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Simultaneous Isolation and Screening of Cellulolytic Bacteria: Selection of Efficient Medium

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The increasing demand for highly active and specific cellulases, especially for bioethanol production, necessitates the selection of potential cellulolytic microbial strains. Present study focused primarily on isolation, screening and selection of efficient cellulose producers based on halozone formation around colonies on agar based screening medium. A total of 105 indigenous cellulolytic bacteria were sought from their natural habitats. Three medium, viz. carboxymethylcellulose-congo red agar, basal agar and CMC agar medium were used and were compared for screening of cellulolytic bacteria using CMC as sole carbon source. Ratio of diameters of colony and halo zone of cellulose hydrolysis around the colony was taken as relative index of cellulase enzyme activity (I_{CMC}). Substantially higher numbers of bacterial isolates produced halo zone around the colony on CMC-congo red agar medium. CMC-congo red agar medium was found superior to other two media for screening of cellulolytic bacteria because of ease of handling, rapid screening and simultaneous screening and selection of cellulolytic bacteria.

Key words: Cellulolytic bacteria, Cellulase, Qualitative screening, Halo zone.

Cellulolytic microorganisms play a major role in recycling the abundant biomass resources on earth through biodegradation of cellulose[1]. Cellulases are group of enzymes that are responsible for catalyzing the hydrolysis of cellulose into sugars. Cellulase system contains endoglucanase (1,4-β-glucanglucanohydrolase, EC 3.2.1.4), exoglucanase (1,4-β-glucan cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D-glucosideglucohydrolase or cellobiase, EC 3.2.1.21). Synergistic effect of these enzymes makes possible the complete hydrolysis of cellulose to glucose[2, 3]. Currently, the demand for cellulases is very high because of their potential biotechnological applications in many industries, such as bio-fuels, textiles, detergent, food, animal feed, paper and pulp, pharmaceutical, and waste management[4, 5, 6].

The initial and the most important step for developing an industrial process for the production of large quantity of enzyme, is the isolation of the potential strains. Isolation and screening of cellulase producing microbes is of immense importance keeping in view their huge demand and the improvement of their biotechnological applications. Halo zone formation on agar medium is a qualitative measure of extracellular cellulase enzyme production by cellulolytic microorgsanisms, is highly influenced by medium components, therefore, easy,

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reproducible, and reliable qualitative method for screening extracellular cellulase producing microorganisms on agar plate was studied by our group. This study concentrates mainly on isolation of indigenous cellulolytic bacteria from natural environmental sources, on effect of incubation period on screening and on selection of hyper-producers of cellulase.

MATERIALS AND METHODS

Different types of samples such as rhizospheric soil, compost, farmyard manure (FYM), and vermicompost were collected from various agro-climatic locations of Kumaon region of North Himalaya (Uttarakhand, India) for isolation of cellulolytic bacteria as shown in Table 1 and stored at 4°C until use. Ten-fold serial dilutions of the samples were used for isolation of cellulolytic bacteria after enrichment with cellulose powder (Sigma-Aldrich, USA) and 0.1 ml diluted sample was spread on the surface of plates containing nutrient agar (NA) medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, and 1.7% agar; pH 7.0). Plates were incubated at 28ºC for 48 h. Morphologically different colonies appearing on the plates were purified and the purified isolates were preserved on agar slants at 4ºC and used during the course of study.

Each bacterial isolate was grown on NA medium for 24 h and then point inoculated with onto three different screening media: basal agar (BA) medium, carboxymethyl cellulose-congo red (CMC-CR) agar medium and CMC medium (Table 2). Cellulase activity assay for each isolate was done qualitatively on each media, using CMC as sole carbon and energy source. Each plate was incubated at 28ºC for 2-3 days, followed by 18 h at 50°C in the dark. After the incubation period was over, halo zone of cellulose hydrolysis around the colony was visualised on BM and CMC plates by staining with 1% Congo red dye (15 min), followed by destaining with 1M NaCl solution for 20 min. However, no staining-destaining was required in case of CMC-CR plates and clear zones were measured directly. Cellulase activities on screening media were recorded as the 'Indices of Relative Enzyme Activity, I_{CMC}' i.e. clear or halo zone ratios, which is a measure of carboxymethylcellulose hydrolysing capacity[7, 8, 9, 10]. Cellulolytic strains were selected on the basis of high I_{CMC} values and stored on slants at 4°C for further studies.

I_{CMC} = diameter of clearing or halo zone / colony diameter

RESULTS AND DISCUSSION

In most investigations, members of the fungal genus *Trichoderma* have been extensively studied due to their hypersectretion of extracellular cellulose-degrading enzymes. Cellulolytic enzymes (endoglucanases, exoglucanases and β-glucosidase) act synergistically during the saccharification of lignocelluloses, which is an important initial step in many applications, including bioethanol production. However, the *Trichoderma* cellulase system is deficient in β-glucosidase, causing the accumulation of disaccharide cellobiose, resulting in repression and end product inhibition of the cellulase complex. The demand for highly active and specific cellulases is continuously increasing, especially for cellulosic bioethanol production, therefore, cellulase systems of other fungi as well as bacteria have also been investigated[11]. Although, initially cellulase research was mainly focused on fungi[12, 13], now a day there has been increasing interest in cellulase production by bacteria because of their fast growth rate[14, 15, 16] and secretion of a complete multi-enzyme system for lignocellulose degradation. For selection of potential cellulolytic microbial strains from a large population or from a new source, quick and reliable screening of extracellular cellulase is very necessary. The diameter of the halo zone is very useful for predicting the enzyme yield, as an aid to select strains with a high level of polysaccharide (such as cellulose) degrading activities. An even more accurate and convenient measure for in vitro cellulolytic potential is the clear zone ratio (I_{CMC} index).

During present investigation, cellulolytic bacteria were sought from Kumaon region of North Himalaya, which is well known for its immense microbial diversity. Total 105 bacterial strains were isolated from various samples viz. rhizospheric soil, compost, FYM and vermicompost as shown in Table 1. The overall bacterial population (cfu/g dw) in these samples varied from 2.8×10^3 to 2.4×10^7 in vermicompost and compost from Almora,

Table 1. Diversity and population dynamics of total and cellulolytic bacteria in various sources

S. No.	Location, source of isolation	Total bacterial population* (cfu/g dw)	Total population* of cellulolytic bacteria (cfu/g dw)	Total strains isolated	Cellulolytic bacterial isolates	Frequency of cellulolytic bacteria in total isolates (%)
1	Almora, rhizospheric soil	8.1×10^3	1.69×10^3	21 (ARCB-1 – ARCB-21)	14	66.7
2	Almora, compost	2.8×10^7	6.6×10^4	15 (ACCB-1 – ACCB-15)	9	60
3	Almora, FYM	4.6×10^4	7.9×10^2	26 (AFCB-1- AFCB-26)	11	42.3
4	Almolra, vermicompost	2.4×10^3	9.1×10^2	33 (AVCB-1 – AVCB-33)	11	33.3
5	Pantnagar, FYM	1.22×10^4	8.6×10^2	10 (PFCB-1 – PFCB-10)	2	20

*population dynamics in various samples was studied by serial dilution plate count method using nutrient agar (for total population) and CMC-CR agar (for cellulolytic population) medium at 28°C.

respectively; while, the cellulolytic population varied from 7.9×10^2 to 6.6×10^4 cfu/g dw in FYM and compost, from Almora, respectively. Though, high population dynamics of cellulolytic bacteria was recorded in all the samples, substantially high total as well as cellulolytic bacterial diversity was found in compost of Almora. Therefore, it can be concluded that natural habitats of microbes such as compost, vermicompost, FYM, and rhizosphere are among the best sources for isolation of cellulolytic bacteria. The isolation of higher numbers of cellulolytic microbes on CMC containing media clearly shows that CMC is a favourable carbon source for screening and selection of hyper cellulase producing microbes.

Although, a total of 51 bacterial isolates grew well on screening media (CMC-CR), not all strains revealed halo zone of cellulose hydrolysis around their colonies on CMC-CR media. Among the three media tested for screening of the isolates for cellulase activity CMC-CR medium was found superior, as it showed a maximum number (47) of halo zone producing bacteria (Fig. 1 and 2). While, only 30 bacterial strains showed halo zone formation on CMC media, followed by 23 strains on BM. While looking for I_{CMC} index, it was observed that all the bacterial isolates showed higher relative cellulase activity on CMC-CR medium as compared to that on the other two media. On CMC-CR medium 12 bacterial isolates had the I_{CMC} values greater than 3.0, and 26 isolates greater than 2.0. Most interestingly, none of the halo zone producing strains showed I_{CMC} less than 1.0. Significantly high clear zone ratio was shown by the isolate AVCB-2, with an I_{CMC} near 18 (Fig. 3). On CMC medium none of the strains had I_{CMC} greater than 2.0 and maximum strains (25) had values less than 1.0. On BA medium, only 3 strains had I_{CMC} greater than 2.0, while 14 formed smaller clearing zones. Moreover, those isolates either showing none or a very small halo zone on CMC and BA medium also had higher I_{CMC} values on CMC-CR medium.

The effect of incubation period (Fig. 4) on halo zone formation was also studied using the best suited medium (CMC-CR). It was found that nearly all of the strains showed maximum I_{CMC} values at 48 h incubation. A decline in clear zone ratio was found with further incubation i.e. at 72 h. It may be concluded that 48 h is the suitable time for screening of cellulolytic bacteria as further

Table 2. Chemical composition of the three screening media used for evaluation of halo zone formation

Constituents	Concentration (%, w/v)		
	Basal media	CMC-CR agar	CMC agar
K_2HPO_4	-	0.05	0.1
$MgSO_4.7H_2O$	0.15	0.025	0.05
KCl	-	-	0.1
Congo red	-	0.02	-
CMC-Na salt	1	0.188	0.5
Gelatin	-	0.2	-
Yeast extract	0.4	-	0.05
$NaNO_3$	-	-	0.1
Glucose	-	-	0.1
Asparagine	0.4	-	-
Agar	1.75	0.5	1.75

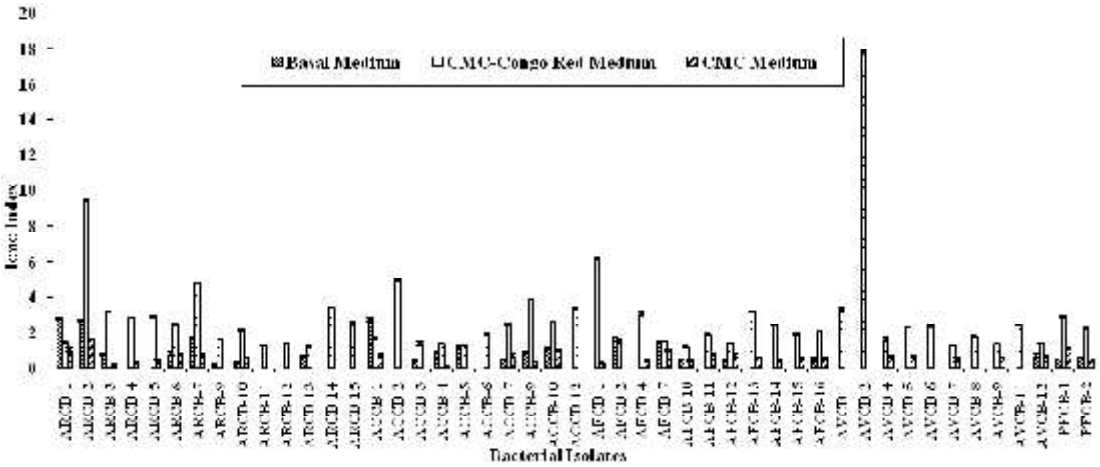


Fig. 1. Comparative evaluation of three different media for screening of cellulolytic bacteria, based on I_{CMC} Index. Error bars indicate SD at 95% confidence level

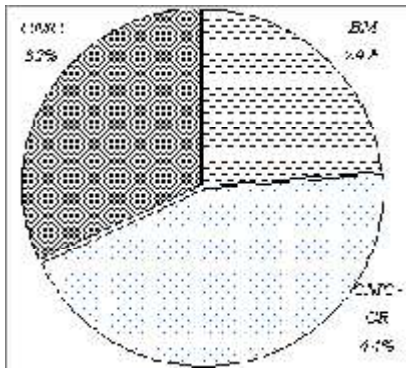
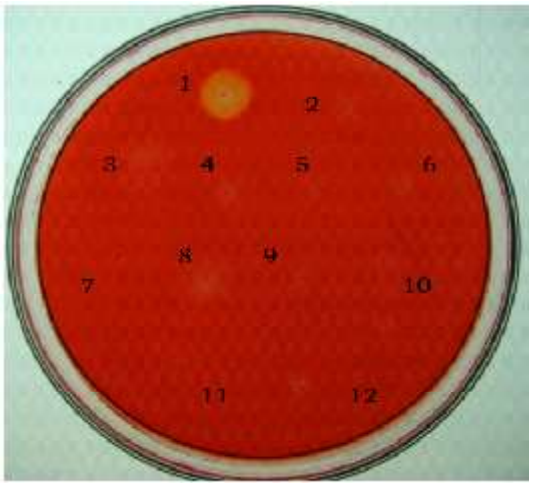


Fig. 2. Frequency (%) of halo zone producing cellulolytic bacterial strains on different media. CMC, CMC media; BM, basal media; & CMC-CR, CMC-Congo red media

incubation results in increase of colony size more than that of the halo zone, therefore, causing a decrease in clearing ratio.

It is also obvious from our results that, halo zone producing capacity through cellulase activity is affected by increasing concentration of the substrate, CMC, in the media. The increase in CMC concentration is accompanied with an increase in the viscosity of the medium, making CMC less available for hydrolysis due to the decrease in diffusion of cellulase enzymes. Among the three media used for the screening of cellulolytic bacteria, CMC-CR medium containing lowest CMC concentration is better than others. Due to the low percentage of agar and CMC in this media cellulase enzyme was able to diffuse more rapidly, resulting in large and distinctively visible clearing zone. Hence, maximum number of isolates formed halo zone of cellulose hydrolysis despite the relatively low amount of CMC in this medium. Therefore, CMC-CR medium can be used for rapid and cost-effective screening of cellulolytic bacteria, as it is easy to prepare, gives prompt results with less chemicals and doesn't involve the time consuming staining-destaining protocol. With the incorporation of lower amount of congo-red dye, this medium can be utilized for enumeration, as well as screening and selection of cellulolytic bacteria at the same time.



Isolates: 1. (AVCB-2); 2. (ARCB-9); 3. (ARCB-11) ; 4. (ARCB-13); 5. (ACCB-4); 6. (ACCB-5); 7. (AFCB-2); 8. (AFCB-7); 9. (AFCB-10); 10. (AVCB-7); 11. (PFCB-1) & 12. (PFCB-2)

Fig. 3. Photo image of clearing zone around colony of cellulose-hydrolysing bacteria on CMC-CR agar after 24 h

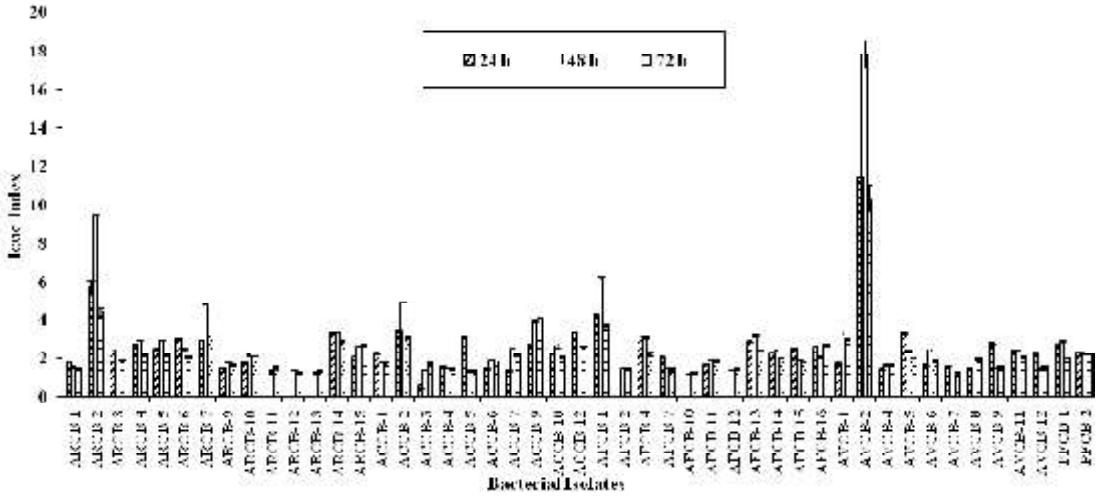


Fig. 4. Influence of incubation period on relative cellulase enzyme activity (I_{CMC} Index) of cellulolytic bacteria on CMC-CR medium. Error bars indicate SD at 95% confidence level

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