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SCREENING OF ENDOPHYTIC ACTINOMYCETES FOR CELLULOSE DEGRADATION

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Abstract:

Actinomycetes or Actinobacteria are Gram positive, filamentous, spore forming a high GC containing (60-75% in their DNA) prokaryotes. Endophytic Actinomycetes are the microbes living in the inner part of plant tissues and important for production of the bioactive molecules. Much of the work on these group were restricted on antibiotic potentiality. In the present study an exploration was carried out with the available six endophytic isolates (from Dept of Biotechnology, Atmiya University Rajkot) for cellulase synthesis by primary Screening and secondary screening. Three types cellulase activity (Endoglucanase-0.368IU at 72h, FPase-0.06IU at 96h and cellobiase-0.321 IU at 96 h) of incubation period were found to be recorded by employing the strain *Nocardopsis alba* EA23. Important morphological, biochemical & physiological identification at preliminary level found to reveal both branched and un branched type of actinomycetes whose genus and species identification yet under progress.

Keywords: endophyte, actinomycetes, endoglucanase, cellobiase, FPase

Introduction:

Actinomycetes an unique group of microorganisms which lies between the true Bacteria and fungi. (Duddu and Guntuku, 2015). They are responsible for an impressive number of secondary metabolites including enzymes, antibiotics, immunomodulators, plant growth promoting substance, vitamins, antioxidants and so on a huge natural repository of about half of the discovered bioactive secondary metabolites (Alharbi et al., 2012). Being a widely distributed group in soil and in myriad harsh extreme physiological niches (pH, temp., salinity, heavy metals, radioactive zones etc) their applicability is so profoundly established in the research and industrial world. Besides the dominant genus *Streptomyces*, other non – streptomyces actinobacteria are also there covering a huge range of taxa (Bouizgarne & Ait Ben Aouamar, 2014). Endophytic actinomycetes generally resides in part or whole inside the plants (Snipes et al., 2007). They are a good source of biocontrol agents (Mohan and Rajamanickam, 2018), industrially important bioactive compounds such as phytohormones, antibiotics, antiprotozoal and antitumor substances, enzymes (protease, laccase, lipase, amylase etc) and recently found to be recorded its lignocelluloses degrading potentiality (Robi et al., 2019). Cellulolysis is processed by cellulase enzyme (Gupta et al,



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2012), and specificity Endoglucanases, Exoglycanases, beta glucosidases, Cellobiohydrolases (Pulgar and Saadeddin,2014).Endoglucanase is generally responsible for the cleavage of the glycosidic bond along the cellulose chains (Gupta et al.,2012). Cellulase being an important biocatalyst used widely for in paper, pulp, biorefineries etc. Microbial sources besides bacteria, fungi, , yeast, actinomycetes also play an important role in lignocellulose degradation, useful in generating varied fermented sugars from cellulose degradation and implied for the growth and in worldwide industrial applications. Endophytes also plays an important role for Cellulose degradation, as cellulose is an polymer with wide Applications (Duddu and Guntuku, 2015).

In the present context, an attempt was made to explore the cellulose degrading potentiality of the already isolated endophytic actinomycetes by primary and secondary screening. The potent strains were also attempted to undergone preliminary characterization for tentative genus identification.

Materials and Methods:

Strains selection

All total six pure endophytic actinomycetes strains *Nocardiosis alba* EA23 (Pre identified taxonomically), EA13, EA30, EA12, EA35, EA14 obtained from the available repository of Department of Biotechnology, Atmiya University Rajkot, were sub cultured on starch casein agar and labelled as per the given codes. All the plates were incubated at 35°C for 16 days and preserved for future studies at 4°C respectively (Singh and Dubey, 2015)

Qualitative screening of selected strains by rapid plate assay (CMCase)

Rapid plate assay was carried out of the six selected strains in a dye based method employing modified Mandel's medium (in g/L: KH_2PO_4 ,1.5; $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$,2.5; $(\text{NH}_4)_2\text{SO}_4$,1.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,0.3; CaCl_2 ,0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$,0.005; MnSO_4 , 0.0016; ZnCl_2 ,0.0017; and CoCl_2 , 0.002; pH 7.0), supplemented with CMC (1%) as a sole carbon source and 0.1% Congo red (15min).After incubation for a week at 35°C, followed by destaining with 1M NaCl for 20 min, catalytic zone was recorded as the index of Relative Enzyme Activity (I_{CMC}).assay (Liang et al.,2014).



Broth culture assay and extraction of crude enzyme

Selected strains EA23, EA13, EA30, EA12, EA35, EA14 chosen from qualitative assay had undergone quantitative assay employing spore suspensions (0.01% Tween 80) of 7 days old SCA culture slants in CMC (Himedia) broth at pH 7.0 & incubated at 35°C, 100 rpm (Shaker Genie) (Aly et al, 2011). The flasks were incubated in orbital shaker incubator (Genei) at 35°C at 120 rpm for a week. At an interval of every 24 hr approx. 5-10 ml of spent broth was withdrawn aseptically and followed by centrifugation at 10,000 rpm in cooling centrifuge (Eppendorf) for 10 min, the supernatant was collected to be used for enzyme assay and the pellet was discarded (Aly et al., 2011)

Quantitative assay of FPase and Endoglucanase

Whatman filter paper strip and 2% Carboxymethyl Cellulose CMC were used as substrates for both the assays respectively. The rest of the process was followed as per the standard assay protocol by Ghosh et al. (1981). The absorbance was recorded at 540 nm in a UV-Visible spectrophotometer (Shimadzu, UV-1800). A glucose standard curve was prepared for measuring liberated glucose concentration and enzyme activity as per standard formula Ghosh et al. (1981). One unit of Filter Paper assay is based on International Unit (IU). 1 IU corresponds to $1 \mu\text{mol min}^{-1}$ of substrate (filter paper) converted. For enzyme unit calculation a linear graph of glucose was constructed using the absolute amount of glucose (0.5 mg/ml) plotted against 540 nm.

$$\text{FPU activity} = \frac{0.37}{\text{Enzyme concentration to release 2.0mg glucose}} \text{units ml}^{-1}$$

One Unit of CMCase is based on International Unit (IU). 1 IU corresponds to $1 \mu\text{mol min}^{-1}$ of liberated hydrolysis product i.e. glucose

$$\text{CMC activity} = \frac{0.185}{\text{Enzyme concentration to release 0.5 mg glucose}} \text{units ml}^{-1}$$

Quantitative Assay of Cellobiase:

For quantitative assay of exocellulase /cellobiase, 15 ml cellobiose was added in 0.05 M citrate buffer (pH 4.8) with 1 ml of enzyme, diluted in citrate buffer to a small test tube and at



least two dilutions were made of each enzyme sample investigated. The assay was performed by the standard procedure.(Ghose, 1987).One Unit of Cellobiose is based on the International Unit (IU), which stands as 1 IU corresponds to $1\mu\text{mol min}^{-1}$ of substrate being converted. For enzyme unit calculation glucose concentrations (mg ml^{-1}) from standard curve determined and according to the following formula enzyme activity calculated.

$$\text{Cellobiase activity} = \frac{0.0926}{\text{Enzyme concentration to release 1mg of glucose}} \text{units ml}^{-1}$$

Morphological identification of selected endophytes

Colony morphology, important microscopic features namely Gram staining (Gram,1884), slide culture (Wijedasa and Liyanapathirana,2012), cover slip culture (Jeyasekaran,2016) for studying mycelial branching and sporulation pattern of the potent strains (EA12, EA30,EA35)were carried out and compared with Bergey's Manual of Systematic Bacteriology,(Goodfellow and O'Donnell, 1989).*Nocardiopsis alba* EA23 with complete taxonomic characterization priorly done (previous research) while, strainsEA13, EA14,EA30 undergone morphological studies in prior study by researcher. .

Biochemical Characterization of selected endophytes

Major biochemical tests such as Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Hydrogen Production, reduction of nitrate , Urea hydrolysis, were performed with the isolates (EA 12 ,EA 30 & EA 35) and recorded as per the standard methods prescribed in the Bergey's Manual of Systematic Bacteriology and described by (Shirling and Gottlieb,1966). Rest of the isolates were priorly identified by biochemical identification tests.

Physicochemical Characterization of selected isolates

Three physical physicochemical parameters namely temperature, pH and percentage of NaCl were studied on growth of selected strains *Nocardiopsis alba* EA23, EA12,EA13, EA14, EA30& EA35 respectively as per the standard protocol of Shirling and Gottlieb (1966).For studying effect of temperature Starch casein (HI media) broth was prepared, and poured in sterilized (121°C for 10 min) tubes. The tubes were cooled to room temperature and the



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selected endophytic cultures were inoculated into them. Tubes were incubated in a range of temperatures from 20°C- 50°C with an increment of 10°C for 5 days. In the similar way, for checking effect of pH on growth of the strains, sterilized starch casein broth containing tubes having different pH in a range from 2-8 with an increment of 2.0 (pH adjusted with 0.1N NaOH and 0.1N HCl) were inoculated with the strains and kept for incubation at 37°C for 5 days. For assessing growth change by the influence of NaCl percentage change sterilized starch casein broth tubes priorly adjusted with a range of NaCl percentage from 1%, 3%, 5%, 7%, 9% were inoculated with the selected cultures kept for incubation at 37°C for 5 days. After 5 days in all the cases the tubes were examined for growth pattern recording optical density (OD) against 480nm in Spectro. (Shimadzu, UV-1800).

Results:

Strain properties:

an already prior characterized endophyte *Nocardiosis alba* EA 23 and other endophytic actinomycetes EA13, EA30, EA12, EA35, EA14 after 16 days incubation had shown in Starch casein agar a characteristic compact, leathery, dry, powdery, punctiform, small to medium, raised elevation, regular to irregular margin, brownish, whitish, greyish, opaque colonies. A crateriform appearance was also noticed on the surface of colony of EA 12. Fig.4.1. has shown the typical colony morphology and aerial mycelia colour in SCA by one of the representative actinomycetes *Nocardiosis alba* EA 23 & EA 13 respectively.



Nocardiosis alba EA 23



Endophytic strain EA13

Fig 4.1. Colonies of representative endophytic actinomycetes on starch casein Agar



Selection of potent endophytes by rapid plate assay of CMCCase:

In the present study, the initiation in the increase in intensity of zone of hydrolysis (in cm) by CMCCase had started from day 7 (Table 4.2) by all the tested strains and reached its peak at Day 16. The significant activities (2.2 cm, 2.7 cm & 2.0 cm) had been exhibited by the strains *Nocardopsis alba* EA 23, EA13 & EA12 respectively. Moderate activity was exhibited by EA30 (1.9cm) and EA14 (1.8 cm) respectively. Least zone of catalysis (0.6cm) was recorded by EA 35. At the same time there were no significant activity observed prior to day 6 or 7 by many of the strains. Most significant increase in hydrolytic zone was observed by EA 13 and *Nocardopsis alba* EA 23 from day 13 onwards till day 16 in arrange from 1.8-2.7 cm.

Table 4.2. Selection of potential endophytic actinomycetes through primary screening of CMCCase in CMC-Na-Congo red media

Strain code	Zone of hydrolysis by CMCCase (cm) at every 24 hr of incubation period															
	D ay 1	D ay 2	D ay 3	D ay 4	D ay 5	D ay 6	D ay 7	D ay 8	D ay 9	D ay 10	D ay 11	D ay 12	D ay 13	D ay 14	D ay 15	D ay 16
<i>Nocardopsis alba</i> EA 23	-	0.1	0.2	0.3	0.5	0.7	0.9	1.1	1.2	1.3	1.5	1.7	1.8	1.9	2.2	2.2
EA 13	-	0.2	0.3	0.5	0.6	0.8	1.1	1.1	1.2	1.5	1.7	1.9	2.2	2.2	2.5	2.7
EA 30	-	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.1	1.2	1.3	1.4	1.5	1.6	1.8	1.9
EA 12	-	0.1	0.2	0.4	0.5	0.6	0.7	0.8	0.9	1.1	1.2	1.3	1.5	1.7	1.9	2.0
EA 35	-	0.2	0.4	0.5	0.6	0.8	1.1	1.3	1.5	1.7	1.8	1.7	1.5	1.3	0.8	0.6

EA 14	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.2	1.3	1.4	1.6	1.8
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FPase, endoglucanase and cellobiase assay:

The selected endophytic strains *Nocardiopsis alba* EA23, EA12,EA13,EA30 and EA14 had shown FPase activity 0.05IU/ml at 120 h, 0.056IU/ml at 120 h, 0.058 IU/ml at 96 h, 0.06 IU at 96 h and 0.056 IU/ml at 96 h of incubation period respectively (Fig.4.3).While, all the strains had shown decrease in activity 6th day i.e.144 h onwards and at7th day i.e.at 168 h of incubation maximum downfall was noticed (Fig.4.3.a). An optimum endoglucanase activity exhibited by *Nocardiopsis alba* EA23, EA12,EA13,EA30 and EA14 were 0.228 IU/ml,0.266 IU/ml,0.213 IU/ml,0.2 IU/ml,0.257 IU/mlat144h,72h, 120 h, 120 h, 96 h of incubation period respectively (Fig.4.3.a).In the similar manner, an optimum Cellobiase activity exhibited by *Nocardiopsis alba* EA23, EA12,EA13,EA30 and EA14 were found to be recorded 0.189 IU/ml, 0.266 IU/ml, 0.213 IU/ml, 0.2 IU/ml and 0.257 IU/ml at 96h,72h,120h, 120 h and ,96 h of incubation period respectively (Fig.4.3.b). For both the enzymes (endoglucanase and cellobiase) activities the decrease in activity was exhibited from day seven i.e.168 h of incubation period.A standard glucose curve employed in the study is represented in Fig.4.3.c.

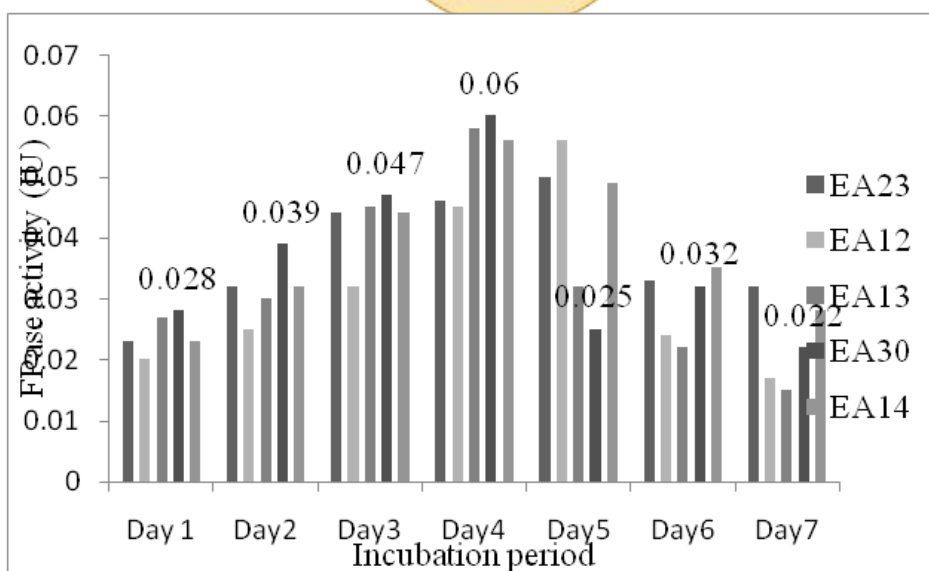


Fig 4.3: FPase activity shown by different actinomycetes isolates



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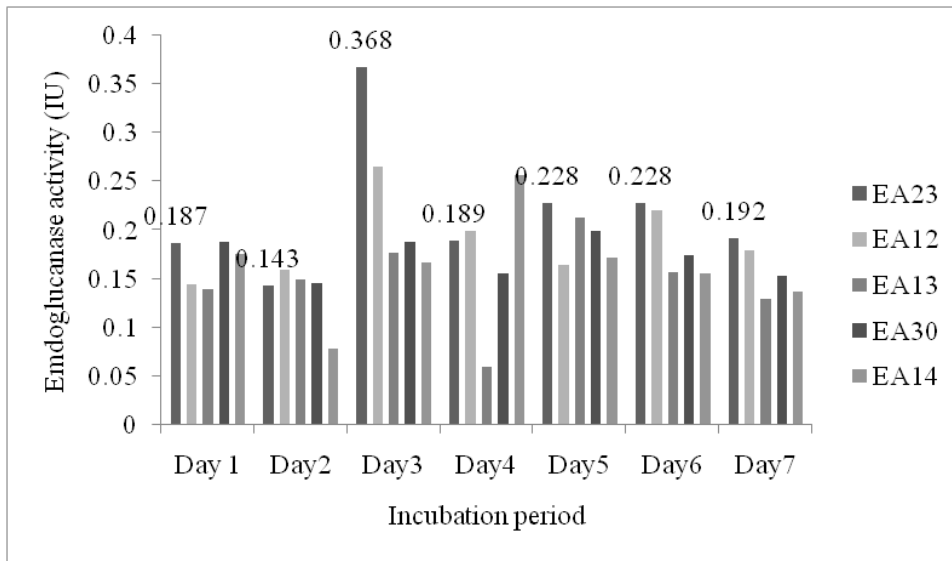


Fig 4.3.a. Cmcase/endoglucanase activity shown by different actinomycetes isolates

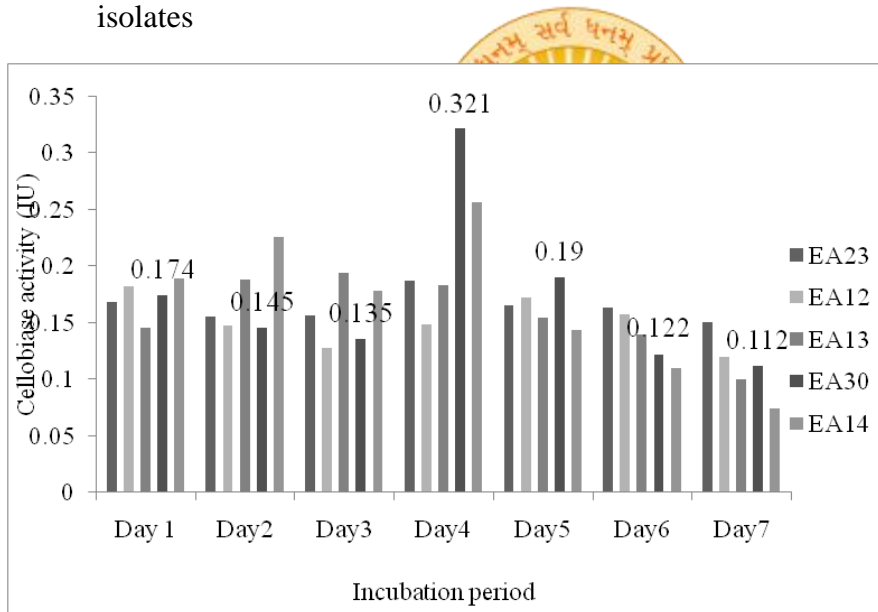
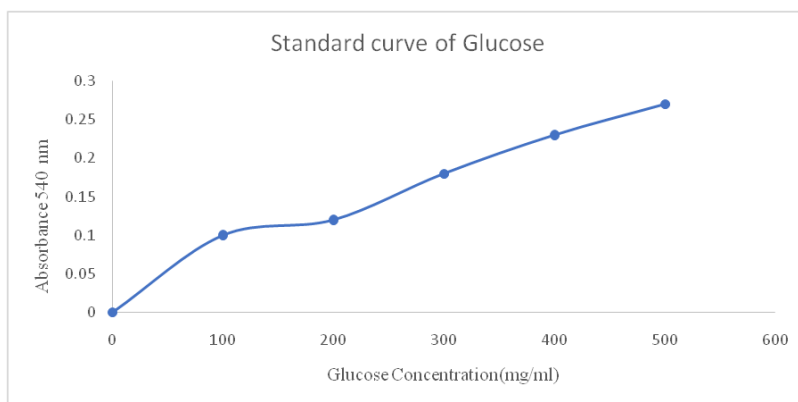


Fig 4.3.b. Cellobiase activity shown by different actinomycetes isolates.

**Fig 4.3.c.** Standard glucose curve

Preliminary identification properties of potential endophytic actinomycetes

The present study had exhibited in Table 4.4 the typical colony morphology of which ranged from small to medium size ; fairly round in shape ;regular to irregular margin; white to off white pigmentation of aerial mycelia; dry powdery, rough texture and consistency; opaque and raised elevation. Fig.4.4. has exhibited the Gram staining property of the strains whose representative images (Fig.4.4) depicts the branching pattern, sporophore, coilingsu, position of spores on sporophore etc. Substrate mycelia was absent with spores & absence of complex branching pattern, whereas aerial mycelia containing spores and simple mycelia structures.

Table 4.4. Colony Characteristics of selected endophytic actinomycetes

Colony Type	EA12	EA30	EA35
Size	Small	Medium	Small
Shape	Round	Round	Round
Margin	Irregular	Irregular	Regular
Pigment	Off-White	Off-White	White
Texture	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque



Consistency	Powdery	Powdery	Dry
Elevation	Raised	Raised	Raised

Table 4.4.1 exhibited the biochemical properties of the selected strains where a range of tests reduction of nitrate, starch hydrolysis and gelatine liquefaction found to be positive for all the strains. Except EA 30 rest had shown negative result for melanin pigmentation. Casein hydrolysis was shown positive by EA12 and EA30, negative by EA35; while all strains had exhibited negative result. For production of H₂S and urea hydrolysis Physiological identifications are exhibited by Table 4.4.2,4.4.3 and 4.4.4 showing influence of change in temperature , pH and salinity on growth of the selected strains. Table 4.4.2 reveals 30°C as an optimum temperature for growth of all tested strains, while least or moderate growth pattern was observed at 20°C & 50°C followed by optimum growth at 40°C respectively. The growth in form of turbidity was well intensified for all the strains more or less at pH 4.0,36.0 and 8.0; while least to moderate turbidity of growth was observed at pH 2.0 for all the strains tested. At pH 10 except EA 12 all strains had shown good growth in form of intense turbidity. The change in NaCl percentage from 3 to 7 had shown good result for almost all the selected strains, while at 1.0 % ,3.0% and 9.0% the result was little random.Only EA 13 and EA 30 had shown moderate growth at 1.0 % salinity, whereas except EA 30, EA 12,EA35,EA13 and EA 14 had exhibited moderate to good growth at 9.0% salinity .

Table 4.4.1.Biochemical Characteristics of potent endophytes

TESTS	EA30	EA12	EA35
Melanin production	+	-	-
Nitrate reduction	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	+	+	-



Gelatin hydrolysis	+	+	+
H₂S production	-	-	-
Urea hydrolysis	-	-	-

('+' indicates Positive result of the test ; '-' indicates Negative result of the test)

Table 4.4.2. Effect of temperature on growth of potent endophytes

Temp. (°C)	<i>Nocardiosis alba</i> EA23	EA13	EA30	EA12	EA35	EA14
20	++	++	++	++	+	+
30	++++	+++	+++	+++	++++	+++
40	+++	++++	++++	++++	+++	+++
50	++	++	++	++	++	++

Note: (++++Excellent , +++Good , ++Moderate ,+Poor)

Table 4.4.3. Effect of pH on growth of potent endophytes

pH	<i>Nocardiosis alba</i> EA23	EA13	EA30	EA12	EA35	EA14
2	++	++	++	+	+	+
4	++++	+++	+++	+++	++++	+++
6	++++	++++	++++	++++	+++	+++
8	++++	++++	+++	++++	++++	+++
10	+++	+++	+++	++	+++	+++

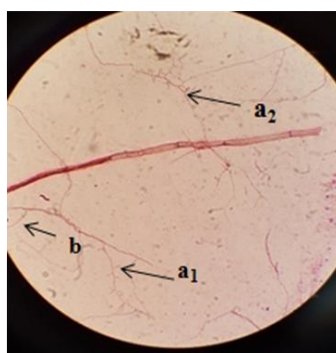
Note: (++++Excellent , +++Good , ++Moderate ,+Poor)

Table 4.4.4. Effect of NaCl percentage on growth of potent endophytes

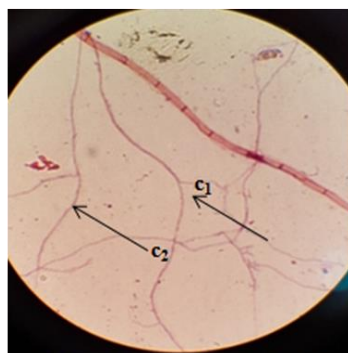
NaCl (%)	<i>Nocardiosis alba</i> EA23	EA13	EA30	EA12	EA35	EA14
1	+	++	++	+	+	+
3	+++	++	++	+++	+++	+++
5	++++	++++	++++	++++	+++	+++

7	++++	++++	+++	++++	++++	+++
9	++	+++	+	++	++	+++

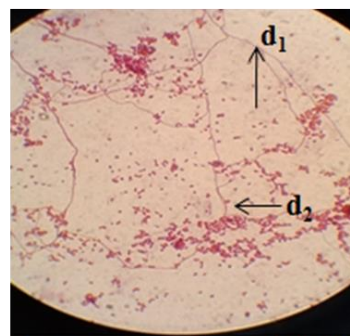
Note: (++++Excellent , +++Good , ++Moderate ,+Poor



Aerial mycelia of EA 13



Substrate mycelia of EA 23



Gram staining view of EA 12

Fig.4.4. Gram staining view of aerial (a₁,a₂- spore positions ; b- simple branching) and substrate mycelia (c₁-simple branching c₂-no spore) of endophytic Actinomycetes (EA13,23) and EA 12 (d₁- branching pattern; d₂-intercalary spore)

5. Discussions:

Endophytic Actinomycetes has capacity to produce secondary metabolites and also an best source for novel compounds (Segaran et al., 2017) and specially few good reports (Duddu and Guntuku,2015) for cellulose degradation are also there. In the present study out of selected actinomycete *Nocardopsis alba* EA23 a well characterized strain (NCBI GenBank Accession No. kx832935) and colony features of rest of the strains (EA13,EA14) had identified as reported by Kumar and Jadeja, (2016). They all were found to exhibit good cellulase activity (Endoglucanase, Cellobiase and FPase). Report by Kumar and Jadeja, (2016) had shown the information of niche of most of these isolates from medicinal plant parts (leaf and root) which also a reason to conduct this study to check diverse metabolic functionality. Table 4.2 also reveals that, prior to Day 6 or 7 many of the strains did not turned up to exhibit a significant increase in development of hydrolytic zone of CMCase and that also can be assumed as the property of slow growing nature and a delayed attainment of stationery or sporulation phase due to which the secondary metabolite like enzyme production got delayed. EA35 had shown continuous promising result in comparison to



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EA13, and *Nocardioopsis alba* EA 23. Report by Passari et al. (2017) had shown 29.6 % cellulase producers as entophytic actinomycetes with maximum activity recorded as 75.2 IU/ml. The difference in optimum quantitative enzyme activity may be due to slow growth period and delayed attainment of stationary phase or the phase of secondary metabolite production like enzymes (Bibb, 2005). *Nocardioopsis alba* EA 23 and other selected strains except EA 30 had shown less FPase activity and in delayed optimum incubation period. While EA 30 had exhibited lesser endoglucanase / CMCase activity in delayed optimum incubation period than *Nocardioopsis alba* EA 23 showing the highest activity at early hours of optimum incubation (72 h) period. Enzyme activity exhibited by EA 12, EA 13 and EA14 strains were little in concurrence with the earlier strain EA23 but not the same. Similarly, in the present study, strain EA 30 had exhibited significant cellobiase activity, at an early 96 h of incubation period which was in comparison to *Nocardioopsis alba* EA 23 and other strains much higher and optimum incubation period also attained early. *Nocardioopsis* sp. and many more genera like *Micromonospora* sp., *Micrococcus* sp. and many endophytic actinobacteria isolated from native herbaceous plants of Korea (Kim et al., 2011) had been thoroughly studied for many bioactive molecule synthesizing ability, out of which strains *Micromonospora* (HW05-01, HW05-02, HW05-05 and HW05-11) and few streptomycetes had shown cellulase and other hydrolytic enzyme activities. Endophytes are always well known for antibiotic and other biocontrol agents production (Kuzniar et al., 2019) more than enzymes like cellulase/ FPase/ cellobiase in specific but still there are reports and quite a better avenue of research ahead to study these group in this aspect may be more in advanced way.

Phenotypic characterization is always regarded as basic foundation besides the advanced system of polyphasic taxonomy (Qinuan et al., 2016). Out of the selected tested strains most of all (EA 13, EA 14) are priorly identified by Kumar and Jadeja, (2016) and one strain *Nocardioopsis alba* EA 23 already taxonomically characterized (NCBI GenBank Accession No.-kx832935). Remaining strains EA 12, EA 30 and EA 35 had exhibited similar morphological, biochemical observations as reported by Kumar and Jadeja, (2016). Slide culture and coverslip culture study of representative strains (EA 13 and *Nocardioopsis alba* EA 23 in Fig.4.4. are reported same as reported by Qinuan et al. (2016). While the physiological characterization had revealed the optimum growth of the selected endophytes at



mesophilic temperature, alkaline pH and moderate to little high salinity. Similar studies on revelation of biologically important endophytic actinomycetes taxa was also stated by Passari et al. (2017); Qin et al. (2009).

Conclusions:

The potent strains *Nocardiosis alba* EA 23 and EA 35 (endoglucanase), EA 30 (cellobiase and FPase) was selected followed by primary and secondary screening for the three type of cellulase activity. The optimum incubation period was not constant for all the enzymes and 72 hr and 120h were found as optimum incubation period for the maximum enzyme activities represented in the study. The best screened strains of endophytic actinomycetes along with others showing less activity successfully undergone preliminary level identification by morphological and biochemical studies. But yet genus identification under progress by molecular and taxonomic characterization study. This work is a preliminary exploration of searching potential endophytic actinomycetes for cellulose degradation as an out of the box study pertaining to enzymology research with endophytes.

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