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USE OF MOLASSES AS A CARBON SOURCE FOR THE GROWTH OF FUNGI AND PRODUCTION OF PECTINASE

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ABSTRACT

Present study was carried out to utilize sugar industry byproduct (molasses) as a carbon source for the growth of different fungi and production of pectinase enzyme. Four types of fungi (*P. lilacinum*, *M. geophillus*, *A. niger* and *A. fumigatus*) were used in this study. The maximum production rate of pectinase was observed by *A. niger* when grown in 2.5% and 5% molasses in comparison to other fungi used. Highest rate of pectinase 10.13units/ml was produced after 72 hours when A. niger was grown on 5% molasses.

Key words: Biosynthesis, Pectinase enzyme, Filamentous fungi, Molasses.

INTRODUCTION

Pectinases are industrially important enzymes in processing of agricultural products. They are used as processing aids for extraction, clarification and maceration of fruits and vegetables by breaking down pectin, (complex heteropolysaccharide) [Kashyap et al., 2001)]. Pectin is primarily found in the middle lamella and primary cell wall of higher plants consisting of α – 1–4 glycosidically linked galacturonic acid residues and α–1–2 linked rhamnose (Schols and Voragen, 2002). Due to complex structure of pectin, its modification or complete breakdown requires many different enzymes. Pectolytic enzyme complex contains depolymerizing and demethoxylating enzymes. Depolymerizing enzymes are polygalacturonase which clears α–1–4–glycosidic bonds between two galact-uronic acid residues, while pectin lyase catalyze a β–elimination reaction between two metylated residues (Stutzenberger, 1992). Desterifying enzyme Pectinesterase catalyze the demethoxylation of methylated pectin, producing methanol and pectin (Delgado et al., 1992). Commercial enzyme preparations used in food processing are almost exclusively derived from *Aspergillus* sp. and are traditionally mixture of polygalact-uronases, pectatelyase and pectin esterase (Marie et al., 2002). Pectin degrading enzymes have been extensively used to improve the stability of fruit and vegetable nectar and in the clarification of fruit juices and wines (Marie et al., 2002; Lang and Doemenburg, 2000; Bailey and Pessa, 1990; Priest, 1984; Fogart and Kelly, 1983). Currently, they are widely used in industry for setting of natural fibers and extraction of oils from vegetables and citrus peels (Ros et al., 1993; Sreekantiah et al., 1971). The enzyme preparations used in the food industry are of fungal origin because fungi are potent producers of

pectic enzyme and the optimal pH of many fruit juices ranges from pH 3 – 5.5 (Bascat et al., 1989). Such preparation are not suited for the production of vegetables purées or other preparations in which pH values are close to neutral (Fonseca and Said, 1995). Furthermore due to the relatively low temperature stability of the fungal enzyme preparation maceration needs to be carried out at temperature not exceeding 45°C, necessitating the incorporation of a pasteurization step to limit the growth of mesophillic microorganisms (Ueda et al., 1982).

Homogenous polygalacturonases preparations are preferred for the separation of whole cells in the manufacture of baby food, vitamins, color and aroma (Chesson and Codner, 1978). Novel applications can be envisaged for the production of polygalacturonases as functional food components. Oligogalacturonides are sugars possess properties that are beneficial to the health of consumers and are termed as prebiotics (Silley, 1986). Prebiotics combine the effects of both the endogenous intestinal bacterial flora and the new probiotic organisms in a minimally processed plant food and represent a perspective for one of the future trends of food biotechnology in the plant product market (Lang and Doernenburg, 2000). As the beneficial functional properties of oligogalact-urosidase become more widely understood, the use of purified polygalactoronase preparation will be necessary for their large scale production in good yields.

New enzymes for use in commercial applications with desirable biochemical and physico chemical characteristics and a low cost of production have been the focus of much research. Application of agro-industrial waste as carbon source in enzyme production process reduces the cost of production and also helps in solving problems with their disposal (Crittenden and Planyne, 1996). In this study different concentration (2.5% and 5%) of molasses (by product of sugar industry) was used as a carbon source for the growth of fungi and production of Pectinase through submerged fermentation process.

MATERIALS AND METHODS

Microorganisms: *Mucor geophillus* and *Penicillium lilacinum* were obtained from Research laboratory Department of Chemistry, Shah Abdul Latif University Khairpur, whereas *Aspergillus niger* and *Aspergillus fumigatus* were isolated and identified in this laboratory. The stock cultures of *A. niger, A. fumigatus, M. geophillus and P. lilacinum were maintained* on agar slants, containing (g/L) glucose 20, peptone 10, agar 20 and distilled water. The ingredients were thoroughly mixed and kept in culture tubes sterilized in autoclave at 15psi for 20 minutes. The sterilized slants were inoculated with *A.niger, A.fumigatus, M.geophillus* and *P.lilacinum* and incubated at 37º C for 5 days to obtain luxuriant growth.

Inoculums: A spore suspension was prepared by adding sterilized water to stock culture to get $50x10^6$ spoes/ml

Mineral medium: The mineral medium for the growth of fungi and production of enzyme was used as reported by Bhatti *et al*., (2004) with slight modification, which contains the following reagents per litre of, KH_2PO_4 1.0g, $FeSO_4 7H_2O$ 6.32mg, $MgSO_4.7H_2O$ 0.25g, $ZnSO_4.7H_2O$ 1.1mg, $MnCl_2.2H_2O$ 3.54 mg, $CaCl_2$ $2H_2O$ 46.7 mg and NH_4NO_3 2.4 g. The pH of medium was adjusted at 6.5

Fermentation medium: 50ml of mineral medium supplemented with 2.5 and 5% molasses as carbon source was taken in 250ml flask. The pH of medium was adjusted at 6.5. The flasks were plugged with cotton wool and sterilized at 121ºC and 15psi for 25minutes. The flasks were inoculated with 1.0 ml of inoculums containing $50x10^6$ spores/ml and incubated at 31± 2ºC in shaking incubator at 90rpm.The culture broth was filtered from mycelium after an interval of 24 hours incubation period, through Whatman No.1 filter paper. The recovered mycelium was dried at 80ºC and weighed.

Determination of Reducing Sugar: Reducing sugar content of broth was determined according to Miller (1959) Dinitrosalicylic acid (DNS) method.

Determination of Total sugar: Total Sugar content of broth was determined according to the method reported by Montgomery (1961).

Determination of Protein: The protein content in the culture broth was determined by the method of Lowry *et al*., (1951) using bovine serum albumin as a standard.

Determination of pectinase Activity: The enzyme activity was determined by DNS method (Miller, 1959). I ml of culture broth was added to I ml of 1% soluble citrus pectin solution (Sodium citrate buffer pH 5) and incubated for 15 minutes. The reaction was stopped by adding 2.0 ml of DNS (3, 5-dinitrosalicylic acid) reagent. The reaction mixture was heated in boiling water bath for 5 minutes. After cooling the absorbance of reaction color was read at 540 nm.

One unit of pectinase activity is defined as one micromole of reducing sugar released per milliliter under the assay condition.

RESULTS AND DISCUSSION

In this study four fungal species (*Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium lilacinum* and *Mucor geophillus*) were selected and grown in media containing molasses for the production of pectinase. The pectinase production capacity by *P. lilacinum* was observed when grown in a culture medium supplemented with 2.5% molasses as a carbon source. The maximum production of pectinase [6.98Units/ml] was obtained at 72 hours and then decreased with the increase of time period. The concentration of total sugars, reducing sugars and protein were decreased with the increase of fermentation time period. Whereas, pH values of culture medium initially decreased and then increased throughout fermentation period. The capacity of microorganism to produce extracellular enzymes is greatly influenced with different factors

like carbon and nitrogen sources, temperature and pH (El-Refi et al., 1984).

Table-1: *Penicillium lilacinum* was grown in mineral medium supplemented with 2.5% molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	Total Protein mg/ml	Total Sugar mg/ml	Reducing Sugar mg/ml	Pectinase Activity U/ml
24	3.8	0.967	0.915	8.46	7.59	4.154
48	3.4	0.988	1.198	8.17	6.84	5.54
72	2.7	1.029	1.212	7.85	6.3	6.98
96	2.8	1.078	1.317	6.1	5.94	5.76
120	2.6	1.132	1.32	5.98	5.51	5.13
144	3.1	1.137	1.336	5.3	4.76	4.35
168	3.8	1.145	1.342	4.72	4. 25	3.3
192	3.7	1.205	1.415	3.54	3.2	2.82
216	4.8	1.255	1.245	2.45	2.39	1.4
240	4.9	1.358	1.225	1.3	0.62	1.2

The results of pectinase production by *Aspergillus niger* when grown in a culture medium with 2.5% molasses as a carbon source are presented in Table - 2. The maximum production of pectinase [7.76Units/ml] was recorded at 72hours while the increase of time period decreases the rate of production. The results of present study are grater then the results of Amande and Adebayo -Tayo (2012) in case of *A. niger* grown on different carbon sources for the production of pectolytic enzyme. The concentration of total sugars, reducing sugars and proteins were decreased with increase of fermentation time. The fluctuations were noted in the final pH values of culture medium during the growth of microorganism.

Table-2: *A. niger* was grown in mineral medium supplemented with 2.5% molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	Total Protein mg/ml	Total Sugar mg/ml	Reducing Sugar mg/ml	Pectinase Activity U/ml
24	4.57	1.113	0.995	8.87	7.93	4.67
48	5.52	1.124	1.192	8.62	7.21	5.36
72	5.13	1.276	1.253	7.8	7.1	7.76
96	6.95	1.325	1.25	6. 7	5.915	5.71
120	6.75	1.25	1.219	5.53	4.98	5.1
144	5.2	1.26	1.212	4.2	3.71	4.28
168	5.8	1.345	1.195	3.6	2.82	3.13
192	3.62	0.28	1.189	2.31	1.75	2.5
216	4.405	0.295	1.167	1.36	1.1	2.25
240	4.475	0.27	1.155	0.93	0.64	1.4

The effect of 2.5% molasses on the production of pectinase and growth of *Aspergillus fumigatus* are shown in Table–3. The maximum production of pectinase [6.69Units/ml] was recorded at 72 hours while the increase of time decreases the rate of production. The concentration of T. sugar, R. sugar and proteins decreased with the increase of time period. The pH values of culture medium increased with the increase of fermentation period.

Table-3: *A. fumigatus* was grown in mineral medium supplemented with 2.5 % molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	T. Protein mg/ml	Total Sugar mg/ml	R. Sugar mg/ml	Pectinase Activity U/ml
24	5.4	0.985	1.01	8.67	8.1	4.8
48	5.9	0.990	1.11	7.95	6.96	4.58
72	6.1	1.089	1.13	7.2	6.12	6.69
96	6.45	1.033	1.14	6.61	5.92	5.9
120	6.15	1.045	1.2	5.45	4.68	4.63
144	6.2	1.042	1.29	3.85	3.27	3.37
168	6.25	1.205	1.2	2.8	2.28	3.2
192	6.35	1.206	1.17	1.62	1.49	2.21
216	6.5	1.203	1.16	1.25	0.99	1.81
240	6.75	1.165	1.15	0.98	0.75	0.789

The results of pectinase Production by mixed culture of *A. fumigatus* and *A. ngier* grown on mineral medium supplemented with 2.5% molasses are presented in Table-4. The highest pectinase production [6.16 Units/ml] was noted at 72 hours. The increase of fermentation time decreased the rate of pectinase production, concentration of total sugar, reducing sugar and protein. The gradual increase in pH values was also noted during the growth of microorganisms.

Table-4: A mixed culture of *A. niger* + *A. fumigatus* was grown in 2.5 % molasses Mineral medium when incubated at 30±2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	Total Protein mg/ml	Total Sugar mg/ml	Reducing Sugar mg/ml	Pectinase Activity U/ml
24	5.4	0.819	0.918	9.1	8. 32	3.94
48	5.65	0.922	0.925	8. 18	7.74	4.52
72	5.5	0.962	0.898	7.9	6.87	6.16
96	6.25	1.057	0.872	6.74	6.23	5.82
120	6.25	1.122	0.853	5.7	5.17	3.98
144	6.55	1.189	0.842	4.72	3.8	3.16
168	6.8	1.197	0.831	2.97	2.5	2.76
192	7.6	1.242	0.818	2.37	1.85	2.43
216	8.15	1.32	0.811	1.27	1.16	1.76
240	7.45	1.447	0.802	0.95	0.73	1.22

As shown in Table–5, the highest rate of pectinase production [6.58 Units/ ml] was recorded at 96 hours when *Mucor geophillus* was grown in 2.5% molasses mineral medium. The concentration of total sugar, reducing sugar and protein was decreased with the increase of fermentation time. The pH of the culture medium was changed from acidic to slightly basic with the increase of time.

Table-5: *M. geophillus* was grown in mineral medium supplemented with 2.5% molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	Total Proteins mg/ml	Total Sugar (mg/ml)	Reducing Sugars mg/ml	Pectinase Activity U/ml
24	5.55	0.87	1.119	8.248	7.67	3.31
48	5.57	0.89	1.142	7.5687	7.17	4.97
72	5.95	0.97	1.218	6.98	6.21	5.67
96	5.45	1.1	1.225	5.91	4.86	6.58
120	5.45	1.11	1.228	3.85	3.44	4.88
144	6.35	1.14	1.239	3.49	3.12	3.94
168	6.35	1.17	1.241	2.78	2.21	3.45
192	6.5	1.2	1.249	1.63	1.26	2.77
216	7.15	1.21	1.232	1.98	0.89	1.879
240	7.45	1.22	1.116	0.89	0.653	0.78

Table-6 shows the results of *P. lilacinum* grown on mineral medium supplemented with 5% molasses. The highest production [9.89Units/ml] of pectinase was noted at 72 hours. Whereas, the increase of time period decreases the rate of pectinase production and concentration of Total sugar, Reducing sugar and Protein. The pH of culture medium was decreased throughout fermentation period.

Table-6: *Penicillium lilacinum* was grown in mineral medium supplemented with 5 % molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	T. Protein mg/ml	T. Sugar mg/ml	R. Sugars mg/ml	Pectinase Activity U/ml
24	5.8	0.970	2.01	8.52	7.93	5.13
48	6.1	0.975	2.59	8.13	7.38	6.83
72	6.15	1.083	1.96	7.86	6.89	9.89
96	6.0	1.040	1.86	6.97	5.57	7.14
120	5.8	1.151	1.86	5.65	4.55	5.84
144	5.4	1.170	1.77	4.60	3.97	4.12
168	5.75	1.220	1.84	3.69	2.94	3.20
192	6.4	1.250	1.85	2.48	2.13	2.74
216	6.65	1.300	1.82	1.86	1.70	2. 50
240	6.8	1.320	1.88	0.97	0.69	1.12

Aspergillus niger grown in mineral medium supplemented with 5% molasses as carbon source produced the highest pectinase production [10.35 Units/ml] after 72 hours (Table -7). The increase of time period decreases the rate of pectinase production, sugar and protein concentration. The pH of culture medium increased with the increase of fermentation period.

Table-7: *A. niger* was grown in mineral medium supplemented with 5 % molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	Total Proteins mg/ml	Total Sugar mg/ml	Reducing Sugars mg/ml	Pectinase Activity U/ml
24	5.4	1.142	1.19	9.66	8.13	5.53
48	5.9	1.19	1.27	8.53	7.1	6.2
72	6.1	1.22	1.29	8.12	6.94	10.35
96	6.4	1.23	1.29	7.9	6.23	8.16
120	6.1	1.25	1.31	5.75	4.98	6.45
144	6.8	1.3	1.33	4.18	3.43	5.19
168	7.35	1.33	1.33	3.84	2.95	4.37
192	7.3	1.41	1.39	2.23	1.76	2.61
216	7.1	1.42	1.44	1.91	1.69	1.76
240	7.8	1.45	1.45	0.98	0.97	1.18

The results of pectinase production by *A. fumigatus* are shown in Table-8. The highest production [8.62 Units/ml] was recorded at 72 hours and production decreased with increase in time. The concentration of T.sugar, R. sugar and T. Protein were decreased when fermentation time increased. The medium pH changed from acidic to basic with the increase of time.

Table-8: *A. fumigates* was grown in mineral medium supplemented with 5 % molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	Total Proteins mg/ml	Total Sugar mg/ml	Reducing Sugars mg/ml	Pectinase Activity mg/ml
24	4.9	0.997	2.18	8.145	7.98	5.89
48	4.4	1.206	2.25	7.78	6.74	6.76
72	4.3	1.295	1.8	6.98	5.87	8.62
96	4.7	1.285	1.98	6.32	5.31	6.78
120	4.9	1.338	1.97	5.38	4.78	6.27
144	5.3	1.360	1.96	4.89	4.24	5.785
168	5.4	1.378	1.95	3.6	3.29	4.38
192	5.45	1.391	1.9	2.62	2.35	3.58
216	5.85	1.316	1.88	1.94	1.31	2.16
240	6.4	1.408	1.86	0.78	0.62	1.71

Table-9 represents the results of mixed culture of *A.niger* and *A.fumigatus* when grown in culture medium supplemented with 5% as a carbon source. Highest production of pectinase [8.54 Units/ml] was noted at 72 hours while production of pectinase decreased with the increase of fermentation time total sugar, reducing sugar and total Protein concentration decreased with the increase of time period. The pH of the medium becomes basic with the increase of fermentation period during the growth of microorganism.

Table-9: A mixed culture of *A .niger* and *A. fumigates* was grown in mineral medium supplemented with 5 % molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	T. Protein mg/ml	T. Sugar mg/ml	R. Sugars mg/ml	Pectinase Activity U/ml
24	4.2	1.165	1.476	8.96	7.94	4.43
48	4.75	1.656	1.97	8.49	7.32	6.1
72	5.4	1.684	1.97	7.96	6.83	8.54
96	6.15	1.68	1.84	6.88	6.15	7.87
120	6.25	1.875	1.83	5.97	4.96	6.49
144	6.45	1.78	1.79	4.61	3.52	5.44
168	6.6	1.78	1.75	3.95	3.27	4.77
192	6.7	1.75	1.7	2.9	2.47	2.26
216	6.5	1.77	1.65	1.68	1.43	1.19
240	6.1	1.77	1.6	1.23	0.84	0.99

The results of pectinase production depicted in Table-10, when *M.geophillus* was grown in medium supplemented with 5% molasses as a carbon source. The highest pectinase production (8.14units/ml) was achieved at 72hours. The rate of pectinase production decreases with the increase in fermentation time. The concentration of Total sugar, Reducing sugar and protein decreases with the increase of time period of fermentation process.

Table-10: *M. geophillus* was grown in mineral medium supplemented with 5 % molasses at 30 ± 2ºC and pH was adjusted at 6.5

Time Hours	Final pH	Biomass g/50ml broth	T. Protein mg/ml	T. Sugar mg/ml	R. Sugars mg/ml	Pectinase Activity U/ml
24	5.65	1.37	1.921	9.9	8. 97	4.54
48	5.6	1.452	1.929	9.29	7.8	6.54
72	6.05	1.512	1.922	7.861	7.59	8.14
96	6.6	1.677	1.876	6.28	5.83	6.9
120	6.5	1.681	1.865	4.97	4.74	5.34
144	6.95	1.705	1.857	3.9	3.48	4.63
168	6.35	1.737	1.854	3.87	2.93	3.7
192	7.15	1.782	1.8	3.3	2.8	2.67
216	7.55	1.79	1.85	1.9	1.5	2. 9
240	7.7	1.823	1.88	1.16	0.831	1.15

The comparison of pectinase production capacity of different is shown in Table-11, when the fungi gown in mineral medium supplemented with 2.5 and 5% molasses. The highest amount of pectinase was produced by *Aspergillus niger* 7.76 units/ml and 10.13 units/ml respecttively in comparison to other tested fungi. This observation is in agreement with the earlier report by Kutateladze et al., (2009) regarding pectinase production by the genera *Aspergillus*. The use of sugar industry byproduct (molasses) to enzyme production is a useful way and similar opinion is also shared by Bayoumi *et al.,* (2009). It is seen that correlation is not observed in-between the growth of microorganism, enzyme production and utilization of different agricultural wastes as a carbon source (Hoa and Hung, 2013, Martínez-Trujillo, et al., 2008). The findings of this study might be applied in a pilot scale for production of pectinase through submerged fermentation with cost effective using cheap substrate (molasses).

Table-11: Comparison of pectinase production by different fungi when grown on 2.5 and 5% molasses mineral medium at 30±2 and pH was adjusted 6.5

Filamentous Fungi	Biomass g/50ml broth	Pectinase Activity U/ml	Biomass g/50ml broth	Pectinase Activity U/ml
	2.5% Molasses		5% Molasses	
P. lilacinum	1.029	6.98	1.08	9.89
A. fumigatus	1.089	6.69	1.29	8.62
A. niger + *A. fumigatus*	0.962	6.16	1.68	5.97
A. niger	1.27	7.76	1.23	10.13
M. geophilous	0.998	6.58	1.512	8.14

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