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Pectinase production from immobilized and free cells of *Geotrichum candidum* AA15 in galacturonic acid and sugars containing medium

Asia Ahmed, Uroosa Ejaz, Muhammad Sohail *

Department of Microbiology, University of Karachi, Karachi 75270, Pakistan

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ABSTRACT

Immobilization of microbial cells over agricultural residue is an approach to reduce the cost. In this study, cells of *Geotrichum candidum* AA15 were immobilized on corncob and the effect of simple sugars on pectinase production by immobilized and free cells was investigated. The results indicated that the simple sugars, except for glucose, had positive effect on the pectinase production. Moreover, presence of galacturonic acid in the reaction mixture did not inhibit the pectinase activity. The data further revealed that the pectinase titers reached its peak in 5 h of cultivation and remained unaffected even after the addition of galacturonic acid. Indeed the titers were several folds higher when the immobilized cells were placed in galacturonic acid containing medium. Generally, galacturonic acid repressed pectinase production by the free cells. The study explored the advantages of immobilized cells for pectinase production as it did not exhibit the catabolite repression.

1. Introduction

Yeasts play diversified roles in the preparation and processing of foods. Several bioactive metabolites from yeasts have been described as nutraceuticals, whereas many yeast enzymes are commercially employed for the processing of foods such as fruit juices are clarified using pectinases (Gummadi and Panda, 2003). Although, various yeast strains have been reported for the industrial production of pectinases, studies on immobilized *Geotrichum candidum* are scarce in the literature.

Immobilization of microbial strain is one of the strategies that has been reported to obtain higher yields of enzymes with substantially reduced expenditures (Verbelen et al., 2006). Some agro-waste materials, such as corncob (CB) can be used as a matrix for immobilization. Porous property of CB provides high surface area and microtubes for the entrapment of the microbial cells and can be used as a potential low-cost support carrier for immobilization of microorganisms (Djordjević et al., 2016).

Earlier, CB was used for the immobilization of *G. candidum* AA15 and higher yield of pectinase was reported (Ejaz et al., 2018) with the considerable recycling. Although, optimization of parameters influencing production of pectinase by free (Ahmed et al., 2019) and immobilized cells of *G. candidum* AA15 (Ejaz et al., 2018) was carried out, however, the influence of simple sugars on the production of pectinase was needed to be elucidated.

Presence of simple sugars in the growth medium influences pectinase production variably in different strains as the production is repressed in some of the strains while in few cases induction was detected. Moreover, such studies were carried out using free cells. In spite of the fact that the regulation of pectinase synthesis was found to differ in immobilized cells than in the free cells, the role of media containing simple compounds in pectinase production by immobilized yeast cells has yet to be established. Therefore, it was worthwhile to study the effect of simple sugars on pectinase production by free and immobilized cells of *G. candidum* AA15.

2. Material and methods

2.1. Microorganism and inoculum preparation

The yeast strain, *G. candidum* AA15, was retrieved on Sabouraud's Dextrose agar (SDA) plates and inoculum was prepared by transferring an isolated colony into 250 mL Erlenmeyer flask containing 50 mL SDB (Oxoid). The flask was incubated at 30 °C for 24 h. The density of the culture was adjusted

* Corresponding author.
E-mail address: msohail@uok.edu.pk (M. Sohail).
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to 1.0 by taking optical density (OD) at 600 nm using spectrophotometer. For pectinase production by using free cells, 5% inoculum was transferred to mineral salt medium (MSM). Mineral salt medium was prepared by dissolving 10 mL of 10X solution A (2% KH_2PO_4, 1.4% $(NH_4)_2SO_4$, 0.3% $MgSO_4{\cdot}7H_2O$, 0.1% $CaCl_2$, 1% Peptone, 2% Tween 80) and 100 μL of 100X solution B (0.5% $FeSO_4{\cdot}7H_2O$, 0.16% $MnSO_4{\cdot}H_2O$, 0.14% $ZnSO_4{\cdot}7H_2O$, 0.29% $CoCl_2{\cdot}6H_2O$) in 98.9 mL of distilled water containing suitable substrate. Inoculum for immobilized cells was prepared using pretreated CB of 0.5 ± 0.1 cm^3 size. After washing with tap water, the pieces of CB were boiled for 5 min and then dried at 45 °C for 24 h (Lakkana and Pattana, 2012). The alkali pretreatment to CB was followed by immobilization as reported elsewhere (Ejaz et al., 2018). Two pieces of immobilized CB were taken as inoculum.

2.2. Pectinase production in the presence of different sugars

Initially the effect of presence of sugars was investigated by transferring the inoculum of free and immobilized cells to the MSM containing 2% of different sugars including xylose, glucose and galactose and incubated at 28 °C for 72 h. After incubation, the media was centrifuged at 3500 rpm for 15 min and cell-free culture supernatant (CFCS) was assayed for pectinase by determining reducing sugars. The substrate, 0.5% pectin (Sigma-Aldrich) (in 50 mM sodium citrate buffer, pH 4.8) was mixed with CFCS and incubated at 37 °C in a water bath for 30 min. The reaction was quenched by the addition of Dinitrosalicylic acid and was boiled for 5 min. After chilling on ice bath, distilled water was added. OD_{540} was measured against blank. The blank was prepared using heat inactivated CFCS. One international unit of pectinase was defined as the amount of enzyme that liberates 1 μmol of galacturonic acid per min under the standard assay conditions.

2.3. Determination of pectinase activity using pectin and pectin with galacturonic acid as substrate

Subsequent to the finding that galacturonic acid stimulated the production of pectinase, in another experiment, the free and immobilized cells were transferred separately to the flasks containing MSM supplemented with 1% pectin only or 1% pectin with 2% galacturonic acid and incubated at 28 °C for 72 h. CFCS was collected as described above and assayed for pectinase activity, both in the presence and in the absence of 1% galacturonic acid. The ODs of control tubes were also taken into consideration by adding galacturonic acid in the reaction mixture.

2.4. Effect of the presence of galacturonic acid in production medium on pectinase production

The effect of the presence of simple nutrient, galacturonic acid, was studied by challenging the immobilized and free cells of AA15 with galacturonic acid at different time intervals. Briefly, immobilized and free cells were inoculated in 5 different sets of flasks (4 test sets containing MSM with 1% pectin and 1 control set containing MSM with 1% galacturonic acid) and incubated at 28 °C for 72 h. In 1 test set of flasks, 1% galacturonic acid was added at the start of cultivation (at 0 min). In another set, 1% galacturonic acid was added after 1 h of inoculation, while in the third test set 1% galacturonic acid was transferred after 30 h of inoculation. The samples were withdrawn from all the flasks intermittently and pectinase activity was determined.

A control of CB without yeast cells was also incubated for each set of flasks.

All the experiments were conducted in triplicate and mean values have been presented. Standard deviation was calculated using Microcal Origin 6.0.

3. Results and discussion

3.1. Production of pectinase on different carbon sources

The impact of additional carbon sources such as glucose, galactose, xylose and pectin on pectinase production by *G. candidum* AA15 was studied. It was observed that the strain produced pectinase in presence of all the tested carbon sources (pectin, xylose, glucose and galactose) suggesting the constitutive expression of the enzyme (Oskay and Yalçin, 2015). Pectinase production by immobilized yeast strain was generally much higher than in the free cells (Table 1). The production was not observed in the control flask (matrix without immobilized cells). Among all the added carbon sources, xylose proved to be best for optimal production of pectinase indicating the role of this sugar as inducer. Moreover, the effect of presence of xylose was not so pronounced in case of free cells compared to immobilized cells.

Whereas, negative effect of glucose on pectinase production by immobilized cells of *G. candidum* AA15 was evident. It is possible that the presence of glucose caused a repressive effect on pectinase production. In addition, the absence of glucose may have limited the growth and growth-dependant pectinase production was suppressed. In contrast, Poletto et al. (2017) studied the pectinase production by *Aspergillus niger* LB-02-SF and have suggested that, under a growth limiting condition, pectinase production is increased. It was previously reported that if the monosaccharides (such as glucose) are used over a certain concentration, they have a repression effect on the production of pectinolytic enzymes (Akimitsu et al., 2004). Teixeira et al. (2000) reported that pectin esterase activity from *Aspergillus japonicus* 586 was susceptible to catabolic repression with high glucose concentrations. Indeed the composition of the pectinase production medium influences the pectinolytic activity.

3.2. Pectinase activity in the presence of pectin and pectin with galacturonic acid

Immobilized cells of *G. candidum*AA15 produced a titer of 0.308 IU mL^{-1} in presence of pectin and galacturonic acid compared to the 0.218 IU mL^{-1} when galacturonic acid was not present in the production medium. However, the activity of pectinase was enhanced to 0.4 IU mL^{-1} when the enzyme assay was performed in presence of galacturonic acid. It indicated the absence of stringent catabolite repression mechanism and end product tolerance of the enzyme in the given strain that was also found active in some other yeast strains (Moyo et al., 2003). The enzymes with tolerance to their end-products do not lose their activity when the reaction is prolonged and end-product is accumulated, hence, can be exploited for large-scale applications. While the free cells produced 0.137 and 0.241 IU mL^{-1} of pectinase in absence and in presence of

Table 1
Production of pectinase on different carbon sources. Free and immobilized cells were cultivated in mineral salt medium containing sugars and pectinase activity was detected in cell free culture supernatant.

Pectinase production on	Pectinase activity (IU ml^{-1})*	
	Immobilized cells	Free cells
2% xylose	0.246	0.081
2% glucose	0.107	0.075
2% galactose	0.215	0.046
1% Pectin	0.218	0.137

* The values represent average of triplicate with insignificant standard deviation.

Table 2
Effect of addition of galacturonic acid to the pectin containing medium on pectinase production by free and immobilized cells of *G. candidum* AA15. Galacturonic acid was added at the start of cultivation (at 0'), at the commencement of pectinase production (after 1 h) and logarithmic phase of the growth (after 30 h).

Time interval (hour)	Pectinase activity (IU ml^{-1})*									
	1% galacturonic acid was added at 0'		1% galacturonic acid was added after 1'		1% galacturonic acid was added after 30'		1% pectin in MSM		1% galacturonic acid in MSM	
	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells
0′	0.06	0	0	0	0	0	0.115	0	0.06	0
2'	0.803	0	0.912	0	0.548	0	0.458	0	0.927	0.07
5′	1.074	0	1.208	0	0.851	0	0.797	0.121	1.173	0
22'	0.84	0	0.863[a]	0	0.511	0.059[a]	0.495	0.228	1.173	0
28'	0.976	0	1.408	0	0.524	0.124[a]	0.489	0.077	1.667	0
31'	0.587	0	0.709	0	0.801	0	0.396	0.07[a]	0.488	0.08[a]
46'	0.49	0	0.43	0	0.49	0	0.285	0.274	0.86	0.181
72'	0.14	0	0.29	0	0.4	0	0.218	0.137	0.2	0.28

* The values represent average of triplicate with insignificant standard deviation.
[a] The values had significant Standard Deviation.

galacturonic acid in MSM, respectively, however, the activity remained unaffected by the presence of galacturonic acid in assay mixture (data not shown).

3.3. Regulation of pectinase production

We studied the effect of the addition of galacturonic acid on the production of the pectinolytic activities by *G. candidum* AA15. The data revealed that the incorporation of galacturonic acid in production medium at different time interval enhanced the pectinase production by immobilized *G. candidum* AA15. The production of the pectinolytic activity as measured by reducing groups was not negatively affected when galacturonic acid was added to growing culture of *G. candidum* AA15 regardless of the time of addition. It indicated that galacturonic acid acts as an inducer for pectinase. Generally, pectinase production by AA15 initiated after 2 h of cultivation of immobilized cells and increase until 5 h, whereupon the pattern varied with the change in presence of carbon source in the production medium (Table 2). When the immobilized cells were challenged with the galacturonic acid after 30 h of cultivation a transient increase in the titers of pectinase was noted. Moreover, the pectinase titers remained higher until 28 h when galacturonic acid was present as sole source of carbon endorsing about the positive effect of galacturonic acid on the ability of immobilized cells to produce pectinase.

Interestingly, pectinase from free AA15 appeared distinct than from immobilized cells as the incorporation of galacturonic acid in pectin containing medium at any stage of fermentation resulted in complete inhibition of the pectinase production by the free cells. The gene expression in immobilized yeast is indeed vary greatly than in free cells as reported earlier for encapsulated cells of *Saccharomyces cerevisiae* BY4722 (Nagarajan et al., 2014). The authors observed interruption in cell division in immobilized cells, however, extracellular amount of glucose and ethanol varied continuously hinting about metabolic status of the cells. Moreover, productivity of ethanol by immobilized cells was five folds higher than by free cells. In this work, galacturonic acid acted as an inducer for immobilized cells but causes self-catabolite repression in free cells when the concentration is increased beyond the utilization capability of the organism. An optimized production medium is indeed required for the strain; any variation from the optimal concentration of carbon source caused decrease in the productivity of the enzyme (Abdullah et al., 2018).

4. Conclusion

It is concluded that the immobilized cells not only yield higher titers of pectinase as compare to free cells with recyclability, rather catabolite repression is relieved in the immobilized cells of AA15, hence immobilized yeast can be used for cost-effective production of this industrially important enzyme.

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Competing interests

This publication is approved by all authors and they do not have any conflict of interest regarding any financial, personal or other relationships with any other people or organizations.

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