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Screening and Optimization for Cellulase Production by Soil Bacterial Isolates JRC1 and JRC2

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ABSTRACT

Cellulase production by soil bacteria can be beneficial as the cellulase production rate is comparatively advanced due to the hasty bacterial rate and bacteria are competitively less or nil pathogenic as compared to fungi. Screening of bacteria and optimization of fermentation conditions such as substrate concentration, pH, temperature, incubation period are imperative for successful production of cellulase. The present study is conducted to produce cellulase by local isolates JRC1 and JRC2 using Carboxymethylcellulose (CMC) as substrate. Following that, cellulase produced from isolates were partially purified using ammonium sulphate precipitation method for the characterization of cellulase. Cellulase was successfully produced and optimized with substrate concentration 3%, pH 7, and temperature 35 ^{0}C and inoculum size 10%. It provides current data for cellulase production by soil bacteria as well as its optimization procedures and an acumen into the various mechanisms bacteria uses to tolerate, survive and carryout processes that could potentially lead to the eco-friendly tactic for bio-conversion of cellulose. It also provides biotechnological aspects, application of cellulase research.

KEY WORDS: BIOCONVERSION, CELLULASE, CELLULYTIC BACTERIA, ENZYME PRODUCTION, OPTIMIZATION.

INTRODUCTION

Cellulase play significant role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic bacteria. The bioconversion of cellulose into glucose is nowadays recognized to comprise of two steps in the enzyme system. In the initially stage , β-1, 4 glucanase breakdowns the glucosidic linkage to cellobiose, which is a glucose dimer with a β-1, 4 linkage as contrasting to maltose, a complement with a α-1, and 4 linkage. Subsequently in the second stage, this β-1, 4 glycosidic linkage is fragmented by β-glycosidase.

Cellulase is one of the most convenient enzyme found on the earth. Which is used in several industries now a days for various purposes. Cellulase is mainly used to break such a complex polysaccharide substance that is known as cellulose. Which is the most lavish and most leading biomass on earth.it is the primary product of photosynthesis in terrestrial environments and most copious renewable bio resource produced in biosphere. Cellulose accounts for 50% of the dehydrated mass of herbal biomass and approximately 50% of the dehydrated weight of subordinate sources of biomass

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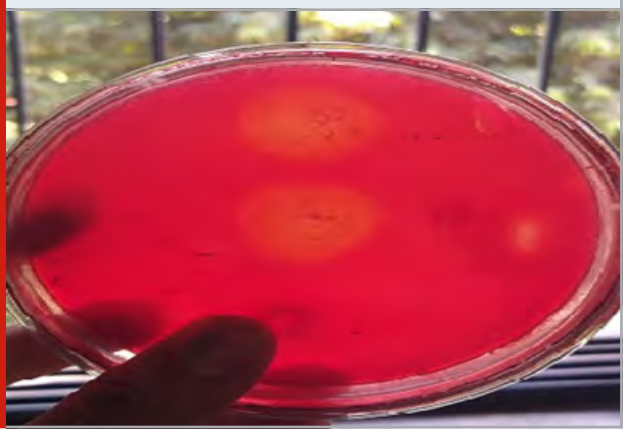
such as agricultural trashes(Patagundi, Shivasharan, & Kaliwal, 2014).

Cellulase is mainly produced by the several organisms such as bacteria, fungi, protozoa, and actinomycetes. However the growth rate, genetic engineering is so concerned bacteria are more preferential as their growth rate is normally higher and it is to manipulate bacterial gene as compared to other organisms also it has the nil or least pathogenicity as compared to fungus. The cellulolytic property of some bacterial genera such as Cellulomonas (Saratale, Saratale, Lo, & Chang, 2010), Cellvibrio, (Berg, von Hofsten, & Pettersson, 1972) Pseudomonas (Kasana, Salwan, Dhar, Dutt, & Gulati, 2008) Bacillus (Shankar & Isaiarasu, 2011) and Micrococcus (Mmango-Kaseke, Okaiyeto, Nwodo, Mabinya, & Okoh, 2016) has been reported.

Now a days cellulase is tremendously used in various industries including paper and pulp, detergent, fermentation, food, (Bhat & Bhat, 1997; Phitsuwan, Laohakunjit, Kerdchoechuen, Kyu, & Ratanakhanokchai, 2013; Ryu & Mandels, 1980) and many more as described in this research. So, cellulase enzyme will be the furthermost thrilling technology of future. Continuous research for developments in marked features for cellulase production (such as rate, substrate specificity, and specific activity) is looked for to accomplish improved techno economic feasibility. The present work was performed to optimize the nutritive and environmental constraints for cultivating cellulase production by bacterial isolates JRC1 and JRC2.

Figure 1: Zone of clearance on CMC Agar plate



MATERIALS AND METHODS

Screening and Isolation of Cellulase Producing Bacteria: Cellulolytic bacteria were isolated from soil by the dilution pour plate or spread plate method using CMC (Carboxymethyl cellulose) agar media. Tenfold serial dilutions of each sample was taken. The modified medium composition of CMC agar (g/L) E: CMC 10; KH_2PO_4 1.0; $MgSO_4.7H_20$ 0.5; NaCl 0.5; $FeSO_4.7\ H_2O$ 0.01; NH_4NO_3 0.3; agar 12 (Kuhad, Gupta, & Singh, 2011) and the pH is adjusted to 7.0; the plates were then incubated at 30 0C, 35 ^{0}C, 40 ^{0}C, and 45 ^{0}C for 24-48 hours. To envisage the clear zone of cellulose hydrolysis, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 % NaCl (Sethi, Datta, Gupta, & Gupta, 2013). To specify the cellulolytic activity of the organisms, diameter of the clear zone around colonies on CMC agar was measured. Beside, a more quantitative assay method was used to determine the cellulose activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a 3,5-Dinitrosalicylic acid (DNSA) method (Miller, 1959). Bacterial isolates showing highest activity were selected for optimization of cellulase production.

Bacterial Identification on the Basis of Morphological and Biochemical Characteristics: The bacterial isolates JRC1 and JRC2 were preliminarily identified by means of morphological, cultural and biochemical tests. The examination includes colony morphology, gram's reaction and various biochemical tests such as methyl red, VP, Sugar fermentation, citrate utilization, starch utilization, lipid utilization, urea utilization, gelatine hydrolysis, H2S production, indole production, casein hydrolysis, catalase test and dehydrogenase test. The results were compared to the bergey's manual of determinative bacteria for partial identification (Sobur et al., 2019).

Table 1. Morphological and cultural characterization of cellulase producing isolates JRC1 and JRC2

Isolates	Colony characteristics On Nutrient Agar medium	On CMC Agar plate	Morphological characteristics
JRC1	Small, circular, entire, raised, smooth, Opaque with no pigment	Growth observed with small , circular, smooth colonies	Gram positive Big rods arranged single and in chains Actively motile
JRC2	Medium, circular, entire, raised, smooth, moist, Translucent with no pigment	Growth observed with medium, circular, moist colonies	Gram positive Big rods arranged in long chains. Actively motile

Cellulase enzyme production by submerged fermentation: Cellulase enzyme produced was checked by the submerged fermentation (Tolan & Foody, 1999) process with the modified medium composition that is (g/100mL) CMC 2g; Peptone 0.2g; $FeSO_4$ 0.1; KH_2PO_4 0.1; $MgSO_4$ 0.1 and pH adjusted to 7.0; 50ml of the medium is taken in the 250 mL Erlenmeyer's flask. The flasks were sterilized in autoclave at 121 ^{0}C at 15 lbs pressure for 20 minutes. The inoculum medium was incubated at 37 ^{0}C in rotatory shaker for 24 hour. Then it is transferred to fermentation medium, the composition of the fermentation medium is same, as the inoculum medium except the concentration of CMC is 1% instead of 0.5%; after the successful transformation of inoculum medium into fermentation medium, fermentation medium was kept at 37 ^{0}C in rotatory shaker for 72 hours with 150 RPM.

Table 2. result of biochemicaltests

NO	Name of the test	JRC1	JRC2
1	Methyl red test	-	
	-		
2	VP test	-	-
3	Sugar fermentation		
	1.glucose	⊥	⊥
	2.manitol	⊥	⊥
	3.xylose	⊥	⊥
	4.lactose	⊥	⊥
	5.maltose	-	-
	6.sucrose	-	-
4	Citrate utilization test	-	-
5	Starch utilization	+	+
6	Lipid utilization		
	+	-	
7	Urea utilization	-	-
8	Indole production	-	-
9	H2s production	-	-
10	Gelatine hydrolysis	-	-
11	Casein hydrolysis	+	+
12	Catalase test	+	+
13	Dehydrogenase test	+	+
NOTE:	(-)Negative, (+)Positive, (⊥) presence of only acid		

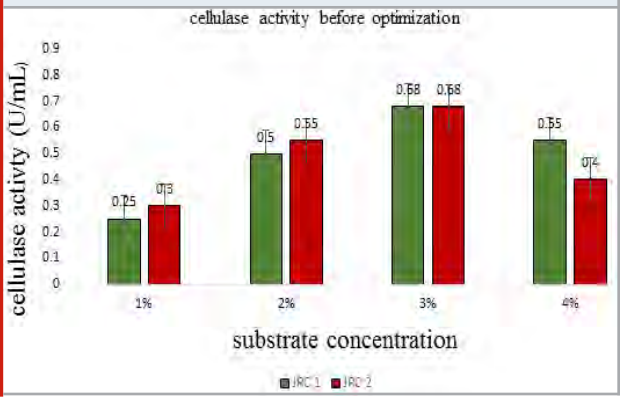
Production of Crude Enzyme: After the end of fermentation period the culture medium was centrifuged at 1500 rpm for 15 minutes to obtain crude enzyme(Shanmugapriya, Saravana, Krishnapriya, Mythili, & Joseph, 2012).

Cellulase Enzyme Assay: Cellulase enzyme activity was measured by using DNSA method (Miller, 1959). CMCase activity, which uses Carboxymethylcellulose as substrate in brief reaction mixture composed of 1mL of crude enzyme plus 1mL of 1% of CMC solution made up in the phosphate buffer pH 7 as substrate. The reaction was carried out at 55 ^{0}C for 20 minutes. The reaction was determined by adding 3 mL of DNSA reagent. The colour was then developed by boiling mixture for 5 minutes. OD of samples was measured at 575 nm against a blank having all other reagents except the crude enzyme. The quantity of reducing sugar unconfined by the hydrolysis was measured. The amount of CMCase required to release 1µmole of reducing sugar per mL per minute under given assay condition is determined as enzyme unit (EU) (Lokhande & Pethe, 2017).

Optimization for cellulase production: Furthermore optimization methods were carried out for different parameters cited following to improvise the cellulase activity, different modified methods have been adapted from the references cited following as well: pH Fermentation broth containing the optimum concentration of substrate and carbon source are taken and the pH of the broth is adjusted to 5.0, 7.0, 9.0 and 11.0 in different flasks with the help of 1 N HCl and 1 N NaOH (Acharya, Acharya, & Modi, 2008). The cultures are inoculated and incubated at optimum temperature. At the end of incubation period, the cell-free culture filtrate is taken and used as crude enzyme.

Temperature: Fermentation medium with pH 7 was inoculated with 24 hour grown selected bacterial strain. The broth was incubated at different temperatures ranging from 30 ^{0}C, 35 ^{0}C, 40 ^{0}C, 45 ^{0}C and 50 ^{0}C for 24 hour (Liang, Zhang, Wu, Wu, & Feng, 2014). After sufficient incubation period, the cell-free culture extract is gained and used as crude enzyme.

Figure 2: cellulase activity before optimization



Substrate concentration: Fermentation medium with pH 7 was inoculated with 24 hour grown selected bacterial strain with different concentration of CMC as substrate such as 1%, 2%, 3% and 4% which is incubated at optimum temperature for 24-72 hours. After 72 hours of successful incubation period, the cell-free culture extract is obtained and used as enzyme source.

Inoculum size: Inoculum size to be transferred in to production medium is checked for the optimization by taking different inoculum size of 6%, 8%, 10% and 12 % which is further transferred to an ideal production medium with overnight grown culture and incubated at optimum temperature for 24-72 hours.

RESULTS AND DISCUSSION

Bacteria with cellulase production potential were isolated from soil and on the Basis of their morphological, cultural and biochemical characteristics, the efficient two isolates were designated as JRC1 & JRC2. As shown in the figure 1 cellulose degrading bacteria were isolated on CMC agar which shows the clear zone of hydrolysis with diameter of 8.1 mm and 8.7 mm of JRC1 and JRC2 respectively. Screening of bacteria was accompanied by using the Congo red test as a initial study for identifying cellulase producers. After 3 days of incubation, both the two isolates JRC1 and JRC2 confirmed positive results in the Congo red test. Since the sole carbon source in CMC agar was cellulose, therefore the result of the test were a strong manifest that cellulase was produced in order to degrade cellulose. Partial Bacterial Identification was done on the basis of morphological, cultural and biochemical characteristics as described below in the table. As mentioned in figure 2 preliminary cellulase enzyme assay for cellulase activity shows maximum cellulase activity about 0.68 U/mL with the substrate concentration of 3 % and the standard pH of 7.0, and temperature of 37^{0}C. this result was taken before the optimization. As compared to 0.07 U/mL (Ariffin, Abdullah, Umi Kalsom, Shirai, & Hassan, 2006) isolates JRC1 and JRC2 gives comparatively higher activity.

Figure 3: Effect of pH on cellulase activity

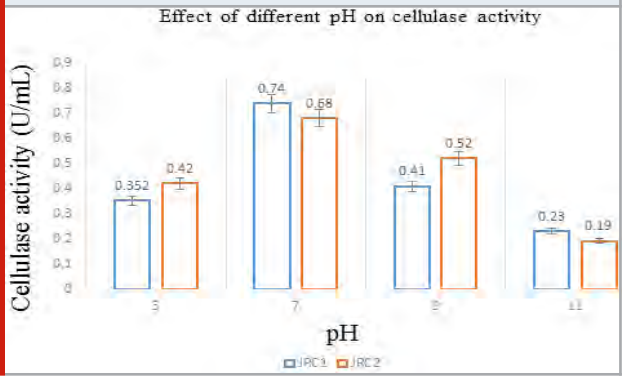


Figure 4: Effect of Temperature on cellulase activity

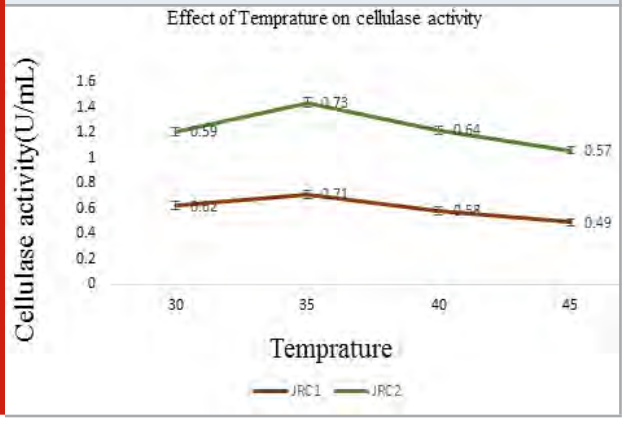


Figure 5: Effect of Substrate concentration on cellulase activity

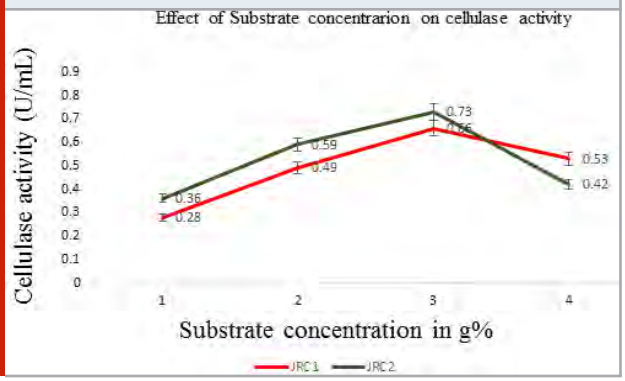
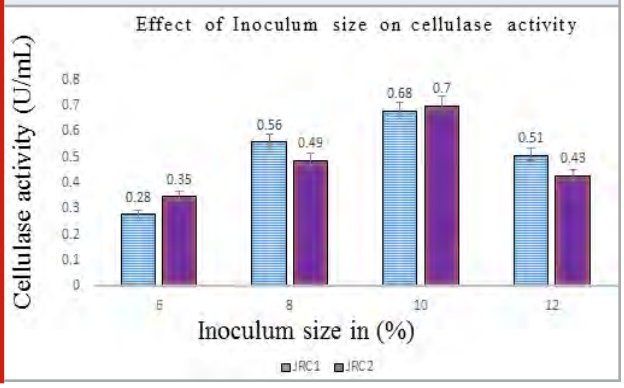


Figure 6: Effect of Inoculum size on cellulase activity



Optimization for cellulase production: Effect of pH: Both the isolates were indorsed to grow in medium of various pH ranging from 5.0 to 11.0 in odd digits. Maximum enzyme activity up to 0.74 U/mL and 0.68 U/mL was observed at pH of 7.0 in case of JRC1 and JRC2 respectively (Figure: 3). which is found to be fairly higher than (Ariffin et al., 2006) which was 0.6 U/mL in case of *B. pumilus*.

Effect of Incubation Temperature: cellulase activity substantiated at various temperatures exposed that both the isolates JRC1 and JRC2 generated determined cellulase production up to 0.71 U/mL and 0.73 U/mL respectively at 35 0C (Figure: 4). Where in case of ASN 2 (Irfan, Safdar, Syed, & Nadeem, 2012) it was nearly 0.15 U/mL .

Effect of Substrate concentration on cellulase activity: to check the maximum enzyme activity by means of substrate concentration, various amount of CMC (in g %) was taken ranging from 1 to 4 %. it is proved that isolates JRC1 and JRC2 shows maximum enzyme activity 0.66 U/mL and 0.73 U/mL respectively at substrate concentration of 3 % (Figure: 5). Which is comparatively higher than the *micrococcus spp*. Which is around 0.02 U/mL with substrate concentration of 2 % (Immanuel, Dhanusha, Prema, & Palavesam, 2006).

Effect of Inoculum size on cellulase activity: cellulase enzyme activity was also been influenced by the inoculum size ranging from 6 to 12 (in g %). Where

results obtained showed that 10% inoculum size bought the highest cellulase production compared to other inoculum size (in g %) (Figure: 6). In this existing work we are trying to determine the effect of inoculum size on cellulase activity. First time we are reporting the optimum inoculum size for cellulase production which is 10%.

CONCLUSION

The objective of existing work was to isolate cellulose degrading bacteria with potential of more cellulase production capability from soil. This study clearly demonstrate the production of cellulase from isolates JRC1 and JRC2 which produced maximum yield of cellulase. Optimum temperature and pH were determined as 35 ^{0}C and pH 7.0, substrate concentration and inoculum size were found to be 3 % and 10% respectively. Cellulase production is carefully dignified in bacteria and for improving its production capacity these controls can be amended. Cellulase yields appear to relay on complex association having a range of factors like inoculum size, pH, temperature, substrate concentration, presence of inducers and so forth.

Conflict of interest: There is no conflict of interest regarding the data published and authorship.

REFERENCES

Acharya, P., Acharya, D., & Modi, H. (2008). Optimization for cellulase production by Aspergillus niger using saw dust as substrate. African Journal of Biotechnology, 7(22). doi: 10.5897/ajb08.689

Ariffin, H., Abdullah, N., Umi Kalsom, M., Shirai, Y., & Hassan, M. (2006). Production and characterization of cellulase by Bacillus pumilus EB3. Int. J. Eng. Technol, 3(1), 47-53. ISSN 1823-1039

Berg, B., von Hofsten, B., & Pettersson, G. (1972). Growth and cellulase formation by Cellvibrio fulvus. J Appl Bacteriol, 35(2), 201-214. doi: 10.1111/j.1365-2672.1972.tb03691.x

Bhat, M., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnology advances, 15(3-4), 583-620. doi: 10.1016/s0734-9750(97)00006-2

Immanuel, G., Dhanusha, R., Prema, P., & Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. International Journal of Environmental Science & Technology, 3(1), 25-34. doi: 10.1007/BF03325904

Irfan, M., Safdar, A., Syed, Q., & Nadeem, M. (2012). Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. Turkish Journal of Biochemistry/Turk Biyokimya Dergisi, 37(3). DOI: 10.5505/tjb.2012.09709

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current microbiology, 57(5), 503-507. doi: 10.1007/s00284-008-9276-8

Kuhad, R. C., Gupta, R., & Singh, A. (2011). Microbial cellulases and their industrial applications. Enzyme research, 2011. doi: 10.4061/211/280696

Lokhande, S., & Pethe, A. (2017). Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production. International J. of Life Sciences, 5(2), 277-282. ISSN 2320-7817

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31(3), 426-428. doi: 10.1021/ac60147a030

Mmango-Kaseke, Z., Okaiyeto, K., Nwodo, U. U., Mabinya, L. V., & Okoh, A. I. (2016). Optimization of cellulase and xylanase production by Micrococcus species under submerged fermentation. Sustainability, 8(11), 1168. doi: 10.3390548111168

Patagundi, B. I., Shivasharan, C., & Kaliwal, B. (2014). Isolation and characterization of cellulase producing bacteria from soil. International Journal of Current Microbiology and Applied Sciences, 3(5), 59-69. ISSN 2319-7706

Phitsuwan, P., Laohakunjit, N., Kerdchoechuen, O., Kyu, K. L., & Ratanakhanokchai, K. (2013). Present and potential applications of cellulases in agriculture, biotechnology, and bioenergy. Folia Microbiol (Praha), 58(2), 163-176. doi: 10.1007/s12223-012-0184-8

Ryu, D. D., & Mandels, M. (1980). Cellulases: biosynthesis and applications. Enzyme and Microbial Technology, 2(2), 91-102. doi: 10.1186/s12934-016-0507-6

Saratale, G. D., Saratale, R. G., Lo, Y. C., & Chang, J. S. (2010). Multicomponent cellulase production by Cellulomonas biazotea NCIM-2550 and its applications for cellulosic biohydrogen production. Biotechnol Prog, 26(2), 406-416. doi: 10.1002/btpr.342

Sethi, S., Datta, A., Gupta, B. L., & Gupta, S. (2013). Optimization of cellulase production from bacteria isolated from soil. ISRN Biotechnol, 2013, 985685. doi: 10.5402/2013/985685

Shankar, T., & Isaiarasu, L. (2011). Cellulase production by Bacillus pumilus EWBCM1 under varying cultural conditions. Middle-East Journal of Scientific Research, 8(1), 40-45. ISSN 1990-9233

Shanmugapriya, K., Saravana, P., Krishnapriya, M. M., Mythili, A., & Joseph, S. (2012). Isolation, screening and partial purification of cellulase from cellulase producing bacteria. Int J Adv Biotechnol Res, 3, 509-514. ISSN 0976-2612

Sobur, M. A., Sabuj, A. A. M., Sarker, R., Rahman, A. T., Kabir, S. L., & Rahman, M. T. (2019). Antibiotic-resistant Escherichia coli and Salmonella spp. associated with dairy cattle and farm environment having public health significance. Veterinary world, 12(7), 984. doi: 10.14202/vetworld.2019.984-993.

Tolan, J. S., & Foody, B. (1999). Cellulase from submerged

fermentation Recent progress in bioconversion of lignocellulosics (pp. 41-67): Springer. Acharya, P., Acharya, D., & Modi, H. (2008). Optimization for cellulase production by Aspergillus niger using saw dust as substrate. African Journal of Biotechnology, 7(22). doi: 10.5897/ajb08.689

Ariffin, H., Abdullah, N., Umi Kalsom, M., Shirai, Y., & Hassan, M. (2006). Production and characterization of cellulase by Bacillus pumilus EB3. Int. J. Eng. Technol, 3(1), 47-53. ISSN 1823-1039

Berg, B., von Hofsten, B., & Pettersson, G. (1972). Growth and cellulase formation by Cellvibrio fulvus. J Appl Bacteriol, 35(2), 201-214. doi: 10.1111/j.1365-2672.1972.tb03691.x

Bhat, M., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnology advances, 15(3-4), 583-620. doi: 10.1016/s0734-9750(97)00006-2

Deswal, D., Khasa, Y. P., & Kuhad, R. C. (2011). Optimization of cellulase production by a brown rot fungus Fomitopsis sp. RCK2010 under solid state fermentation. Bioresour Technol, 102(10), 6065-6072. doi: 10.1016/j.biortech.2011.03.032

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current microbiology, 57(5), 503-507. doi: 10.1007/s00284-008-9276-8

Kuhad, R. C., Gupta, R., & Singh, A. (2011). Microbial cellulases and their industrial applications. Enzyme research, 2011. doi: 10.4061/211/280696

Lokhande, S., & Pethe, A. (2017). Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production. International J. of Life Sciences, 5(2), 277-282.ISSN 2320-7817

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31(3), 426-428. doi: 10.1021/ac60147a030

Mmango-Kaseke, Z., Okaiyeto, K., Nwodo, U. U., Mabinya, L. V., & Okoh, A. I. (2016). Optimization of cellulase and xylanase production by Micrococcus species under submerged fermentation. Sustainability, 8(11), 1168. doi: 10.3390548111168

Patagundi, B. I., Shivasharan, C., & Kaliwal, B. (2014). Isolation and characterization of cellulase producing bacteria from soil. International Journal of Current Microbiology and Applied Sciences, 3(5), 59-69. ISSN 2319-7706

Phitsuwan, P., Laohakunjit, N., Kerdchoechuen, O., Kyu, K. L., & Ratanakhanokchai, K. (2013). Present and potential applications of cellulases in agriculture, biotechnology, and bioenergy. Folia Microbiol (Praha), 58(2), 163-176. doi: 10.1007/s12223-012-0184-8

Ryu, D. D., & Mandels, M. (1980). Cellulases: biosynthesis and applications. Enzyme and Microbial Technology, 2(2), 91-102. doi: 10.1186/s12934-016-0507-6

Saratale, G. D., Saratale, R. G., Lo, Y. C., & Chang, J. S. (2010). Multicomponent cellulase production by Cellulomonas biazotea NCIM-2550 and its applications for cellulosic biohydrogen production. Biotechnol Prog, 26(2), 406-416. doi: 10.1002/btpr.342

Sethi, S., Datta, A., Gupta, B. L., & Gupta, S. (2013). Optimization of cellulase production from bacteria isolated from soil. ISRN Biotechnol, 2013, 985685. doi: 10.5402/2013/985685

Shankar, T., & Isaiarasu, L. (2011). Cellulase production by Bacillus pumilus EWBCM1 under varying cultural conditions. Middle-East Journal of Scientific Research, 8(1), 40-45. ISSN 1990-9233

Shanmugapriya, K., Saravana, P., Krishnapriya, M. M., Mythili, A., & Joseph, S. (2012). Isolation, screening and partial purification of cellulase from cellulase producing bacteria. Int J Adv Biotechnol Res, 3, 509-514. ISSN 0976-2612

Sobur, M. A., Sabuj, A. A. M., Sarker, R., Rahman, A. T., Kabir, S. L., & Rahman, M. T. (2019). Antibiotic-resistant Escherichia coli and Salmonella spp. associated with dairy cattle and farm environment having public health significance. Veterinary world, 12(7), 984. doi: 10.14202/vetworld.2019.984-993.

Tolan, J. S., & Foody, B. (1999). Cellulase from submerged fermentation Recent progress in bioconversion of lignocellulosics (pp. 41-67): Springer. DOI:10.1007/3-540-49194-5_3