Standard IAA, b) Purified IAA sample of *B. paranthracis* and c) Purified IAA sample of *S xylosus*

4 Conclusion

Endophytic bacteria are the more likely hotspot for the synthesis of indole acetic acid and other supplement factors for plant development and promotion. In this work, *Bacillus thuringiensis*, B*acillus paranthracis, Staphylococcus xylosus* and *Bacillus cereus* are gram positive endophytic bacteria isolated from leaves of *Kalanchoe pinnata* (Lam.) subjected for the production of IAA using Yeast Maltose Dextrose media. The optimization of media results in the maximum production of IAA for various endophytes. Though optimized media used for IAA production of all four endophytes, *B. paranthracis* and *S. xylosus* produce potential amount of IAA but there was no influence on *B. thrungiensis* and *B.cereus*. The characterization of IAA with Salkowski's reagent produces pink colour for *Agrobacterium sp, Acinetobacter sp, Bacillus sp* and *Pseudomonas sp* as per research report. In this study, *B. paranthracis*, *S. xylosus* and *B.cereus* forms pink colour with Salkowski's reagent. Previous study reports revealed an identical Rf values for standard IAA and an endophytic extracts. TLC report showed that *Bacillus paranthracis* and *Staphylococcus xylosus* were producing potential IAA with retention factor 0.91 and 0.95. The extract of *Bacillus paranthracis* and *Staphylococcus xylosus* of HPLC analysis is shown to produce 48.63μg/ml and 57.45μg/ml with retention time of 5.053 minutes and 5.58 minutes compare to standard retention time, but there was no significant IAA peak has shown for *B. thrungiensis* and *B. cereus* in this study. This study concludes that such as *Bacillus paranthracis* and *Staphylococcus xylosus* isolated from leaves of *Kalanchoe pinnata* (Lam.) have efficiency to produce IAA similar to its host plant but *B. thrungiensis* and *B.cereus* did not produce IAA since IAA production by endo bacteria varies greatly between species and strains within the same species. *Bacillus paranthracis* and *Staphylococcus xylosus* endophytes can be used as effective bioinoculants in the Agri-field to make chemical free use for enhanced yields.

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