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Isolation, Identification and Media Augmentation for Cellulase Producing Forest Isolate *Bacillus velezensis* SSV-2

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A B S T R A C T

There has always been an increasing demand for an alternative to fossil fuels and for effective biomass utilization. Microbial cellulases a class of hydrolytic enzymes can convert cellulose, the most abundant biopolymer on Earth into useful products. In this study, 50 different bacterial isolates were isolated from forest soil samples collected from distinct regions of Kerala, India. Different screening techniques have been conducted to find out the potent cellulolytic isolates. 32 isolates showed positive results on Congo-red staining and they were subjected to quantitative screening by submerged fermentation in CMC medium followed by cellulase assay. Isolate SSV-2 collected from Peechi forest area was observed to be the best cellulase producer and that was identified as *Bacillus velezensis* based on morphological, biochemical evaluation and 16S rRNA gene sequencing analysis. In order to enhance the cellulase producing capacity of the strain, optimization of different physical and chemical parameters were carried out. Maximum cellulase production was observed when the medium pH was at 7 and CMC was found as the best carbon source. Peptone is observed to be the better nitrogen source with a cellulase activity of 22.67 U/mL. Maximum CMCase activity was observed after 72 hours of growth by *Bacillus velezensis* SSV-2.

***Key words*:** Isolation, *Bacillus velezensis*, cellulase, CMC, Optimization

Cellulose is one of the most important biological compounds on the ecosystem and is an integral part of plant biomass. Cellulose is a linear polysaccharide made of D-glucose residues, which are interlinked by β-1, 4-glycosidic linkages [5]. Being the vital component of plant cell wall, it is one among the major lignocellulosic wastes produced on earth. Plentiful availability of cellulose makes it a fascinating candidate for the preparation of many industrially important products. Unfortunately, much of the lignocellulosic wastes are disposed in unscientific ways like biomass burning. Since cellulose is not freely soluble in water, the biodegradation of lignocellulosic waste materials needs the enzymatic conversion of cellulose.

Cellulolysis is catalyzed by cellulases, which are inducible enzymes produced mainly by microorganisms for their growth and survival in the cellulosic materials. Cellulase is a group of three synergistic enzymes which act together for the complete breakdown of cellulose into simple sugars. These include β-1,4 endoglucanase (EC 3.2.1.4) that randomly cleaves the β-1,4 glycosidic linkages of cellulose polymer. Second one β-1,4 exoglucanase (EC 3.2.1.91) attack the non-reducing ends of cellulose chain and release cellobiose. β-glucosidase (EC 3.2.1.21) is the third one that hydrolyses the cellobiose and other short-chain oligosaccharides to glucose [4-5], [27].

Many microorganisms have been isolated and identified from various sources ranging from soil to hot springs which are efficient in cellulolysis. These include bacteria, fungi, yeasts and actinomycetes [13]. Among these,*Trichoderma*, *Aspergillus*, *Pencillium* and *Phanerochete* are the potential fungal candidates producing cellulases in large quantities[5]. Cellulase from different microbial origin shows major differences regarding their stability at various catalytic conditions [1]. There is extensive research in the area of bacterial cellulases also because of their fast growth rate, their resistance to extreme environments and easiness in genetic manipulations [3]. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Bacillus* and *Acetovibrio* genera are known for producing cellulase of different types. Because of the enormous applicability of cellulase enzyme in many industries like bioethanol production, agricultural and plant waste management, cotton industry, detergent industry, brewery and wine, starch processing and extraction and processing of fruit juices [9], [11], [14] the research activities in the field of cellulase enzyme is very high. Soil which is considered to be the richest sources of microbial

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diversity is comparatively less explored. The present work aims on the screening and isolation of a novel high cellulase producing soil bacteria from samples collected from different forest areas of Kerala state, its identification and optimization of cultural conditions of the strain for maximum enzyme production.

MATERIALS AND METHODS

Isolation of bacteria producing cellulase activity

Soil samples (10 g each) were collected from different forest regions of Kerala including Thattekadu, Vazhachaal, Agasthyamala, Peechi, Nedumangadu, Pambadum Shola and Aaralam. Cellulolytic bacteria were isolated by serial dilution method, which were later grown in culture medium containing CMC as the selective carbon source. The components of the media in which the bacteria were isolated consist of $MgSO_4$ 0.02 g; K_2HPO_4 0.1 g; Yeast Extract 0.06 g; Peptone 0.06 g; Carboxy methyl Cellulose 1 g; and Agar; 2 g per 100mL medium. Congo- red staining in the bacterial colony was used as the preliminary screening procedure. The occurrence of clear zone around the colony was taken as positive, cellulose- degrading ability of the isolate [28], [30]. The diameter of hydrolysis zone gives an indication of the best cellulase producers. These colonies were further selected for secondary screening and fermentation studies.

Enzyme production

The selected isolates of primary screening were cultured at room temperature on a rotary shaker at 120 rpm in an enzyme production media composed of $MgSO_4$ 0.02 g; $K_2HPO_4$0.1 g; Yeast Extract 0.06 g; Peptone 0.06 g; Carboxy methyl Cellulose 1 g and distilled water 100 mL at pH 7. Broth culture was collected after every 24 hours of inoculation and exposed to centrifugation at 7000 rpm for 15 minutes at 4℃. Supernatant was collected and preserved as crude enzyme preparation at 4℃ for further enzyme studies.

Enzyme assay

The cellulase activity was quantified by estimating the reducing sugar produced during the enzymatic activity by 3, 5-dinitrosalicyclic acid (DNS), [19] using glucose as standard. The reaction mixture consists of 1mL 0.5% CMC in 0.2M phosphate buffer (pH 7), and 0.5mL crude enzyme which are incubated at 50℃ for 10 minutes and the reaction was stopped by adding 3mL DNS reagent. After boiling the mixture for 5 minutes and sudden cooling absorbance was measured at 540 nm using a spectrophotometer. The activity was measured as U/mL which indicates the amount of CM Case necessary to release 1µmole of reducing sugar per mL per minute.

Identification of the selected strain

The maximum cellulase producing bacterial strain after the primary and secondary screening was identified on the basis of morphological and biochemical characterization according to the Bergey's Manual of Systematic Bacteriology [12] and the results were confirmed by16S rRNA gene sequencing method from the Institute of Microbial Technology (IMTECH), Chandigarh, India followed by BLAST search and phylogenetic tree construction.

Optimization of cultural conditions for enhanced cellulase secretion

Effect of different carbon sources on CM Case production by the strain

Influence of different carbon sources namely lactose, sucrose, glucose, starch, maltose, fructose, xylose, xylan, CMC, glycerol and filter paper were studied by culturing the identified strain in the enzyme production medium without CMC. 0.5% of eleven different carbon sources mentioned above were added as a substitute to CMC in the cellulase production medium and CM Case production pattern was noticed. Later influence of different concentration of the optimum carbon source was analyzed, by adding concentrations ranging from 2.5-15g/L of the best carbon source in the cellulase production medium.

Influence of different nitrogen sources on CM Case production

Effect of eleven distinct nitrogen sources namely urea, NH_4SO_4, yeast extract, peptone, casein, KNO_3, thiourea, $NaNO_3$, NH_4Cl, tryptone and gelatin at a concentration of 5 g/L and different concentrations ranging from 2.5-15g/L of the optimal nitrogen source was also monitored.

Influence of initial medium pH on CM Case production pattern by the strain

To identify the influence of initial medium pH on the cellulase production capability of the strain, the identified strain was cultured in the cellulase production media with different pH ranging from 4-11.

Effect of inoculums size on CM Case production

The influence of inoculum size on the cellulase production ability of the bacteria was tested by adding pre inoculums of different sizes (2.5%, 5%, 7.5%, 10%, 12.5% and 15%) into the cellulase production medium at pH 7.

Effect of agitation speed on CM Case production

The influence of agitation speed on the CM Case production profile of the bacteria was analyzed by culturing the strain in cellulase production medium with pH7 at different agitation speeds ranging from 100-160 rpm.

RESULTS AND DISCUSSION

Screening and isolation of cellulolytic bacteria

Bacteria producing considerable levels of cellulolytic enzymes were best suited for industries with growth capabilities in bioreactors. Fifty different bacteria were isolated from the soil samples collected from different forest areas using CM Case agar plates, which is the particular media component for the isolation of cellulolytic bacteria. The primary screening by Congo-red staining revealed that 32 out of 50 strains shows positive results by developing clear zones around the bacterial colonies. Cellulose degrading capacity of the positive isolates was carried out by calculating the diameter of the zone of clearance around the bacterial colony after Congo-red staining and the remaining 18 isolates were excluded from further studies. Among the 32 isolates, the isolate SSV-2 obtained from the soil sample collected from Peechi forest area in Thrissur district of Kerala, India showed maximum clearance zone of 3.2 cm (Fig 1). To confirm the result, all the 32 isolates from Congo-red positive screening were used for submerged fermentation studies in the cellulase production medium.

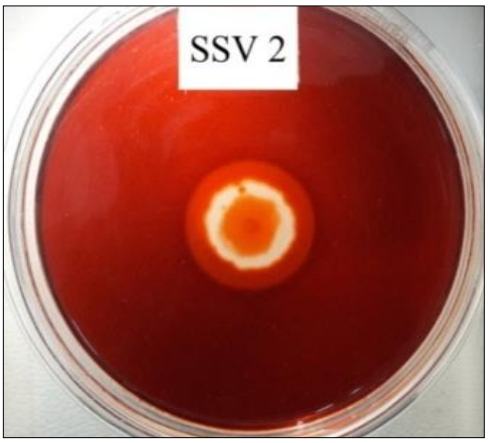

Fig 1 Clearance zone produced by SSV-2

CMC was provided as the carbon source in the medium and their respective cellulolytic activity was estimated. The cellulase enzyme assay was done at every 24-hour interval for a period of six days. The cellulase activity on CMC substrate was observed to be highest for the strain SSV-2. It showed maximum activity (15.56 U/mL) after 72 hours and the least activity was found at 24 hours.

Congo-red staining technique has been utilized in many studies to identify the cellulolytic potential of microorganisms. Teather and Wood explained the relation between the diameter of clearance zone and the log enzyme concentration but this kind of correlation can't always explain the cellulase synthesis capability of the microorganism [30]. In this study itself, certain strains that produced good clearance zones in cellulose agar plates, almost clear and large as that of SSV-2, failed to show substantiate cellulolytic activity under submerged fermentation. This may be either due to the poor enzyme secreting capacity of the strain or due to negligible production to quantify after cultivation in the liquid medium. Sadhu and Maiti have also stated that the size of clearance zone always doesn't represent the cellulolytic ability of the strain [25].

Identification of SSV-2

The strain SSV-2 was identified on the basis of morphological, biochemical characterization and its confirmation was carried out by 16S rRNA gene sequencing. Many cellulolytic bacterial strains are identified from different environments with novel properties and high cellulase activities. A novel salt tolerant cellulase enzyme was obtained from *Vibri*o sp. G21 isolated from mangrove soil collected from the coastal areas of Xiamen, China [10]. An organic solvent stable alkaline cellulase was identified from a marine isolate of *Bacillus aquimaris* by Nitin *et al*. [31]. The morphological peculiarities of the bacterial colony of SSV-2 (Fig 2) is represented in the (Table 1). The growth characteristics of SSV-2 at different temperature, pH, and NaCl concentration were also studied. Temperature ranging from 10-65℃ was studied and it was found that no growth was observed at 10-15℃and beyond 55℃.The growth pattern was studied for pH values ranging from 5-9 and NaCl concentration ranging from 2-9% and the strain showed good growth in all these pH ranges and NaCl concentrations. The results of biochemical tests conducted according to Bergy's Manual are represented in the (Table 2), which indicates that the strain shows positive results for methyl red test, casein test, starch hydrolysis, gelatin hydrolysis, nitrate reduction, catalase, oxidase and urease test. The strain failed to grow on MacConkey agar, and showed negative results for indole test, Voges Proskauer test, and H_2S gas production. (Table 3) represents the results of acid production from carbohydrates.

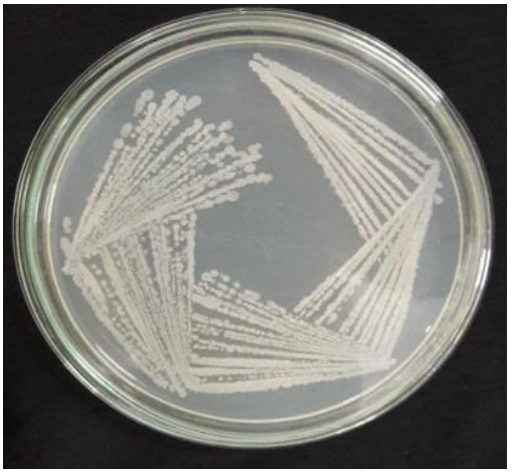

Fig 1 SSV-2 on CMC- agar medium

Table 1 Colony morphology of SSV-2

Configuration	Circular
Margin	Entire
Elevation	Raised
Density	Transparent
Pigmentation	Creamish
Gram reaction	+ve rods
Spore and motility	+ve, motile

Table 2 Biochemical characterization results of SSV-2

Growth on MacConkey agar medium	-
Indole test	-
Methyl red test	+
Voges Proskauer test	-
Citrate test	±
Casein test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Nitrate reduction	+
Catalase	+
Oxidase	+
H_2S gas production	-
Urease	+

Table 3 Acid production from carbohydrates

Glucose	+
Fructose	-
Salicin	+
Mannitol	-
Raffinose	+
Sucrose	+
Rhamnose	+
Galactose	-
Innositol	+
Arabinose	+
Xylose	+

From the morphological and biochemical characterization analysis, it is evident that the isolate SSV-2 is showing maximum similarity with the bacterial genus *Bacillus.* To confirm the results and to identify the strain up

to species level 16S rDNA gene sequencing has been done, and the rDNA sequence (Fig 3) has been used to perform BLAST search in NCBI database to compare the sequence with all other available sequences in the database. The results of BLAST search has been used to construct phylogenetic tree (Fig 4). The BLAST search of 16S rDNA sequence of the strain showed that it has 99.85% of sequence similarity with *Bacillus velezensis*, indicating that our strain of interest SSV-2 is belonging to the genus *Bacillus* and species *velezensis.* Similar result is obtained from the phylogenetic tree construction by neighbor joining method also. The isolate SSV-2 is aligned on the same branch along with *Bacillus velezensis* strain. Many reports are available on the isolation and characterization of extra cellular cellulase production from *Bacillus velezensis* [18], [20-21].

>SSV2

CGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTA
AGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAA
AGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
GGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGT
ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCG
GAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGG
AGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGT
TTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTG
CCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAAT
CATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAG
GTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAC
GAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTAAGGAGCCAGCCGCCGAAGGTGGGACAGATGAT
TGGGGTGAAGTCGTAACA

Fig 3 16S rDNA sequence of the strain SSV-2

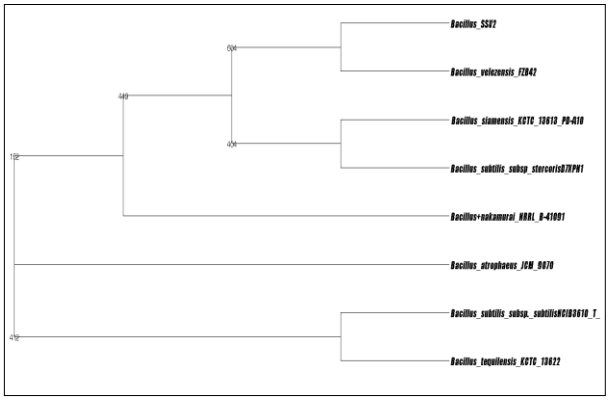

Fig 4 Phylogenetic tree created using the 16S rDNA sequence of the strain SSV-2 and closely ranked sequences from BLAST search

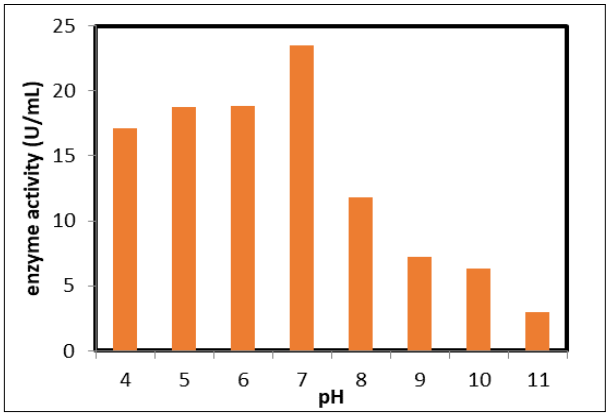

Fig 5 Influence of medium pH on cellulase production by SSV-2

Optimization of cultural conditions for enhanced production of cellulase from SSV-2

The initial pH of the enzyme production medium is observed to be one among the best incubation conditions for the increased production of cellulase. In the present study the highest cellulase production was observed when the initial medium pH was kept as 7. There is no drastic change in cellulase activity when the medium pH changes from 4 to 7, there is a slow increase in enzyme activity up to the pH 7 and after that the enzyme activity suddenly falls off when medium pH reaches 8 and the activity becomes negligible when the medium becomes more alkaline (Fig 5). Similar observation has been recorded in *Streptomyces sp* F2621 by Tuncer *et al.* [32]. Sadhu *et al.* [24] also reported the maximum cellulase activity in a *Bacillus* species (MTCC10046) isolated from cow dung when the medium pH was kept at 7. Medium optimization works in *Streptomyces griseorubens* reported the similar result as there was a hike in cellulase activity up to pH 7 and after that a sudden decline is observed and very negligible cellulase activity at higher alkaline pH like 11 [22].

Different carbon sources were used to induce cellulase production in the strain SSV-2. The least cellulase production was noticed when 0.5% fructose was used as the sole carbon source and the maximum activity was shown when CMC was used as the carbon source followed by filter paper (Fig 6a). Apart from CMC and filter paper, lactose and glycerol were also found to have a positive influence on the release of cellulase from the strain whereas the remaining sources had very negligible influence on cellulase

production. 5g/L CMC is calculated to be the best optimal concentration (Fig 6b). Use of CMC as the sole carbon source gives the best result for CM Case production [8]. Similar results have been shown by Sadhu *et al*. [24] were also the maximum cellulase activity was shown when 5g/L CMC was used as the sole carbon source.

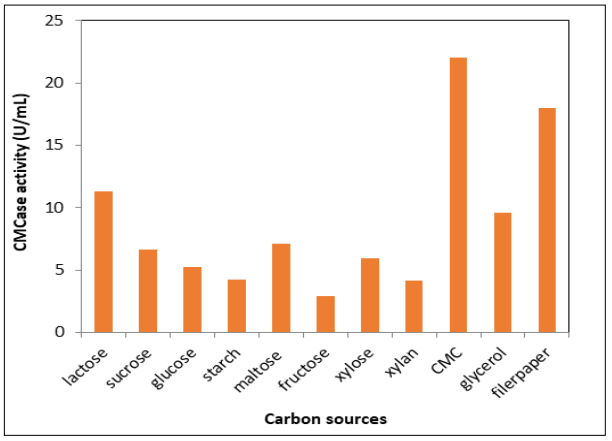

Fig 6a Influence of carbon sources on CM Case production by SSV-2

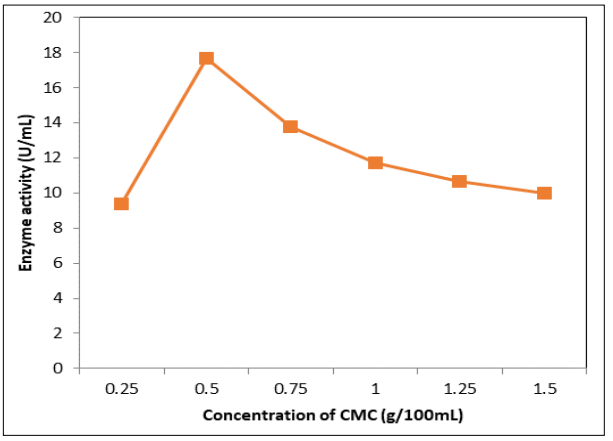

Fig 6b Influence of different concentrations of CM Case

Considering the influence of different nitrogen sources on cellulase production, peptone is observed to be the better nitrogen source (22.67 U/mL cellulase activity), which is followed by yeast extract and tryptone (Fig 7a). No CM Case was produced when KNO_3, thiourea and $NaNO_3$ were used as the only source of nitrogen. The optimal concentration of peptone was found as 15g/L (Fig 7b). Many studies suggest that organic nitrogen sources are better compared to inorganic nitrogen sources in promoting cellulase production in microbial strains [7], [15], [29]. The current study is also confirming the same where inorganic nitrogen sources are having very less influence on cellulase production except NH_4Cl. In a study by Liang *et al.* [17] where the optimization of cellulase production by *Paenibacillus terrae* ME27-1 was carried out and the better nitrogen source for enhanced cellulase production from the strain was the inorganic NH_4Cl. A few other reports are also supports the same observation [16].

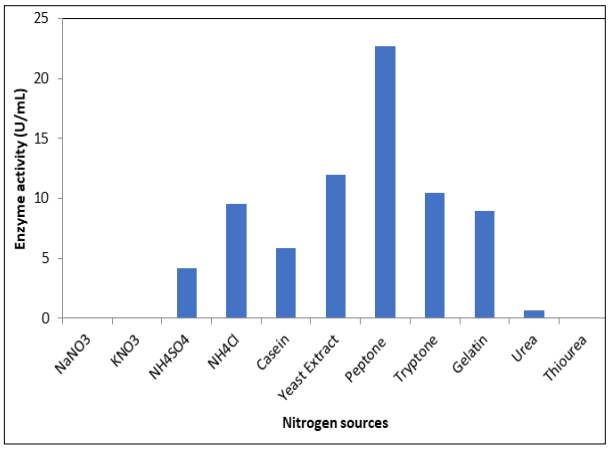

Fig 7a Influence of nitrogen sources on cellulase production by SSV-2

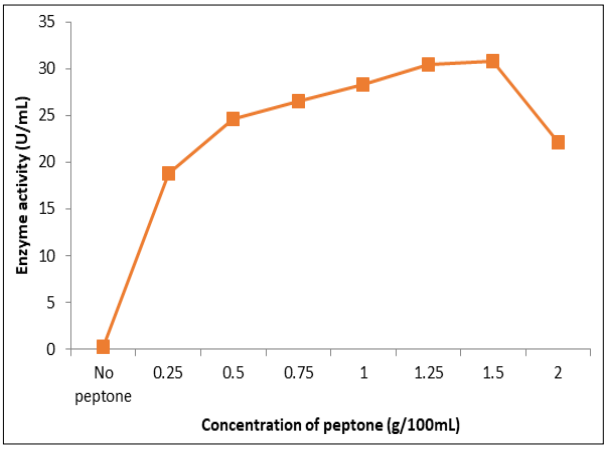

Fig 7b Influence of different concentrations of peptone

The influence of inoculums size on cellulase production by the strain SSV-2 was found very negligible and almost similar enzyme activities were shown by all volumes. Among different inoculums sizes tested, 10% inoculum gave maximum CM Case activity after the incubation period (Fig 8). In a study by Shaikh *et al.* [28], a soil isolate of *Bacillus* has been characterized and that produces maximum CM Case activity when 2% inoculums size was used and in that case also almost same activities were produced by other inoculums volumes also. In this study the bacterial strain SSV-2 produced maximum CM Case activity after 72 hours of incubation (Fig 9). The enzyme production gradually increases from 24 hour up to 72 hours of incubation and after that the enzyme activity gradually falls off. The change in growth and pH parameters of the enzyme production medium during the incubation period is represented in the (Fig 10). In the beginning stages the low cellulase activity may be due to the reason that the strain may use other simpler nutrients supplemented in the growth medium and after that only the cellulose was used and start releasing cellulase. In a study by Chellapandi and Himanshu [6] suggest that native strains of *Streptomyces* species in submerged fermentation produces maximum cellulase activity after 72-88 hours of incubation. There are other similar reports suggesting the late-stage synthesis of cellulase [26]. On the contrary, reports are there suggesting the earlier enzyme secretion like, maximum CM Case activity of *S. viridobrunneus* SCPE-09 was observed after 48h of incubation [7], which is earlier than that noted for the strain SSV-2.

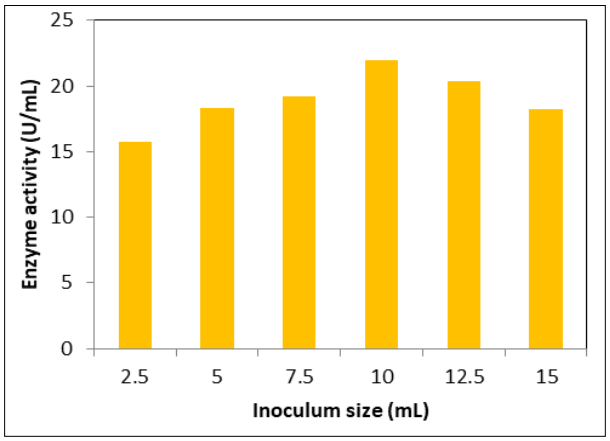

Fig 8 Influence of inoculum size on cellulase production by SSV-2

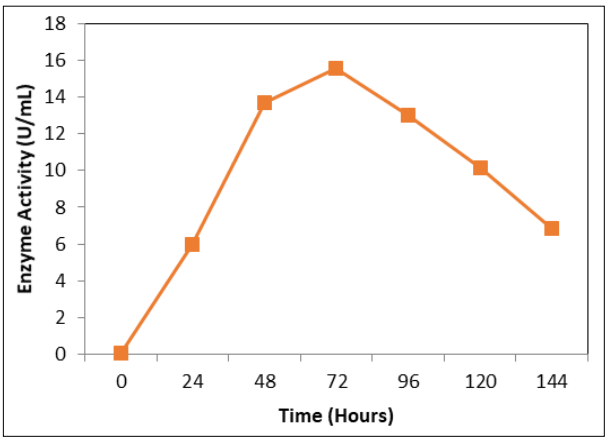

Fig 9 Cellulase production pattern in SSV-2 over different incubation period

While studying agitation speed as a factor in controlling cellulase production in the strain SSV-2, maximum activity was observed at 150 rpm (Fig 11). The factors influencing antimetabolite production by an actinobacterial isolate, *Amycolatopsis sp*. ST-28 from a tea garden soil [2].

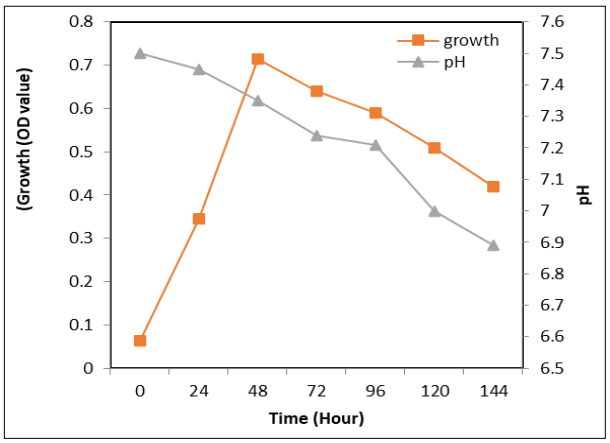

Fig 10 The change in growth and pH of themedium during the incubation period.

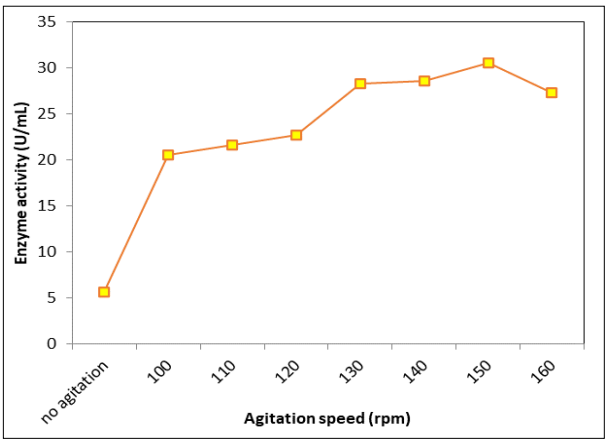

Fig 11 Influence of agitation speed in cellulase production by SSV-2

CONCLUSION

In the present study 32 different cellulolytic soil bacterial strains were isolated from different forest areas of Kerala state and which were further screened for cellulase activity in multiple levels and the strain SSV-2 was identified finally as the top producer of cellulase by submerged fermentation studies. The strain produced a notable quantity of cellulase (15.56 U/mL after 72 hours of incubation in CMC medium). Later the strain was identified by morphological, biochemical and 16S rRNA gene sequencing as *Bacillus velezensis*. The different cultural conditions of the strain were optimized to enhance the cellulase production by the strain SSV-2. The optimized cultural parameters of the strain are 0.5% CMC as the carbon source, 1.5% of peptone as nitrogen source, initial medium pH as 7, inoculums size as 10% and agitation speed as 150 rpm.

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Conflicts of interest

The authors hereby declare "no conflicts of interest.

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