Isolation and Characterization of Solventogenic Clostridia for Biobutanol Production

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***Abstract*— Screening of solventogenic Clostridia was carried out by enrichment of different samples such as compost, soil and mangroves in DRCM broth and thioglycollate broth anaerobically. On Subsequent isolation, 38 different catalase negative and weakly positive isolates were obtained on DRCM agar. Two isolates BS1 obtained from soil of rhizosphere near cactus plant produced maximum of 7.5 g/L of butanol in AAM medium. Butanol, butyric acid and sugar tolerance BS1 was found to be 18 g/L, 4 g/L and 200 g/L respectively. The ability of organism to produce butanol in medium such as RCM, ANS and TYE was tested, RCM was found to be equally effective like AAM, but was found to be uneconomical then AAM. An isolate CD1 obtained from cow dung was also able to produced 6.1 g/L of butanol did not give reproducible butanol thus BS1 was selected for further study. Growth curve of BS1 was studied in AAM medium to determine the onset of acidogenic and solventogenic phases for optimum butanol production. Growth of BS1 was observed after 16 hours of incubation and it was in the log phase of growth till 36 hours. Maximum pH drop was observed and reached to a steady pH. When the smears of endospore staining were observed under microscope it was observed that cells of BS1 entered sporulation stage after 36hours. At the same time gas chromatography of withdrawn fermentation revealed onset of solvent production and maximum 7.54 g/L butanol was produced by metabolizing 42 g/L of glucose on 68hours of incubation. Biochemical and molecular characterization of BS1 was carried out. It was identified as Clostridium beijerinckii and it shared 99% homology with strain E092 accession no JX267108**

***Index Terms*— Biobutanol, Clostridium, Gas chromatography, Fermentation**

I. INTRODUCTION

There are several renewable energy resources non carbon based and carbon based energy sources are available. Many of these sources are non-renewable and are decreasing due to continuous use. Depletion of these organic resources results in need of organic renewable energy resources such as biofuel production using microorganisms like ethanol, butanol, glycerol etc. In anticipation of the depletion of fossil fuels, increasing price of transportation oils and climatic changes research interest gained in the production of biobutanol from renewable resources.

Butanol (butyl alcohol or n-butanol) is a four carbon straight chained alcohol with a molecular formula of C_4H_9OH (MW 74.12). It is a colorless, flammable alcohol. It has a high calorific value, which is 29.2 MJ/dm, with lesser melting point and boiling point of –89.5°C& 117.2°C, respectively. The flash point is 36ºC with self-ignition temperature is at 340ºC [1]

Butanol has several industrial application such as it is widely used in industry and applied as a solvent, used as a feedstock chemical in the plastic, paints and polymer industry and as a food-grade extractant in the food and flavor industry [2] [3]. Currently; butanol is used only as an additive to gasoline because there is no engine working exclusively on this alcohol [1] [4] [5]. It is also used as solvent in the perfume industry and for the manufacturing of antibiotics, vitamins and hormones and many others [4] [6].

Butanol has certain advantages over other fuel making it superior biofuel compare to others. It has a relatively low heat of vaporization and corrosive, less miscible in water, less flammable, less hazardous, and more miscible in gasoline. It is with slightly higher octane number as compare to ethanol [3] [7]. Therefore butanol is preferred biofuel in comparison with ethanol. Research by Petrochemical industries also finds butanol as superior fuel additive in improving quality of petrol as compare to ethanol.

Presently butanol is synthetically derived from a petrochemical route based on propylene oxo synthesis in which aldehydes from propylene hydro formylation are hydrogenated to yield 1-butanol. The Butanol is also made by a fermentation process called ABE (a name given on the basis of initial letters of the product formed i.e acetone, butanol, ethanol), carried out mostly by solventogenic bacteria *Clostridia species*, known as biobutanol. In the early 1900s, acetone–butanol was the second largest fermentation process, behind only to ethanol. Chemical method of synthesizing butanol from petrochemical-based processes started taking over in the 1950s which substituted biochemical method of butanol production using fermentation. Continuous use from petrochemical resulted into insufficient petroleum supply and have renewed interest in obtaining butanol using fermentation process from renewable resources and therefore also created interest in screening of solventogenic *Clostridia*. It is expected that production of biobutanol can reduce consumption of oil and natural gas by the automobile industry. It reduces emissions of harmful gases into the atmosphere; the features enhance its usefulness both as an additive to gasoline, as well as biofuels. Considering the need and current interest in biobutanol aims to screen solventogenic *Clostridia* capable of producing butanol.

II. MATERIAL AND METHOD:

Enrichment & isolation of *Clostridium* species

Different samples such as dung, composts, soil, plant roots & mangroves were collected in sterile screw cap tubes & processed immediately or stored in deep freezer at -20^{o}C till processing.1gram of the sample was mixed thoroughly in 10 ml of saline & heated at 80^{o}C for 10 minutes with cooling for

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inactivation of vegetative form of microorganisms. Solid particles were allowed to settle & 1 ml of supernatant was added in 10 ml of Differential reinforced Clostridium media &Thioglycollate broth for enrichment of *Clostridium* [8]. Anaerobiosis was created by agar plugs wherein sterile 4% agar was overlaid on broth and incubated at 37^{o}C for one week. *Clostridium* grows anaerobically imparting turbidity to broth and shifts agar plug due to gas production. A loop full enrichment broth was isolated on DRCM agar plates which were incubated anaerobically at 37^{0}C for 72 hours. Colonies were then checked for catalase activity. Weakly positive & negative colonies were selected and biochemical identification of genus *Clostridium* was carried out [9]. Using biochemical identification as a part of screening the isolate was identified as *Clostridium beijerinckii*. This was further confirmed using 16srRNA partial identification (Amnion Biotech, Bangalore).

The *Clostridia* strains were maintained under anaerobic conditions as spore suspensions in ddH_2O at room temperature. Suspension was also preserved in 20% glycerol stored in deep freezer at -20^{o}C [3]

The isolate obtained were inoculated in *Clostridium* culture medium (Dextrose, 20 g; K_2HPO_4, 1 g ; KH_2PO_4, 1g; $FeSO_4$, 0.5 g ; $MgSO_4$, 1 g; CH_3COONH_3, 3 g; Para amino benzoic acid, 0.1; Yeast extract, 5 and d/w, 1 liter), named as Ammonium Acetate Medium, AAM in this study [10]. Tubes were incubated until endospore formation was observed and sporulated cells were harvested by centrifuging the broth at 3000 rpm for 15 minutes. The cells were washed twice using sterile D/W & suspended in D/W to attain final O.D of 1 at 620 nm [11]. Suspension was given a heat shock treatment at 80^{o}C for 10 minute, cooled immediately & used as inoculum [8, 11]. 10 % of inoculum was used in study [12] and fermentation was carried in AAM medium with 6% glucose in screw cap tubes incubated anaerobically at 37^{0}C. The AAM medium for fermentation contained 6 % glucose, which was higher than the growth media (2%), as lower concentration of carbon source could extend the lag phase & make fermentation unsuccessful [13].

Selection of media for butanol production

Along with AAM, Reinforced Clostridial medium (RCM), Tryptone yeast extract acetate medium (TYA) & Anaerobic sugar (ANS) medium were used for butanol production [14] , 1 ml of aliquot was withdrawn after 72 hours of incubation & processed for butanol detection by gas chromatography. Based on these results, media & isolate was selected to study growth curve of isolate to determine optimum time of production and concentration of butanol attained.

Growth curve of BS1 (an isolate obtained from black rhizosphere soil of cactus plant) to determine acid break point & course of butanol production

Growth curve of BS1 was studied by inoculating 10 % inoculum of BS1 with O.D 1 at 620 nm in AAM media (6 % glucose). A set of identical tubes were prepared, 0.5ml inoculum of BS1 was inoculated into in 4.5 ml medium. Tubes were then incubated at 30^{o}C anaerobically for 4 to72 hours of incubation; the different sets were prepared to be sampled at every 4 hours of interval. The tubes were opened after specified time interval & 3ml of sample was centrifuged at 3000 rpm for 15 minutes. The clear supernatant was stored at - 20 °C until analyzed for pH, glucose & butanol production. Cells were suspended in saline to measure optical density [15]. Clear supernatant was processed to detect pH, reducing sugars & butanol concentration. Growth was measured as absorbance at 620 nm and cell biomass as dry weight was calculated [16] & protein in mg/ml. Cell morphology was analyzed (vegetative & endospore stage) by endospore staining of cell suspension.

O.D is related to dry weight of cell by the formula [17]

Dry cell weight/ Dry cell mass (DM) = 0.3X O.D 600

Analytical procedure

For detection of butanol 2ml aliquot was centrifuged at 3000 rpm for 15 minutes. 900µl of aliquot was derivatized with 100µl of phosphoric acid. 2µl of sample was injected at 150^{o}C injector temperature & is detected using FID at 170^{o}C with nitrogen as carrier gas at pressure 4 psi [8]. Quantification of butanol was carried out using gas chromatography (NUCON gas chromatogram with Carbovac column) after preparing 1 to 5% of butanol in glass d/w. Chromatograms were observed using computer software Winchrom06EX. Retention time for butanol & areas under the peaks of different concentration were recorded, a calibration curve was plotted & used for quantitative analysis of butanol in further study. The selected isolates were tested for butanol tolerance, butyrate tolerance & glucose tolerance. All the tests were done in triplicates in AAM fermentation medium.

Butyric acid tolerance: Standard 20 g/l butyric acid (LOBA) was used as stock & butyric acid concentration were prepared in a range of 1 to 14 g/l

Butanol tolerance: Dilution of butanol within a range of 2-24 g/l was carried out using 50 g/l concentration of butanol stock

Glucose tolerance: Glucose tolerance was determined using 500 g/l stock glucose of concentration at a range of 0 to 240 g/l of glucose

III. RSEULT AND DISCUSSION:

Enrichment & isolation of *Clostridium* species and testing potential of butanol production of isolates:

Enrichment of *Clostridia* was done in DRCM & thioglycollate broth. Gas production due to growth of anaerobic microorganisms shifted agar plug upwards in the tubes (Fig 1). 38 different catalase negative isolates were obtained on DRCM agar plate after 48 hours of incubation. Maximum isolates were obtained from dung (37 %) followed by compost (29 %) & mangroves (21 %), soil (10 %) & plant roots (3 %) (Fig 2).

All the isolates obtained on enrichments and isolations were able to grow anaerobically in AAM with 2 % dextrose but only 9 were able to ferment 6% glucose and on gas chromatography and four isolates showed ABE peaks.

Ability of the four isolates to produce butanol was further confirmed and quantified by butanol fermentation medium containing 6 % of glucose. As isolate CD1 (6.1 g/l) & BS1 (4.9 g/l) gave higher butanol compare to MN2 (3.25 g/l) & GG1 (1.7 g/l) (Table 1), therefore parameters related to butanol production such as butanol, sugar & butyric acid tolerance of the former 2 isolates were determined. The

primary reason for low butanol concentration appears to be its toxicity. Isolates BS1 had maximum butanol tolerance of 18 g/l whereas CD1 could tolerate 14 g/l, which is higher than conventional butanol toxicity level of 13 g/l as reported to be the primary reason for low butanol concentration [18] [19]. Thus these isolates obtained from nature are having potential of butanol production. Glucose & butyric acid tolerance of these isolates were determined. 200 g/l glucose & 4 g/l of butyric acid tolerance was observed for isolate BS1, whereas CD1 showed 220 g/l glucose & 3 g/l of butyric acid tolerance respectively (Table 2).

Selection of media for butanol production using isolates CD1 & BS1

Four different media viz Ammonium Acetate Medium (AAM), Reinforced Clostridial medium (RCM), Tryptone yeast extract acetate medium (TYA) & Anaerobic sugar (ANS) medium were used for selection of suitable nutrient. Isolate BS1 showed maximum butanol production of 7.5 g/l in AAM after 72 hours of incubation, followed by RCM (6.025 g/l). Poor growth in TYE was observed & ANS did not support the growth of isolates. CD1 showed maximum butanol in TYE media (3.95 g/l) followed by AAM (3.23 g/l) whereas no growth of CD1 was observed in ANS, no butanol production in RCM broth was detected. Statistical analysis using paired t test was carried out between the maximum butanol produced by BS1 (7.5 g/l) & CD1 (3.95 g/l) for further selection among the two (Table 3). The $t_{0.10,\ 4}$ was found to be 5.8 which is greater than‘t’ table value of 1.533. Thus there was a significant difference in butanol production by the two isolates & BS1 produced higher concentration of butanol, it was selected for further characterization.

As both RCM & Ammonium Acetate Media both on fermentation produced butanol, statistical analysis using paired ‘t’ test between butanol produced by Ammonium Acetate Medium; 7.5 g/l & RCM media, 6.025 g/l was carried out for selection of basic media composition for further studies. The $t_{0.10,\ 3,}$ of 1.22 was found with insignificant difference between butanol productions in AAM & RCM, however as the basic composition of AAM, is more economical, the medium was found to be more suitable for butanol production.

Growth curve of BS1 to determine acid break point & course of butanol production

Growth curve of isolate BS1 was studied to assess the time taken by isolate BS1 to attain acidogenic phase & further the onset of solventogenic phase therefore pH, reducing sugar concentration, butanol levels were determined (Fig. 3B).

Growth of BS1 initiated after 16 hours of incubation, a steady state growth was attained after 36 hours of incubation with slow increase in protein content (exponential phase of growth with acediogenesis) that remained constant up to 68 hours of incubation (Stationary phase of growth for solventogenesis) (Fig 3A). During solventogenesis the vegetative cells sporulated thus were unable to reproduce [20].

Apart from these parameters, optical density of cells was measured; an increase in optical density (620 nm) was observed which was expressed as increase in that dry weight content in cell biomass. A gradual increase in dry weight of biomass was observed and exponential phase of growth was observed at this period of growth, till 36 hours of incubation explains the acidogenic phase in life cycle of solventogenic *Clostridium, BS1*.(Fig 3A,& B)

At the end of exponential phase endospore staining was performed along with cell mass and protein measurement. The smears examined under bright field microscope at 100X showed cells of BS1 which were thin long & without spore till 36 hours. The observations made were in concurrence to cell biomass (Fig 4). Cell biomass nearly remained constant till the end of study i.e 72 hours with increase in solvent production, as explained above thus showing the second phase of solventogenesis.

Though the biomass was constant but a decrease in protein content was observed after 60 hours of incubation, which may be due to release of spores due to depletion of sugars. The cells during solventogenesis becomes thick & after 60 hours spores bulged out from the cells. Many cells remained in the endospore state till 72 while some converted themselves into spores also revealed by decrease in protein content of the biomass (Fig 3A & 4). Similar observations were reported for *Clostridium acetobutylicum* grown on mixture of sugars glucose, xylose arabinose and xylose obtained on hydrolysis of lignocellulosic biomass [21].

Initial pH of the medium was adjusted to 7 which with onset of growth in both test and control media by 16 hours decreased to 5.6 revealing acidogenic phase of BS1 (*Clostridium sp*). The pH remained in the range of 5.0 to 5.6 there on up to 64 hours and further decreased to 4.7 on 72 hours of incubation. The pH drop is majorly due to butyric acid & acetic acid production, with small quantity of propionic acid. This is the phase for solvent production by *Clostridia* known as acid break point wherein cells from acidogenic phase enters into solventogenesis & these acids converted to solvent acetone, butanol & ethanol due to activity of enzyme acetoacetate decarboxylase, butanol dehydrogenase and ethanol dehydrogenase respectively [22] (Fig 3B).

On set of butanol production was observed after 28 hours of growth with 0.72 g/l butanol that increased gradually to 7.54 g/l on 68 hours of incubation. This phase of growth from 28 to 72 hours was solventogenic, wherein there was a steep raise in solvent was production was observed which maximize to7.54 g/l on 68 hours of incubation. Initial pH of the medium was adjusted to 7 which with onset of growth in both test and control media by 16 hours decreased to 5.6 revealing acidogenic phase of BS1 (*Clostridium sp*). The pH remained in the range of 5.0 to 5.6 there on up to 64 hours and further decreased to 4.7 on 72 hours of incubation.

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Glucose concentration decreased with increased time internal, overall 4.2 % of reducing sugars were metabolized for production of 7.54 g/l butanol (Fig 3B). The pH decreased further on and after 72 hours of incubation where in conversion of solvent to acids begin and high acidity the solventogenic phase due to deactivation of solventogenic enzyme [4] [23].

Identification of isolate BS1

Morphological and cultural characteristics

Strain BS1 was isolated on DRCM agar plate, white, irregular translucent colonies of catalase negative BS1 were observed. Microscopic examination & biochemical test such as anaerobic fermentation of sulphate, growth above 45 ^{O}C, Gal/Lac fermentation and litmus milk and microaerophilic growth of organism for identification of genus were performed. Gram staining and endospore staining of these isolates were done, they were found to be Gram positive terminal, and sub-terminal spore bearers. On the basis of genus specific biochemical tests and morphology all the isolates were identified as *Clostridium.* Biochemical tests such as Litmus milk, SO_4 reduction, formate & pyruvate reduction, gelatinase activity and sugar fermentation test for lactose, galactose, starch, cellobiose, arabinose, dulicitol, xylose to dentify the species. Biochemical tests were performed for species identification for isolates CD1 (an isolate from cow dung), BS1 (an isolate from root of cactus plant), MN2 (an isolate from mangrove) & GG1 (an isolate from compost of gober gas plant). All four isolates were identified as genus *Clostridia* on primary identification. On biochemical identification, isolate MN2 & BS1 showed similarity to *Clostridium beijerinckii* & CD1 & GG1 were found to be similar to *Clostridium acetobutylicum.* 16S ribosomal partial identification was carried out. On observation of the aligned sequences by NCBI blast, BS1 showed identity with ***Clostridium beijerinckii*** **strain E092 Accession number JX267108.1** with total score and query score of 2503 with 99 % cover across (Table 4).

DNA sequencing results for isolate BS1:

Forward:

CGGGGGGGGGTGCTTACCTGCAAGTCGAGCGATGA
AGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTG
AGTAACACGTGGGTAACCTGCCTCATAGAGGGGAA
TAGCCTTTCGAAAGGAAGATTAATACCGCATAAGA
TTGTAGTGCCGCATGGCATAGCAATTAAAGGAGTA
ATCCGCTATGAGATGGACCCGCGTCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACATTGGG
ACTGAAACACGGCCCAAACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGGGAAACCCTGAT
GCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGA
TTGTAAAGCTCTGTCTTCAGGGACGATAATGACGG
TACCTGAGGAGGAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAGGCGGGTGT
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GGGTGCTGCATTCCAAACTGGATATCTAGAGTGCA
GGAGAGGAAAGTAGAATTCCTAGTGTAGCGGTGAA
ATGCGTAGAGATTAGGAAGAATACCAGTGGCGAAG
GCGACTTTCTGGACTGTAACTGACACTGAGGCTCG
AAAGCGTGGGGAGCAAACAGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGATACTAGTGTAGGGG
TTGTCTGACCTCTGTGCCGCGCTAACGCATTAGTAT
TCCGCCTGGGGAGTACGGTCGCAGATAAACTCAAA
GATGACGGGGCCCGCACAGCAGCGGAGCATGTGGT
TTTATTCGAAGCAACCGCGAAAGAACCTACT

Reverse:

GTCGCTGGACCTACCTTAGGTCGCTGCCTCGCTTAC
GCGTTAGCTCACGAACTTTGGGTATTGCCAACTCTC
ATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA
CGTATTCACCGCGACATTCTGATTCGCGATTACTAG
CAACTCCAGCTTCATGTAGGCGAGTTTCAGCCTACA
ATCCGAACTGAGACTGGTTTTAAAGTTTGGCTCCAC
CTCGCGGTTTAGCATCTCTCTGTACCAGCCATTGTA
GCACGTGTGTAGCCCTAGACATAAGGGGCATGATG
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CATGCACCACCTGTCTTCCTGCCCCGAAGGGCTTCC
CCGATTAAGGGTAATTCAGGAGATGTGGAGTCTAG
GTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT
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GAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAA
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GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACG
CTTTCGAGCCTCAGTGTCAGTTACAGTCCAGAAAGT
CGCCTTCGCCACTGGGTATTCTTCCTAATCTCTACG
CATTTCACCGCTACACTAGGAGTTCTACTTTCCTCT
CTGCACTCTAGATATCAGTTGGATGCAGCACCAGTT
GAGCCGGAGGTAGTTCACATCCACTAATATCCACC
TACGGCCTCCCTTTACCGCCCCCAGGTTAAAC

Aligned Data (1413bp)

CGGGGGGGGGTGCTTACCTGCAAGTCGAGCGATGA
AGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTG
AGTAACACGTGGGTAACCTGCCTCATAGAGGGGAA
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GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACATTGGG
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TTGTAAAGCTCTGTCTTCAGGGACGATAATGACGG
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GGGTGCTGCATTCCAAACTGGATATCTAGAGTGCA
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TTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
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GGGGAAGCCCTTCGGGGCAGGAAGACAGGTGGTGC
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TAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTT
GCTACCATTTAGTTGAGCACTCTAGCGAGACTGCCC
GGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAA
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TACAATGGCTGGTACAGAGAGATGCTAAACCGCGA
GGTGGAGCCAAACTTTAAAACCAGTCTCAGTTCGG
ATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAG
TTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC
CATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTA

ACGCGTAAGCGAGGCAGCGACCTAAGGTAGGTCCA GCGAC

On observation of the aligned sequences by NCBI blast, organism showed identity of ***Clostridium beijerinckii*** **strain E092 Accession number JX267108.1** with total score and query score of 2503 with 99% cover across (Table 4)

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TABLES AND FIGURES

I. Butanol production by different isolates on primary screening (Avg ±Stdev)

Isolate	**CD1**	**BS1**	**MN2**	**GG1**
Butanol g/L	6.1± 0	4.9±0. 1	3.25±0.3 5	1.7±0. 1

II. Butanol, glucose & butyric acid tolerance of culture CD1&BS1

Butanol tolerance (g/L)		**Glucose tolerance (g/L)**		**Butyric acid tolerance (g/L)**	
CD1	BS1	CD1	BS1	CD1	BS1
14	18	220	200	3	4

III. Amount of butanol in g/L produced by BS1& CD1 on different media within 72 hours (Average± Stdev)

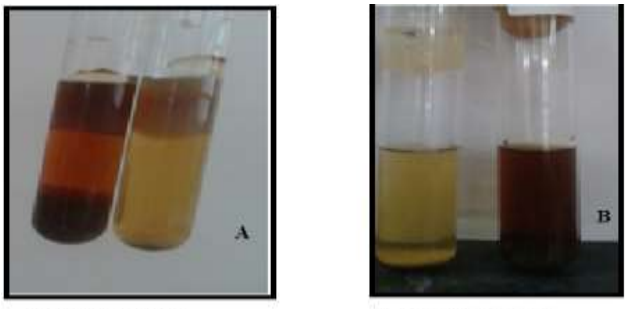
Isolates	**Butanol in g/l in different media**			
	AAM	**RCM**	**ANS**	**TYE**
BS1	7.5±0	6.025±1.76	**-***	0.001
CD1	3.23±0.7023	**-***	**-***	3.95±0.05

Key: -*, Could not ferment the media.
AAM, Ammonium acetate medium
RCM, Reinforced Clostridial medium
TYA, Tryptone yeast extract acetate medium
ANS, Anaerobic sugar

IV. Description of aligned sequence of BS1 on 16S ribosomal RNA gene

Description Max	**score Total**	**score Query**	**cover**	**E value**	**Ident**	**Accession**
Clostridium beijerinckii strain E092	2503	2503	99 %	0.0	99 %	JX267108. 1

The data is showing maximum homologous similarity *Clostridium beijerinckii* strain E092 16S ribosomal (partial sequence) RNA gene across 99% cover.



A. Before incubation **B. After incubation**

Fig 1. Agar plug technique to create anaerobiosis for enrichment of *Clostridia*

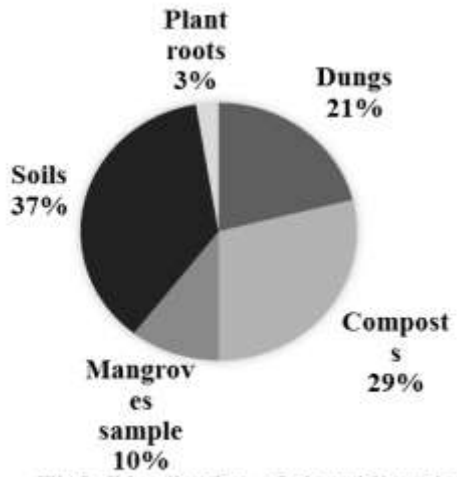


Fig2. Distribution of clostridium isolates obtained from various samples

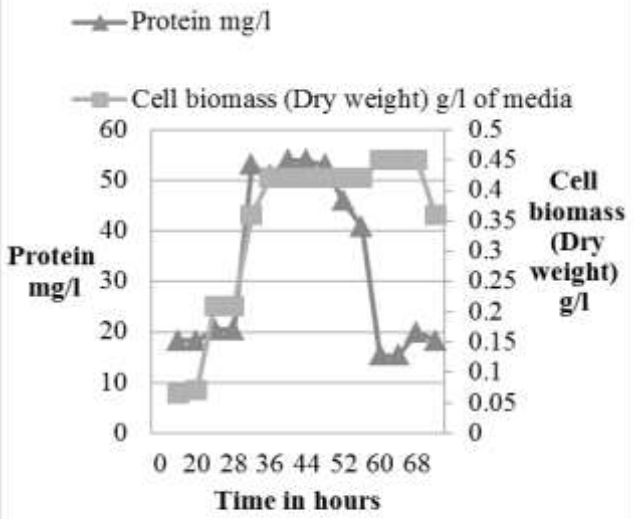


Fig. 3A.Growth Curve of BS1 in AAM medium

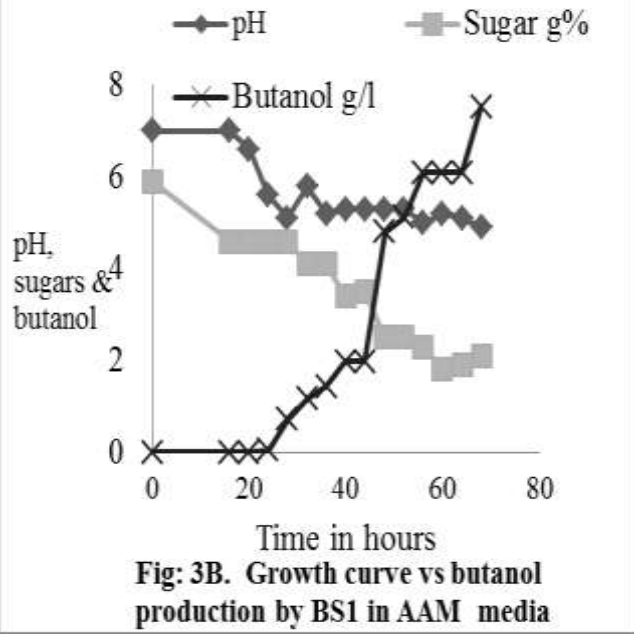


Fig: 3B. Growth curve vs butanol production by BS1 in AAM media

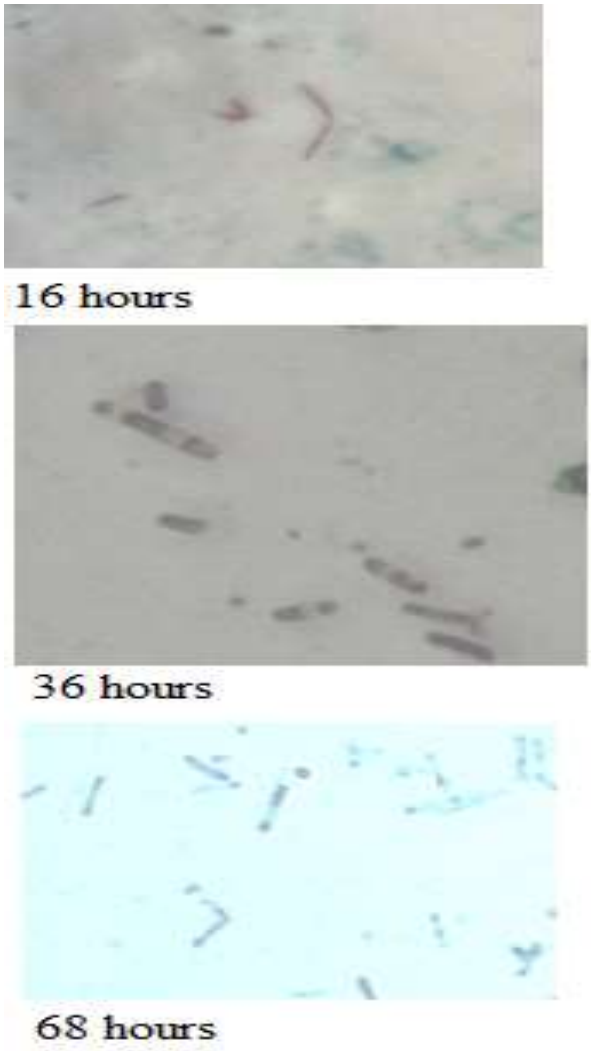


Fig 4. Endospore staining of isolate BS1 during growth curve