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Optimization of pectinase production by *Aspergillus niger* using orange pectin based medium

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ABSTRACT

Pectinases are heterogeneous group of enzymes which are used to hydrolyze the pectic substances and thus becomes of high interest in many industries. The objective of this study is to develop cost effective industrial medium for pectinase production. The effects of medium composition on the production of pectinase using a newly isolated Aspergillus niger strain were studied using one factor at a time (OFAT) approach. At first, different media which were reported before for their high potency to support pectinase production were screened. The medium which support higher pectinase production was composed of (g L^{-1}): Sucrose, 30; K_2HPO_4, 1 and Capek concentrate (10 mL) containing per 100 mL: $NaNO_3$,3; KCl ,0.5; $MgSO_4 \cdot 7H_2O$,0.5; $FeSO_4 \cdot 7H_2O$, 0.01. This medium yielded cell biomass and total pectinase activity of 2.15 g L^{-1} and 26.85 U mL^{-1}, respectively. Attempts were done to change the carbon source to cost effective source (mandarin pectin) which considered also as agriculture waster. After this step, other medium ingredients were also changed in terms of types and concentrations to develop new medium formula for high pectinase production. The optimal medium composition for pectinase production obtained in this work was composed of (g L^{-1}): Mandar in orange pectin, 30; ammonium sulphate, 3.33; and K_2HPO_4, 1.0. This medium supported maximal enzyme production up to 74.35 U mL^{-1}after 96 hours cultivation.

Keywords: *Aspergillus niger*, Medium optimization, Pectinase, Mandarin orange peel.

INTRODUCTION

Pectinase is a generic term that used from derivation of the pectin. Pectin is a complex class of carbohydrates polymer which composed of member galacturonic acid that linked through the α-1-4-glycosidic linkage and it is widely found in the primary cell walls or at the middle lamella of higher plants [1]. Application of the pectinases is based on the pH requirement either it is acidic or alkaline for optimum enzymatic activity. The acidic pectinases are usually applied for extraction, clarification and liquefaction of fruit juices or wines whereas for alkaline pectinases are widely used in the fabric industry for retting of plant fibres such as flax, hemp and jute. In addition, it also used for solving the retention problem in mechanical pulp bleaching in paper industry [2]. The potential and capability of true filamentous fungi in the processes or particularly in the secretion and production of pectinases have been revealed by in many studies [3]. Thus, the filamentous fungi provide a potentially high yielding and relatively cheap option and the genus of *Aspergillus* has been used with a success as a production host [4]. *A. niger* is a normally known as cell factory for the production of organic acids and different types of industrial enzymes [5,6].This based on the versatile metabolism of *A. niger* which allow to grow on a wide ranges of substrate under various environmental conditions [7-9].

In general, pectinase production is highly influenced by growth medium composition[10]. However, the most crucial fermentation medium is the carbon source which is essential to support cell growth and expression of the enzyme production [11].Carbon sources are ranged from simple small molecules like sugars, organic acids, and alcohol up to

complex structure high molecular weight polysaccharides. Usually, industrial bulk enzymes such as pectinases, are produced using agriculture feedstocks as source of carbon to reduce the production cost and also for better enzyme induction [12]. According to this study, citrus pectin gives the highest pectinase yield with 14.00 U mL^{-1} while fructose and poygalacturonic acid give the lowest pectinase yield with 3.00 U mL^{-1}. Furthermore, nitrogen source plays important role in both cell growth and pectinase biosynthesis as reported in previous study [13].Additions of phosphate in fermentation medium can improve the enzyme biosynthesis and its production [14].Therefore, the main objective of this study is to formulate new production medium for high pectinase production of *A.niger* suitable for industrial application using one factor at a time (OFAT)methods.

EXPERIMENTAL SECTION

Microorganism, preparation of inoculums, and medium composition

The fungus was identified as *A. niger* NRC1ami was obtained from National research centre, Dokki, Cairo, Egypt. Once received, stock culture in form of master cell bank and working cell bank were carried out, deposited under strain number WICC F29 in Wellness Industry Culture Collection (Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Johor, Malaysia).For activation, *A.niger* was first cultivated on potato dextrose agar and incubated for 7 days at 30^0C.The obtained spores were harvested and suspended in sterile saline solution to obtain 1×10^4 spore mL^{-1} for inoculum preparation.

Shake flask cultivation

The initial pH condition for the basal medium was adjusted at 5.5, prior to sterilization at 121 °C for 15 min. 2.5 mL of spore suspension were used to inoculate 250mL Erlenmeyer flask of 50mL working volume. The inoculated flasks were incubated for 96 hours on rotary shaker at 150 rpm and 30 °C. After incubation, the cells were separated using cooling centrifuge at 4000 rpm for 10 min. The supernatant was taken for further analysis and the cell pellets were washed using distilled water and centrifuged twice. The washed precipitate was dried in vacuum oven for constant weight.

Screening of media for cell biomass and pectinase production by *A. niger.*

From previous studies, different media have been applied for optimization of pectinase production by *A. niger*[9,15-19] (Table 1). As starting point for this research, the previously published optimized medium have been tested with the new fungal strain in this study.

Table 1 Different media used for production of pectinase by *A. niger*

No	Medium composition (g L^{-1})	References
1	Citrus pectin, 15.00; KH_2PO_4, 0.5.; $MgSO_4$, 0.5.; KNO_3,2.50	[15]
2	Citrus peel, 15.00; K_2HPO_4, 0.50; $MgSO_4.7H_2O$, 0.50; yeast extract, 20.00	[16]
3	Pectin, 10.00; K_2HPO_4, 2.00; KH_2PO_4, 2.00; $(NH_4)_2SO_4$, 2.00	[17]
4	Sucrose,30.00; K_2HPO_4, 1.00; $NaNO_3$, 3.00; KCl ,0.50; $MgSO_4.7H_2O$,0.50; $FeSO_4\cdot 7H_2O$, 0.01	[9]
5	Glucose, 34.60; KH_2PO_4, 2.00; corn powder, 20.97 and $(NH_4)_2SO_4$, 8.42	[18]
6	Citrus pectin, 3.00; K_2HPO_4, 2.00; $MgSO_4.7H_2O$, 0.10;$(NH_4)_2SO_4$, 1.00.	[19]

Improvement of medium composition for cell biomass and pectinase production by *A. niger*

The best medium screening for cell biomass and pectinase production was chosen as the basal medium during one factor at a time (OFAT) method as an optimized medium composition. Hence, the aim of this study was the effect of an individual parameter toward the cellbiomass and pectinase production. Thus, the parameters included different types of carbon source which were classified as monocarbon (glucose, fructose, xylose, glycerol, and galacturonic acid), disaccharides (sucrose and lactose), polysaccharides (malt extract, apple pectin, citrus pectin, and mandarin orange pectin). Then, this follow study of selected carbon source different concentrations of carbon source when growth in the (between 10 and 50 g L^{-1}), different types of nitrogen source (sodium nitrate (control), ammonium sulphate, ammonium nitrate, ammonium chloride, urea), different concentrations of selected nitrogen source (between 1.33 and 5.33 g L^{-1}) and different concentrations of phosphorus salt (between 0.5 and 3.0 g L^{-1}). All experiments were performed in triplicates.

Orange peel treatment

Mandarin Orange peel were cut into small pieces, washed by tap water several times and dried at 50°C [15,16].Finally, the dried mandarin orange peel was milled and sieved [20].

Comparison study on the growth kinetics of *A.niger* when growth in the medium of unoptimized and optimized OFAT medium

The growth kinetics of unoptimized medium and optimization medium through OFAT method by using one factor at a time was compared by analysed the cell biomass and pectinase production after 4 days of cultivation at rotary shaker with 30 °C and 150 rpm (Table 2). All experiments were triplicates and the samples were analysed.

Table 2Unoptimized and optimized medium components

Unoptimized medium (modified)		Optimized medium (OFAT)	
Component	g L^{-1}	Component	g L^{-1}
Sucrose	30	Pectin industrial	30
K_2HPO_4	1.00	K_2HPO_4	1.00
$NaNO_3$	3.00	Ammonium sulphate	3.33
KCl	0.50	KCl	0.05
$MgSO_4.7H_2O$	0.50	$MgSO_4.7H_2O$	0.05
$FeSO_4 \cdot 7H_2O$	0.01	$FeSO_4 \cdot 7H_2O$	0.1

Analysis

Pectinase activity determination

Pectinase activity was determined using 1% (w/v) citrus pectin as a substrate. The culture supernatant (0.3 ml) was incubated with 0.7 ml substrate and mixed together for 15 min at 40 °C. The liberated galacturonic acid was determined according to Miller [21] and modification assays from Esawy*et al.*[16] by dinitrosalicylic acid (DNS).One unit (U) of pectinase activity was defined as the amount of enzyme producing 1µmolof galacturonic acid per min.

Biomass Determination

The biomass production was measured by the cell dry weight of the sample. After the sample was filtered, it was dried at temperature 60 °C-80 °C to a constant weight[22].

RESULTS AND DISCUSSION

Screening of production medium on the cell biomass and pectinase productionby *A. niger*

In this experiment, the six media (of different formulation obtained previous studies) were investigated for suitability to produce pectinase enzyme with the strain under study. Samples were collected after 96 hours cultivation and proceeds for the analysis. The results in figure 1 clearly demonstrate that

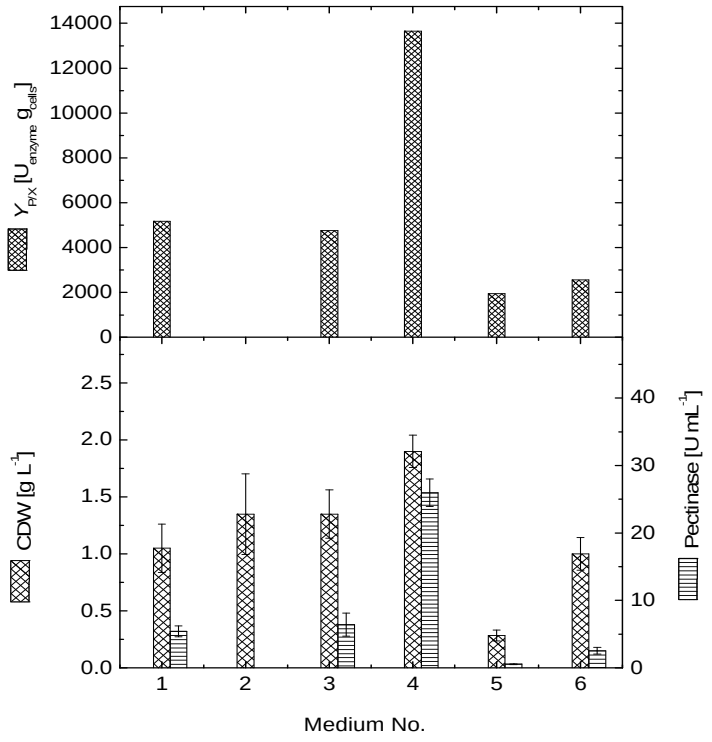

Figure 1 Cell growth and pectinase production by *A. niger* using different media after 96 hours cultivation (The data present the mean values and standard deviation of two independent experiments)

The growth of *A. niger* production on different media screening in Figure 1 showed maximum cell biomass of 1.90 g L^{-1} from medium 4 compared with cell biomass of 1.35 g L^{-1} from medium 2 and 3.Followed by 1.05 g L^{-1} from medium 1, 1.00 g L^{-1} from medium 6 and 0.29 g L^{-1} from medium 5.Medium 4 has the highest pectinase, followed by medium 3, 1, and 6with Pectinase production of25.95 U mL^{-1}, 6.42 U mL^{-1}, 5.43 U mL^{-1}, and 2.56 U mL^{-1},

respectively. In conclusion, medium 4 (Table 1)was choose because it can produce the maximum cell biomass and pectinase activity due to the presence of adequate growth substrate in culture medium which include carbon source, nitrogen sources, phosphorus and trace metal which is important cell for growth of *A.niger* to secrete the maximum pectinase. Medium 4 composed of medium with some modification in g L^{-1} which include sucrose, 30.00; K_2HPO_4, 1.00; and Capek concentrate (10 mL) containing per 100 mL: $NaNO_3$, 3.00; KCl, 0.05; $MgSO_4.7H_2O$, 0.50; $FeSO_4 \cdot 7H_2O$, 0.01 (Table 2)[9].

Effect of different types of carbon source on the cell biomass and pectinase production by *A. niger*

All of the carbon sources used in this study classified as the mono carbohydrates, disaccharides and polysaccharides. Various sources from mono carbohydrates used which include glycerol, glucose, xylose, fructose and galacturonic acid. Furthermore, lactose and sucrose were used as sources from disaccharides whereas; malt extract and pectin excreted form apple, citrus and industrial waste were used as polysaccharides sources in this studies. The result was shown in figure 2. Among the mono carbohydrate carbon sources, the galacturonic acid was achieved the higher pectinase and specific yield of enzyme production which were 58.56 U mL^{-1} and 44196.23 U_{enzyme} g_{cell}^{-1}, respectively. The cell biomass production was 1.33 g L^{-1} and the pH of the medium after cultivation was pH 7.18 which increased from the initial that was pH 5.5. Similar to observation by Aguilar and Huitrón[12], the galacturonic acid give the higher activity of exo-pectinase and were released by *Aspergillus* sp. when growing on the galacturonic as a carbon source. However, the selection of the best carbon sources was not depend on the higher production only but also lower in economic value. The galacturonic acid was highly costing and it is not suitable in a large scale industry as now many industries like the lower economic value with the higher production. Therefore, the selection of the best carbon source was on the second higher pectinase production and specific yield of enzyme which was from the pectin industrial which is classified as polysaccharides produced about 37.68 U mL^{-1} and 22836.36 U_{enzyme} g_{cell}^{-1}, respectively. The cell biomass production of *A.niger* when cultivated in pectin industrial was 1.65 g L^{-1} and the pH of the medium after cultivation was 7.19. Pectin from industrial was the source from the orange peel waste. It is not only low in economic value but utilizes all the waste that produced by others industries. This work is in agreement with other work which showed superiority of orange peel waste (as source of pectin) to support pectinase production compared to other carbon sources [15]. However, other studies showed also that pectinase production can be enhanced through proper selection of the carbon source in the cultivation medium[16].

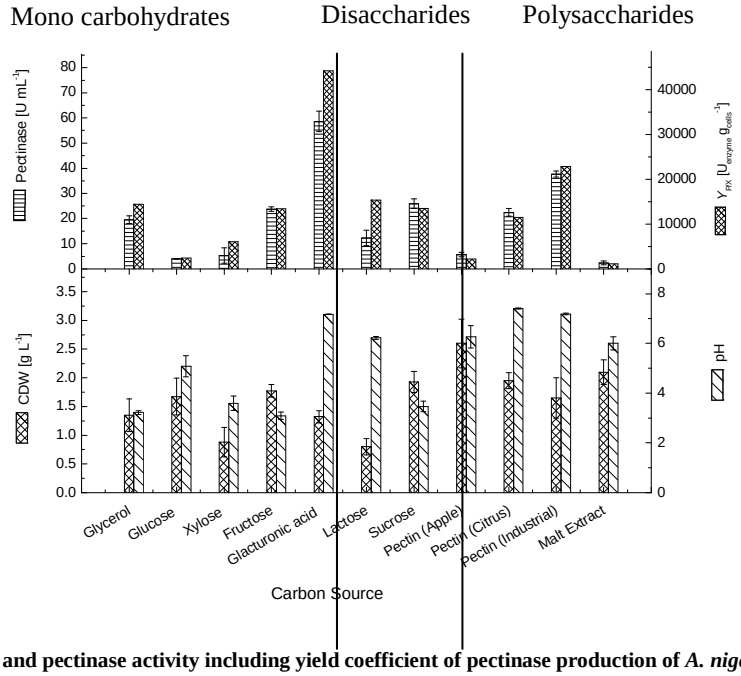

Figure 2The cell biomass and pectinase activity including yield coefficient of pectinase production of *A. niger* in the different types of carbon sources

Effect of different concentrations of carbon source on the cell biomass and pectinase production by *A. niger*

The different concentrations of pectin from the Mandarin orange peel were studied to find the optimum concentration for cell growth and producing higher pectinase production. The concentration of the pectin industrial in the range of 10 to 30 g L^{-1} was used in this study. Figure 3 shows that increasing the pectin concentration from 10 up to 30 g L^{-1} increased both of volumetric and specific enzyme production and decreased gradually thereafter. At 30 g L^{-1} pectin the maximal volumetric and specific pectinase production of 40 U $mL^{-1}$and 24169.18U_{enzyme} g_{cell}^{-1}, respectively, were obtained. In addition, this pectin concentration produced biomass of 1.66 g L^{-1}. From the result obtained, it showed that, the further increased of pectin industrial decreased the cell biomass and pectinase

production. Hence, pectin at 30 g L^{-1} was used as a carbon source for next experiments for further medium optimization study

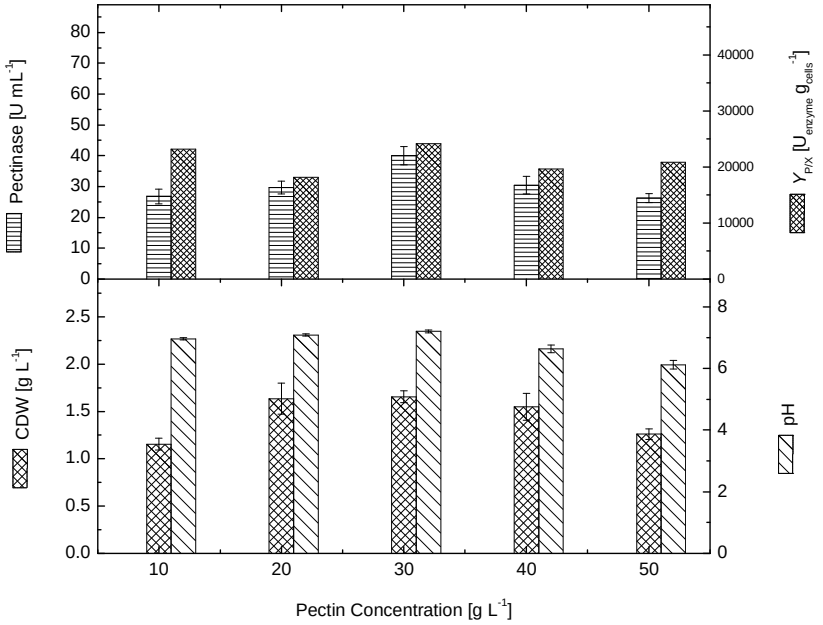

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Figure 3The cell biomass and pectinase activity including yield coefficient of pectinase production of *A. niger* in the different concentrations of pectin

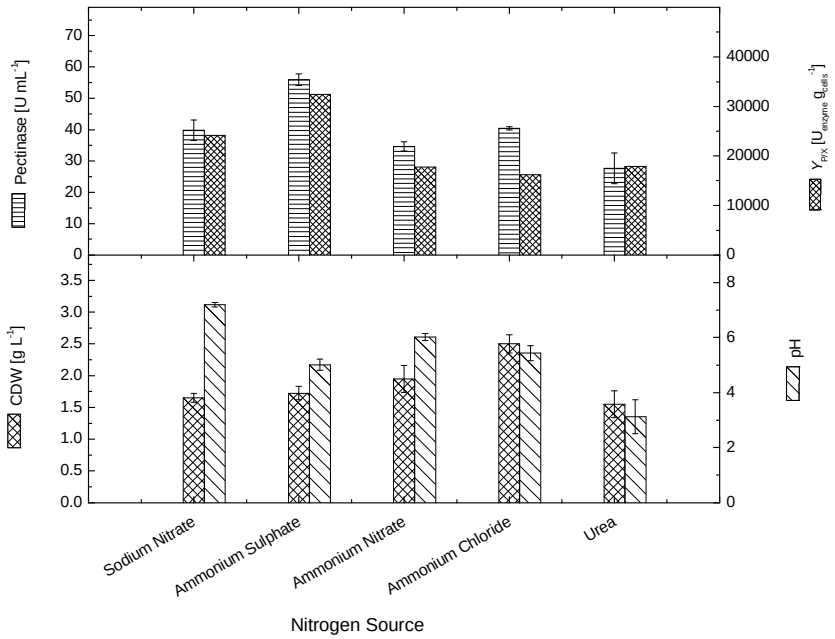

Figure 4The cell biomass and pectinase activity including yield coefficient of pectinase production of *A. niger* in the different types of nitrogen sources

Effect of different types of nitrogen source on the cell biomass and pectinase production by *A. niger*

Subsequently, different types of nitrogen sources were used which are sodium nitrate, ammonium sulphate, ammonium nitrate, ammonium chloride and urea. From Figure 4, ammonium sulphate gave the best result in producing the pectinase and specific yield of pectinase which were 55.90 U mL^{-1}, 32405.79 U_{enzyme} g_{cell}^{-1}, respectively. Whereas, the cell biomass production for ammonium sulphate was 1.73 g L^{-1} and the pH condition

after the cultivation was pH 5.02. Similarly, in the studies of Weihong *et al.* [14],they proved that ammonium sulphate was preferred to produce higher pectinase productivity and mostly used as a nitrogen sources for fungal fermentation. The lowest production of pectinase and specific yield of pectinase was from the urea with 27.65 U mL^{-1} and 17838.71 U_{enzyme} g_{cell}^{-1}, respectively at pH 3.26. Urea gives the minimum cell biomass which was 1.55 g L^{-1}. In this study; the reducing of the pH in urea was due to the releasing of the ammonium ion by urea inside the medium. It is known that, ammonium ion was element of an inhibitory enzyme and produced the enzyme in the lowest value [23].Thus, ammonium sulphate was preferred and used as a nitrogen source for this study.

Effect of different concentrations of nitrogen source on the cell biomass and pectinase productionby *A. niger*

The different concentrations of ammonium sulphates were in the range of 1.33 to 4.33 g L^{-1}. Figure 5 showed the best concentration of nitrogen source was 3.33 g L^{-1}. It produced maximum pectinase, specific yield of pectinase and the cell biomass with 74.35 U mL^{-1}, 36898.26 U_{enzyme} g_{cell}^{-1} and 2.02 g L^{-1}, respectively. At this concentration, the pH condition was pH 4.56. These findings were in correlation with the study of Gummadi and Panda [24]showing the optimal pH for the *A. niger* was around 4.00.As a result, ammonium sulphate at concentration of 3.33 g L^{-1} was chosen. The minimum production of pectinase, specific yield of pectinase and cell biomass was obtained when the concentration of ammonium sulphate at 6.33 g L^{-1} which were 48.20 U mL^{-1}, 32901.02 U_{enzyme} g_{cell}^{-1} and 1.47 g L^{-1}respectively. The pH condition for cultivation with ammonium sulphate of 6.33 g L^{-1} was 4.64. Studies by Puri *et al.* [23], stated that the higher concentration of nitrogen source was not significantly contributed to the higher yield of pectinase. As a result, ammonium sulphate with 3.33 g L^{-1} was preferred and used as optimized concentration of nitrogen source for further optimization study.

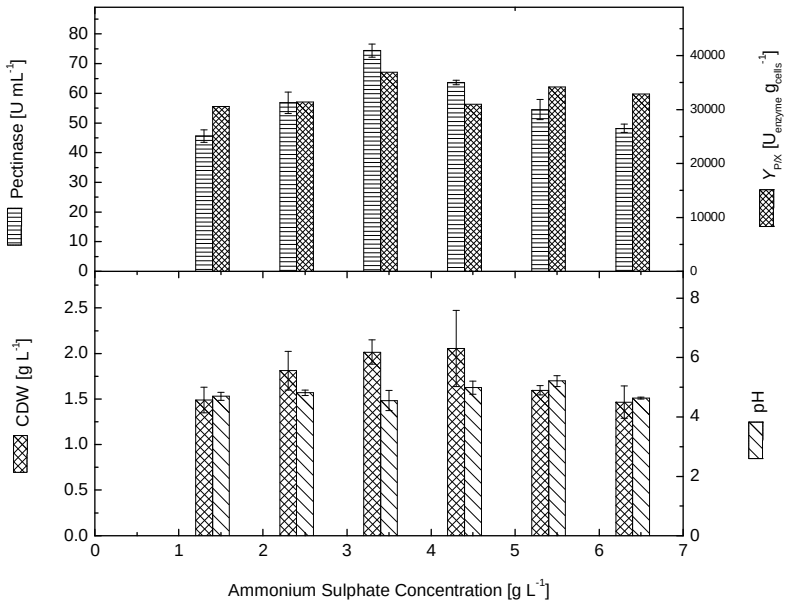

Figure 5The cell biomass and pectinase activity including yield coefficient of pectinase production of *A. niger* in the different concentrations of ammonium sulphate

Effect of different concentrations of phosphorus salt on the cell biomass and pectinase production by *A. niger*

In this study, the phosphorus salt used was dipotassium hydrogen phosphate. The concentration ranges were studied in a range of 0.50 g L^{-1} to 3.00 g L^{-1} and the result was demonstrated in Figure 6. The highest concentration of dipotassium hydrogen phosphate used was3.00 g L^{-1} gives the lowest pectinase and specific yield of pectinase which were 48.20 U mL^{-1} and 32901.02 U_{enzyme} g_{cell}^{-1}, respectively. The cell biomass production for this concentration was 3.18 g L^{-1} with the final pH of 5.32. This is due to the growth of the *A.niger* were delayed and the maximum of cell mass concentration were obtained in the late hours and at that time the cell concentration was rapidly diminished [25]. Therefore, the best concentration of dipotassium hydrogen phosphate was at 1.00 g L^{-1} with the highest pectinase and specific yield of pectinase were 74.35 U mL^{-1} and 36898.26 U_{enzyme} g_{cell}^{-1}, respectively. Whereas, the cell biomass production was 2.52 g L^{-1} with the pH 4.87 which was lower compared to the others. In conclusion, dipotassium hydrogen phosphate with 1.00 g L^{-1} was used in the medium cultivation throughout this study.

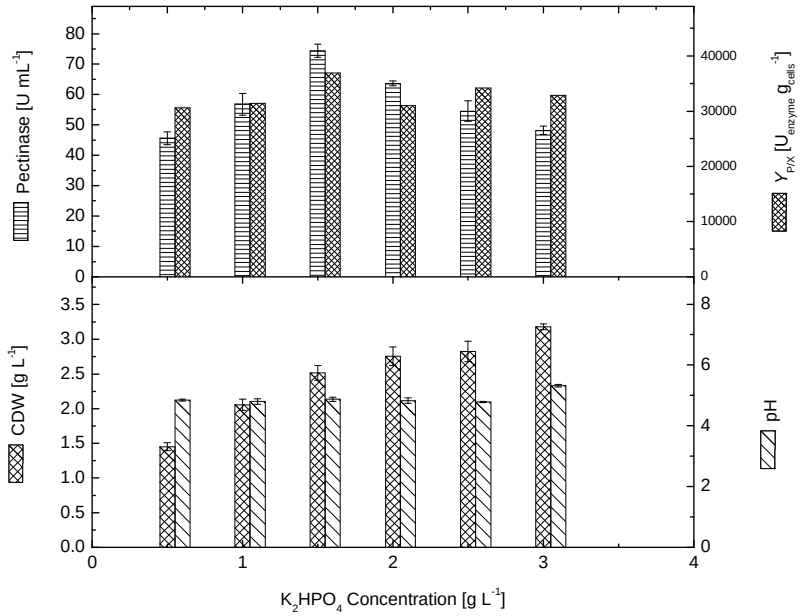

Figure 6The cell biomass and pectinase activity including yield coefficient of pectinase production of *A. niger* in the different concentrations of dipotassium hydrogen phosphate

Growth kinetic of pectinase production and cell biomass between unoptimized and optimized medium by using one factor at a time (OFAT) method

The complete growth of microbial during the cultivation in unoptimized medium and optimized medium was shown specifically in pectinase production, cell biomass, yield coefficient and pH changes (Figure 7 and 8). In general, kinetic studies are important in order for better understanding the microbial growth and metabolites production behaviour during cultivation under different cultivation conditions. Through the study of the optimization of one factor at a time (OFAT) of the main medium components, it is clearly obvious that the new optimized medium give much higher cell biomass and volumetric and specific pectinase production when compared with unoptimized medium (table 3). In the new optimized medium, using pectin as natural carbon source is more supportive substrate for pectinase production over the defined carbon source. The high induction of pectinolytic enzymes in pectin supplemented culture was also reported before [14,15,20].The nitrogen sources used in the optimized medium was ammonium sulphate. For fungal cell cultivation, it was also reported that ammonium sulphate usually used as a nitrogen source to support cell growth and different metabolites production as it also contain utilizable sulphur [26].This is also clear in this study that carbon and nitrogen sources influenced the pectinase activity. From Table 3, it clearly showed that, the cell mass production in optimized medium was quite less compared to the unoptimized media. the maximum biomass in the new optimized production was about 2.15 g L^{-1}, which was decreased by about 23.21% when compared to the growth in unoptimized medium. On the other hand, the pectinase production in the new optimized production medium was increased up to 26.85 U mL^{-1}after 54 hours. This value is alsmot141% higher compared to the volumetric enzyme producction in unoptimized medium (11.11 U mL^{-1} after 42 hour). As a result, the production rate of pectinase in unoptimized medium was very slow of only 0.26U mL^{-1} h^{-1} whereas, production of pectinase in optimized medium reached up to 0.50U mL^{-1} h^{-1} .These results are likely to be related to the study from Tong and Rajendra [27],who showed that the amount of mycelial growth are not correlation towards the production of enzyme.

As shown in Figure 7, the pH started to decline after 32 hours in the unoptimized medium. After 66 hours the pH dropped to 5.89 to 4.78 and stayed low (pH 4.58-4.15) during the growth remaining period. Besides, in the optimized medium (Figure 8) the pH started declining soon after inoculation, fell to 4.48 after 6 hours and stayed low (pH 4.20-4.33) during the growth cycle. Both condition of pH be evidence for decreasing of pH and proved the pectinase activity were actively produced [29].The yield of pectinase production in this study showed that the new optimized medium gave the higher value about12517.48 U_{enzyme} g_{cell}^{-1} compared to unoptimized medium only 3967.86 U_{enzyme} g_{cell}^{-1}. In conclusion, the new optimized medium produced more pectinase enzyme during the cultivation compared to unoptimized medium.

Table 3: Kinetics parameters for cell growth and pectinase production when *A. niger* cultivated in unoptimized and optimized medium using OFAT method

Parameters	Unoptimized medium	Optimized medium by OFAT method
Biomass, X_{max} (g L^{-1})	2.8	2.15
Pectinase , P_{max} (U mL^{-1})	11.11 at 42 hour	26.85 at 54 hour
Yield ,$Y_{P/X}$ (U_{enzyme} g_{cell}^{-1})	3967.86	12517.48
Specific growth rate, μ (h^{-1})	0.031	0.024
Production (U mL^{-1} h^{-1})	0.26	0.50

CONCLUSION

Through this study, the low cost of nutrient which was mandarin orange peel waste was used as natural substrate for the pectinase production. Furthermore, the usage of ammonium sulphate as nitrogen source can support the cell growth and pectinase production by *A. niger*. Ammonium sulphate was one of the components that always been used in industry as a nitrogen sources due to its ability to provide utilizable sulphur. Thus, it is not only used as a nitrogen sources but also used as a sulphur sources. Hence, all medium used in this study are applicable not only in the shake flask study but also in industries condition as all the components in the medium has undergoes the optimization process for efficient fermentation that benefit for producing higher pectinase activity.

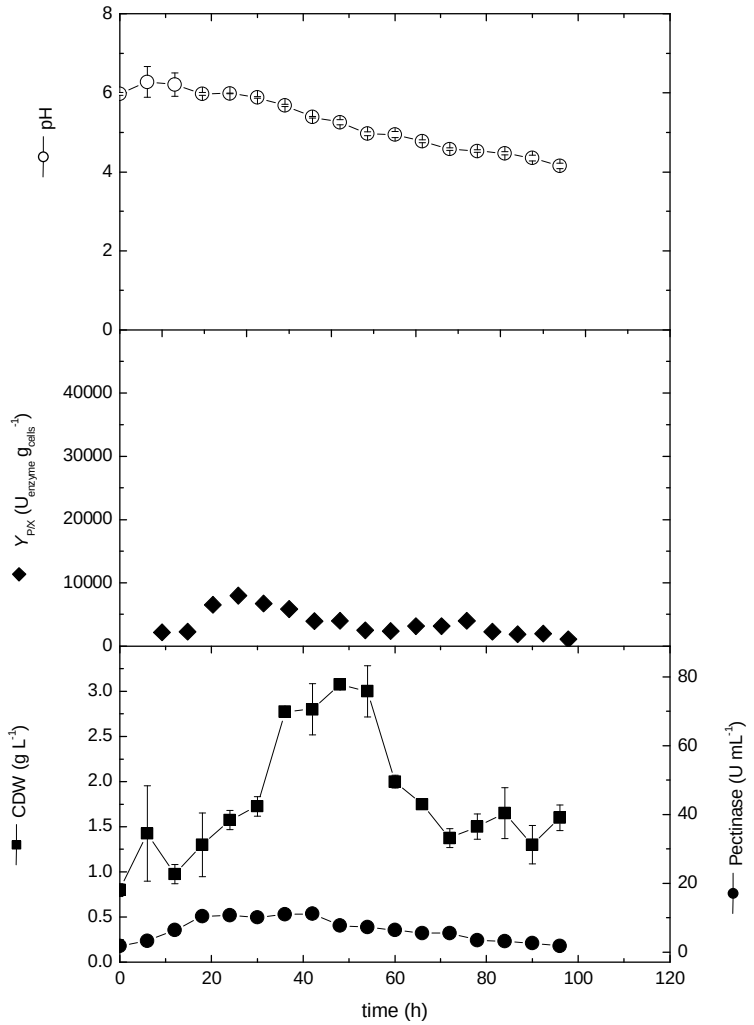

Figure 7 The cell biomass and pectinase production including yield coefficient of in*A. niger* growth in shake flask cultures of using the unoptimized medium

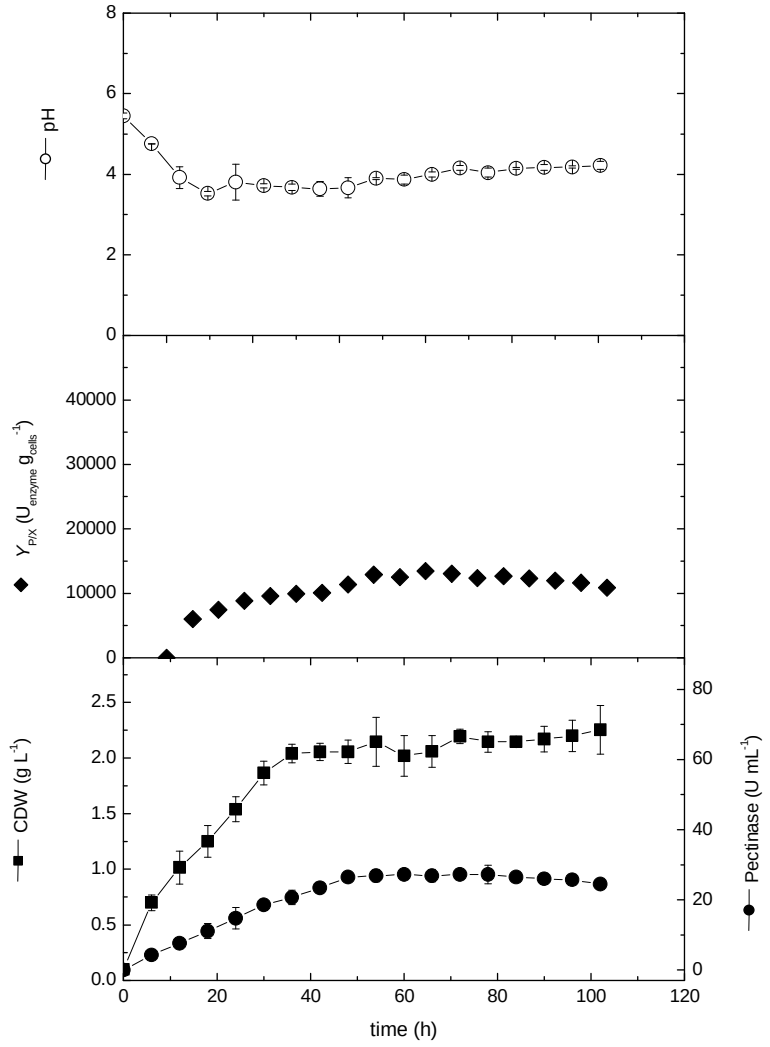

Figure 8The cell biomass and pectinase production including yield coefficient of in *A. niger* growth in shake flask cultures of using the optimized medium (OFAT)

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