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Production of extra cellular enzymes by microbial strains in molasses and additives supplemented fermentation media

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This work investigates the use of molasses (a cheap substrate) together with additives in fermentation medium to produce extra cellular protease, amylase and lipase, with the aid of *Aspergillus oryzae* and *Bacillus subtilis.* The concentration of molasses (0.1 – 1.0%) used for enzyme production by *A. oryzae* significantly influenced the extracellular lipase, amylase and protease biosynthesis/ secretion. The highest extracellular enzyme activities were found at 96 h of processing (72, 52 and 65 U/ml for lipase, amylase and protease, respectively). As additives, two different carbon sources: *Avena sativa* and *Cicer arietinum* were tested for production of extracellular enzymes by *A. oryzae.* Among the different concentrations of *A. sativa,* high enzyme activity (61, 83 and 121 U/ml, respectively for lipase, amylase and protease) was noted at concentration of 2.5 g/L. For another additive, *C. arietinum,* highest extracellular enzyme activity was found at 96 h (71; 61, 80 U/ml for lipase, amylase and protease). In this study, it is concluded that the production of extracellular enzyme using molasses with additives had maximum value compared to using molasses alone in 96 h fermentation by *Aspergillus oryzae.*

Key words: Molasses, extracellular enzyme, *Aspergillus oryzae.*

INTRODUCTION

Enzymes are proteins that function as specialized catalysts for chemical reactions. They have contributed greatly to the traditional and modern chemical industry by improving existing processes (Rao et al., 1998). The use of enzyme-mediated processes can be traced to ancient civilizations. Today, more than 3000 enzymes are known, and of these, about 600 are in commercial use; the majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly (Godfrey and West, 1996; Wilke, 1999; Gilbert and Dupont, 2011). Because of improved understanding of production biochemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in methods of using enzymes have greatly expanded demand. Furthermore, because of the many different transformations that enzymes can catalyze, the number of enzymes used in commerce continues to multiply (Haq et al., 2006). The world enzyme demand is satisfied by

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12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe (Hasan et al., 2006). Global enzymes market is estimated to rise by 7% at a healthy pace to $8.0 billion in 2015. Gains will reflect a continued world economy rebound from the global financial crisis of 2009. Enzymes are employed in a diverse array of applications in industries and scientific research, ranging from the degradation of various natural substances to biosynthesis of different compounds (Wilke, 1999).

Molasses is an interesting raw material; it is rich in nutrients and minerals, cheap in price as well as abundant; hence a by-product in sugar industries. Molasses as nutrient medium can be used as a relatively inexpensive and economic alternative to synthetic medium for the production of industrial important enzymes (Johnvesly and Naik, 2001). This leads to the present study.

MATERIALS AND METHODS

The strains of *A. oryzae* and *B. subtilis* were obtained from University of Madras, Chennai and were cultured in Potato Dextrose Agar and Nutrient Agar respectively. Agro-industrial by product was tested as substrate: sugarcane molasses. The molasses was added until total sugars reached 10 g/L in the minimum medium and the other particular residues were used at 50 g/L. As nitrogen source, urea (300 g/L) was filtered (0.22 µm Micropore membrane) and added to the autoclaved medium in order to get 100 mM.

Effect of different concentrations of molasses on enzyme production using *A. oryzae* and *B. subtilis*

A. oryzae and *B. subtilis* were inoculated into production medium with different concentrations of molasses: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0%. The experiments were conducted for a period of 144 h for *A. oryzae* and 48 h for *B. subtilis*. Every 24 h of interval for *A. oryzae* and 4 h for *B. subtilis* cultures the samples were collected, centrifuged and used for the estimation of enzymes (amylase, protease and lipase) and protein.

Effect of different concentrations of additives on enzyme production from molasses using *A. oryzae* and *B. subtilis*

In addition to molasses and other carbon sources, two additives namely *A. sativa* and *C. arietinum* were amended separately at the concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 g at pH 7 in the medium; the experimental flasks were inoculated with tested cultures and incubated at 30±2°C for 144 h (*A. oryzae*) and 37±1°C for 48 h (*B. subtilis*). The samples of cultures were collected and centrifuged at regular interval; the supernatant was used for the estimation of enzymes (amylase, protease and lipase) and protein.

Analytical methods

The protein concentration was determined according to the method of Bradford (1976). Lipase activity was measured using various *p*-nitrophenyl esters as described by Hasan et al. (2006), with some modifications. One volume of a 10 mM solution of each substrate in 2-propanol was mixed with 9 volumes of 100 mM Tris-HCl buffer (pH 8). When long acyl chain *p*-nitrophenyl esters (C14-C18) were used, PVA 0.25% (w/v) was also incorporated into this buffer. This mixture was then pre-warmed at 40ºC in a water bath and immediately distributed (1 ml) into 1.5 ml cells. The reaction was started by adding 0.5 ml of enzyme solution at an appropriate dilution in 10 mM Tris-HCl (pH 8). The absorbance at 410 nm of the assay against a blank without any enzyme was continuously monitored for 2–5 min using a UV-vis spectrophotometer (ShimadzuUV-160A, Shimadzu Corporation, Kyoto, Japan). The reaction rate was calculated from the slope of the curve absorbance versus time, using a molar extinction coefficient of 12,750 cm^{-1} M^{-1} for *p*-nitrophenol. One enzyme unit was defined as the amount of protein releasing 1µmol of *p*-nitrophenol per minute under the above reaction conditions.

Amylase activity (Miller, 1959)

The activity of amylase was analyzed by incubating 0.3 mL enzyme with 0.5 mL soluble starch (1%, w/v) prepared in 0.05 M phosphate buffer, pH 6.5. After incubation at 90ºC for 10 min, the reaction was stopped and the released reducing sugars were analyzed colorimetrically by the addition of 1 mL of 3-5- dinitrosalicylic acid reagent. An enzyme unit is defined as the amount of enzyme releasing 1 mM of glucose from the substrate in 1 min at 90ºC.

Assay of protease activity

Protease activity was measured by the method of Johnvesly and Naik (2001) using casein as a substrate. Half milliliter of the diluted enzyme was mixed with 0.5 ml 100 mM Tris-HCl (pH 8.5) containing 1% casein, and incubated for 15 min at 500°C. The reaction was stopped by addition of 0.5 ml trichloroacetic acid (20%; w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 rpm for 15 min to remove the precipitate. The acid soluble material (aromatic aminoacids) was determined spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0-50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine per min under the experimental conditions used.

RESULTS AND DISCUSSION

In the present study, the concentration of molasses (0.1 – 1.0 %), used for enzyme biosynthesis by *A. oryzae* and *B. subtilis,* significantly influenced the extracellular lipase, amylase and protease secretion. In *A. oryzae*, the highest extracellular enzyme was at 96 h (72, 52, 65U/ ml respectively (Table 1). It was evident that the concentration of molasses (0.1 – 1.0%) used for enzyme production by *B. subtilis* significantly influenced the extracellular protease, amylase and lipase. The content respectively was 149, 113 and 89 U/ml with a highest extracellular enzyme at 36 h (Table 2).

Two different carbon sources of *A. sativa* and *C. arietinum* were tested for extracellular lipase, amylase and protease secretion by *A. oryzae* and *B. subtilis.* Among the different concentrations of *A. sativa,* 2.5 g/l allowed high enzyme activity in *A. oryzae* fermentation: 61; 83; 121 U/ml for lipase, amylase and protease

Table 1. Effects of molasses concentration on extracellular enzyme biosynthesis by Aspergillus oryzae in submerge fermentation.

Concentration of mollases (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	48 h	96 h	144 h	48 h	96 h	144 h	48 h	96 h	144 h
0.1	15	29	22	12	29	23	22	48	29
0.2	16	31	22	12	31	23	24	52	33
0.3	16	33	26	14	34	28	26	56	35
0.4	18	36	30	16	36	30	26	58	39
0.5	19	39	30	18	38	30	30	60	41
0.6	19	41	31	18	41	32	32	62	43
0.7	21	44	32	21	44	32	32	62	43
0.8	22	46	40	23	46	40	36	68	45
0.9	23	50	40	26	50	42	38	71	45
1	25	52	42	28	52	44	40	74	45

Table 2. Effect of molasses concentration on extracellular enzyme biosynthesis by *Bacillus subtilis* in submerge fermentation.

Concentration of mollases (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
0.1	55	90	65	50	68	60	58	98	68
0.2	40	55	40	50	80	60	68	118	88
0.3	48	62	48	52	82	70	70	120	84
0.4	48	70	58	50	88	68	78	122	94
0.5	52	80	68	60	92	82	80	138	100
0.6	56	90	62	70	110	90	96	150	130
0.7	60	92	78	60	105	84	80	120	90
0.8	52	80	68	70	95	80	75	120	90
0.9	50	75	60	60	80	70	65	105	95
1	48	70	60	50	90	75	65	105	75

Table 3. Effects of Avena sativa on extracellular enzyme biosynthesis by Aspergillus oryzae in submerge fermentation media.

Concentration of *A. sativa* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	48 h	96 h	144 h	48 h	96 h	144 h	48 h	96 h	144 h
0.5	24	52	30	15	49	30	28	58	32
1	25	58	31	18	52	32	30	62	34
1.5	29	62	33	20	56	32	34	64	34
2	36	78	45	26	62	40	39	80	50
2.5	32	68	42	25	58	39	36	76	50
3	29	59	34	22	54	32	29	62	40
3.5	22	52	32	20	50	32	27	52	34
4	22	44	32	16	44	30	24	49	30
4.5	22	46	26	14	42	24	20	42	30
5	18	38	27	14	32	21	16	39	30

respectively. The second substrate, *Cicer arietinum* (2.5 g/l) allowed biosynthesis of 71; 61, 80 U/ml lipase, amylase and protease respectively with highest extracellular enzymes at 96 h (Tables 3 and 4). With *the addition of B. subtilis* and *A. sativa* (3.5 g /L), protease (167 U/ml), lipase (113 U/ml) and amylase (150 U/ml) activity was higher. However, maximum enzyme secretion was obtained at the concentration of 3.0 g. The second additive, *Cicer arietinum (*2.5 g/l) allowed to obtain 149 U/ml of lipase, 121 U/ml of amylase and

Table 4. Effect of *Cicer arietinum* on extracellular enzyme biosynthesis by *Aspergillus oryzae* in submerge fermentation.

Concentration of *C. arietinum* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	48 h	96 h	144 h	48 h	96 h	144 h	48 h	96 h	144 h
0.5	18	38	28	19	40	34	22	60	46
1	20	42	30	21	54	38	30	62	52
1.5	22	44	34	26	56	38	36	80	60
2	30	56	50	30	64	42	40	84	62
2.5	34	60	50	42	82	62	44	88	70
3	38	66	62	46	86	59	46	100	72
3.5	32	64	58	32	62	50	48	119	102
4	30	56	42	29	58	40	46	98	80
4.5	28	52	40	26	52	38	44	82	64
5	26	48	40	20	44	36	38	79	56

Table 5. Effects of Avena sativa on extracellular enzyme biosynthesis by Bacillus subtilis in submerge fermentation.

Concentration of *A. sativa* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
0.5	51	70	50	50	71	59	98	144	130
1	53	80	58	48	79	59	64	116	90
1.5	53	82	69	50	88	64	64	120	98
2	58	88	70	58	88	71	80	130	96
2.5	60	97	82	81	131	95	90	140	120
3	70	106	80	90	150	130	96	152	132
3.5	71	113	91	81	131	95	96	164	152
4	67	97	79	75	117	83	70	150	140
4.5	63	90	67	75	130	95	70	140	95
5	50	88	71	50	88	71	70	119	80

Table 6. Effects of Cicer arietinum on extracellular enzyme biosynthesis by Bacillus subtilis in submerge fermentation.

Concentration of *C. arietinum* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
0.5	49	71	60	56	97	71	73	123	101
1	50	88	71	67	116	88	81	132	116
1.5	58	88	70	71	119	89	93	152	99
2	67	97	79	81	137	101	108	176	141
2.5	71	121	91	96	149	130	96	167	150
3	60	97	82	79	127	95	96	150	130
3.5	70	106	80	64	117	87	70	132	95
4	53	82	69	69	108	80	66	119	80
4.5	63	90	67	64	105	74	70	106	80
5	50	79	59	78	125	91	66	100	74

protease 176 U/ml. With the concentration of 2.0 g/l, highest extracellular enzyme activity was observed at 36 h (Tables 5 and 6). The addition of additives in molasses shows effective production of enzymes compared with without additive molasses fermentation.

The use of agro-industrial byproducts allows bio-processes development for the production of large quantities at viable cost enzymes. The growing demand for lower cost in industrial processes that are also highly specific and environmentally safe has stimulated the search for new enzymes. The use of agro-industrial residues in bioprocesses has enabled the production of enzymes employing alternative substrates at low cost as well as reducing environmental degradation caused by

the disposal of these residues (Elisashvili et al., 2008; Karp et al., 2013).

The sugar and ethanol industry produces different residues and many of them have great potential for bioprocesses application (Pandey et al., 2000). Sugarcane molasses is dark syrup obtained during sugar production from sugarcane or beets, resulting from the final stage of crystallization from which further recovery of sugar is no longer economically viable (Arakaki et al., 2011). Sugarcane molasses has an average of 50% total sugars in which sucrose predominates (Arakaki et al., 2011). The production of sugarcane in Brazil in 2012/2013 harvest reached 589 million tons, yielding from 23 to 35 tons of molasses (CONAB, 2013). Its composition and abundance makes this residue a potential substrate for the development of biotechnological processes, including the production of enzymes and other products (Miranda et al., 1999).

Conclusion

Sugarcane molasses is the best substrate for protease, amylase and probably lipase secretion by *Bacillus subtilis* and *Aspergillus orzyae* in submerged cultivation. Enzyme production is negatively affected by the increase in molasses concentration. The highest level of protease activity (74 and 150 U/ml) occurs in a cultivation medium containing of 1% and 0.6 to 0.7% of sugarcane molasses, respectively for *A. oryzae* and for *B, subtilis* process. In addition to molasses, the carbon sources *Avena sativa* (2%) and *Cicer arietinum* (3%) added medium enhance the enzyme producing activity of strain *A. oryzae.* Similarly, in addition to molasses, the carbon sources *Avena sativa* (2%) and *Cicer arietinum* (3%) added medium enhance the enzyme producing activity of strain *B. subtilis.* The results indicate the versatility of *B. subtilis* in production of significant levels of enzymes in submerged cultures based on abundant and inexpensive agro-industrial by products.

Conflict of interests

The authors did not declare any conflict of interest.

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