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USING SOME AGRORESIDUES FOR AMYLASE PRODUCTION BY SOME LOCAL *Bacillus* ISOLATES IN SOLID STATE FERMENTATION

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ABSTRACT

The production of extracellular amylases by some local *Bacillus* isolates was studied in solid state fermentation (SSF) using wheat bran (WB), rice bran (RB), corn bran (CB), potato waste (PW) individually as well as in combinations (ratio of 1:1 w/w). The appropriate incubation period was examined. Among the four substrates tested highest enzyme production was observed with WB by all *Bacillus* isolates. Maximum enzyme production (425.26 U/g) was observed with WB after 72 h by *B. licheniformis* and decreased with further incubation. Maximum enzyme production (384.27 U/g) was observed with RB after 96 h incubation period by *B. licheniformis,* while maximum enzyme production (396.53 U/g) was observed with CB by *B. subtilis* 10 at 72 h and maximum enzyme production (389.35U/g) was observed with PW by *B. brevis* 11 after 72 h. All combination treatments of substrates (ratio 1:1 w/w) gave significant decreases in the enzyme production compared to individual substrates. Although, RB, CB and PW were proved promising substitutes for wheat bran in amylase production.
Keywords: amylase, agroresidues, solid state fermentation.

INTRODUCTION

Bacterial amylase is produced by different fermentation techniques. Solid state fermentation (SSF) has numerous advantages including productivity and may be preferred to submerged fermentation (SmF) due to simple technique, low capital investment, lower levels of catabolite repression and better product recovery (Babu and Satyanarayana, 1995). The SSF technique is mainly confined to process involving fungi (Haq *et al*., 2002). It is believed that this technique is not suitable for bacterial cultures because of higher water activity requirements (Lonsane *et al.*, 1985). However, successful bacterial growth by using the SSF technique is known in many natural fermentations (Ramesh and Lonsane, 1991).

The production of amylases by SSF is to the limited genus *Bacillus* and *B. subtilis* , *B. polymyxa* , *B. mesentericus* , *B. vulgarus*, *B. megaterium* and *B. licheniformis* have been used for amylase production in SSF (Babu and Satyanarayana, 1995). The production of bacterial α-amylase using the SSF technique requires only a fermentation time of 24-48 h (Ramesh and Lonsane, 1987). Thus, with reduced batch time, the use of SSF technique for bacterial α-amylase production leads to considerable reduction in capital and recurring expenditure.

Various solid substrates, such as wheat bran, corn bran, rice bran, etc. have been used in SSF. These substrates were employed individually without any supplementation with other carbon and nitrogen sources. Among these,

WB was superior as reported by Lonsane and Ramesh, (1990). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and the moisture level of the substrate is therefore most important.

There are several factors, which affect SSF processes. Among these, selection of a suitable strain, substrate and selection of process parameters are crucial (Pandey *et al.*, 2000). The nature of solid substrate is the most important factor in SSF. This not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells. Therefore, the particle size and the chemical composition of substrate are of critical importance (Lonsane *et al.*, 1985).

Both natural as well as synthetic substrates can be used in SSF. The selection of a substrate for SSF process depends upon several factors mainly related with cost and availability and thus may involve the screening of several agro-industrial residues. An ideal solid substrate provides all necessary nutrients to the microorganism for optimum function. However, some of the nutrients may be available in suboptimal concentrations, or even not present in the substrates. In such cases, it would be necessary to supplement them externally. It has also been a practice to pretreat some substrates before use in SSF processes which makes them more easily accessible for microbial growth (Sato and Sudo, 1999).

The aim objective of this research was to optimize amylase production by some local *Bacillus* isolates by SSF technique using some agriculture wastes.

MATERIALS AND METHODS

Bacteria used:

Eleven *Bacillus* isolates were isolated from the farm of Faculty of Agriculture, Mansoura University and used as biological materials. The isolates were maintained on starch agar medium.

Inoculum preparation: *Bacillus* isolates were grown on starch agar at 37 ºC for 24 h. A loopful of the growth was transferred to 100 ml starch-broth liquid medium contains (g L^{-1}) 5 g peptone, 3 g beef extract and 2 g soluble starch (Skerman, 1967) and incubated for 24 h at 37 ºC.

Enzyme production in SSF:

In an attempt to choose a potential substrate for SSF which supports amylase production, various agroresidues i.e. wheat bran(WB), rice bran (RB), corn bran (CB) and potato waste (PW) were screened individually as well as in combinations (mass ratio of 1:1 w/w). SSF was carried out by taking 5 g of dry substrate in a 250-mL Erlenmeyer flask moistened with 5 ml distilled water. The contents of the flasks were autoclaved at 121 °C for 20 min. Each fermentation medium was inoculated using 2.5 ml of culture broth and incubated at 37 °C for 24, 48, 72, 96 and 120 h.

Enzyme extraction:

The crude enzyme was extracted by mixing a fermented bacterial substrate with 50 ml distilled water on a rotary shaker (350 rpm) for 15 min. The slurry was squeezed through double gauze. Extracts were pooled and centrifuged at 5000 x g for 20 min. to separate small substrates particles, cells and spores. The brown clear supernatant was used as crude enzyme for enzyme assays.

Determination of enzyme activity:

Amylase activity was determined by modified iodine method described by Hernandez and Pirt (1975). 2.5 ml of 0.4% soluble starch in phosphate buffer, pH 6.5 were mixed with 0.25 ml crude enzyme and incubated for 10 min. at 40 °C. The reaction was stopped by adding 1 ml 1 N HCl. 0.5 ml from each reaction tube mixed with 1 ml of a 0.2% iodine – 0.4% KI solution and 2 ml distilled water , allowed to stand for 15 min. at room temperature and the color intensities were measured at 620 nm. One unit of amylase activity was defined as the amount of enzyme which hydrolyze 0.1 mg of soluble starch at 40 °C in 10 min.

RESULTS AND DISCUSSION

Data presented in Table (1) show the variations of amylase production using wheat bran as nutrient medium and anchorage for Bacillus isolates in SSF during five-days with 24 h interval time. The amylase production pattern indicates that the enzyme was induced during the growth phase and that the maximum enzyme production was observed at 72 h of incubation except *Bacillus brevis* 6 the maximum production was observed at 120 h. Continuing the incubation further resulted in a decline of the enzyme yield, the reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium (Ramesh and Lonsane, 1987). The highest production (425.26 U/g) was achieved after 72 h by *Bacillus licheniformis*.

Table 1: Effect of wheat bran as nutrient medium and anchorage for Bacillus isolates for amylase production using SSF technique throughout 120 h.

Bacillus isolates	Enzyme activity (U/g) Incubation period				
	24 h	48 h	72 h	96 h	120 h
Bacillus subtilis 1	364.01	401.56	408.56	383.72	374.27
Bacillus subtilis 2	335.01	404.08	406.29	391.71	378.64
Bacillus brevis 3	314.18	394.02	401.66	400.24	397.56
Bacillus brevis 4	285.64	396.32	404.70	390.02	383.38
Bacillus brevis 5	377.14	400.41	405.13	397.23	389.44
Bacillus brevis 6	270.42	337.66	390.66	398.71	403.27
Bacillus brevis 7	359.06	377.77	418.91	411.15	410.44
Bacillus licheniformis	393.06	398.03	425.26	408.77	402.42
Bacillus brevis 9	316.35	398.83	403.20	396.77	390.75
Bacillus subtilis 10	398.05	406.18	408.22	403.46	396.84
Bacillus subtilis 11	394.51	415.26	420.32	400.14	391.09
• LSD at 5% : 0.67					

Data recorded in Table (2) show variations of amylase production of *Bacillus* isolates when rice bran was used as nutrient medium and anchorage for Bacillus isolates in SSF during five-days with 24 h interval time. The maximum enzyme production was observed at 72 h of incubation by *B. subtilis* 1, *B. subtilis* 11, *B. brevis* 4, *B. brevis* 7 and *B. brevis* 9 and at 96 h by *B. subtilis* 10 and at 120 h by *B. subtilis* 2, *B. brevis* 3, *B. brevis* 5 and *B. brevis* 6 and the highest enzyme production (384.27 U/g) was observed at 96 h by *B. licheniformis*. Results are in agreement with those obtained by Haq *et al.,* (2003) on *Bacillus licheniformis*. The enzyme production have a significant decrease with using rice bran as substrate in solid state fermentation compared with WB, the presence of bran particles along with broken rice in the wastes might have resulted in lower growth and enzyme production compared to wheat bran.

Table 2: Effect of using rice bran as nutrient medium and anchorage for Bacillus isolates for amylase production by SSF technique throughout 120 h.

Bacillus isolates	Enzyme activity (U/g)				
	Incubation period				
	24 h	48 h	72 h	96 h	120 h
Bacillus subtilis 1	169.32	266.78	270.32	253.17	250.31
Bacillus subtilis 2	115.09	207.45	315.87	347.83	370.09
Bacillus brevis 3	105.33	212.99	321.84	336.17	361.94
Bacillus brevis 4	118.84	189.85	276.48	250.59	242.95
Bacillus brevis 5	205.38	284.41	300.89	331.01	364.83
Bacillus brevis 6	149.26	245.68	316.76	321.98	364.83
Bacillus brevis 7	220.00	272.29	325.58	296.72	290.22
Bacillus licheniformis	204.21	297.13	376.80	384.27	382.49
Bacillus brevis 9	101.62	269.17	345.78	339.86	334.64
Bacillus subtilis 10	185.47	269.65	284.63	316.83	247.18
Bacillus subtilis 11	169.32	262.49	274.70	247.51	244.96
• LSD at 5% : 0.67					

The ability of isolates to produce amylase from corn bran is given in Table (3). It shows sharp reduction in enzyme production by some isolates, *B. subtilis* 2, *B. brevis* 3, *B. brevis* 5 and *B. brevis* 6. A gradual increases in enzyme production by the other isolates were observed, the maximum enzyme production was observed at 72 h by *B. subtilis* 10, *B. brevis* 7, *B. brevis* 9, while it was observed at 96 h by *B. subtilis* 1 and at 120 h by *B. brevis* 4, B*. licheniformis*, and *B. subtilis* 11.The highest enzyme production (396.53 U/g) was achieved by *B. subtilis* 10 at 72 h.

Results in Table (4) show the effect of potato waste and incubation period on amylase production, it revealed that there is a reduction in enzyme production by *B. licheniformis* and *B. subtilis* 10 but the isolates of *B. brevis* 3, *B. brevis* 4, *B. brevis* 5, *B. brevis* 6, *B. brevis* 7 and *B. subtilis* 2 show high productivity after 72 h of incubation, *B. subtilis* 1 and *B. brevis* 9 reached a peak after 120 h. The highest enzyme production (389.35U/g) was observed by *B. brevis* 7 after 72 h.

Table 3: Effect of using corn bran as nutrient medium and anchorage for Bacillus isolates for amylase production by SSF technique throughout 120 h:

Bacillus isolates	Enzyme activity (U/g)				
	Incubation period				
	24 h	48 h	72 h	96 h	120 h
Bacillus subtilis 1	79.17	172.53	315.20	369.84	332.56
Bacillus subtilis 2	9.15	50.56	175.90	126.57	115.64
Bacillus brevis 3	14.53	75.10	171.50	42.71	32.31
Bacillus brevis 4	67.73	146.04	290.96	323.07	325.86
Bacillus brevis 5	13.46	68.96	83.02	176.48	176.41
Bacillus brevis 6	11.27	91.46	123.21	138.67	82.71
Bacillus brevis 7	335.69	371.99	393.31	382.06	380.45
Bacillus licheniformis	254.76	297.78	340.82	348.98	381.77
Bacillus brevis 9	47.18	252.86	383.74	379.57	299.58
Bacillus subtilis 10	250.93	355.99	396.53	385.53	330.49
Bacillus subtilis 11	135.75	291.56	327.00	330.47	386.35
• LSD at 5% : 0.67					

Tables (1, 2, 3 and 4) show the differences in amylase production by eleven *Bacillus* isolates using four agroresidues individually as nutrient medium and anchorage in SSF during five-days with 24 h interval time. Statistical analysis shows that the best isolate for amylase production is *Bacillus brevis* 7 followed by *B. licheniformis*, *B. subtilis* 11, *B. subtilis* 1, *B. brevis* 9, *B. subtilis* 10, *B. brevis* 5 and *B. brevis* 4 respectively, and there is no significant difference between *B. brevis* 6, *B. subtilis* 2 and *B. brevis* 3.Wheat bran proved to be the best source for amylase production followed by potato waste, rice bran and corn bran respectively and the best incubation period is 72 h.

Table 4: Effect of using potato waste as nutrient medium and anchorage for Bacillus isolates for amylase production by SSF technique throughout 120 h:

Bacillus isolates	Enzyme activity (U/g)				
	Incubation period				
	24 h	48 h	72 h	96 h	120 h
Bacillus subtilis 1	276.97	321.26	360.76	369.57	378.02
Bacillus subtilis 2	262.83	287.15	321.70	294.25	256.63
Bacillus brevis 3	287.76	312.18	355.47	321.43	307.15
Bacillus brevis 4	262.56	286.46	317.62	285.12	274.70
Bacillus brevis 5	287.34	309.76	358.86	356.90	341.98
Bacillus brevis 6	298.43	324.35	379.79	332.10	305.70
Bacillus brevis 7	261.12	318.54	389.35	375.27	374.66
Bacillus licheniformis	236.23	248.34	224.22	185.84	148.96
Bacillus brevis 9	176.94	219.20	280.24	322.86	328.72
Bacillus subtilis 10	138.15	150.65	169.54	191.63	155.25
Bacillus subtilis 11	243.87	302.10	360.94	375.73	375.11
• LSD at 5% : 0.67					

Tables (5, 6, 7 and 8) show the difference in amylase production by four Bacillus isolates using six agroresidues combinations as nutrient medium and anchorage in SSF during five-days with 24 h interval time.

Table(5): Effect of using different combinations between the agroresidues for amylase production by *B. subtilis* 1 by SSF technique at 37° C throughout 120 h:

Incubation period			Relative activity			
			WB	**RB**	**CB**	**PW**
	Combination treatment		**364.01**	**169.32**	**79.17**	**276.97**
24 h	WB+RB	**259.51**	-28.71	53.27	-	-
	WB+CB	**215.73**	-40.74	-	172.49	-
	WB+PW	**332.35**	-8.70	-	-	19.99
	RB+CB	**257.78**	-	52.24	225.60	-
	RB+PW	**259.69**	-	53.37	-	-6.24
	CB+PW	**294.84**	-	-	272.41	6.45
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٠١.٥٦	٢٦٦.٧٨	١٧٢.٥٣	٣٢١.٢٦
48 h	WB+RB	**301.65**	-24.88	13.07	-	-
	WB+CB	**319.64**	-20.40	-	85.27	-
	WB+PW	**348.25**	-13.28	-	-	8.40
	RB+CB	**278.85**	-	4.52	61.62	-
	RB+PW	**297.93**	-	11.68	-	-7.26
	CB+PW	**360.88**	-	-	109.17	12.33
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٠٨.٥٦	٢٧٠.٣٢	٣١٥.٢	٣٦٠.٧٦
72 h	WB+RB	**328.45**	-19.61	21.50	-	-
	WB+CB	**322.82**	-20.99	-	2.42	-
	WB+PW	**362.6**	-11.25	-	-	0.51
	RB+CB	**286.9**	--	6.13	-8.98	
	RB+PW	**325.27**	-	20.33	-	-9.84
	CB+PW	**371.96**	-	-	18.01	3.10
			WB	**RB**	**CB**	**PW**
	Combination treatment		٣٨٣.٧٢	٢٥٣.١٧	٣٦٩.٨٤	٣٦٩.٥٧
96 h	WB+RB	**340.44**	-11.28	34.47	-	-
	WB+CB	**336.62**	-12.27	-	-8.98	-
	WB+PW	**357.97**	-6.71	-	-	-3.14
	RB+CB	**292.75**	-	15.63	-20.84	-
	RB+PW	**337.35**	-	33.25	-	-8.72
	CB+PW	**364.87**	-	-	-1.34	-1.27
			WB	**RB**	**CB**	**PW**
	Combination treatment		٣٧٤.٢٧	**250.31**	٣٣٢.٥٦	٣٧٨.٠٢
120 h	WB+RB	**290.3**	-22.44	15.98	-	-
	WB+CB	**308.19**	-17.66	-	-7.33	-
	WB+PW	**348.7**	-6.83	-	-	-7.76
	RB+CB	**287.76**	-	14.96	-13.47	-
	RB+PW	**281.03**	-	12.27	-	-25.66
	CB+PW	**312.19**	-	-	-6.13	-17.41
• LSD at 5% : 0.907						

As shown in Table (5), production of amylase by *B. subtilis* 1 using combinations of WB with any another agroresidues (ratio 1:1 w/w) resulted in significant decreases in enzyme production compared with WB separately, while using combinations of RB with any another agroresidues (ratio 1:1 w/w)

resulted in significant increases in enzyme production compared with RB separately. Using combinations of CB with another agroresidues (ratio 1:1 w/w) folded the production of enzyme at 24 and 48 h of incubation and the production decreases with further incubation. Also using combinations of PW with WB or CB (ratio 1:1 w/w) resulted in significant increase in the enzyme production by *B. subtilis* 1 compared to PW as an individual substrate at 24 and 48 h and significant decreases with further incubation but using combination of WP+RB resulted in significant decreases in the enzyme production. Data clearly show that the best combination for amylase production by *B. subtilis* 1 is CB+PW followed by WB+PW which achieved 371.96 and 362.6 U/g respectively at 72 h.

Results in Table (6) reveal that production of amylase by *B. brevis* 7 using combinations of WB with any other agroresidues (ratio 1:1 w/w) resulted in significant decreases in enzyme production compared with WB separately, while using combinations of RB with WB or PW (ratio 1:1 w/w) resulted in significant increases in enzyme production compared with RB separately. Using RB+CB gave significant decrease in enzyme production compared with RB separately and using combinations of CB with any other agroresidues (ratio 1:1 w/w) resulted in significant decreases in the production of enzyme compared with CB separately. Combinations of PW with any other agroresidues (ratio 1:1 w/w) resulted in significant increase in the enzyme production by *B. brevis* 7 compared to PW separately at 24 and 48 h and significant decreases with further incubation. The best combination for amylase production by *B. brevis* 7 is WB+PW followed by CB+PW which achieved 380.86 and 372.41 U/g at 72, 96 h respectively.

Results in Table (7) show that production of amylase by *B. licheniformis* using combinations of WB with any other agroresidues (ratio 1:1 w/w) resulted in significant decreases in enzyme production compared with WB separately, while using combinations of RB with WB or PW (ratio 1:1 w/w) resulted in significant increases in enzyme production at 24 h and decreases with further incubation compared with RB separately. On the other hand using RB+CB gave significant decreases in enzyme production compared with RB separately and using combinations of CB with RB or PW gave significant decreases in the production of enzyme compared with CB separately but using WB+CB gave significant increases at 24, 72 h and the rate of increase decreased at 120 h. Using combinations of PW+WB gave significant decreases at 24, 48 h and the enzyme production increased with further incubation but using RB+PW gave significant increases at 24, 48, 72 h and the enzyme production decreased with further incubation while using CB+PW resulted in significant decreases compared with PW separately.The best combination for amylase production by *B. brevis* 7 is RB+CB followed by WB+CB which achieved 363.42 and 360.78 U/g at 120, 96 h respectively.

Table(6): Effect of using different combinations between the agroresidues for amylase production by *B. brevis* 7 by SSF technique at 37˚ C throughout 120 h:

Incubation period			Relative activity			
			WB	**RB**	**CB**	**PW**
	Combination treatment		**359.06**	٢٢٠	٣٣٥.٦٩	٢٦١.١٢
24 h	WB+RB	**283.85**	-20.95	29.02		
	WB+CB	**277.4**	-22.74		-17.36	
	WB+PW	**345.89**	-3.67			32.46
	RB+CB	**285.67**		29.85	-14.90	
	RB+PW	**299.65**		36.20		14.76
	CB+PW	**344.43**			2.60	31.90
			WB	**RB**	**CB**	**PW**
	Combination treatment		٣٧٧.٧٧	٢٧٢.٢٩	٣٧١.٩٩	٣١٨.٥٤
48 h	WB+RB	**309.37**	-18.11	13.62		
	WB+CB	**318.91**	-15.58		-14.27	
	WB+PW	**350.7**	-7.17			10.10
	RB+CB	**292.75**		7.51	-21.30	
	RB+PW	**339.08**		24.53		6.45
	CB+PW	**363.51**			-2.28	14.12
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤١٨.٩١	٣٢٥.٥٨	٣٩٣.٣١	٣٨٩.٣٥
72 h	WB+RB	**336.99**	-19.56	3.50		
	WB+CB	**338.99**	-19.08		-13.81	
	WB+PW	**380.86**	-9.08			-2.18
	RB+CB	**301.99**		-7.25	-23.22	
	RB+PW	**340.71**		4.65		-12.49
	CB+PW	**367.51**			-6.56	-5.61
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤١١.١٥	٢٩٦.٧٢	٣٨٢.٠٦	٣٧٥.٢٧
96 h	WB+RB	**330.45**	-19.63	11.37		
	WB+CB	**338.53**	-17.66		-11.39	
	WB+PW	**369.41**	-10.15			-1.56
	RB+CB	**312.92**		5.46	-18.10	
	RB+PW	**363.42**		22.48		-3.16
	CB+PW	**372.41**			-2.53	-0.76
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤١٠.٤٤	**290.22**	**380.45**	٣٧٤.٦٦
120 h	WB+RB	**298.56**	-27.26	2.87		
	WB+CB	**324.82**	-20.86		-14.62	
	WB+PW	**359.06**	-12.52			-4.16
	RB+CB	**319.18**		9.98	-16.10	
	RB+PW	**323**		11.29		-13.79
	CB+PW	**326**			-14.31	-12.99
• LSD at 5% : 0.907						

Table (8) shows that production of amylase by *B. subtilis* 1 using combinations of WB with any another agroresidues (ratio 1:1 w/w) resulted in significant decreases in the enzyme production compared with WB separately, while using combinations of RB with any another agroresidues (ratio 1:1 w/w) resulted in significant increases in enzyme production compared with RB separately and using combinations of CB with any other agroresidues (ratio 1:1 w/w) resulted in increase the production of enzyme at

24 h of incubation compared with CB separately and the rate of increase decreased with further incubation.

Table(7): Effect of using different combinations between the agroresidues for amylase production by *B. licheniformis* by SSF technique at 37˚ C throughout 120 h:

Incubation period			Relative activity			
			WB	**RB**	**CB**	**PW**
	Combination treatment		٣٩٣.٠٦	٢٠٤.٢١	٢٥٤.٧٦	٢٣٦.٢٣
24 h	WB+RB	**299.47**	-23.81	46.65	-	-
	WB+CB	**280.4**	-28.66	-	10.06	-
	WB+PW	**208.82**	-46.87	-	-	-11.60
	RB+CB	**189.02**	-	-7.44	-25.80	-
	RB+PW	**277.67**	-	35.97	-	17.54
	CB+PW	**194.74**	-	-	-23.56	-17.56
			WB	**RB**	**CB**	**PW**
	Combination treatment		٣٩٨.٠٣	٢٩٧.١٣	**297.78**	٢٤٨.٣٤
48 h	WB+RB	**324.91**	-18.37	9.35	-	-
	WB+CB	**330.36**	-17.00	-	10.94	-
	WB+PW	**218.39**	-45.13	-	-	-12.06
	RB+CB	**296.84**	-	-0.10	-0.32	-
	RB+PW	**279.4**	-	-5.97	-	12.51
	CB+PW	**194.83**	-	-	-34.57	-21.55
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٢٥.٢٦	٣٧٦.٨	٣٤٠.٨٢	٢٢٤.٢٢
72 h	WB+RB	**249.33**	-41.37	-33.83	-	-
	WB+CB	**341.26**	-19.75	-	0.13	-
	WB+PW	**226.9**	-46.64	-	-	1.20
	RB+CB	**287.21**	-	-23.78	-15.73	-
	RB+PW	**307.65**	-	-18.35	-	37.21
	CB+PW	**152.87**	-	-	-55.15	-31.82
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٠٨.٧٧	٣٨٤.٢٧	٣٤٨.٩٨	١٨٥.٨٤
96 h	WB+RB	**136.16**	-66.69	-64.57	--	--
	WB+CB	**360.78**	-11.74	--	3.38	--
	WB+PW	**238.52**	-41.65	--	--	28.35
	RB+CB	**356.33**	--	-7.27	2.11	--
	RB+PW	**173.03**	--	-54.97	--	-6.89
	CB+PW	**113.63**	--	--	-67.44	-38.86
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٠٢.٤٢	٣٨٢.٤٩	٣٨١.٧٧	١٤٨.٩٦
120 h	WB+RB	**84.47**	-79.01	-77.92	--	--
	WB+CB	**290.12**	-27.91	--	-24.01	--
	WB+PW	**251.79**	-37.43	--	--	69.03
	RB+CB	**363.42**	--	-4.99	-4.81	--
	RB+PW	**117.45**	--	-69.29	--	-21.15
	CB+PW	**151.69**	--	--	-60.27	1.83
• LSD at 5% : 0.907						

Using combinations of PW with any other agroresidues gave significant increase in the enzyme production at 24, 48 h and decreases with further incubation compared with PW separately. The best combination for amylase

production by *B. subtilis* 11 is CB+PW followed by WB+PW which achieved 365.33 and 352.16 U/g at 96, 72 h respectively.

Finally Statistical analysis shows that the best isolate for amylase production is *Bacillus brevis* 7 followed by *B. subtilis 1*, *B. subtilis* 11 and *B. licheniformis* respectively, with no significant difference between *B. subtilis* 1 and *B. subtilis* 11.WB+WP proved to be the best for amylase production followed by WB+CB, CB+WP, RB+PW, WB+RB and RB+CB respectively, and the best incubation periods is 48, 72, 96 h with no significant difference between them.

Among the various substrates screened for SSF, WB gave the highest enzyme production (425.26 U/g) at 72 h by *B. licheniformis* (Table 1) and CB was the next by producing (396.53 U/g) at 72h by *B. subtilis* 10 and PW gave (389.35 U/g) at 72 h by *B. brevis* 7, finally RB which gave (384.27 U/g) at 96 h by *B. licheniformis.*

All combinations of substrates (ratio 1:1 w/w) gave significant decreases in the enzyme production by the selected isolates compared to individual substrates. Although, RB, CB and PW were proved promising substitutes for wheat bran in amylase production.

Generally, results obtained with the used isolates and different substrates are in harmony with those obtained by previous investigators. Baysal *et al.,* (2003) found that the optimum incubation time for amylase production from *B. subtilis* was at 48 h. On the other hand Gangadharan *et al.,* (2006) reported that the optimum incubation time for amylase production from *B. amyloliquefaciens* was at 72 h. Hashemi, Maryam *et al.,* (2010) reported on *Bacillus sp.* KR-8104 that the maximum enzyme production was observed at 72 h on wheat bran medium. Baysal *et al.*, (2003) found that the production of α-amylase by *B. subtilis* reached its maximum after 48 h for WB and 24 h for RB. The production of enzyme decreased after 48 h for WB but similar to those at 72, 96 and 120 h for RB. Researchs are in agreement with present data. Anto *et al.,* (2006) reported that the best incubation time for maximal enzyme production by *Bacillus cereus* MTCC 1305 is 72 h, and that the yield of enzyme decreased with further incubation.

Incubation		Relative activity

			WB	RB	CB	PW
	Combination treatment		٣٩٤.٥١	١٦٩.٣٢	١٣٥.٧٥	٢٤٣.٨٧
24 h	WB+RB	**266.14**	-32.54	57.18		
	WB+CB	**245.79**	-37.70		81.06	
	WB+PW	**305.56**	-22.55			25.30
	RB+CB	**200.47**		18.40	47.68	
	RB+PW	**248.97**		47.04		2.09
	CB+PW	**325.72**			139.94	33.56
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤١٥.٢٦	٢٦٢.٤٩	٢٩١.٥٦	٣٠٢.١
48 h	WB+RB	**311.92**	-24.89	18.83		
	WB+CB	**330.63**	-20.38		13.40	
	WB+PW	**332.54**	-19.92			10.08
	RB+CB	**289.75**		10.39	-0.62	
	RB+PW	**305.74**		16.48		1.20
	CB+PW	**344.98**			18.32	14.19
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٢٠.٣٢	٢٧٤.٧	٣٢٧	٣٦٠.٩٤
72 h	WB+RB	**317**	-24.58	15.40		
	WB+CB	**329.36**	-21.64		0.72	
	WB+PW	**352.16**	-16.22			-2.43
	RB+CB	**293.26**		6.76	-10.32	
	RB+PW	**315.37**		14.81		-12.63
	CB+PW	**355.97**			8.86	-1.38
			WB	**RB**	**CB**	**PW**
	Combination treatment		٤٠٠.١٤	٢٤٧.٥١	٣٣٠.٤٧	٣٧٥.٧٣
96 h	WB+RB	**320.36**	-19.94	29.43		
	WB+CB	**336.17**	-15.99		1.72	
	WB+PW	**345.89**	-13.56			-7.94
	RB+CB	**299.02**		20.81	-9.52	
	RB+PW	**329.99**		33.32		-12.17
	CB+PW	**365.33**			10.55	-2.77
			WB	**RB**	**CB**	**PW**
	Combination treatment		٣٩١.٠٩	٢٤٤.٩٦	٣٨٦.٣٥	٣٧٥.١١
120 h	WB+RB	**299.47**	-23.43	22.25		
	WB+CB	**333.54**	-14.72		-13.67	
	WB+PW	**326.45**	-16.53			-12.97
	RB+CB	**295.93**		20.81	-23.40	
	RB+PW	**295.2**		20.51		-21.30
	CB+PW	**318.18**			-17.64	-15.18

- LSD at 5% : 0.907

Table(8): Effect of using different combinations between the agroresidues for amylase production by *B. subtilis* 11 by SSF technique at 37˚ C throughout 120 h:

The reason for enzyme decreasing might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium (Ramesh and Lonsane, 1987). It could have been also be due to the fact that the microorganisms reached their stationary phase and have started producing secondary metabolites, resultingin a lower yield of enzyme.

In this study wheat bran proved to be most suitable substrate for the colonisation of *Bacillus* isolates, as indicated by the maximum visible growth on the surface of substrate and the highest enzyme yield which is possibly

due to the presence of various suitable nutrients in wheat bran and/or due to its most suitable particle size and consistency required for anchorage, colonisation and enzyme exertion by the present *Bacillus* strains. Also Haq *et al.* (2002) have reported wheat bran as the best substrate for amylase production by *Bacillus licheniformis* using different agricultural by-products.

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استخدام بعض المخلفات الزراعية لإنتاج إنزيم الأميليز بواسطة بعض العزلات المحلية من جنس الباسيلس بواسطة طريقة التخمير الصلب

عبدالله العوضي ابراهيم سليم ، فتحي اسماعيل على حوقه ، عايده حافظ عفيفي عامر و عبدالرحمن محمد محمود العتابي

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تم استخدام احدى عشرة عزلة محلية لجنس الباسيلس لإنتاج إنزيم الأميليز من أربعة مخلفات زراعية – وهى ردة القمح ،رجيع الأرز، ردة الذرة ومخلفات البطاطس – بطريقة التخمير الصلب. تم استخدام المخلفات بصورة فردية وفى مخاليط (بنسبة ١:١ و/و). وتم اختبار فترة الحضانة المناسبة للإنتاج. ومن بين هذه المخلفات المختبرة أعطت ردة القمح أعلى إنتاجية للإنزيم مع كل عزلات الباسيلس المستخدمة ، و كانت أقصى إنتاجية للإنزيم (٤٢٥,٢٦ وحدة/ جم) باستخدام ردة القمح مع *B. licheniformis* بعد ٧٢ ساعة من التحضين وقد انخفض الإنتاج بزيادة فترة التحضين. أما استخدام رجيع الأرز قد أعطى أعلى انتاجية (٣٨٤,٢٧ وحدة/ جم) بعد ٩٦ ساعة من التحضين مع *B. licheniformis* بينما ردة الذرة أعطت أعلى إنتاجية (٣٩٦,٥٣ وحدة/جم) بعد ٧٢ ساعة من التحضين مع *B. subtilis* 10 ، وكانت أعلى إنتاجية من مخلفات البطاطس (٣٨٩,٣٥ وحدة / جم) بعد ٧٢ ساعة من التحضين مع *B. brevis* 11 .

استخدام مخاليط من المخلفات بنسبة ١:١ و/و أدى الى انخفاض معنوي لإنتاج الإنزيم مقارنة باستخدام المخلفات منفردة. ومن هذه الدراسة نجد أن مخلفات البطاطس وردة الذرة ورجيع الأرز تعتبر بدائل واعدة لردة القمح في إنتاج إنزيم الأميليز.

قام بتحكيم البحث

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