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Research article

Two layered strategy for cost effective production of pectinase: immobilization of yeast and utilization of crude substrate

Uroosa Ejaz *, Hurmat Hanif, Muhammad Sohail

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

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ABSTRACT

The wide scale application of pectinase is generally hindered by high cost of the enzyme production. In this work, a two dimensional strategy was adopted to reduce cost of pectinase production by *Geotrichum candidum* AA15. The strain was immobilized in alginate beads. The optimum concentration for bead formation was found to be 3.5% of sodium alginate (NA) with 4% calcium chloride ($CaCl_2$). Such immobilized cells retained the ability to produce 0.115 IU mL^{-1} of pectinase for up to 6^{th} production cycle in citrus pectin containing medium while free cells produced only 0.046 IU mL^{-1} of pectinase. For the cultivation of immobilized cells on orange peels (OP), a combination of 4.5% NA and 4% $CaCl_2$ was found effective to prepare beads. *Geotrichum candidum* AA15 produced 0.220 IU mL^{-1} pectinase by fermenting OP as a substrate for up to 3^{rd} production cycle. The results revealed that the process of immobilization can be used as a promising strategy in combination with the use of naturally available waste biomass.

1. Introduction

Microbial enzymes have many applications in commercial sector. One of the enzymes, pectinases, degrade pectin present in primary cell wall and middle lamella of plants (Mojsov, 2016). Pectinases are applied in various industrial settings such as in food and feed processing to increase the yield of juice and its clarity (Ahmed and Sohail, 2020). However, high cost of enzyme production is generally considered as a major limiting factor for wide scale application of the enzyme. Immobilization of microbial cells to inert solid matrix provides an advantage to recycle the producer strain thereby reduce the production cost substantially (Ejaz and Sohail, 2020). There are also other advantages associated with immobilization such as lower risk of microbial contaminations, improvement in product yield, increased substrate uptake and easier product recovery (Verbelen et al., 2006). However, no single method and a matrix is best for immobilization of all the microorganisms. The methods can be judged by their merits and demerits, such as some are simple, cheap and effective while some others are effective and durable but expensive. Alginate gel has widely been used for the immobilization of cells (Kregiel et al., 2013), however, the optimal ratio of the Na-alginate and $CaCl_2$ vary from organism to organism and even influenced by the fermentation of raw material. Cell encapsulation offers many advantages and therefore considered as an attractive method of immobilization. Cells are enclosed within a thin semipermeable membrane. Nutrients and products can pass through the membrane of bead. Encapsulation also improves the stability of cell and inhibitor tolerance. Furthermore, cells can move in the inner liquid core of the bead (Ylitervo et al., 2011).

In addition to immobilization, utilization of crude waste materials as generated by agro-industrial practices also prospects to reduce the production cost. Being rich in carbohydrates, proteins and minerals, such wastes can serve as a source of nutrition for microorganisms. Pakistan is amongst the largest producer of oranges and holds a large number of fruit juice industries which produce several million tons of wastes in the form of peels. Although, orange peels (OP) have been used by several workers to produce pectinase, however, suitability of the substrate has not been explored for immobilized yeast cells. This work describes immobilization of a less-studied yeast, *Geotrichum candidum*, in alginate beads and pectinase production was monitored using OP as substrate.

2. Experimental

2.1. Orange peel powder

Orange peels (OP) were collected from a local fruit juice seller. Dried OP was ground to 100 μm mesh size.

* Corresponding author.
E-mail address: uroosaejaz24@gmail.com (U. Ejaz).

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Table 1. Effect of different concentrations of Na-alginate and $CaCl_2$ on consistency of beads.

Aqueous solution of $CaCl_2$	Beads formation using aqueous solution of Na-alginate*					
	2%	3%	3.5%	4%	4.5%	5%
2.5%	-	+	+	+	-	+
3%	-	+	+	+	-	+
3.5%	-	++	+	+	+	+
4%	-	++	+++	-	+	+

* Consistency of the beads was evaluated visually and graded in terms of +, ++ and +++ signs, the later being perfectly spherical and stable.

2.2. Na-alginate beads formation

Na-alginate (NA) solution (2%) prepared in distilled water was dropped separately into aqueous solutions of $CaCl_2$ with variable concentration (2.5%, 3%, 3.5% and 4%). The beads were left for 30 min at 30 °C. The same procedure was repeated using 3%, 3.5%, 4%, 4.5% and 5% Na-alginate solution and examined visually (Unver et al., 2015).

In yet another experiment, NA solution was prepared in 0.9% saline and the protocol mentioned above was repeated. Similarly, another experiment was conducted using $CaCl_2$ solution prepared in 50 mM sodium citrate buffer (pH 4.8). Likewise, different combinations of NA and $CaCl_2$ solutions were prepared in different solvents to investigate the most suitable combination (Unver et al., 2015).

2.3. Inoculum

Geotrichum candidum AA15 was revived on Sabouraud's Dextrose agar (SDA) plates. Culture, from SDA slant was transferred to seed broth (Sabauraud's Dextrose broth) (Oxoid, USA) and incubated at 30 °C for 24 h. Optical density of the inoculum was measured at 600 nm and was adjusted to 1.0.

2.4. Pectinase production

The inoculum corresponded to A_{600} 1.0 was centrifuged and 3.5% aqueous NA solution was added into pellet. The vortexed suspension was then dropped into 4% aqueous solution of $CaCl_2$ and was left for 30 min at 30 °C. The beads were then transferred to 0.38% chitosan (prepared in 1% acetic acid) for 30 min at 30 °C and then washed with distilled water. The beads were then kept for 30 min in 50 mM Na-citrate buffer (pH 4.8).

Ten immobilized NA beads were transferred to 50 mL of mineral salt medium (MSM) (Ahmed et al., 2019) containing 0.75% pectin and incubated for 24 h at 25 °C. A control of free cells was also used by adopting the same protocol for pectinase production. For control, inoculum was transferred into the MSM as used for immobilized cells. After incubation period, the content was centrifuged at 4000×g for 25 min and cell free culture supernatant was used for pectinase assay, whereas, the immobilized beads and free cells pellet were used for the next production cycle.

As mentioned above, different concentrations of NA and $CaCl_2$ were used for making the immobilized beads for pectinase production by using orange peels (OP) powder instead of commercial pectin. The fermentation protocol was same for OP as mentioned for commercial pectin.

2.5. Enzyme assay

Pectinase assay was performed by estimating reducing sugars (Miller 1959; Ejaz et al., 2018).

2.6. Statistical analysis

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

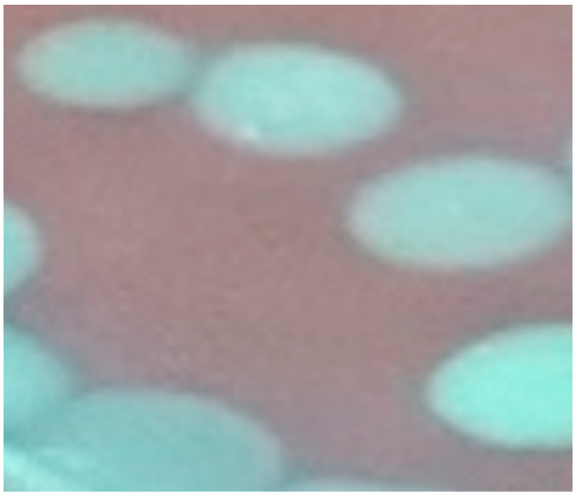

Figure 1. Alginate beads formed by mixing 3.5% aqueous solution of sodium alginate and 4% aqueous solution of $CaCl_2$.

3. Results and discussion

3.1. Pectinase production by immobilized yeast

Pectinases are of great value from the industrial perspective as they are used in various processes including plant fiber processing, tea and coffee fermentation, fruit mash treatment, improvement of essential oils, in the production of baby food, degumming fiber crops, extraction of oils, treatment of industrial wastewater etc (Alimardani-Theuil et al., 2011). Pectinase is largely obtained from molds or yeasts (Poondla et al., 2016). However, high cost of production and low yield hinders the mass production of pectinase. Immobilization of microbial cells is one of the strategies that can be adopted to reduce the enzyme production cost (Ejaz and Sohail, 2020). For this purpose, the carrier or matrix cost represents a significant part of the investment and therefore, search for a cheap and easy to regenerate support material is still relevant (Laopaiboon and Laopaiboon, 2012). So far, in industries, delignified cellulosic and diethylaminoethyl cellulosic material have been used as an immobilization matrix of yeast for the continuous production of beer and wine, respectively (Almeida et al., 2003). The present study was commenced with screening of sodium alginate (NA) beads for their suitability to immobilize *G. candidum* AA15 for pectinase production. Previously, alginate beads were used to immobilize *Saccharomyces cerevisiae* for the production of succinate dehydrogenase and pyruvate decarboxylase (Kregiel et al., 2013). However, utmost possible literature survey did not reveal any report about the use of alginate beads for immobilization of *G. candidum*. As the technique of the beads preparation needs to be optimized according to the producing strain, therefore, it was studied for the immobilization of the strain AA15.

The mechanical strength of beads is linked with concentration of NA. Alginate beads should have adequate mechanical strength; otherwise cells could leak from it. A too rigid bead can affect the transfer of

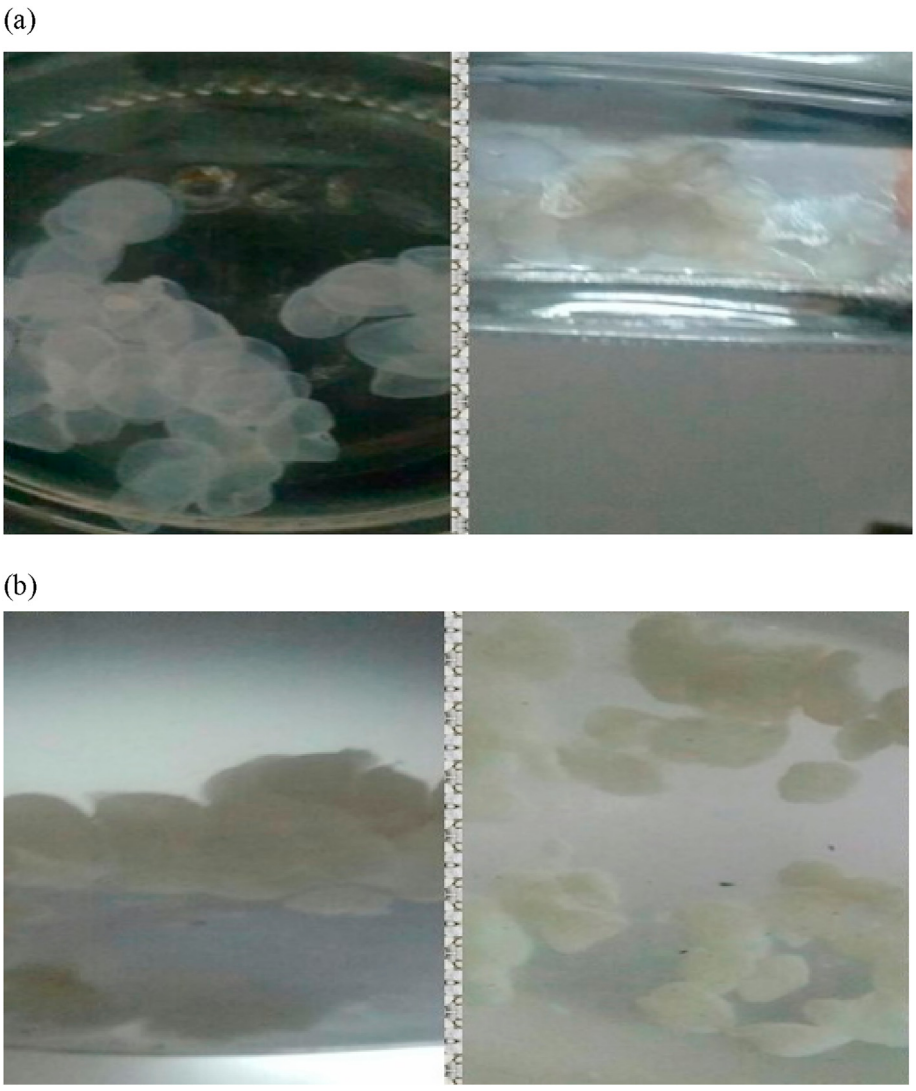

Figure 2. Na-alginate beads (a) Without chitosan coating. (b) With chitosan coating.

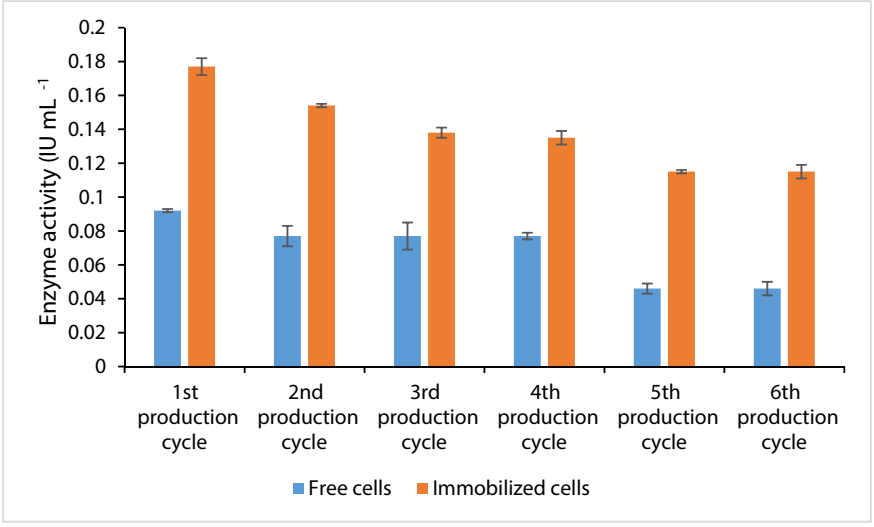

Figure 3. Pectinase production by free and immobilized cells of *G. candidum* AA15 for six production cycles.

Table 2. Pectinase production by immobilized cells using orange peels.

Concentration of Na-alginate and $CaCl_2$	Enzyme productivity (IU mL^{-1})*			
	1st cycle	2nd cycle		3rd cycle
4% $CaCl_2$ and 3.5% Na-alginate	0.207	0.200 96.6%		0.184 88% (Beads dissolved)
4% $CaCl_2$ and 3.5% Na-alginate	0.240	0.223 92.9%		0.215 89% (Beads dissolved)
4% $CaCl_2$ and 4% Na-alginate	0.215	0.214 99%		0.207 96% (Beads dissolved)
4% $CaCl_2$ and 4.5% Na-alginate	0.268	0.237 88%	0.220 (Beads survived) 82%	

* Insignificant standard deviation. The value of IU mL^{-1} obtained in 1st cycle was considered as 100% and other values were compared accordingly.

nutrients and number of immobilized cells, whereas, soft bead would not have enough mechanical strength. Therefore, effect of different concentration of NA and $CaCl_2$ on beads consistency were investigated. Initial experiments revealed that 4% aqueous solution of $CaCl_2$ and 3.5% aqueous solution of NA gave better result than that of the other concentrations (Table 1). Consequently, the best suited concentrations of NA and $CaCl_2$ were prepared in buffer, saline and distilled water to observe the effect of solvent on beads formation. The results affirmed that 3.5% aqueous solution of NA and 4% aqueous solution of $CaCl_2$ produced the beads of appropriate size and shapes (Figure 1). After the optimization of concentration and solvents for NA and $CaCl_2$, the beads were treated with chitosan to provide stability. Post treatment of beads with chitosan resulted in better consistency (Figure 2). Furthermore, cellular composition of encapsulated yeast is different from the free cells (Ahmed et al., 2020). Chitosan coating also makes the immobilized cells more tolerant to heat than the free cells (Ylitervo et al., 2011).

Repeated batch fermentation experiments were carried out to determine the efficiency of immobilization for pectinase production and stability of cells immobilized in NA beads. It is important to realize that the physiology of immobilized cells is affected by the microenvironment (Berlowska et al., 2013). In this research, higher titer of pectinase was obtained by immobilized *G. candidum* AA15 (0.177 IU mL^{-1}) compared to the free cells (0.092 IU mL^{-1}) and the matrix remained effective for up to six cycles with 0.155 IU mL^{-1} pectinase productivity (Figure 3). This observation can be linked with the fact that metabolic activity increases in immobilized cells (Ahmed et al., 2020; Berlowska et al., 2013; Behera et al., 2011). Notably, the cost of free cells recycling is higher than that of immobilized beads recycling hence the significant reduction of the production cost can be achieved by using immobilized cells. The studies conducted by Chen et al. (2013) also highlighted the effectiveness of recycled immobilized *Clostridium acetobutylicum* for butanol production in repeated-batch fermentation of glucose.

3.2. Use of orange peel

Citrus fruits are widely consumed by people worldwide. These are used for the preparation of marmalades, pickles, squashes and juices (Dhillon et al., 2004). Seeds, peels and pulp are the citrus waste product which also cause waste disposal problem. However, citrus peels are rich in sugar content and can be fermented by microorganisms for the production of ethanol, citric acid and many value added enzymes (Poondla et al., 2016). The media composition is one of the important factor for enzyme production and the balance between carbon and nitrogen sources in media influences the secondary product formation and growth rate, thereby influences the distribution and density of immobilized cells (Ejaz et al., 2018). Therefore, pectinase production by immobilized AA15 in presence of citrus waste, orange peels (OP), was studied.

OP has not been previously exploited as a substrate for NA immobilized yeast cells. It is rich in pectin that can induce synthesis of pectinolytic enzymes. Indeed, it has been reported as a substrate for *Bacillus subtilis* (Kohli and Gupta, 2015) and *Geotrichum candidum* (Qadir et al., 2020) for pectinase production. Poondla et al. (2016) observed 9 fold increment in pectinase production by fermentation of OP as a substrate for *S. cerevisiae*. However, it was speculated that the consistency of the beads would be greatly affected in presence of an insoluble substrate. Therefore, various combinations of NA and $CaCl_2$ were tested along with chitosan coating, and stability of the beads was noted for three continuous production cycle. The data showed that the beads formed by mixing 4% $CaCl_2$ and 4.5% NA remained intact with natural substrate for up to 3rd production cycle with a yield of 0.220 IU mL^{-1} or 85% of productivity (Table 2) (Figure 4). The two heterogeneous systems are present which includes NA beads in which yeasts are immobilized, solid substrate which is OP and liquid medium. Particles of OP are transformed into the different oligomeric and monomeric sugars by the action of pectinase because cell produced some basal amount of enzyme constitutively. Theses monomers and oligomers entered inside the NA bead and induced the pectinase production by yeast cells. Some organism produce pectinase both constitutively and inductively (Jain et al., 1990; Dosanjh and Hoondal, 1996). The results were also analyzed by two-way analysis of variance (two-way ANOVA). The ANOVA was run for three production cycle (Table 3). For column, F-value was greater than the F-critical value which reveals that there was a significant difference between the three production cycles and P-value is also <0.05. The beads formed by mixing other combinations (3%, 3.5% and 4% of NA with 4% $CaCl_2$) could retain their productivity for up to 2nd production cycles (Table 2). Earlier, *G. candidum* AA15 has been reported for 0.250 IU mL^{-1} pectinase by using commercial pectin (Ahmed et al., 2019). Hence, the results demonstrated that production of pectinase by using OP is comparable to that of using commercial pectin that was in line with the findings of Nighojkar et al. (2006). The study provides information that could be used to develop a large scale process based on utilization of an abundantly available waste and immobilized yeast cells.

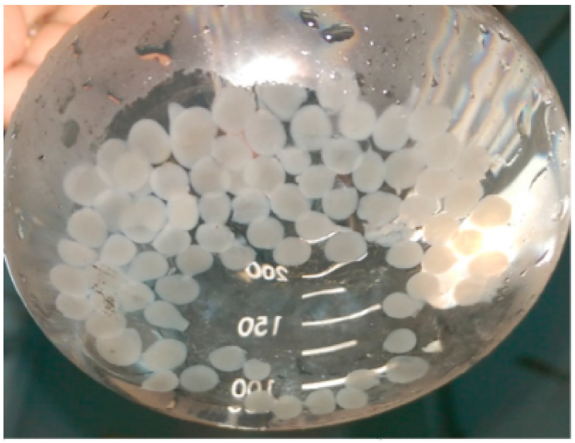

Figure 4. Alginate beads formed by mixing 4% $CaCl_2$ 4.5% Na-alginate.

Table 3. Analysis of variance of pectinase production by immobilized cells.

Source of Variation	Sum of square	Degree of freedom	Mean sum of square	F[a]	P-value[b]	F crit
Sample (Rows)	1.342465	3	0.447488	1.099993	0.398355	3.862548
Columns	15.60796	3	5.202652	12.78889	0.00135	3.862548
Error	3.661294	9	0.40681			
Total	20.61172	15				

[a] F = Fishers's function.
[b] P = Corresponding level of significance.

4. Conclusions

The strain *Geotrichum candidum* AA15 immobilized in Na-alginate produced greater yield of pectinase as compared to the free cells. The different concentration of constituents affecting Na-alginate beads formation were evaluated for pectinase production on commercial substrate as well as on a crude substrate. The cells immobilized on the beads formed by mixing 4% $CaCl_2$ and 4.5% sodium alginate retained (0.220 IU mL^{-1}) productivity for up to three production cycle when orange peel was used as substrate. The study revealed potential of immobilized yeast cells for continuous production of pectinase on orange peels.

Declarations

Author contribution statement

Uroosa Ejaz: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hurmat Hanif: Performed the experiments.

Muhammad Sohail: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing Interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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