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PRODUCTION OF RAW STARCH HYDROLYZING AMYLOLYTIC ENZYMES BY *STREPTOCOCCUS BOVIS* PCSIR - 7B

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Thirty strains of *Streptococci*, isolated from bovine rumen were screened for raw starch digesting activity. Of all the cultures tested *Streptococcus bovis* PCSIR -7B produced the best results (1.50 IU/ml). Alpha amylase production was maximum in medium -M_3 (3.8 IU/ml). Corn starch and peptone were the best sources of carbon and nitrogen for the enzyme production. Cereal starches were hydrolysed more readily as compared to root starches.

Key words: Bovine, Rumen, Hydrolysis.

Introduction

Some amylases digest starch granules without gelatinization [1]. But most of the amylases hydrolyze gelatinized starch granules much quicker than the raw starch granules [2]. Scientists have been searching for such microorganisms which can produce highly active amylase for the hydrolysis of raw starch granules [3-9] in order to develop an effective process for the conversion of renewable biomass resources.

The present study included the following objectives:

(i) Isolation of amylase producing streptococci from bovine rumen. (ii).Production of amylases from selected strain of *Streptococcus bovis* under optimum culture conditions. (iii).Examination of the hydrolytic action of the crude enzyme on various starch granules.

Materials and Methods

Collection of rumen sample. The rumen fluid was obtained from a fistulated cow kept at the college of Veterinary Sciences, Lahore. The fluid was passed through several layers of surgical gaze and was then placed in a sterilised and tightly plugged 250 ml flask.

Isolation and screening of amylase producing microorganisms. 10 ml of freshly collected rumen fluid were added to 90 ml of sterile distilled water. One loopful of the suspension was streaked onto meidum containing % (w/v) corn starch 1.0, peptone 1.0, and agar agar 2.0. The agar plates were incubated anaerobically in a fermentation jar at 37° for a period of 48 hrs. Colonies showing zones of starch hydrolysis were picked-up, sub-cultured in PYSB-medium (poly peptone 1.0%, yeast extract 1.0% and soluble starch 1.0%) and transferred to nutrient agar medium by stab culture.

About thirty colonies from the stab cultures were picked-up and inoculated into Poly Peptone – Yeast extract soluble starch broth for amylase production. The supernatant obtained after centrifugation at 3000 x g and 4° was used as crude enzyme.

Inoculum preparation. The bacterium was routinely maintained on nutrient agar medium, containing (g/l) peptone, 6.0; casein hydrolysate 4.0, yeast extract 3.0, glucose 2.0, beef extract 1.5 and agar agar 20 at 7.0 pH. Preculture was carried out in the same medium without agar. The inoculum was incubated at 37° for 24 hrs. on a rotary shaker, kept at 200 r.p.m.

Fermentation technique. 150 ml each of the culture media given in Table 1 was separately taken in 250 ml capacity flasks, autoclaved at 15-atm. pressure for 30 mins. and inoculated with 2.0 ml of the pre-inoculum. Corn starch when ever used was sterilised separately and then added to the flask containing sterilised nutrient broth. All the cultures were incubated at 37° for 48 hrs and hand shaken for 5 mins. (2-3 times daily) and analyzed for amylase activity.

Enzyme assays and analytical methods. Total carbohydrate of the digest was determined by phenol sulfuric acid method [10]. For the determination of reducing value, Somogyi-Nelson method was employed [11].

Raw starch digesting activity was assayed according to the methods described in our previous publication [3], except that the pH of phosphate buffer used in this case was 6.5 and reaction mixture was incubated at 40° instead of 35° for a period of 60 mins.

Results and Discussions

Strain selection. Table 1 indicates the production of raw starch hydrolysing amylolytic enzymes by the locally isolated cultures of *Streptococci*, using PYSB-medium. The enzyme assay was carried out after incubating the cultures for 48 hrs. on a rotary shaker at 37°. Strain No. 1, 7, 8, 12, 13, 14, 26 and 27 showed better results i.e. 1.11, 1.50, 1.13, 1.07, 1.05, 0.98, 1.18 and 1.23 IU/ml of enzyme activity were produced,

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respectively. Isolate number 7 was therefore, selected for further studies. The morphological and physiological studies on the isolate were conducted according to Bergey's Manual of Determinative Bacteriology [12]. The strain was identified and designated as *Streptococcus bovis* PCSIR -7B.

Selection of fermentation medium. The data given in Table 2, show amylase formation by selected culture of *S. bovis* PCSIR -7B in different culture media. The enzyme yield was maximum in medium-M3 (3.8 IU/ml) while it was low in medium-M1 (3.2 IU/ml) and minimum in medium-M2 (2.5 IU/ml). Medium-M2 was selected for investigating the effect of various carbon and nitrogen sources on amylase production.

Effect of different carbon sources on amylase production. The data given in Table 3 show the effect of different carbon sources on amylase production. The amount of each carbon source was kept at a level of 1.0%. Amylase yield was quite low in case of lactose, fructose and sucrose i.e. 0.9, 1.2 and 1.7 IU/ml of enzyme activity were produced respectively. Better reuslts were obtained with glucose, maltose and soluble starch i.e. 2.0, 2.5 and 2.8 IU/ml were produced respectively. Corn starch, when used as sole carbon source, gave the maximum enzyme yield (3.61 IU/ml).

Mizokami *et al.* [11] had reported the use of raw corn starch as sole carbon source for amylase production by *Streptococcus bovis*, isolated from rumen fluid. Recently Taniguchi *et al.* [13] used potato starch granules sterilised with ethylene dioxide for amylase induction in *Bacillus circulans* F.2. Final pH of the meidum in general either remained neutral or was slightly acidic.

To determine the optimum level of carbon source required for maximum enzyme production, different concentrations of corn starch (ranging from 0.5 to 3.0%) were studied (Table 3). The enzyme production increased with the increase in corn starch concentration, in the begining. The maximum enzyme yield (4.21 IU/ml) was however, produced when corn starch was added at a concentration of 2.0%. Further increase in the starch concentration decreased the rate of enzyme synthesis. This might be the result of the increased viscosity of the culture medium which in turn reduced the rate of aeration and agitation of the broth and hence the enzyme synthesis [13].

TABLE 1. PRODUCTION OF RAW STARCH DIGESTING ALPHA-AMYLASE BY LOCALLY ISOLATED CULTURES OF *STREPTOCOCCUS* SPECIES.

Isolate number	Alpha-amylase activity (IU/ml)	Isolate number	Alpha-amylase activity (IU/ml)
1.	1.11	16.	0.10
2.	0.72	17.	0.25
3.	0.16	18.	0.30
4.	0.23	19.	0.46
5.	0.18	20.	0.52
6.	0.41	21.	0.09
7.	1.50*	22.	0.38
8.	1.13	23.	0.17
9.	0.36	24.	0.41
10.	0.18	25.	0.39
11.	0.27	26.	1.18
12.	1.07	27.	1.23
13.	1.05	28.	0.92
14.	0.98	29.	0.21
15.	0.72	30.	0.07

* Highest amylase yield.

TABLE 2. PERCENTAGE COMPOSITION OF THE DIFFERENT FERMENTATION MEDIA USED FOR THE PRODUCTION OF ALPHA AMYLASE BY *STREPTOCOCCUS BOVIS* PCSIR - 7B.

Constituents	Media		
	M_1	M_2	M_3
Raw corn starch	–	–	1.00
Soluble starch	1.0	1.00	–
$(NH_4)_2SO_4$	-	0.50	-
Peptone	0.50	-	1.00
Yeast-extract	-	0.30	0.50
Cysteine - HCl	0.10	-	-
$KH_2PH_4.2H_2O$	0.25	0.25	
$K_2HPO_4.3H_2O$	0.25	.0.25	-
$MgSO_4..7H_2O$	0.05	0.05	-
$CaCl_2.2H_2O$	0.02	0.02	-
$CaCO_3$	-	-	0.50
NaCl	0.10	0.10	-
Amylase yield (IU/ml)	3.20	2.50	3.80

TABLE 3. EFFECT OF DIFFERENT CARBON SOURCES ON THE ALPHA AMYLASE PRODUCTION BY *S. BOVIS* PCSIR-7B.

S. No.	Carbon source (1.0%)	Final pH	Amylase activity (IU/ml)
1.	Lactose	6.3	0.9±0.03
2.	Fructose	7.0	1.2±0.01
3.	Glucose	7.2	2.0±0.15
4.	Maltose	7.1	2.5±0.22
5.	Sucrose	6.5	1.7±0.14
6.	Corn starch	6.6	3.6±0.07
7.	Soluble starch	6.4	2.8±0.01
8.	None	6.3	Zero

Note: (1). Average of three replicates with standard error. (2). The fermentation was carried out at 37° for 48 hrs on a rotary shaker kept at 300 r.pm..

Effect of different nitrogen sources on enzyme production. The effect of addition of various nitrogen sources on enzyme production was investigated (Table 5). The nitrogen sources were added at a concentration of 1.0 gm nitrogen/liter of the culture medium. Peptone, corn steep liquor and $(NH_4)_2CO_3$ produced better results i.e. 4.46, 4.00 and 3.68 IU/ml were produced, respectively. The enzyme yield in case of inorganic nitrogen sources was in the decreasing order of $(NH_4)_2CO_3$, NH_4NO_3, $(NH_4)_2SO_4$ and $NaNO_3$ i.e. 3.68, 2.24, 2.12 and 1.05 IU/ml, respectively. Similar results were obtained by Wolin *et al.* [14] while working on the use of ammonium salts as sole source of nitrogen for the growth of *S. bovis*. Of the organic nitrogen sources peptone, corn steep liquor, yeast extract and urea produced 4.46, 4.00, 3.35 and 0.78 IU/ml of enzyme activity, respectively. Peptone was therefore, selected as the best nitrogen source for amylase production.

The greater yield obtained in case of peptone might be due to the presence of certain vitamins, amino-acids and poly peptides which might be absent in other compounds, supple-

TABLE 4. EFFECT OF DIFFERENT CONCENTRATIONS OF CORN STARCH ON AMYLASE PRODUCTION BY *S. BOVIS* PCSIR-7B.

S. No.	Corn starch conc. % (w/w)	Amylase activity (IU/ml)
1.	0.5	0.40±0.17
2.	1.0	1.73±0.26
3.	1.5	2.21±0.11
4.	2.0	4.36±0.35
5.	2.5	4.00±0.21
6.	3.0	3.45±0.24

Note: (1). Average of three replicates with standard error. (2). The fermentation was carried out at 37° for 48 hrs. on a rotary shaker kept at 300 r.p.m..

TABLE 5. EFFECT OF DIFFERENT NITROGEN SOURCES ON THE PRODUCTION OF ALPHA AMYLASE BY *S. BOVIS* PCSIR-7B.

S. No.	Nitrogen sources (0.1%)	Final pH	Amylase activity (IU/ml)
1.	$NaNO_3$	6.8	1.05±0.02
2.	$NH_4.NO_3$	7.0	2.24±0.11
3.	$(NH_4)_2SO_4$	6.5	2.12±0.10
4.	$(NH_4)_2CO_3$	6.3	3.68±0.25
5.	Peptone	7.2	4.46±0.04
6.	Corn steep liqour	6.9	4.00±0.27
7.	Yeast extract	7.1	3.35±0.21
8.	Urea	7.4	0.78±0.01
9.	None	6.2	Zero

Note: (1). Average of three replicates with standard error. (2). The fermentation was carried out at 37° for 48 hrs. on a rotary shaker kept at 300 r.p.m.

TABLE 6. EFFECT OF DIFFERENT CONCENTRATIONS OF PEPTONE ON AMYLASE PRODUCTION BY *S. BOVIS* PCSIR-7B.

S. No.	Peptone concentration % (w/v)	Amylase activity (IU/ml)
1.	0.5	3.75±0.21
2.	1.0	5.86±0.11
3.	1.5	4.42±0.25
4.	2.0	4.00±0.18
5.	2.5	3.51±0.37
6.	3.0	3.12±0.13

Note: (1). Average of three replicates with standard error. (2). The fermentation was carried out at 37° for 48 hrs. on a rotary shaker kept at 300 r.p.m..

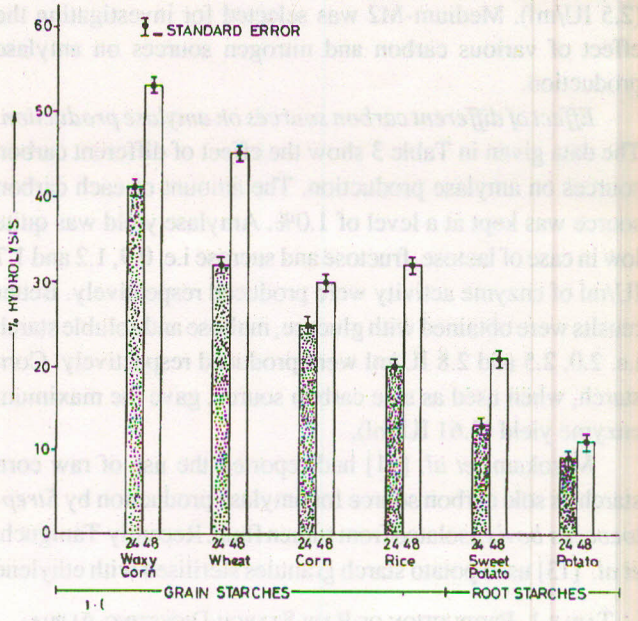

Fig. 1. Degree of hydrolysis of various starches after the action of alpha amylase of *S.bovis* PCSIR-7B. 24 and 48 refers to the time of hydrolysis, in hrs.

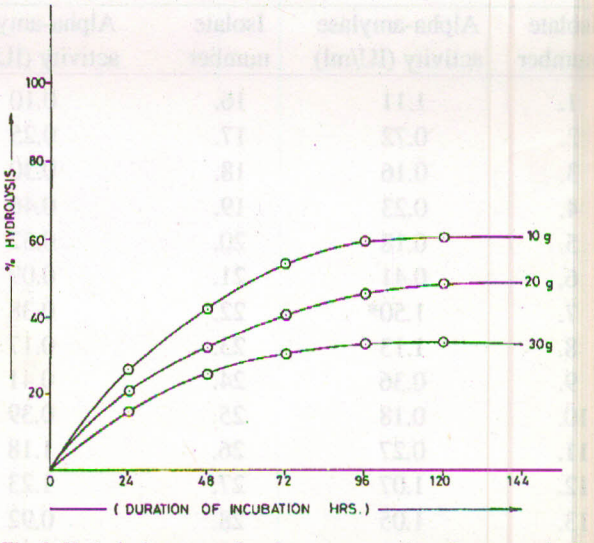

Fig. 2. Hydrolysis curves of various concentration of corn starch after the action of alpha amylase of *S . bovis* PCSIR-7B.

mented in the media [15]. Studies on the nutrition of *S. bovis* by Niven *et al.* [16] showed that the growth of the bacterium was greatly dependent on the presence of certain amino acids and vitamins i.e. Arginine, Glutamine, Glutamic acid, Biotin, Riboflavin and Thiamine, etc. Most of these factors have been reported to be present in peptone, by various workers [14-17]. Final pH of the medium in most of the cases, either remained neutral or was slightly acidic.

To determine the optimum level of nitrogen source required for maximum enzyme synthesis, diferent concentrations of peptone (ranging from 0.5-3.0%) were examined (Table 6). Best results (5.68 IU/ml) were produced at a concentration of 1.0% (w/v). Further increase in the nitrogen concentration did not improve the rate of enzyme synthesis.

Hydrolysis of various starch granules by the crude enzyme. 100 mg of various starch granules were treated with 5.68 IU of raw starch digesting amylase, at pH 5.8 and 35°. The reaction mixture was incubated on a rotary shaker kept at 300 r.p.m. Samples were analyzed for raw starch digesting activity after an incubation of 24 and 48 hrs. The amount of total sugars thus released was determined by Somogyi-Nelson method [8] with maltose as standard.

It is evident from Fig. 1, that the extent of hydrolysis of the waxy corn, wheat, corn and rice starches was 41,32,25 and 20% after 24 hrs. and it increased to 53,45,30 and 32% respectively after an incubation of 48 hrs. The starches of sweet potato and potato exhibited a comparitively lesser degree of hydrolysis (i.e. 21 and 11% respectively, after 48 hrs). The lesser degree of hydrolysis of the last two types may be attributed to the structural characteristic of these starches, which always showed resistance towards enzyme attack [17].

In order to determine the possibility of the use of crude enzyme for the saccharification of raw starch; 10, 20 and 30g of corn starch were added in different flasks which contained 100 ml (568-IU) of crude enzyme, at 5.8 pH. Grains of thymol were added to avoid any contamination by mic-robes. The mixtures were incubated at 35° on a rotary shaker kept at 300 r.p.m. Aliquots of the digests were taken out at intervals and degree of hydrolysis of each samples was determined.

Figure 2. shows that 10 g sample was hydrolyzed to a degree of 60% after five days, while samples of 20 and 30 g were hydrolyzed to an extent of 50 and 35%, respectively. The lower degree of hydrolysis of last two types might have resulted from the increased viscosity of the reaction mixture which in turn made the agitation of the mixture difficult. Thus chances of contact between the starch granules and enzyme were reduced, resulting in a decreased rate of starch hydrolysis [7].

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