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THE RELATIONSHIP OF PELLET MORPHOLOGY TO POLYGALACTURONASE PRODUCTION OF *Rhizopus oryzae* IN VARIOUS MEDIA COMPOSITIONS

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Abstract
Various media parameters affecting the polygalacturonase (PG) production of Rhizopus oryzae, were studied and their relation to pellet morphology was investigated. The basal medium in the absence of Mg^{+2} and in the presence of 4 mg/kg of Zn^{+2} at pH 3, resulted into maximum PG activity (11.53 U/ml). A composition of 14.78 g/l of glucose, 10 g/l of galactose, 5 g/l mannose, 0.5 g/l of arabinose and 19.73 g/l of xylose resulted into maximum PG activity (27.94 U/ml) when used as combined carbon sources. Corn meal as the nitrogen source, promoted PG synthesis where it resulted into 33 % more activity than corn steep liquor (CSL) and $(NH_4)_2SO_4$ which were the next highest promoter. The highest number of pellets with an average mean diameter of 1.25±0.25 mm was obtained with the formulation containing $(NH_4)_2SO_4$ and 25 g/l of glucose. Yeast extract on the other hand resulted into pellet formation with an average mean diameter of 1.75±0.25 mm at higher glucose concentration (50 g/l). The interactive effect of suitable carbon source (glucose) with suitable nitrogen source (corn meal) enhanced the PG activity 4 times more than the basal medium with pellets of 1.44±0.35mm in average diameter.

Keywords: *Rhizopus oryzae*, polygalacturonase, pellet morphology

DEĞİŞİK ORTAM KOMPOSİZYONUNDA *Rhizopus oryzae*'nin POLİGALAKTURONAZ ÜRETİMİ İLE PELLET MORFOLOJİSİNİN İLİŞKİSİ

Özet
Bu çalışmada, ortam kompozisyonunu belirleyen değişik faktörlerin Rhizopus oryzae pellet morfolojisine ve poligalakturonaz (PG) enzimi üretimine olan etkisi incelenmiştir. En yüksek PG aktivitesine (11.53 U/ml) 4 mg/kg Zn^{+2} içeren, Mg^{+2} içermeyen ve pH 3 olan basal ortamında ulaşılmıştır. Karbon kaynağı olarak 14.78 g/l glukoz, 10 g/l galaktoz, 5 g/l mannoz, 0.5 g/l arabinoz ve 19.73 g/l ksilozun birlikte kullanılmıyla da en yüksek PG aktivitesine (27.94 U/ml) ulaşılmıştır. Mısır unu nitrojen kaynağı olarak PG sentezini tetiklemede oldukça etkili olup bir diğer yüksek tetikleyici olan mısır maserasyon sıvısı (CSL) ve $(NH_4)_2SO_4$'den %33 daha fazla aktivite vermiştir. $(NH_4)_2SO_4$ and 25 g/l glukoz içereren formulasyon ortalama çapı 1.25± 0.25 olan en fazla sayıda pellet oluşumunu sağlamıştır. Ortalama çapı 1.75±0.25 mm olan pelletlerin oluşumu ise yüksek glukoz konsantrasyonuna sahip (50g/l) maya ekstraktı içeren formulasyon ile elde edilmiştir. Uygun karbon kaynağının (glukoz) uygun nitrojen kaynağı (mısır unu) ile interaktif etkisi PG aktivitesini basal ortama göre 4 kat daha arttırmış, ortalama çapı 1.44±0.35mm olan pelletler oluşturmuştur.

Anahtar kelimeler: *Rhizopus oryzae*, poligalaktronaz, pellet morfolojisi

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INTRODUCTION

It is well experienced that pellet type of morphology exhibits many advantages over the filamentous type due to its non viscous nature. This is mainly accounted by facilitating the nutrient and oxygen transfer and reducing power consumption of the agitator and thereby easing the downstream processing of the final product. (1). Therefore, this type of morphology is the desired type in many industrial fermentations such as in the production of antibiotics, organic acids and enzymes, (2). However, factors such as pH, temperature, media composition, inoculum type and fermentation conditions affect this morphology, significantly (3). Therefore, one has to optimize these factors for maximum productivity of the desired product and for the control of uniform pellet formation. However, sometimes pellet type of morphology might not be possible due to genetic factors of the fungus. Therefore, different type of morphologies can be observed in the production of different compounds. For example, dispersed growth is preferrred for penicillin production by *Penicillium chrysogenum* and fumaric acid by *Rhizopus arrhizus*, whereas pellet type of growth is observed for the production of pravastatin precursor by *Penicillium citrinum*, citric and itaconic acid by *Aspergillus niger* (4-6). Thus, the studies on the fungal pellet formations are limited to the level of individual strain.

As it is known pectinases are widely used in the textile, paper, chemical and food industry (7). The applications are mainly in the retting of flax and vegetable fibres, increasing the yield of fruit juice extraction, de pectinisation and clarification of fruit juices, extraction of oils from vegetables and citrus peels and pretreatment of pectic waste water. (7, 8). Pectinases account 25% of the global enzyme sales, where this contribution is estimated to increase further by the year 2013 (9). Therefore their production by cost efficient and productive means exhibits tremendous importance.

The objective of this study was to investigate the effect of major factors, such as initial pH, media composition (various carbon and nitrogen sources) and metal ions (iron and magnesium) significant in industrial fermentations, on the pellet morphology of *Rhizopus oryzae* and determine their relation to polygalacturonase (PG) production. Polygalacturonase which attracts the most attention among the pectinases by *Rhizopus oryzae* (considered rarely) was considered for this purpose. Finally, the goal was to propose a medium formulation that could result into desired pellet type of morphology with maximum PG activity using low cost carbon and nitrogen sources. Since the literature on the effect of these factors on the polygalacturonase and morphology of *Rhizopus oryzae* is scare, therefore the current study was thought to close the gap to some degree.

MATERIAL AND METHODS

Culture preparation and fermentation medium

Rhizopus oryzae ATCC 4858 was purchased in the lyophilized form, from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. Stock cultures of this strain was prepared in 20% glycerol water and stored at -80 ^{o}C. The culture was propagated on YME (Yeast Malt Extract) agar slants containing malt extract (10 g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l) for one week at 30^{o}C. Afterwards, spores were transferred on molasses agar slants and incubated at 30 ^{o}C for another week and later harvested using 0.02%(v/v) Tween-80 water solution (10)

For all runs, the basal fermentation medium consisted of glucose (50 g/l), urea (2g/l), KH_2PO_4 (0.6 g/l) and Fe^{2+} (100 µg/kg). All media components were dissolved in total volume of 100 ml distilled water in 250 ml cotton-plugged Erlenmeyer flasks, inoculated with $7x10^5$ total spores and incubated at 30 ^{o}C and 170 rpm for 96 h. The initial pH of the basal medium was adjusted to pH 3, if not otherwise mentioned. After the fermentation time (96 h) each flask was assayed for morphological measurements, carbohydrate content and polygalacturonase (PG) activity.

Analytical methods and morphological measurements

PG (polygalacturonase) activity was assayed according to the procedure given by Panda et al. (11) using 2.4 g/l of polygalacturonic acid as substrate at pH 6.6 and 26^{o}C with a reaction time of 20 min. Total carbohydrate concentrations were determined by the phenol sulfuric acid method (12).

Pellet particles were analyzed by counting the number of pellets per given volume and measuring the pellet size using the diameter corresponding to a circular area equivalent to the pellet projected area. Pellet morphology was characterized using image analysis (13). The image was captured with a eurocam (Euromax, Holland) mounted on a phase contrast microscope (Novex, Holland). Image analysis was performed with the software package Image-Pro Plus 4.5.1. (Media Cybernetics Inc., Silver Spring, MD,USA).

Effect of various media parameters (pH, metal ions, carbon and nitrogen sources)

To determine the effect of initial pH and metal ions, the basal medium was prepared at different initial pH (3.0, 3.5, 4.5 and 5) at different concentrations of Mg^{+2} (0, 25, 50 and 75 mg/kg) and Zn^{2+} (0, 2, 4 and 6 mg/kg) by changing each factor one at a time. The fermentation conditions were such as described in material and methods.

The effect of carbon sources first as single and later as combination was investigated by conducting two different set of experiments. In the first part five different carbon sources as hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose) were used first as single carbon source in the amount of 25 g/l in the basal medium to replace glucose. In the second part, glucose in the basal medium was replaced with a combination of sugars selected by Mixture Design by Design Expert 7 trial version program (Table not shown). In this design total carbohydrate concentration was kept constant at 50 g/l. Concentrations of sugars changed in the range of 0-25 g/l glucose, 0-10 g/l galactose, 0-5 g/l mannose, 0-5 g/l arabinose and 0-20 g/l xylose. The fermentation conditions for both experimental sets were such as described in material and methods. Design Expert 7.0.0 Trial Version software was used to analyze the data, where the significances of all terms were judged statistically according to their p-values.

The effect of nitrogen sources on the response parameter was investigated using various sources such as peptone, corn steep liquor, soybean meal, corn meal, yeast extract, whey and $(NH_4)_2SO_4$. These were used at the concentration of 2 g/l in order to replace the urea in the basal medium. Furthermore, to determine the concentration of glucose on different nitrogen sources, two glucose concentrations (25, 50 g/l) were studied. Finally, the interactive effects of nitrogen sources (corn meal and CSL) were used in combination with glucose and xylose at different concentrations replacing the nitrogen and carbon sources in the basal medium. The fermentation conditions were such as described in material and methods.

RESULTS AND DISCUSSIONS

Effect of pH and metal ions

Initial pH of the medium and certain metal ions are well known to have great influence on enzyme production and morphology of the culture (14). Since their effect changes with type of the organism as well as with the fermentation conditions, these factors have to be well determined for each and every strain under consideration. Hence, the effect of pH was investigated using basal medium containing 4 mg/kg of Zn^{+2} and 50 mg/kg of Mg^{+2} (Table 1). Increasing the pH from 3.5 to 5 did not cause a significant change in PG activity, however it did have profound effect on the morphology. For example a big clump was observed at pH 5, whereas at lower pH a pellet type of morphology was seen. In fact, this could be explained by the effect of pH changes, which alters the surface properties of spores and thereby their ability to coagulate (15). At an initial pH of 3, there was no requirement for Zn^{+2} in the medium since the maximum polygalacturonase (PG) activity of 5.22 U/ml was achieved in the absence of this metal ion. Thus increasing the level to 2 mg/kg reduced the PG activity, significantly. This issue brought up the idea that this metal ion may act as an inhibitor on the PG activity but not necessary on the growth of the organism itself. The morphology of Rhizopus oryzae in the corresponding runs (2, 3, 8 and 11) changed from being fluffy loose pellets to few small clumps. Obviously, the morphology was affected by the concentration of Zn^{+2} ion, which in turn might have affected the PG production where maximum PG was synthesized with fluffy loose pellets. Increasing Mg^{+2} from 0-75 mg/kg (Table 1) at an initial pH of 3 reduced the PG activity tremendously from 11.53 U/ml to almost 0 U/ml. Similar to Zn^{+2}, PG activity was inhibited in the presence of Mg^{+2}. The type of morphology was strongly influenced by the concentration of Mg^{+2} ranging from clump to pellet and mycelium form. As a result it can be concluded that maximum PG activity (11.53 U/ml) can be obtained at 4 mg/kg of Zn^{+2} and in the absence of Mg^{+2} at pH 3 with a single clump formation. PG activity was reduced from 11.53 U/ml to 9 U/ml when both Zn^{+2} and

Mg^{+2} were excluded from the basal medium. This was still acceptable if the cost of Zn^{+2} should be a concern. However, if the interest is to compromise the PG activity for pellet type of morphology then it is recommended to add 50 mg/kg of Mg^{+2} to the basal medium but exclude the Zn^{+2} . This still would result into PG activity of 5.22 U/ml. The current results were in close agreement with the studies reported in the literature (16, 17).

Table 1.

Run Order	Zn^{+2} (mg/kg)	Mg^{+2} (mg/kg)	Initial pH	PG activity (U/ml)	Morphology
1	0	0	3.0	9.0	few small clumps
2		50	3.0	5.22	fluffy pellets with mycelium
3	2	50	3.0	0.66	clump with small pellets
4	4	0	3.0	11.53	one single small clump
5		25	3.0	0.66	small pellets and clump
6		50	3.0	0	clump
7			3.5	0.05	small pellets
8			4.0	0.03	medium sized pellets and clump
9			5.0	0.09	big clump
10		75	3.0	0.52	mycelium
11	6	50	3.0	0.35	Few small clumps

Table 1. Effect of pH and metal ions ($Zn^{+2}$and Mg^{+2}) on polygalacturonase (PG) activity and morphology

Effect of carbon sugars

In order to determine the individual effect of carbon sugars, single carbon sources in the form of hexoses (glucose, galactose and mannose) and pentoses (xylose and arabinose) were used to replace the carbon source in the basal medium in the amount of 25 g/l. This was done first to identify the preference of this organism for these sugars and second to determine the sugars that would induce pellet type of morphology preferred mainly by the industry. In any industrial fungal fermentation raw material cost makes up the major cost. Therefore, finding cheap resources containing these sugars can significantly reduce the costs. The effect of each sugar on PG activity was in the increasing order, such as mannose, arabinose, galactose, glucose and xylose. Xylose and glucose (6.69 and 6.24 U/ml, respectively) were the two sugars increasing the PG activity, almost 1.94 times more than mannose (3.36 U/ml). The final morphology of the culture was affected profoundly by single sugars. For example arabinose resulted into mycelial fragments including lots of hyphal elements, whereas mannose and glucose resulted into clump type of morphology. Fluffy pellets of different sizes and numbers were obtained by xylose and galactose (Fig 1). These resembled a normal distribution.

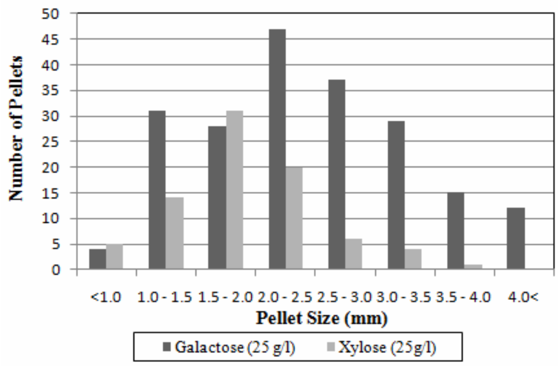

Fig. 1 Effect of different carbon sugars on pellet size distribution in the basal medium at 30 ºC and 170 rpm incubated for 96 h.

A Mixture D-optimal design with 5 center points was used to determine the linear and interactive effect of 5 (pentoses) and 6 (hexoses) carbon sugars such as glucose, galactose, mannose, arabinose and xylose on PG activity and morphology (Table not shown). According to the analysis of variance (not shown), none of the variables (carbon sugars) were found significant (p>0.05) on the response parameters under the ranges studied. Physically this meant, that the fungus did not have special preference, as long as the total carbon amount was constant. In fact, clump to clump mixed with fluffy pellets was the observed morphology. The size and density of pellets and clumps were quite different. A media composition of glucose (19.88 g/l), galactose (6.20 g/l), mannose (4.92 g/l), xylose (4.93 g/l) and arabinose (14.07 g/l) resulted into pellet type morphology, only. Maximum PG activity (27.94 U/ml) was obtained at a composition of 14.78 g/l of glucose, 10 g/l of galactose, 5 g/l mannose, 0.5 g/l of arabinose and 19.73 g/l of xylose. In fact, this composition promoted 3 times more PG synthesis compared to the basal medium in previous section where single glucose source at a concentration of 50 g/l was used. Therefore, the presence of these sugars in the same medium caused a synergistic effect on the production of polygalacturonase enzyme. As a result, this study showed that the fungus does not have special preference for certain sugars and it can assimilate even five carbon sugars (pentoses) such as xylose and arabinose. This conclusion, is important, because there are number of renewable cheap resources like, paper pulp sulfite a by product of the paper industry and wood hydrolyzates or rice straw which can be used using this organism in the production of various commercially valuable products of (18, 19).

Effect of nitrogen sources

Different nitrogen sources peptone, corn steep liquor (CSL), soybean meal, corn meal, yeast extract, whey and $(NH_4)_2SO_4$, were used to replace the urea in the basal medium, at two different glucose concentrations (25 and 50 g/l). As can be seen from Fig. 2, glucose concentration at 50 g/l resulted in the highest PG activity. Corn meal was very effective in promoting PG synthesis resulting into 33% more activity than CSL and $(NH_4)_2SO_4$, which were the next highest promoter.

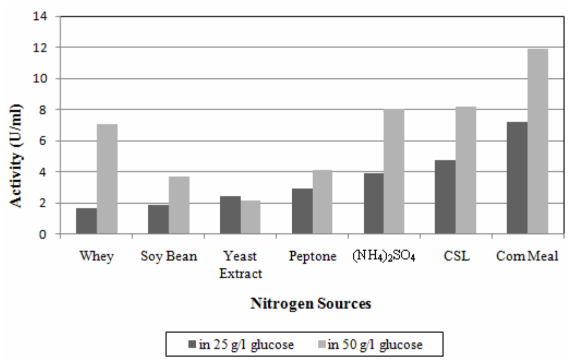

Fig. 2 Effect of different nitrogen sources on PG activity at two different glucose levels (25 g/l and 50 g/l) in the basal medium at 30 °C and 170 rpm incubated for 96 h.

As it may be followed from Fig. 3 at two different concentrations of glucose (25 and 50g/l) the type of nitrogen source had a significant effect on the type of morphology. It ranged from being only clump to various sized and shaped pellets. Besides, the concentration of glucose together with the nitrogen source determined the size and number of pellets formed. The highest number of pellets with an average mean diameter of 1.25±0.25 mm was obtained with the formulation containing $(NH_4)_2SO_4$ and 25 g/l of glucose. However, $(NH_4)_2SO_4$ the same nitrogen source in the presence of 50g/l of glucose resulted into clump formation. Yeast extract on the other hand was the preferable nitrogen source that resulted into pellet formation with an average mean diameter of 1.75±0.25 mm at higher glucose concentration (50 g/l). Therefore, the nitrogen sources have to be selected by taking the concentration of the carbon source into account when considering this morphological parameter. Similar results were obtained in a study reported by Du et al., (5) in the investigation of morphological changes of *Rhizopus chinesis* 12 in submerged culture and its relationship with antibiotic production.

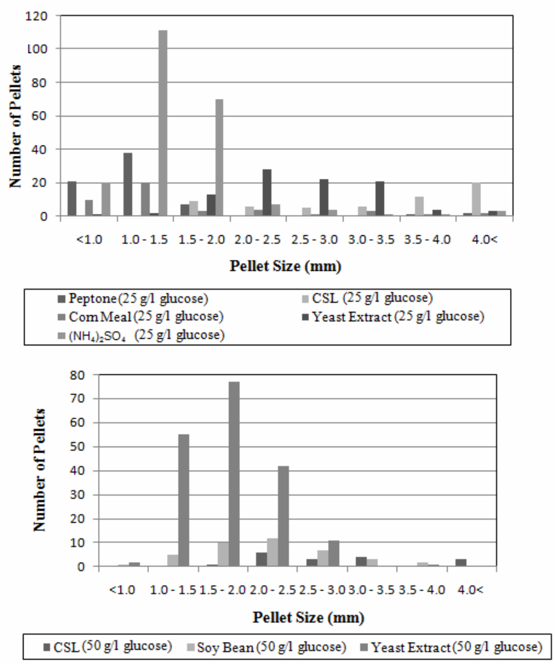

Fig. 3 Effect of different nitrogen sources on pellet size distribution at glucose levels (25 g/l and 50 g/l) in the basal medium at 30 °C and 170 rpm incubated for 96 h.

Interactive effect of carbon and nitrogen sources

The carbon and nitrogen sources which resulted into highest polygalacturonase (PG) activity in previous sections were combined in order to determine if their interactive effect would promote enzyme activity further. Hence, glucose and xylose as carbon sources together with corn meal and CSL as nitrogen sources were chosen to replace the carbon and nitrogen sources in the basal medium at pH 3. The main goal in this section was to determine a low cost media formulation that could result into highest PG activity, since the sources used were cheap resources. As can be observed (Fig. 4) the highest PG activity (34.68 and 36.94 U/ml) was achieved either in a formulation composed of CSL (2 g/l) as nitrogen source and xylose (50 g/l) as carbon source with clump type of morphology or in a formulation composed of corn meal (2g/l) and glucose (100 g/l) with a pellet type of morphology with an average size of 1.44 ±0.35 mm in diameter. This result revealed that CSL was complimentary with xylose, whereas corn meal was complimen-

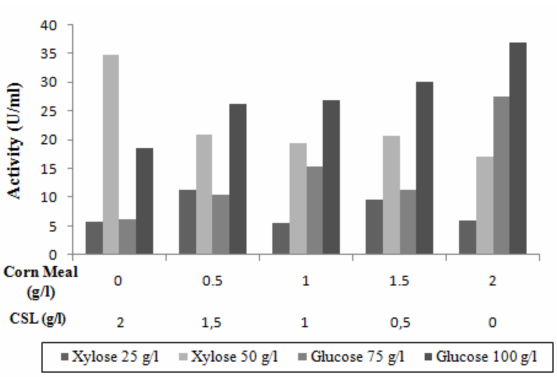

Fig. 4 Interactive effect of different nitrogen and carbon sources on PG activity in the basal medium at 30 °C and 170 rpm incubated for 96 h.

tary with glucose. It was amazing to notice that, the assimilation of the sugar type (pentose or hexose) was a function of the nitrogen source used. The metabolic pathways towards enzyme synthesis and the type of morphology associated with it apparently were directed based on these combinations. This set of experiments also demonstrated, that if the preference was to use CSL and corn meal in combination at different concentrations, then it was recommended to use glucose at 100 g/l as opposed to xylose. The xylose assimilation was adversely affected by the presence of corn meal possibly due to some inhibitors present in corn meal that were influencing the synthesis of some metabolites responsible in the enzyme synthesis. Overall, the interactive effect of suitable carbon source (glucose) with suitable nitrogen source (corn meal) enhanced the PG activity 4 times more with a pellet type of morphology with an average diameter of 1.44±0.35mm compared to the basal medium (Table 1 run 1 and Fig.4). In fact the exclusive preference of the culture for nitrogen sources in combination indicated that the necessary elements were not present in sufficient amounts in sole nitrogen sources. CSL and corn meal therefore were complimentary. Numerous studies investigated the sole effect of carbon and nitrogen sources on the production of various enzymes by different organisms, however there are very few studies on the combined effect of these sources on the enzyme synthesis in relation to the morphology of the culture (5, 6, 18, 19). Therefore, this study will provide additional information by closing the gap in the literature to some degree and serve as reference in the low cost media formulations for fungal organisms in various industrial application

CONCLUSION

Effect of initial medium pH, metal ions, carbon sources and nitrogen sources were used to investigate their effect on polygalacturonase and its relation to morphological changes. The dependency of each product for these sources was different, therefore it was recommended to apply the corresponding condition for the responses of interest, separately. Besides, the combination of various carbon sugars together resulted into higher PG activity compared to a single carbon source indicating the suitability of cheap resources to be utilized by this organism. This study may also benefit fungal fermentations in process design by applying the conditions and media formulation that can result in desired pellet type of morphology for an easy fermentation and subsequent down stream processing. Moreover, researches developing mathematical models describing fungal systems may benefit from the data on pellet sizes and corresponding enzyme synthesis.

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Yeni Kitap

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