



ISOLATION AND CHARACTERIZATION OF BETULIN TOLERANT MICROBIAL ISOLATES

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ABSTRACT

Betulin and betulinic acid are naturally occurring lupane type pentacyclic triterpenes is widely isolated from about 25 plants around the globe. On commercial scale betulin, is specifically collected from the bark of *Betula pendula* and *Betula alba* L. The aim of our current work was to isolate and characterize betulin tolerant strains which are potentially active for the bioconversion of betulin. The experiments were aimed for screening and isolating microorganisms from a natural source in addition from culture collection centre for utilizing betulin as substrate. Three hundred fifty isolates were screened for their tolerance to betulin in selective medium containing up to 0.003mg/ml of betulin. Isolates were tested based on their morphological, cultural characteristics, biochemical and molecular (16S rDNA gene sequence analysis) characterization. The growth (PCV=packed cell volume mg/ml) of betulin tolerant isolate was considered as bioconversion efficiency of selected isolate. Three indigenous betulin tolerant bacteria (KD109, KD 136 and KD 235) which were able to use betulin were isolated, and further characterized by biochemical methods. As a validation of betulin tolerance of isolate KD235, the fermented broth was extracted as a product and analyzed for the presence of betulinic acid by RP-HPLC which revealed similar results as available in previous literature. Present work represents a pioneer study showing prokaryotic system for betulin biotransformation to give betulinic acid as a significant compound for pharmacological applications having anti-cancer and anti-HIV activity.

Key-words: Betulin; Biotransformation; *Bacillus megaterium*; HPLC; pharmacological properties; Betulinic acid; microbial system.

INTRODUCTION

Since ancient times mankind have been using selected plants and their parts as crude medication for the curing certain disease. As the civilization progressed we started using the refined forms of the plant parts as fine powders and decoctions (Abubakar & Loh, 2016; Shaffer, 2005). Plants have numerous bioactive constituents as alkaloids, flavanoids, steroids, terpenes and many more in them that serves as effective drugs against death causing diseases like cancer, malaria and many more (Gupta, Sharma, & Rao, 2012; Jang, Kim, Lee, Inn, & Lee, 2015). Betulin and its derivatives have attracted more attention because it has wonderful pharmacological activities,

such as, antitumor, antiviral, and anti-inflammatory activities and many more (Alakurtti, Mäkelä, Koskimies, & Yli-Kauhaluoma, 2006). Researchers have shown that betulinic acid as a derivative of betulin is significantly cytotoxic against non-melanoma human tumor varieties. Because of its selective cytotoxicity against tumor cells and favourable therapeutic mode of action, betulinic acid is a very promising newer chemotherapeutic agent for the treatment of cancer and HIV infections (Aiken & Chen, 2005; Saxena et al., 2006). For commercial use through chemical synthesis, Betulin is used as precursor to Betulinic Acid but due to the specific reaction conditions, environmental safety and pollution issues it is not advisable. All the biocatalytic reactions can be carried out under certain safer, healthier, environmental friendly and economical conditions (Csuk, Schmuck, & Schäfer, 2006; Kim, Pezzuto, & Pisha, 1998; Pervaiz, Ahmad, Madni, Ahmad, & Khaliq, 2013). The biotransformation is more beneficial and appropriate key factor for obtaining bioactive molecules such as betulinic acid, betulone and other derivatives of betulin that's why biotransformation is advisable (Nielsen et al., 2014; Prasad, Girisham, & Reddy, 2010; Zhang, Cheng, Yu, Cordell, & Qiu, 2005). Betulinic acid (BA) is the vital molecules of our interest. Betulin (Fig 1a- 3β -lup-20(29)-ene-3, 28-diol) was one of the first natural products isolated in 1788 from the bark of the white birch, *Betula alba* (Csuk, 2014). Betulin from birch bark is a triterpene that can be extracted by different methods as organic solvents, thermal sublimation or supercritical carbon dioxide up to the yield of 30 % of total dry weight. A small amount of betulinic acid is often present during extraction of betulin (Csuk, Schmuck et al. 2006). Betulinic acid (Fig 1b) was immobilized on acrylamide matrix for phospholipase activity of snake venom from *Naja nigricollis*, shown a good immobilizing agent for some basic proteins identified such as c-cardiotoxin, indicated the usefulness of immobilized betulinic acid for exploring cellular targets (Tseng & Liu, 2004). Another study investigated the mechanisms responsible for the protection by betulin against cadmium induced cytotoxicity in human hepatoma cell lines. The protection of betulin against cadmium cytotoxicity was lesser effective in the Hep3B cells than in the HepG2. Cadmium can induce apoptosis via Fas-dependent and independent apoptosis pathways which shown protective effects against cadmium induced cytotoxicity (Oh, Choi, & Lim, 2006). Another group of researchers suggested the inhibition of SREBP pathway can be employed as a therapeutic strategy to treat metabolic diseases including type II diabetes and atherosclerosis, the study shown that betulin adversely effects the biosynthesis of fatty acids and cholesterol that can inhibit sterol regulatory element-binding proteins (SREBPs) pathway (Tang et al., 2011). Considering the abovementioned specialties of betulinic acid, the target of our work was to found out suitable isolate for the bioconversion of betulin to its derivatives either betulinic acid (BA) or else by suitable betulin utilizing bacteria, as most of the recent betulin transformations were done by fungi as biocatalyst (Chen, Liu, Zhang, He, & Fu, 2009; Feng et al., 2013; Mao, Feng, Bai, & Xu, 2012).

MATERIALS AND METHODS

Chemicals and Reagents

Standard betulin (98%) and betulinic acid (90% technical grade) was purchased from Sigma Aldrich and dissolved in dichloro methane (also known as Methylene Chloride) and methanol (HPLC grade) (1mg/ml) as a stock solution for our experiments. Acetonitrile, Methanol, Millique water (HPLC grade) was obtained from SRL Limited (Mumbai). Rest of the media components and chemicals used in the study were of analytical grades from Himedia, Merck and SRL Mumbai.

Collection, isolation, and acclimation of isolated consortium

The microbial consortium capable of growing under betulin stress condition were grown by long term enrichment culture in 250ml flask similarly as followed by other researchers for utilization and degradation of other molecule (Feng et al., 2013; Schwab, Fuchs, & Huang, 2013). Initially we exposed isolated microbes for a week at 0.1mg/ml betulin stress then as the resistant stable colonies were obtained after 6 weeks then we took these isolates for next generation with gradually higher betulin concentration. As the pure culture was established we identified them according to morphological, cultural and biochemical characteristics using “Bergey’s Manual of Determinative Bacteriology”.

Screening of suitable biocatalyst for betulin biotransformation

Different isolated cultures from betulin enriched medium were separately inoculated to 50 ml betulin screening media (BSM) containing 0.1mg betulin, 2.5 gm of dextrose, 0.25 gm yeast extract, 1.25 gm peptone, 1.25 gm of NaCl, 1.75gm of K₂HPO₄, 0.50 gm of beef extract, in 250 ml of distilled water, at 37°C, pH 7.0 for 48 hours. Further from this, 0.1 ml culture broth was inoculated to solid medium and cultivated for next 07 days for different isolates. The strains were isolated and maintained on self modified LB medium and PDA medium plates for bacteria and fungi respectively for further tests. Stock cultures of microbes were stored on slants of nutrient agar at 4°C. The culture media used for the culture are of typical microbiological substrates, containing organic nitrogen sources (peptones, yeast extracts, tryptone, beef extracts) a carbon source (glucose, maltose, glycerin) at a pH of 4.5 to 8.5, and the incubation temperature ranges from 25°C to 45°C. Cultures were maintained by transferring to fresh slants on every 15 days and only fresh cultures were used for biotransformation experiments.

Identification of the suitable biocatalyst

The morphology of different isolated strains were observed under a microscope (Labomed - Luxedo 4D, camera attached microscope). Gram’s staining was performed; the morphology of the cells and spore chain morphology was identified by spores’ staining technique. The pure cultures were grown on nutrient agar or potato dextrose medium and transferred to Luria-Bertoni, Mac-conkey agar medium, EMB agar medium, and Mannitol salt agar medium for differentiate and identify bacteria. The plates were incubated inverted at 28-37°C in the incubator and growth were observed on every 12 hrs intervals after inoculation for 05 days. The biochemical tests such as Nitrate reduction, Malonate Test, ONPG, Voges Prauskauer Test, and Catalase test were performed for the identification of our isolated strains specially, *Bacillus* spp.

Morphological and cultural characterization

Different isolated strains were grown on Luria-Bertani (LB) agar and some other self modified media were examined for their morphological and cultural characteristics, including cell shape, colonial appearance, endospore formation and pigmentation, after incubation at different pH (4.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) and at different temperatures (25, 30, 35, 40 and 45 °C). All tests were carried out by incubating the cultures at different combinations of condition using design of expert statistical tool.

Biochemical characterization

Variety of biochemical tests are suggested for identification of different groups of microbes. Different microbes carry distinguished characterization as per their group, genera and species i.e. based on Bergey’s Manual Of Determinative Bacteriology (Vos et al., 2011), Voges-Prauskauer

(VP), ONPG, Catalase, Citrate, Arginine utilization, Malonate are some of the key factors that we considered in our studies .

RESULTS AND DISCUSSION

Screening of efficient suitable strains for biotransformation of betulin

The present work deals with the microbial conversion of betulin to pharmaceutically more significant betulinic acid. Betulin has lower solubility and more toxicity than betulinic acid therefore it has been suggested to make derivatives of betulin to be used for pharmaceutical applications (Cao, Zhao, & Yan, 2007; Zhao & Yan, 2008). Here we investigated betulin tolerant isolates responsible for microbial conversion of betulin into more soluble derivative as betulinic acid (BA). Biochemically this bioconversion of betulin into its respective derivative occurred due to the redox activity of microorganisms. The fungi catalyzed biotransformation of betulin to betulone, betulinic acid and other derivatives (Bastos, Pimentel, de Jesus, & de Oliveira, 2007; Liu, Fu, & Chen, 2011; Mao et al., 2012) are previously reported. Based on previous studies this study focused on bacterial isolates and specially bacilli isolates, findings are summarized in Table 1 and 2 (Dubey, Jawed, & Haque, 2011; Vary, 1994). The 350 isolated microorganisms were screened for redox activity out of them 03 isolates (i.e. KD109, KD 136 and KD 235) were found positive and one from these isolates i.e. KD235 shown maximum growth corresponding to higher biocatalytic activity. The isolate KD235 was further grown in betulin containing (0.001 to 0.008 mg/ml) medium with various combination of cultural conditions at shake flask level for 168h. The results showed maximum growth of KD235 at pH 6.5, 30°C, and 0.003mg/ml betulin concentration in 84-86h (Fig 2). The fermented broth samples were extracted following some modifications in earlier reports (Liu et al., 2011). The extracted broth samples were filtered through 0.22µ syringe filters (Axiva, Germany) and analyzed by RP-HPLC (YL9112, South Korea). The HPLC system used throughout current study consists of Yong Ling's 9112 pump, YL9120 UV-Vis detector, ProntoSil C18 HQ105 H column (250mm X 4.6mm X 5mm). Samples were analyzed under following condition: the flow rate was set at 1.0 ml/min under room temperature, mobile phase was composed of acetonitrile-water 91:09 (v: v). The wavelength was set at 210 nm (Feng et al., 2013; Liu et al., 2011). The HPLC results were compared in figure 2 where betulin as substrate and shown peak at the retention time of 3.4 min (Fig 3a) and a new peak found at 12.8 minutes shown biotransformed product as betulinic acid (Fig 3b). This new peak was formed after utilization of betulin as substrate. The fractions of transformed product was collected and further validated by NMR analysis (Fig 4) which found similar results as indicated in previous studies.

Identification of strain KD235

The HPLC results shown encouraging results for filtrates' obtained by KD235 catalyzed fermentation. The biochemical characterization of selected isolate KD235 already grouped it as *Bacillus megaterium*, but further molecular characterization by isolating genomic DNA, PCR amplification of the purified products and 16S rDNA sequencing were outsourced by Elixir Biotechnologies, Ahmadabad (Gujarat) India. The molecular characterization were matched it with existing *Bacillus megaterium* strains (Fig 5). The 16S rDNA analysis of KD235 showed (99%) similarities to reference stains i.e., to *Bacillus megaterium* (GenBank NCBI reference no KR-261097).

CONCLUSIONS

From three decades, betulinic acid has been a promising new chemotherapeutic agent for the treatment of cancer and HIV infections. There are very few studies available on the bioprocess optimization studies for terpene like betulin and betulinic acid. The available literature represents

the extraction process from various plants and their parts of birch plant biology but not more work found on microbial transformation of betulin and betulinic acid. Morphological and biochemical characterization of three hundred fifty isolates were done, 03 out of them (KD109, KD 136 and KD 235), were screened for their higher growth in high betulin containing medium. The isolate KD235 shown maximum transformation of betulin by showing maximum growth at 0.003mg/ml betulin concentration. That's why KD235 was further characterized by molecular analysis which revealed 99% similarities to *Bacillus megaterium* as reference stains. Considering all finding in the current study isolate KD235 was named as *Bacillus megaterium* KD235. Further scale up, biotransformation and downstream studies are going on.

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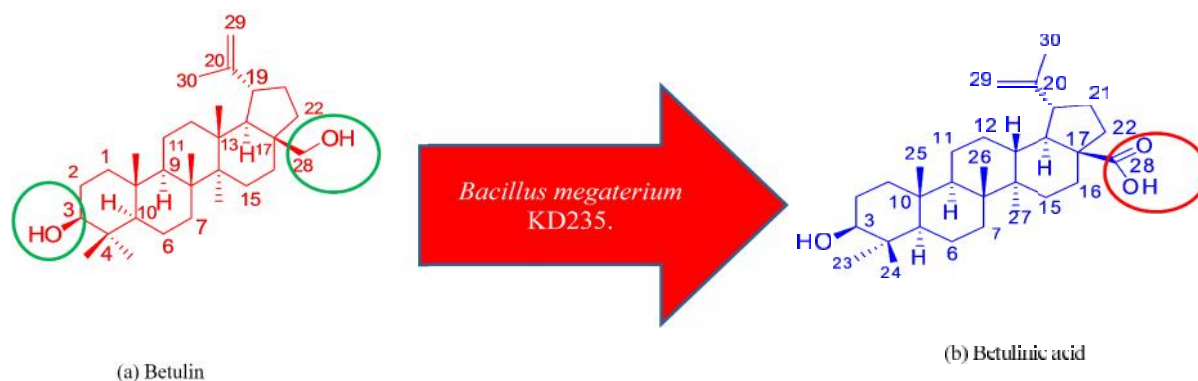


Figure-1 Molecular structure of (a) betulin (b) betulinic acid

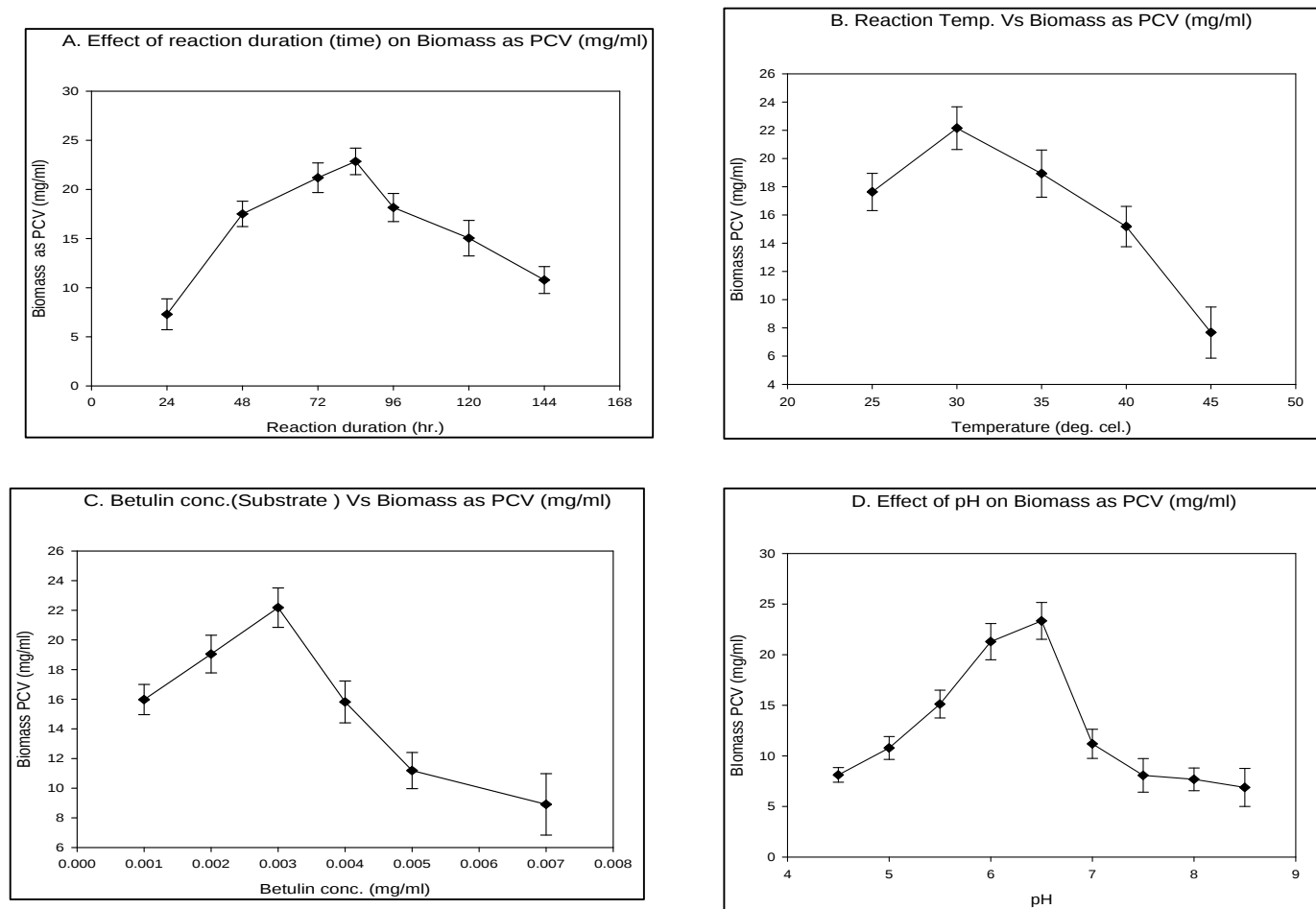


Figure-2 Effects of various process parameters on biomass generation as PCV (mg/ml)
 (a) Effect of reaction duration (time) on Biomass (as PCV mg/ml)
 (b) Reaction Temp. Vs Biomass (as PCV mg/ml)
 (c) Betulin conc. (Substrate) Vs Biomass (as PCV mg/ml)
 (d) Effect of pH on Betulin biomass as PCV (mg/ml)

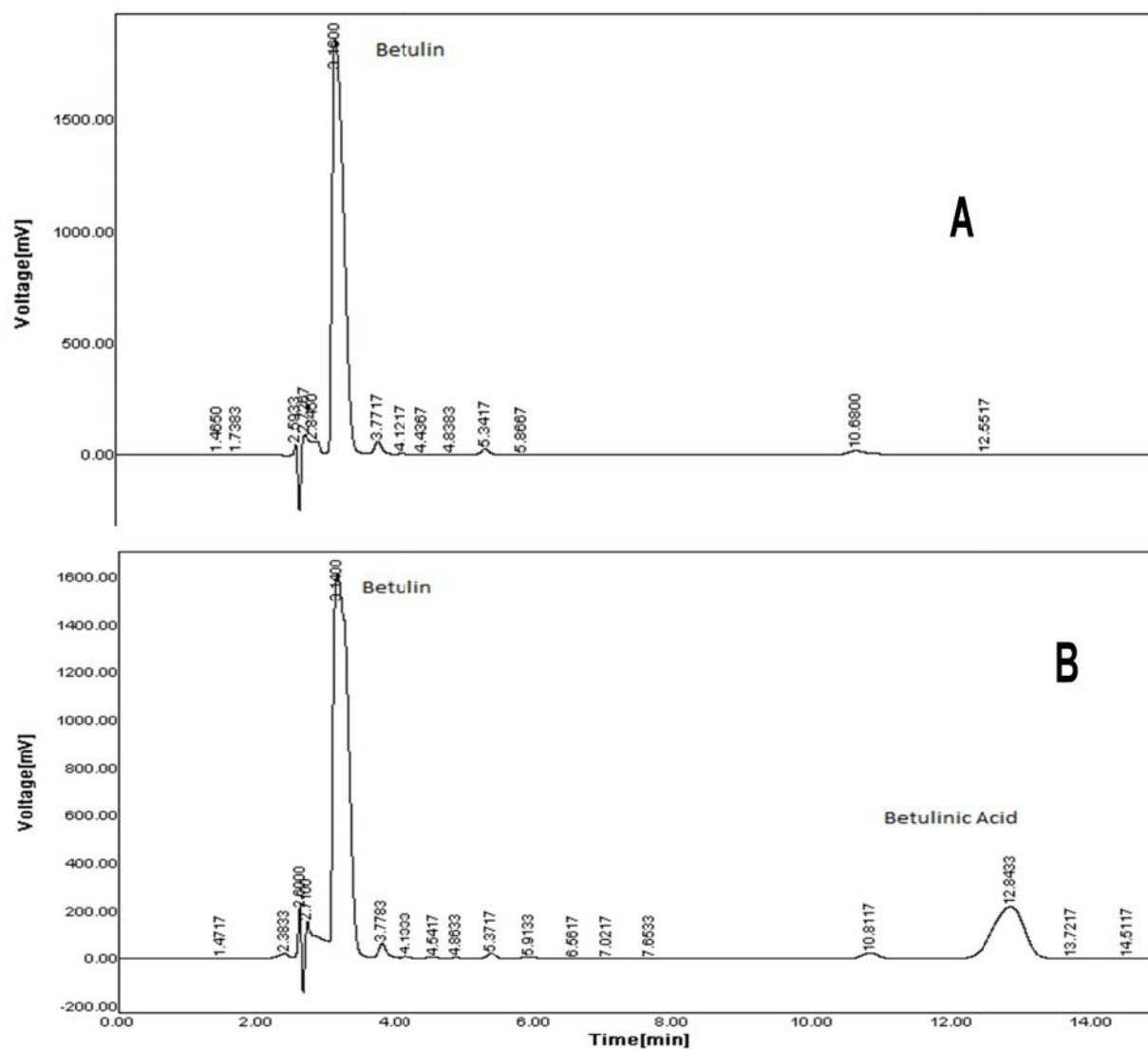


Figure-3 RP-HPLC analysis the peaks are showing (a) betulin (b) betulinic acid

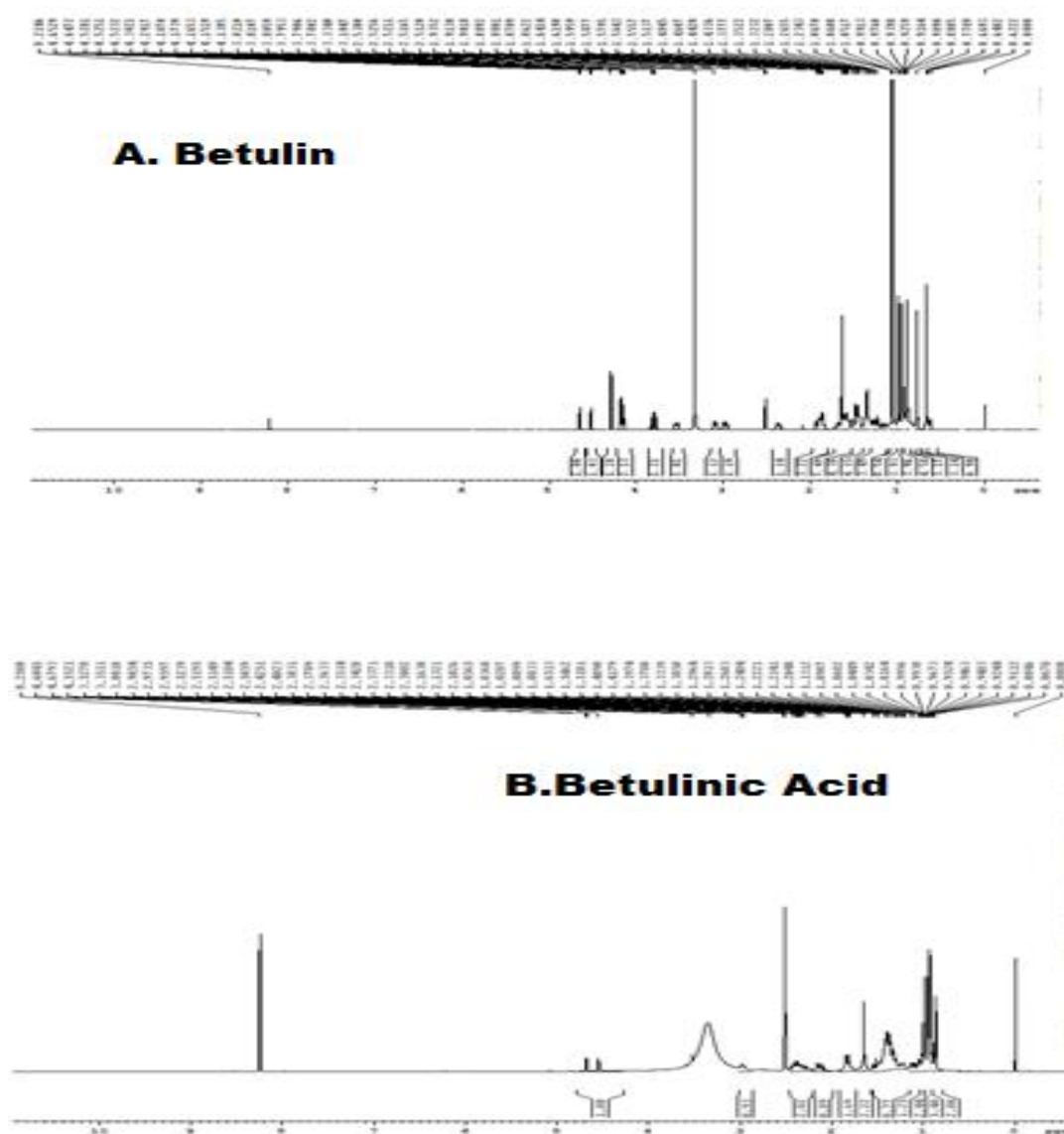


Figure-4 NMR analysis, variation in the peaks is showing biotransformation (a) betulin (b) betulinic acid

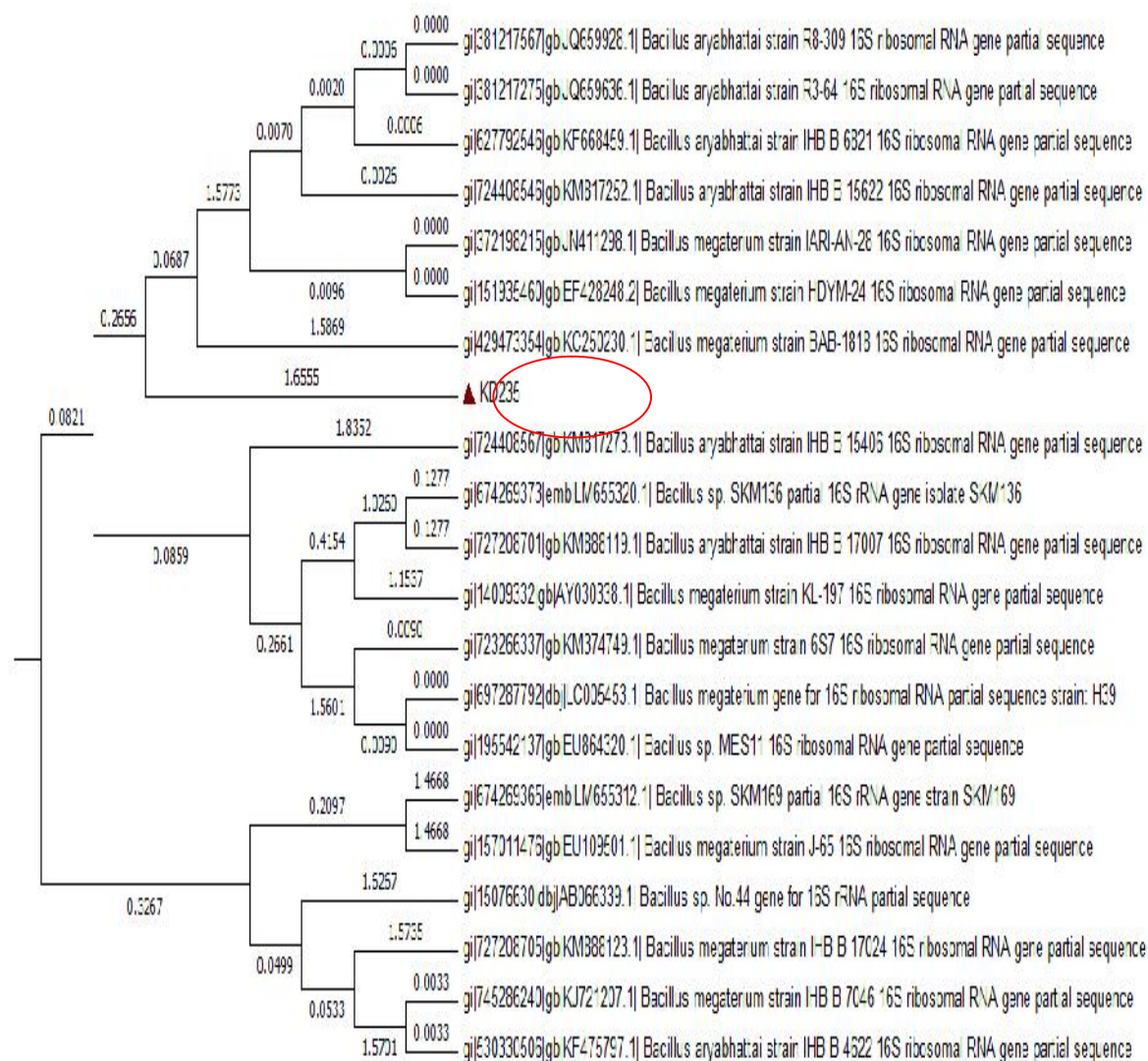


Figure-5 Phylogenetic tree showing inter-relation of betulin tolerant KD235 (NCBI GenBank KR261097)

Table 1 Biochemical characterization and identification of some oil isolates

Sl. No.	Name of the Bacterial Strain	Name of the Biochemical Test						
		Malonate	VP *Test	Citrate	ONPG	Nitrate Red ⁿ .	Catalase	Arginine
1	<i>Bacillus atrophaeus</i>	—	+	+	—	+	+	—
2	<i>B.cereus</i>	—	+	+	—	+	+	+
3	<i>B. coagulans</i>	—	+	—	+	—	+	+
4	<i>B.pumilus</i>	—	+	+	+	+	+	—
5	<i>B.megaterium</i>	+	—	+	+	+	+	—
6	<i>B.Thuringensis</i>	—	+	+	—	+	+	+
7	<i>B.subtilis</i>	—	+	+	+	+	+	—
*Voges-Proskauer's Test		+ positive result			- negative result			

Table 2 Sugar utilization test for identification of some selected soil isolates

Sl. No	Name of the Bacterial Strain	Test for Sugar Utilization				
		Sucrose	Mannitol	Glucose	Arabinose	Trihalose
1	<i>Bacillus atrophaeus</i>	+	+	+	+	+
2	<i>B. cereus</i>	+	—	+	—	+
3	<i>B. coagulans</i>	+	—	+	—	+
4	<i>B. pumilus</i>	+	+	+	+	+
5	<i>B. megaterium</i>	+	+	+	+	+
6	<i>B. thuringiensis</i>	+	—	+	—	+
7	<i>B. subtilis</i>	+	+	+	+	+

+, positive; -, negative; w, weakly positive.