

Production of bioethanol from free and immobilized cellulase produced by *Bacillus subtilis* BTSR1

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In the present situation, entire world is looking forward for an alternate energy source due to increased concern on environmental pollution and energy security. In the present aimed at the production of bioethanol by using free and immobilized cellulase from *Bacillus sp.* In this study, highest cellulase activity was expressed by the bacterial isolate identified as *Bacillus subtilis* BTSR1 (1.817 ± 0.26 U/mL) after 24 hr incubation at 37°C under static condition. In this study, the extracellular cellulose from *B. subtilis* BTSR1 strain was produced, purified and estimated to be 46 kDa with specific activity was 302.27 U/mg. Immobilization process was standardized with respect to concentration of CaCl₂, sodium alginate and bead size in order to obtain maximum efficiency. Different percentages of sodium alginate gel were tried. However, maximum immobilization efficiency was achieved with 4% alginate with 0.2M CaCl₂ and bead size was 10 g. The resultant optimal

conditions for the highest CM Case production by *Bacillus subtilis* under SSF using wheat bran and coffee pulp as substrates were used for the subsequent bioethanol production. Bioethanol production was further optimized by varying substrate concentration (1-5%), Yeast Extract (0.1-0.5%), pH (4-8) and time (2-10 days). Immobilized enzymes produced the highest yield of bioethanol 28 g/l was obtained for 4% of substrate concentration. Immobilized cellulase activity nearly doubled the amount provided from the free enzyme activity. Additionally yeast extract concentration also optimized and ethanol production was found to increase 27 g/l of 0.3% yeast extract concentration. pH and duration were optimized also play a remarkable role in the production of bioethanol. In this study concluded that the maximum amount of bioethanol production was observed in the immobilized enzyme than free enzyme. Thus, immobilized condition was improved the activity of enzyme to produced bioethanol.

Key Words: *Bacillus subtilis*; Immobilization; Bioethanol; Cellulase; Alginate

INTRODUCTION

Lignocellulose forms the major structural component of plants. It is made up of cellulose along with hemicelluloses and lignin strongly interlocked by non-covalent and covalent forces. Annual production of lignocellulosic through photosynthesis is 40 billion tones [1]. The problem of increasing the utility of lignocellulose wastes has been known for decades. It is the most dominating waste material from agriculture and forest [2]. Lignocellulosic biomass is a major resource for the production of bio-fuels since it is largely abundant, inexpensive and eco-friendly. Lignocelluloses are employed for traditional applications (paper manufacture, biomass fuels, composting, animal feed, etc.) since long back and novel markets for lignocellulosic have been identified in recent years. In the field of biotechnology, bioconversion of cellulosic biomass to develop novel and useful products is an important area of research.

Cellulose is degraded by a group of enzymes which acts synergistically to randomly cleave the β -1, 4 glucosidic bonds. The enzyme complex responsible for the hydrolysis of cellulose is cellulases. It is an inducible enzyme secreted by wide variety of fungi, bacteria, protozoan, plants and animals. Cellulose is converted into smaller organic compound called cellooligosaccharides or completely into glucose units by the action of cellulases. Three types of synergistic actions are involved in cellulolysis, i) The synergism between endoglucanase and cellobiohydrolase (endo-exo synergism), ii) The synergism between two cellobiohydrolase (exo-exo synergism), and iii) The synergism between all the three enzymes of the cellulase complex (endo-exo- β -glucosidase). Cellulase are extensively used for the industrial purpose (pulp, textile, paper, food industry, and as an additive in the detergents [3]. Cellulolytic microorganisms can be found in all biota where cellulose biomass accumulates. Cellulase producing bacteria were exploited widely for the extraction, purification and characterization of novel cellulase enzymes, because of their higher growth rate, presence of multi enzyme complex and their wide distribution in different environmental niches. Microorganisms from various environments provides potential source of novel cellulase. Naturally occurring bacterial enzymes from the environment is important in many industrial applications

to help overcome costly hurdles.

Members of the Bacillaceae are Gram-positive, endospore forming bacteria which are known to secrete an extensive array of enzymes, including amylases, proteases, β -glucanases, and hemicellulases. Thus far, these organisms have been found to produce only endo- β -1,4-glucanases and have not been capable of extensively degrading crystalline cellulose. The cellulolytic enzymes of *Bacillus* species are almost entirely extracellular and soluble in form. A variety of species of this genus, including strains of *B. subtilis*, *B. polymyxa*, *B. licheniformis* and *B. cereus*, secrete cellulases. Hence the present aimed at the production of bioethanol by using free and immobilized cellulase from *Bacillus sp.*

MATERIALS AND METHODS

Isolation of cellulolytic bacteria

The soil samples were collected from dump yards regions of Thanjavur, Tamil Nadu, India (Geographical coordinates are 10.726 N, 79.080 E). Serial dilution technique has been used to isolate cellulolytic bacteria from soil sample about 10⁻¹ to 10⁻⁷ dilution factor. The 50 μ L of the diluted sample (10⁻⁵) was plated on the screening medium composed of Carboxymethyl Cellulose (CMC) containing-1.0% carboxymethyl cellulose, 1.0% peptone, 0.2 % (NH₄)₂ SO₄, 0.2 % K₂HPO₄, 0.03% MgSO₄·7H₂O, 0.2% gelatin and 1% agar [4]. The CMC gar plates were incubated for 48 hours at 37°C. For the primary screening of cellulose producing bacteria, point inoculation of the isolates was carried out on the same cellulose screening media plates, followed by incubation at 37°C for 48 hours. The plates were flooded with 0.1% congo red and further washed using 1 M sodium chloride to visualize the zone of clearance [5].

Screening for cellulase enzyme production

The particular strains were further tested for their abilities to produce cellulase under Submerged Fermentation (SMF). The maximum cellulose production isolates were considered for the further study.

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Cellulase production

CMC broth CMC 0.5%, Tyrtone 0.20%, KH_2PO_4 0.4%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, Na_2HPO_4 0.04%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0001%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0004% and pH 7.0 [6] was prepared and allocates 50 ml in 250 ml Erlenmeyer flasks for each of the selected isolated strain. The media were inoculated with 2.5 ml of the selected bacterial isolates from the inoculum media and incubated on 37°C for 48 hours at 150 rpm. After fermentation, the fermented broth was centrifuged to remove unwanted materials at 12000 rpm for 10 minutes at 4°C. Finally, clear supernatant was collected and used to determine enzymatic activity [7].

Partial purification of cellulase

Partial purification was achieved by fractional precipitation using ethanol (50% and 70%). The fractions containing protein pellets were dissolved in 0.01 M phosphate buffer (pH=7), then were examined for cellulase activity and protein content. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE (Laby Instruments Model E-2,)) (8%) for protein molecular weight was estimated according to the standard protocol of Laemmli [8].

Immobilization of enzyme

On calcium alginate beads the basic protocol described by Kierstan and Bucke [9] for immobilization of enzyme on alginate beads was used. Sodium alginate (5 gm) was added slowly to 100 ml of vigorously stirred water. Stirring was continued for 3 hours and thereafter the alginate was left for 24 hours to allow the air bubbles to escape. Next day, 10 ml of buffer A and 2 ml of enzyme was added to the viscous solution of sodium alginate with stirring and the slurry was left overnight to remove air bubbles. The homogeneous slurry was extruded drop wise through a syringe into 0.2 M calcium chloride. Beads were cured in 0.2 M calcium chloride for 4 hours and then washed several times until no protein was detected in the washings. Beads were stored at 4°C in 30 mM calcium chloride solution prior to use.

Bioethanol production

The resultant optimal conditions for the highest CM Case production by *Bacillus subtilis* under SSF using wheat bran and coffee pulp as substrates were used for the subsequent bioethanol production. The reduced glucose sugar, as the product of lignocellulolytic biomass digestions, was used for

fermentation to ethanol by *S. cerevisiae*. Fermentation media was prepared by mixing pretreated substrate with peptone and yeast extract in 100 ml citrate buffer. Media prepared was sterilized and inoculated with baker's yeast and 5 ml of free and Na-alginate immobilized enzyme respectively. The fermentation was carried out at 120 rpm and 30°C. The sample was collected at regular intervals of 24 h for the determination of bioethanol. Bioethanol production was further optimized by varying substrate concentration (1%-5%), Yeast Extract (0.1-0.5%), pH (4-8) and time (2-10 days).

Quantitative measuring of bioethanol

Quantitative estimation of ethanol was carried out by the potassium dichromate method [10] where different concentrations (3-18%) of ethanol (99.9%) were prepared giving 5 mL final volume with distilled water. 1 mL of 10% potassium dichromate was added to each concentration and the final volume of each was kept in ice water followed by gently adding concentrated sulfuric acid on the sidewall giving prepared samples for measuring the Optical Density (OD) at 660 nm. The standard graph was plotted and the unknown sample was estimated [11].

RESULTS AND DISCUSSION

Screening of cellulolytic bacteria

Totally 26 cellulolytic bacterial strains were isolated from the soil sample using the 10-5 dilution plate. Among them, six colonies which produced largest zone and hydrolysis the substrate CMC on CMC-Congo red agar plate by cellulolytic bacteria. Bacterial isolates showed different zones of clearance around the colonies. The zone of clearance around the colony is an indicator of cellulolytic potential of the isolates (Figure 1). The primary screening of potential bacterial isolates such as *Bacillus subtilis* BTSR1, *Pseudomonas aeruginosa* BTSR2, *Serratia fonticola* BTSR3, *Escherichia coli* BTSR4, *Clostridium butyricum* BTSR5 and *Staphylococcus aureus* BTSR6 showed maximum zones like 3.68 ± 0.89 mm, 2.84 ± 0.15 mm, 1.73 ± 0.27 mm, 2.75 ± 0.24 mm, 1.12 ± 0.02 mm and 2.97 ± 0.27 mm respectively (Table 1). Utilization of CMC as carbon source is the best for microbial cellulase production [12]. CMC acts as a good carbon source for the production of cellulase production was also reported by Das et al., [13]. Some investigators showed that additional of cellulose, filter paper, CMC, starch or cellobiose to the fermentation medium forwarded cellulase production of cellulolytic organisms such as *Cellulomonas sp.*, *Clostridium* and *Bacillus sp.*

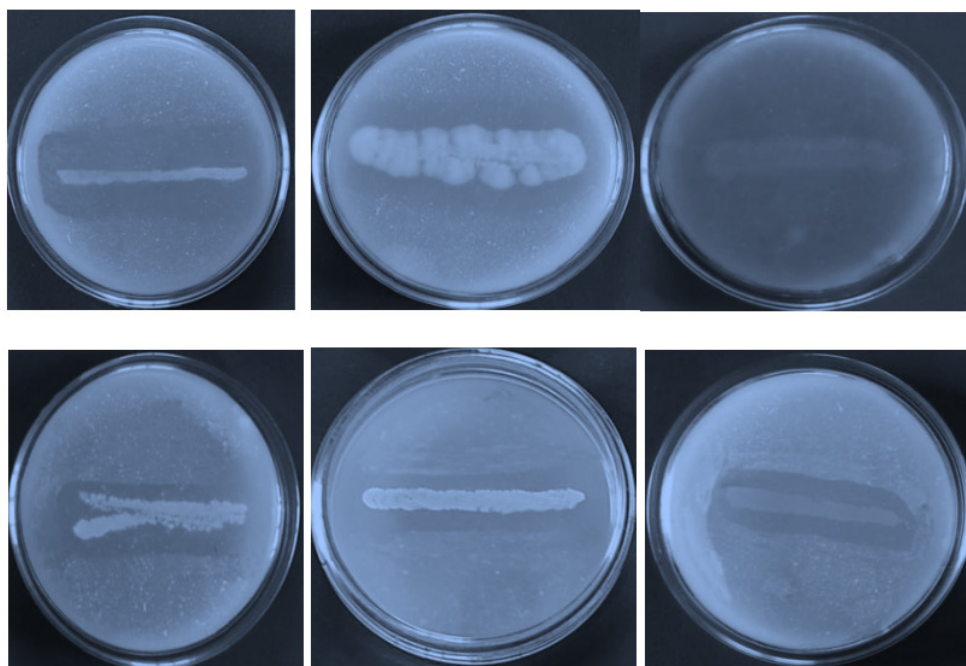


Figure 1) Primary screening of bacteria for cellulase enzyme production

TABLE 1
Primary screening of potential cellulolytic bacterial isolates

S. No	Bacterial isolates	Cellulolytic activity
		Zone of measurement (mm)
1	BTSR1	3.68 ± 0.89
2	BTSR2	2.84 ± 0.15
3	BTSR3	1.73 ± 0.27
4	BTSR4	2.75 ± 0.24
5	BTSR5	1.12 ± 0.02
6	BTSR6	2.97 ± 0.27

Cellulase production

Bacterial isolates were able to produce cellulase under submerged fermentation. The enzyme activity was measured from CMC agar plates. The cellulase yield was recorded by the cellulolytic bacterial isolate such as BTSR1 (1.817 ± 0.26 U/mL), BTSR2 (1.635 ± 0.17 U/mL), BTSR3 (0.427 ± 0.06 U/mL), BTSR4 (1.182 ± 0.15 U/mL), BTSR5 (0.558 ± 0.03 U/mL) and BTSR6 (1.273 ± 0.43 U/mL) after 24 hr incubation at 37°C under static condition (Table 2). Among this, highest cellulase activity was expressed by the bacterial isolate identified as *Bacillus subtilis* BTSR1. So, the strain BTSR1 was considered for the further study. Ojumu et al., [14] investigated about some lignocellulosics, which served as carbon source for the production of cellulase and reported that CMC had a positive influence on the cellulase production. Higher production of cellulase, when CMC served as a substrate may be as a result of induction of the enzyme, since cellulase is known to be a universal inducer of cellulase synthesis [15]. CMC that is the best substrate for cellulase production because it can induce bacteria to produce cellulase enzymes [16]. Screening of potential bacteria from keratinase enzyme activity in basal medium of feather meal broth was screened *Brevibacillus parabrevis* (1.05 ± 0.35 IU/ml) and *Brevibacillus brevis* (1.03 ± 0.14 IU/ml) at 440 nm in spectrophotometer. *Bacillus* species were the predominant isolates encountered in this study [17]. Similar results were reported that the cellulosomal cellulases from *Bacillus megaterium* has both CMCase and avicelase activity; however, the 'cellulases' had a higher activity. The mechanisms by which different types of cellulases enhance each other's activities are complex and not completely understood, and the published data are often inconsistent [18]. A moderately thermophilic *Bacillus* K-12 produced a large amount of cellulase components containing avicelase, xylanase, CMCase and FPase, when grown in avicel medium at 50°C [19].

TABLE 2
Cellulase activity from different isolates

S. No	Bacterial isolates	Enzyme activity (U/MI)
1	BTSR1	1.817 ± 0.26
2	BTSR2	1.635 ± 0.17
3	BTSR3	0.427 ± 0.06
4	BTSR4	1.182 ± 0.15
5	BTSR5	0.558 ± 0.03
6	BTSR6	1.273 ± 0.43

Purification of cellulase

Extra cellular enzyme was purified to homogeneity from the culture extracts. The supernatant containing extra cellular proteins were separated and

extracted by precipitation and it purified by the 40% and 70% ethanol. The cellulolytic protein was 3.27-fold purified with a specific activity of 302.27 U/mg. the total protein content 0.32 mg/mL was obtained from 70% ethanol fraction. 40% ethanol fraction showed the lesser enzyme activity also protein content. In this study, the extracellular cellulase from *B. subtilis* BTSR1 strain was produced, purified and estimated to be 46 kDa with specific activity was 302.27 U/mg (Table 3). In the previous report, Halophilic *A. gracilis* isolated from a hypersaline manmade saltern had the potential to produce extracellular amylase, which was extracted, purified and verified by SDS-PAGE analysis, showing a single band of approximately 35 kDa with a specific activity of 131.02 U/mg [20]. An extracellular α-amylase from the obligate halophilic *A. penicillioideus* TISTR3639 strain was produced, purified, and estimated to be 42 kDa by SDS-PAGE with a specific activity of 118.42 U/mg [21].

TABLE 3
Purification of cellulase from BTSR1 by ethanol fractional precipitation

Purification step	Total activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Purification (Fold)
Ethanol Fraction 40%	38.27	0.08	87.32	1.54
Ethanol Fraction 70%	72.49	0.32	302.27	3.27

Immobilization of enzyme

Immobilization process was standardized with respect to concentration of CaCl₂, sodium alginate and bead size in order to obtain maximum efficiency (Figure 2). Since the cross linking between sodium alginate and calcium ions leads to gelation, the gel entrapment of an enzyme depends on the concentration of both, and the effect of concentration of these constituents was investigated on the efficiency of immobilization. Different percentages of sodium alginate gel were tried. However, maximum immobilization efficiency was achieved with 4% alginate with 0.2M CaCl₂ and bead size was 10 g. Immobilization of enzymes on suitable supports has been observed to overcome these limitations. The enhanced operational stability of the immobilized enzymes/biocatalysts [22] has been of great significance for their exploitation at commercial level. Besides enhanced stability, on immobilization, enzymes can also acquire additional advantageous properties such as their repeated use and their separation from soluble reaction products and untreated substrate. It also improves their application in solvents. Immobilization often stabilizes the structure of enzymes also, thereby allowing their applications even under harsh environmental conditions of temperature, pH and organic solvents [23]. In previous study Xylanase was entrapped in calcium alginate beads by adding enzyme preparation in sodium alginate drop wise with needles into solution of CaCl₂ [24].

Bioethanol production by fermentation

The *S. cerevisiae* was proved to be very effective in converting glucose to ethanol in SSF. In this study, bioethanol production was optimized for the highest yield of bioethanol. The substrate wheat bran and coffee pulp were optimized by different concentration from 1-3% (w/v). Immobilized enzymes produced the highest yield of bioethanol 28 g/l was obtained for 4% of substrate concentration and followed by 3% substrate. Free enzymes showed the 17 g/l for 4% substrate (Figure 3). Immobilized cellulase activity nearly doubled the amount provided from the free enzyme activity. Additionally yeast extract concentration also optimized and ethanol production was found to increase 27 g/l of 0.3% yeast extract concentration (Figure 4). pH optimized also play a remarkable role in the production of bioethanol (Figure 5). The highest ethanol production was found to be at pH 5 (21 g/l) and followed by pH 6 (18 g/l). Duration of fermentation was importance for bioethanol production. In this study, highest amount of ethanol yield on 4th day of fermentation (Figure 6). In this study, maximum amount of bioethanol production was observed in the immobilized enzyme than free enzyme. Thus, immobilized condition improved the activity of enzyme to produced bioethanol.

In previous study reported that bioethanol production by *Aspergillus species*

by incubating amylase producing fungus and baker's yeast (*S. cerevisiae*) at the same time. The evaluated quantity of produced bioethanol was 10.9 g/l within 4 days incubation period [25]. Moreover, *F. oxysporum* was used for converting starch-rich disposal foods to bioethanol with the aid of *S. cerevisiae* cultivation, such combinations were developed by two pathways giving a bioethanol production of 16.3 g/l, and simultaneous fermentation (20.6 g/l) of bioethanol production all in one step [26]. In similar study revealed that the highest enzyme yield (165.24 U/mL) was provided by the immobilized amylase with 1% of covalent bonding agents solution, providing double the sugars' byproduct than from the free amylase form, and double the bioethanol fermentation yield with 0.12 g/g sugar/l. Highly specific activity of such enzyme in the immobilized form could offer a highly effective approach for bioethanol production on the industrial scale than the free and

other reported forms [27].

Cellulase enzymes that are formed will break down the cellulose contained in the carbohydrate substrate into glucose. The glucose formed will be used by microbes for growth. These compounds together with ammonium will form protein, therefore, it is necessary to see the relationship between proteins formed by microbes and the activity of enzymes resulting from hydrolysis [28]. According to Itelima et al., [29] that the protein content of fermentation is varied (depending on the raw material used). Reactor level aerobic batch fermentation with optimized process conditions offered a maximum ethanol concentration of 0.06 g/L from 1 g/L of pretreated sugarcane bagasse in [30].

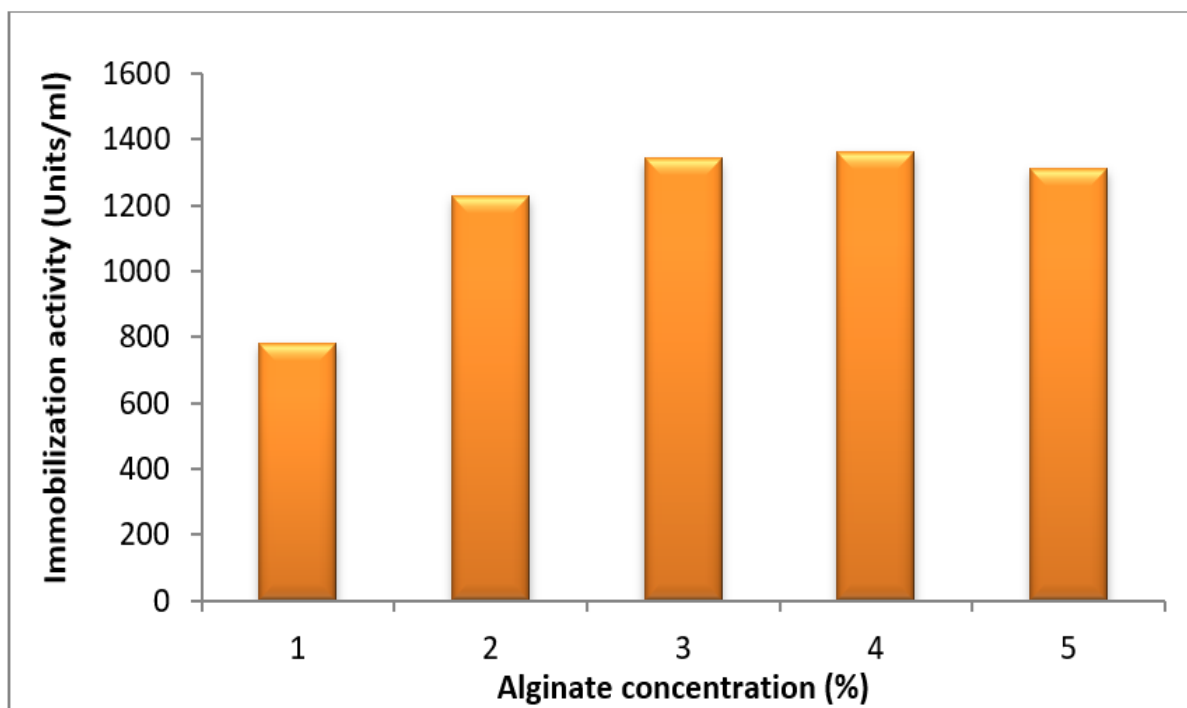


Figure 2) Immobilization activity of cellulase by different concentration of alginate

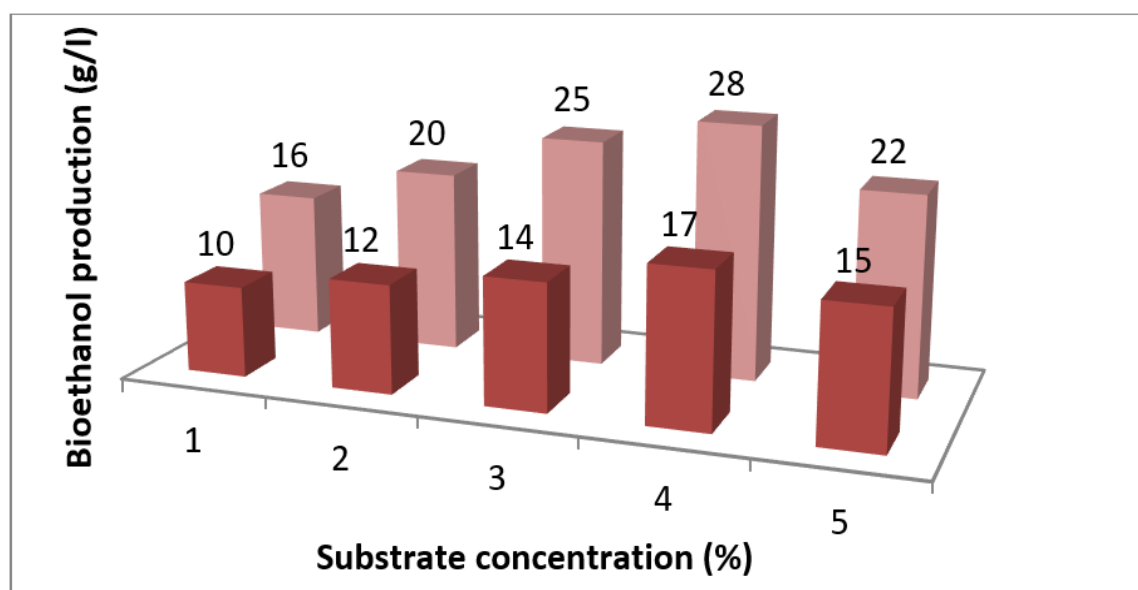


Figure 3) Optimization of bioethanol production by various substrate concentrations of free and immobilized enzymes, Note: (■) Free enzyme, (■) Immobilized enzyme

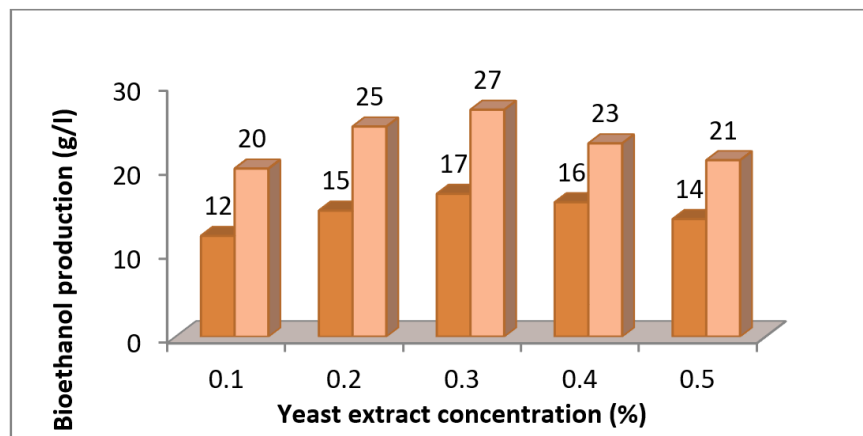


Figure 4) Optimization of bioethanol production by various yeast extract concentrations of free and immobilized enzymes, Note: (■) Free enzyme, (■) Immobilized enzyme

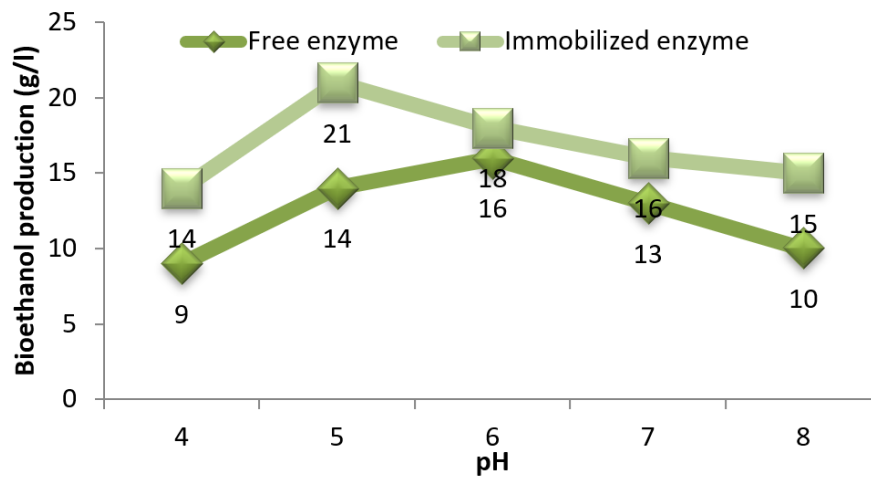


Figure 5) Optimization of bioethanol production by various pH of free and immobilized enzymes

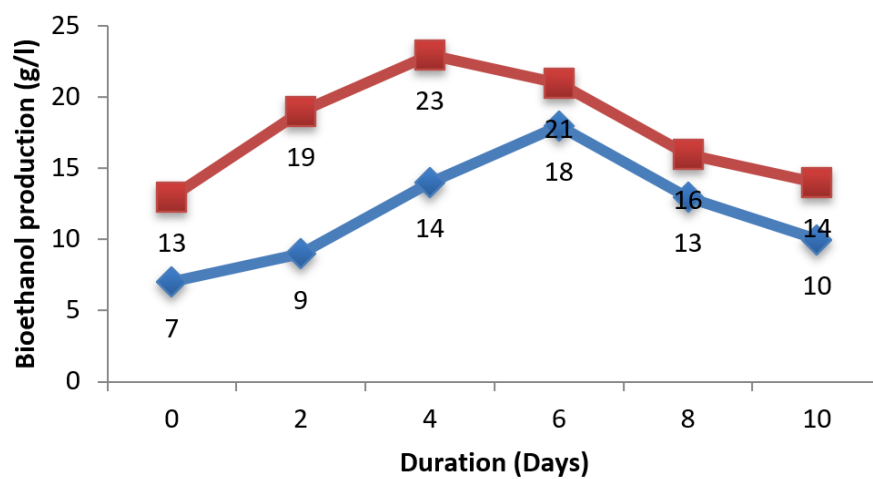


Figure 6) Optimization of bioethanol production by time duration of free and immobilized enzymes, Note: (■) Free enzyme, (■) Immobilized enzyme

CONCLUSION

Cellulase is an industrial enzyme which helps in the conversion of lignocellulosic materials into simpler units. In this study, highest cellulase activity was expressed by the bacterial isolate identified as *Bacillus subtilis* BTSR1. Further, cellulase enzyme was purified and immobilized with sodium alginate. In addition, the results concluded the higher potency of immobilized cellulase than the free enzyme. Thus, the cellulase immobilized can be effectively used for production of bioethanol.

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REFERENCES

- Coughlan MP. The properties of fungal and bacterial cellulases with comment on their production and application. *Biotechnol Genet Eng Rev*. 1985;3(1):39-110.
- Lynd LR, Weimer PJ, Van Zyl WH, et al. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev*. 2002;66(3):506-577.
- Bayer EA, Lamed R, Himmel ME. The potential of cellulases and cellulosomes for cellulosic waste management. *Curr Opin Biotechnol*. 2007;18(3):237-245.
- Maravi P, Kumar A. Isolation, screening and identification of cellulolytic bacteria from soil. *Biotechnol J Int*. 2020;24(1):1-8.
- Bhagat SA, Kokitkar SS. Isolation and identification of bacteria with cellulose-degrading potential from soil and optimization of cellulase production. *J Appl Biol Biotechnol*. 2021;9(6):154-161.
- Irfan M, Safdar A, Syed Q, et al. Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. *Turkish J Biochem*. 2012;37(3).
- Hossain MA, Ahammed MA, Sobuj SI, et al. Cellulase Producing Bacteria Isolation, Screening and Media Optimization from Local Soil Sample. *Am J Microbiol Res*. 2021;9(3):62-74.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680-685.
- Kierstan M, Bucke C. The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotechnol Bioeng*. 2000;67(6):726-736.
- Caputi A, Ueda M, Brown T. Spectrophotometric determination of ethanol in wine. *Am J Enol Vitic*. 1968;19(3):160-165.
- Babu BK, Atiyeh HK, Wilkins MR, et al. Effect of the reducing agent dithiothreitol on ethanol and acetic acid production by *Clostridium* strain P11 using simulated biomass-based syngas. *Biol Eng Trans*. 2010;3(1):19-35.
- Fukumori F, Kudo T, Horikoshi K. Purification and properties of a cellulase from alkalophilic *Bacillus sp.* no. 1139. *Microbiology*. 1985;131(12):3339-3345.
- Das A, Bhattacharya S, Murali L. Production of cellulase from a thermophilic *Bacillus sp.* isolated from cow dung. *AM Eurasian J Agric Environ Sci*. 2010;8: 685-691.
- Ojumu TV, Solomon BO, Betiku E, et al. Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse and corncob. *Afr J Biotechnol*. 2003;2(6):150-152.
- Niranjan AP, Madhou P, Stevenson TW. The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. *Enzyme Microb Technol*. 2007;40(6):1464-1468.
- Abou-Taleb KA, Mashhoor WA, Nasr SA, et al. Nutritional and environmental factors affecting cellulase production by two strains of cellulolytic *Bacilli*. *Aust J Basic Appl Sci*. 2009;3(3):2429-2436.
- Indira M, Rajakumar R. Screening and Characterization of Feather Degrading Bacteria from Poultry Waste and its Potential of Enzyme Activity. *Int J Agric Sci*. 2022;13(2): 455-459
- Beukes N, Pletschke BI. Effect of sulfur-containing compounds on *Bacillus* cellulosome-associated 'CMCase' and 'Avicelase' activities. *FEMS Microbiol Lett*. 2006;264(2):226-231.
- Kim DS, Kim CH. Production and characterization of crystalline cellulose-degrading cellulase components from a thermophilic and moderately alkalophilic bacterium. *J Microbiol Biotechnol*. 1992;2(1):7-13.
- Ali I, Akbar A, Yanwisetpakdee B, et al. Purification, characterization, and potential of saline waste water remediation of a polyextremophilic α -amylase from an obligate halophilic *Aspergillus gracilis*. *BioMed Res Int*. 2014.
- Ali I, Akbar A, Anwar M, et al. Purification and characterization of a polyextremophilic α -amylase from an obligate halophilic *Aspergillus penicillioides* isolate and its potential for souse with detergents. *BioMed Res Int*. 2015.
- Beltagy EA, Abouelwafa A, Barakat KM. Bioethanol production from immobilized amylase produced by marine *Aspergillus flavus* AUMC10636. *Egypt J Aquat Res*. 2022;48(4):325-331.
- Fortin C, Vuilleumard JC. Culture fluorescence monitoring of immobilized cells. *Physiology of immobilized cells*. Amsterdam: Elsevier. 1990:45-55.
- Sidarta E, Syaputra DA, Djafar F. Nilai kadar protein dan aktivitas amilase selama proses fermentasi umbi kayu dengan *Aspergillus niger*. *Fakultas Teknobiologi Universitas katolik Atma Jaya.(Skripsi)*. 2010.
- Kompiang I P. Peningkatan Mutu Bahan Baku Pakan. *Prosiding Seminar Nasional Teknologi Pertanian Ramah Lingkungan*. Kerjasama IPPTP Denpasar, PSE, Balitbangtan dan Universitas Udayana, Denpasar (in Indonesian). 2000.
- Pandey A, Nigam P, Soccol CR, et al. Advances in microbial amylases. *Biotechnol Appl Biochem*. 2000;31(2):135-152.
- Rameswara Reddy Naru, Malarselvi S, Rajakumar R. A Study on dye decolouration in free and immobilized Xylanase and its application in Pulp and Paper mill effluent. *Int J Basic Appl Res*. 2018;8(9): 95-105.
- Sasikumar E, Viruthagiri T. Simultaneous saccharification and fermentation (SSF) of sugarcane bagasse-kinetics and modeling. *Int J Chem Biol Eng*. 2010;3(2):57-64.
- Itelima J, Ogonna A, Pandukur S, et al. Simultaneous saccharification and fermentation of corn cobs to bio-ethanol by co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae*.
- Prasoulas G, Gentikis A, Konti A, et al. Bioethanol production from food waste applying the multienzyme system produced on-site by *Fusarium oxysporum* F3 and mixed microbial cultures. *Fermentation*. 2020;6(2):39.