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Alternative substrates for the amylase and cellulase production with rhizobial isolates

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ABSTRACT

In the present study sixty root nodule bacteria were isolated from *Vigna radiate* and *Cicer arietinum*. As we know that nutrients and growth conditions promote the yield of microbial enzymes. However, carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for commercial production of enzymes. These expensive products could be replaced in the medium with economically available agricultural by-products like corn flour, wheat flour, rice flour, sorghum and baggase as carbon substrate. The aim of this study was the production of amylases and cellulases from rhizobia using cheap carbon sources to reduce the production cost of enzymes .All isolates were screened for the amylase and cellulase production using starch and carboxy methyl cellulase (CMC) hydrolysis respectively .Isolates showing better screening results were further subjected for different starchy carbon substrates for CFU/ml at different time intervals and enzyme (amylase and cellulase) production. The results obtained in the study revealed that wheat flour yields the highest levels of amylase, and baggase promotes the highest amount of cellulase. Statistical analyses between cellulase and amylase data showed that there is a significant difference between their productions. Amount of cellulase (0.1513 u/ml) is significantly greater than that of amylase (0.0091u/ml).

Introduction:

Amylolytic and cellulolytic enzymes are widely used in textile, pharma, food industries and waste water treatment plants [1] The potential applications of amylases [2] and cellulases [3] in biotechnology have already been reported. The majority of the enzymes used in the industry are of microbial origin because microbial enzymes are relatively more stable than the corresponding enzymes derived from plants and animals.

Microbial enzymes production and yield has been highly dependent upon growth conditions and nutrients. Dextrin, fructose , glucose ,lactose ,maltose and starch are common carbon substrates for monitoring amylolytic and cellulolytic activity but these are very expensive for commercial production of enzymes [4,5].These expensive sugars can be replaced by cheap and nutritive agricultural and industrial by-products , is an effective strategy to reduce production cost [6].Therefore to increase the productivity of enzymes from microorganisms brans,straws and flours of different grains and tubers, such as barley, corn, cassava, potato, rice, sorghum and wheat have been used instead of commercially available costly substrates.[7,8].

Microbial enzymes are produced mainly from cultures of *Aspergillus*, *Bacillus*, *Clostridium*, *Pseudomonas*, *Rhizopus* and *Streptomyces* species [9]. Screening of new microorganisms producing enzymes suitable for industrial applications is important [10]. *Rhizobium* being a soil micro-organism produces various enzymes like nitrogenase [11]. Cellulolytic and Amylolytic [12], proteolytic [13] and ureolytic [14] enzyme activities have been detected in pure rhizobial culture. Thus it become imperative to work on these enzyme to suffice the need of on growing demand of enzymes in industry. Studies on rhizobia amylases and cellulases are still scarce. The aim of this study was the

production of amylases and cellulases from rhizobia strains using alternative cheap substrates.

Material and Methods:

Collection of sample:

Root nodule samples of *Cicer arietinum* (Chickpea) and *Vigna radiata* (Mungbean) were collected from Himachal Pradesh Krishi Vishwavidhalya Hill Agriculture and Extention Centre , dhollakaun (H.P.)

Isolation and characterization of root nodulating bacteria:

The collected nodules were first surface-sterilized with 95% ethanol, then with 0.1% mercuric chloride and finally washed thoroughly with distilled water. strains were obtained by streaking the crushed root nodules on YEMA plates and incubated at 28±2°C .After 2 days of incubation, *Rhizobium* colonies were obtained. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure cultures. Pure isolates were used for further study [15]. Biochemical characterization of recovered isolates was done according to Bergey's Manual of Determinative Bacteriology [16]

Qualitative estimation of Amylase and Cellulase activity:

For the estimation of cellulase and amylase enzymes, the isolates were spot inoculated on the media containing carboxymethylcellulase and starch respectively. After an incubation period of 48 hours the plates were observed for the presence of zone of hydrolysis around the colony.

Media preparation for screening procedure:

The growth of isolates were observed on modified medium in which mannitol was replaced with different carbon sources such as maltose, sorghum, wheat flour, corn flour, rice flour, baggase and Tryptone yeast extract agar medium was used as a control.

Effect of starchy substances on growth of Rhizobium and Mesorhizobium:

Serial dilutions of isolates were made in 9ml distill water with 1ml culture and was spread over modified YEMA plates and spreaded plates was incubated at 28C at different intervals (24,48 and 72 hrs) and observed the growth on the medium.

Amylase and cellulase assay for Production of crude enzyme:

Rhizobium strains were inoculated into 100 ml of YEM broth and incubated at 30°C for 48 hours in a shaker maintained at 80 rpm/minute. At the end of the incubation period the culture centrifuged at 6000 rpm for a period of 10 minutes. The supernatant thus obtained was transferred into another tube and used as crude enzyme for measurement of enzymatic activity.

Quantitative estimation of Amylase and Cellulase activity:

Amylase and Cellulase activity was by carried out by estimating reducing sugars formed by the action of the enzyme on suitable substrate. according to the dinitrosalicylic acid(DNSA) method. [17]

In a tube, 0.4ml of enzyme extract , 1.8ml of the substrate and 2ml of dinitrosalicylic acid were added and incubated at 37^0C for 10 min. To stop the reaction, 1ml of 40% solution of sodium potassium tartarate was added. Change in colour was observed and OD was taken at 575nm using a spectrophotometer. OD is proportional to the concentration of the enzyme present. The more the enzyme activity, the more the colour change and thus, the higher the OD. 1% Maltose and 1% glucose solution was used as a enzyme substrate for Amylase and Cellulase activity respectively.

Statistical Analysis:

Data analysis was done using SPSS version 12.0

Results and Discussion:

Isolation of *Rhizobium and Mesorhizobium* sp. From root nodules of *Vigna radiate* and *Cicer arietinum.*

A total of 60 root nodule bacteria were isolated from *Vigna radiate* (50) and *Cicer arietinum* (10) plants growing in field. The isolates were designated as MR1 to MR50 from *Vigna radiate* and CMR1 to CMR10 from *Cicer arietinum*. The isolates were recognized on the basis of their colony morphology on YEMA plates after proper incubation period. The general microscopic characteristics of the selected isolates showed these are rod shaped and gram negative in nature and also similar to those reported earlier [18].

Biochemical characteristics of recovered isolates:

All of the isolates were oxidase, catalase and nitrate reduction positive and could not utilize citrate. None of the isolates was able to grow on medium containing 0.1% methylene blue and gentian violet. These findings are in conformity with the earlier studies of Wei *et al*., 2003, [19] and Hunter *et al*., 2007 [20] on Rhizobial strains. The results on negative gelatinase activity of *Rhizobium* and positive starch hydrolysis were also reported earlier by De Oliveria *et al.,* 2007 [21] .Rhizobial cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *rhizobium*. It is a confirmatory test for *Rhizobium* [22]. . All isolates were fluorescence negative as indicated by growth on King's medium under UV source In the current study all recovered isolates produced yellow slant and red butt showing the utilization of sucrose in TSI [23] (Table1)

Table: 1. Morphological and biochemical characteristics of all recovered isolates from Chickpea and Mungbean:

Characters	Results
Bacterium shape	Rod shaped
Color	Whitish pink and glistering
Elevation	Convex
Opacity	Opaque
Oxygen demand	Aerobic
Spore formation	Non spore forming
Gram staining	Negative
1% Methylene blue treatment	Negative
1% Gentian violet treatment	Negative
Growth on Glucose peptone agar	Positive
Growth on Lactose peptone agar	Positive
Gelatin hydrolysis	Negative
Fluorescence assay	Negative
Nitrate reduction test	Positive
Triple sugar iron test	A/K, A/A, K/A
Catalase test	Positive
Oxidase test	positive
Citrate test	Negative

Screening of amylase and cellulase producing isolates:

Primary screening of amylase on starch agar:

Out of 60 isolates, 9 isolates of *Vigna radiate* and 3 of *Cicer arietinum* were showing better amylase activity .MR-22 showed the highest amylolytic activity with a DCZ of 14 mm and then followed by MR-21, CMR-7, MR-30,MR-3, MR-28, MR-17. MR-8, (Figure 1) Similar results were reported by de oliveira *et.al.*, (2007) [21] that Bacterial isolate INPA R-926 showed the highest amyolytic activity with a DCZ of 21.4 mm. From the figure 1 it may be seen that MR-3, MR-21, MR-30 ,CMR-7 and MR-28 are equal in effect as they fall under the same bar of magnitude.

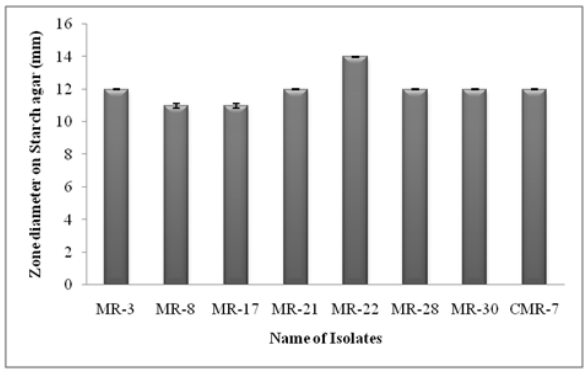

Figure: 1. Primary Screening of recovered isolates on Starch Agar

Primary screening of cellulase on CMC agar:

Out of 60 isolates 9 isolates of *Vigna radiate* and 2 isolate of *Cicer arietinum* were shown cellulase activity. Maximum cellulase activity with a DCZ of 14.6 mm with MR-22 and then followed by CMR-7, MR-3, MR-8, MR-17, MR-28, MR-30, MR-21. From the figure 2 it may be seen that MR-17, MR-21, MR-30 and MR-28 are put under the same bar of equality i.e. these are equal in effect in producing cellulase. Similarly Sudto *et.al.*,2008 [24] observed that *Bacillus subtilis,* and *E.coli* produced cellulase activity with DCZ of 1.07 mm and 1.00 mm in CMC agar respectively.

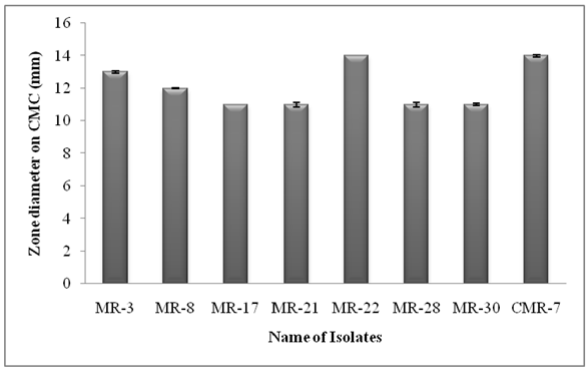

Figure: 2. Primary Screening of recovered isolates on Carboxy Methyl Cellulase Agar

Effect of different carbon substrates on population count of isolates:

In the present study six starchy substrates (maltose, sorghum, wheat flour, corn flour, rice flour and baggase) and two standard media (YEMA and TY) were used to study the population count of isolates at different time intervals. Maximum number of cell count were shown by rice flour and then followed by corn flour, baggase sorghum, YEMA, Wheat flour**,** maltose whereas TY was used as a control. (Table 2 ,3.,4.). Maximum number of cell count was observed in MR-22 and minimum in MR-30. Sudto *et.al.,* (2008) [24] studied growth of *Bacillus subtilis, E.coli* and *Rhizobium* sp. in agricultural waste and found that maximum number of microorganism were obtained in pineapple peels i.e.

$6.50\times10^{8}\pm2.00\times107$,$2.88\times10^{9}\pm2.52\times10^{9}\pm2.52\times10^{7}$

And

$2.11\times10^{9}\pm3.51\times10^{7}$ CFU/ml respectively.

When these materials were used as substrate, the difference in cell growth is affected by various factors such as the presence of activator or inhibitor, vitamins or growth factors, especially sugar content in these substrates. Sugar content affected enzyme production because bacteria could utilize sugar residues as a carbon source.

Table: 2. Population count (log 10 cfu/ml) of recovered isolates on various carbon substrates at 24hrs incubation period.

	Different Type Of Substrates							
ISOLATES	**TYA**	**YEMA**	**Wheat flour**	**Rice flour**	**Corn flour**	**Baggase**	**Sorghum**	**Maltose**
MR-3	2.45	2.16	2.16	2.45	2.36	2.26	2.22	2.13
MR-8	2.46	2.26	2.26	2.44	2.34	2.25	2.29	2.08
MR-17	2.43	2.19	2.19	2.45	2.36	2.23	2.21	2.11
MR-21	2.46	2.25	2.25	2.44	2.41	2.25	2.29	2.08
MR-22	2.43	2.20	2.20	2.44	2.45	2.38	2.24	2.07
MR-28	2.46	2.22	2.22	2.44	2.37	2.30	2.24	2.00
MR-30	2.40	2.13	2.13	2.40	2.33	2.22	2.14	1.09
CMR-7	2.47	2.16	2.16	2.44	2.44	2.19	2.15	2.01

Table: 3. Population count (log 10 cfu/ml) of recovered isolates on various carbon substrates at 48 hrs incubation period

	Different Type of Substrates							
ISOLATES	**TYA**	**YEMA**	**Wheat flour**	**Rice flour**	**Corn flour**	**Baggase**	**Sorghum**	**Maltose**
MR-3	2.46	2.16	2.38	2.46	2.38	2.42	2.28	2.15
MR-8	2.47	2.26	2.42	2.46	2.36	2.42	2.26	2.09
MR-17	2.46	2.19	2.46	2.46	2.40	2.29	2.24	2.13
MR-21	2.47	2.25	2.45	2.45	2.42	2.26	2.27	2.11
MR-22	2.46	2.20	2.46	2.46	2.46	2.42	2.40	2.11
MR-28	2.47	2.22	2.47	2.44	2.39	2.36	2.32	2.04
MR-30	2.46	2.12	2.35	2.44	2.35	2.24	2.20	1.57
CMR-7	2.47	2.13	2.47	2.46	2.45	2.46	2.21	2.04

Table: 4. Population count (log 10 cfu/ml) of recovered isolates on various carbon substrates at 72hrs incubation period.

	Different Type of Substrates							
ISOLATES	**TY broth**	**YEM broth**	**Wheat flour**	**Rice flour**	**Corn flour**	**Baggase**	**Sorghum**	**Maltose**
MR-3	2.47	2.22	2.20	2.47	2.40	2.45	2.30	2.18
MR-8	2.47	2.29	2.25	2.46	2.40	2.43	2.29	2.11
MR-17	2.47	2.21	2.17	2.47	2.44	2.30	2.26	2.14
MR-21	2.45	2.29	2.26	2.46	2.44	2.30	2.29	2.13
MR-22	2.47	2.24	2.17	2.47	2.44	2.46	2.41	2.16
MR-28	2.46	2.24	2.24	2.46	2.42	2.37	2.33	2.09
MR-30	2.42	2.20	2.19	2.46	2.39	2.28	2.24	1.97
CMR-7	2.47	2.15	2.18	2.47	2.47	2.46	2.23	2.07

Production of amylase and cellulase in different carbon substrates by DNSA method:

The data was collected on the production of enzymes – Amylase and Cellulase under the effects of 8 substrates and 8 isolates. The statistical analysis was performed applying factorial method to see the extent of variation among the isolates and among the substrates and their interactions for amylase and cellulase production separately. The findings of the analysis are described below:

Analysis of Amylase production:

The effects of Isolates: The analysis revealed that isolates differ highly significantly (p = 0.001) in their effects on production of amylase.The result indicates (Table 5) that the isolate MR- 22 is most effective as compared to the rest of isolates. MR- 22 produces maximum of amylase (0.1134u/ml) .The isolates may be put in the following order of amylase production.

MR-22	MR-3	MR-21	MR-30	MR- 28
0.1134	0.1030	0.0998	0.0978	0.0976
MR-17	CMR-7	MR-8	CD	
0.0969	0.0961	0.0879	0.007	

The effects of Substrates:

The analysis also revealed that substrates too differ highly significantly (p = 0.001) in their effects on production of amylase. The result indicates in Table 6 that the substrate wheat flour is most effective among the substrates. Wheat flour produces maximum of amylase (0.1292u/ml) .The substrates may be put in the following order of amylase production.

Wheat flour	TYBroth	YEM Broth	Baggase
0.1292	0.1083	0.0951	0.0948
Maltose	Sorghum	Corn flour	Rice flour
0.0944	0.0923	0.0897	0.0888
CD			
0.007			

The effect of interactions:

The combined effects of isolates with substrates vary highly significantly (Table 7). It is found that Wheat flour with isolate MR-22 produces the maximum amylase (0.2150 u/ml)

Analysis of Cellulase production:

The effects of Isolates:

The analysis revealed that isolates differ highly significantly (p = 0.001) in their effects on production of cellulase. The result in Table 5 shows that the isolate MR- 28 is most effective as compared to the rest of isolates. MR- 28 produces maximum of cellulase (0.1929 u/ml) .The isolates may be put in the following order of cellulase production

MR-28	MR-22	MR-30	MR-3	CMR-7
0.1929	0.1776	0.1702	0.1684	0.1513
MR-8	MR-21	MR-17	CD	
0.1479	0.1321	0.1274	0.0102	

The effects of Substrates:

The analysis also revealed that substrates too differ highly significantly (p = 0.001) in their effects on production of cellulase. The result indicates in Table 6 that the substrate Baggase is most effective among the substrates in producing cellulase. Baggase produces maximum of cellulase (0.3538 u/ml) and sorghum produce minimum of cellulase (0.0921 u/ml) .Similar conclusions have been obtained for other microorganisms *Thermoascus aurantiacus* produced the highest levels of CMCase in corncob, grasses and corn straw [25]. *Trichoderma viride* showed CMCase activity in mixed substrate of rice straw and wheat bran [26].

The applied substrates may be put in the following order of cellulase production.

Baggase	Rice flour	TY Broth	Corn Flour
0.3538	0.2538	0.1413	0.1189
Wht flour	Maltose	YEM broth	Sorghum
0.1077	0.102	0.0983	0.0921
CD			
0.0102			

The effect of interactions:

The combined effects of isolates with substrates vary highly significantly. (Table 8) It is found that baggase with isolate MR-28 produces the maximum cellulase (.0.0490 u/ml)

Table: 5. Variation in the effects of isolates in producing the amylase and cellulase

Mean Product(u/ml)	Isolates								level of significance	CD
	CMR-7	MR-3	MR-8	MR-17	MR-21	MR-22	MR-28	MR-30		
Amylase	0.0961	0.103	0.0879	0.0969	0.0998	**0.1134**	0.0976	0.0978	***	0.007
Cellulase	0.1513	0.1684	0.1479	0.1274	0.1321	0.1776	**0.1929**	0.1702	***	0.0102

Table: 6. Variation in the effects of enzymes & substrates in producing the amylase and cellulase

Mean Product (u/ml)	Substrates								level of significance	CD
	Corn Flour	Rice Flour	Baggase	Wheat Flour	Maltose	Sorghum	YEM Broth	TY Broth		
Amylase	0.0897	0.0888	0.0948	**0.1292**	0.0944	0.0923	0.0951	0.1083	***	0.007
Cellulase	0.1189	0.2538	**0.3538**	0.1077	0.1020	0.0921	0.0983	0.1413	***	0.0102

Table: 7. Variation in the joint effects of substrates & isolates in producing the amylase

Isolates	Substrates							
	Corn Flour	Rice Flour	Baggase	Wheat Flour	Maltose	Sorghum	YEM Broth	TY Broth
CMR-7	0.0960	0.0960	0.0920	0.1050	0.0925	0.0915	0.099	0.0965
MR-3	0.0965	0.0945	0.0980	0.1300	0.0980	0.0935	0.0935	0.1200
MR-8	0.018	0.0925	0.0955	0.1200	0.0965	0.0905	0.093	0.0970
MR-17	0.0965	0.0930	0.0935	0.1200	0.0925	0.092	0.0915	0.0965
MR-21	0.0995	0.015	0.0935	0.1400	0.0950	0.0925	0.0925	0.0935
MR-22	0.12	0.0525	0.0930	0.2150	0.0925	0.091	0.0935	0.1500
MR-28	0.0965	0.0970	0.0975	0.0990	0.0945	0.095	0.104	0.0975
MR-30	0.0945	0.0930	0.0950	0.1050	0.0980	0.0925	0.094	0.1150
			CD=0.0198				**p = 0.001**	

Table: 8. Variation in the joint effects of substrates & isolates in producing the cellulase

Isolates	Substrates							
	Corn Flour	Rice Flour	Baggase	Wheat Flour	Maltose	Sorghum	YEM Broth	TY Broth
CMR-7	0.1400	0.3400	0.1800	0.0985	0.0980	0.0940	0.1100	0.1500
MR-3	0.0960	0.2250	0.4600	0.1300	0.1350	0.0920	0.0945	0.1150
MR-8	0.1100	0.1350	0.4400	0.0980	0.0965	0.0925	0.0945	0.1450
MR-17	0.0945	0.2450	0.1350	0.0955	0.0965	0.0905	0.0920	0.1700
MR-21	0.0960	0.2350	0.1850	0.0940	0.0960	0.0925	0.0930	0.1650
MR-22	0.1300	0.3100	0.4650	0.1150	0.0955	0.0915	0.0935	0.1200
MR-28	0.1550	0.3050	**0.4900**	0.1350	0.1050	0.0935	0.1150	0.1450
MR-30	0.1300	0.2350	**0.4750**	0.0955	0.0935	0.0905	0.0350	0.1200
	CD=0.0288			**p = 0.001**				

Conclusions:

According to our results wheat flour yields the highest levels of amylase, and baggase induces the highest levels of cellulase**.** It was concluded that wheat flour was good substrate for amylase and baggase for cellulase production. MR-22 and MR-28 was better isolate in terms of growth and enzyme production in different substrates. Wheat flour and Baggase as an alternative to mannitol used for the growth of *Rhizobium* in commercial preparation of enzymes which will considerably reduce the production cost.

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