OJVR™

Online Journal of Veterinary Research©

Established 1994
ISSN 1328-925X

Volume 22 (10):933-940, 2018.

Production of pepsin by bacteria and fungi from soil and rumen cultured in whey and brans.

Omer Dhiaa Al-Deen Salah, Dhafer Fakhri Al-Rawi, Harith Kamil Buniya.

College of Education, University of Anbar, Iraq.

ABSTRACT

All-Deen Salah OD, Al-Rawi DF, BuniyaHK., Production of pepsin by bacteria and fungi isolated from soil and rumens of goat, sheep and cultured in whey and brans, Onl J Vet Res., 22 (10):933-940, 2018. Pepsin producing bacteria and fungi from 10 soil and goat, sheep and cattle rumen content samples were identified by Biomerieux VITEK 2d and standard methods, and mixed with pasteurized whey or brans to determine optimal pepsin production. Our results suggested that *Staphylococcus sciuri* and *Pythium sp* produced optimal clotting at ~15 min at 50C˚, pH 2.0 in 10% whey with 1.0 ml inoculum incubated 24h yielding 4.6 units/ml pepsin.

Keyword : Pepsin production, *Staphylococcus sciuri, Pythium sp,* wheys, brans.

INTRODUCTION

Proteases account for ~25% of commercial enzymes (Temiz et al., 2007; Gildberg, 1992). Pepsin is highly effective In acidic environments (Northrop, 1930) and found at concentrations of >400 mg/L in mammalian digestive tracts. Pepsin cuts peptide chains for absorption in the small intestine (Kageyama, 2002). Pepsin is secreted by gastric glands as inactive pepsinogen or zymogen but in hydrochloric acid becomes active pepsin (Walsh, 1979). Pepsin has been isolated from stomachs of (Sogawa et al., 1983), rabbits (Kageyama et al., 1990), fish (Gildberg , 1992), plants, bacteria, fungi and viruses (Mandujano-Gonzalez, V. et al., 2016).

Pepsin is used to coagulate cheese (Tavano, 2013), dissolve proteins in juices (Brandelli, A. et al., 2010), in detergents, tanning leathers (Kanth, S.V. et al., 2009) and treatment of digestive and metabolic disorders (Benjakul, S. et al., 2010). Animal and fodder/grain wastes can be used for production of enzymes, antibiotics, insecticides, animal fodder and other biological products (Al-khfaji, 1990). We describe optimal production of pepsin by bacteria and fungi from soil and rumens cultured in pasteurized whey and brans.

MATERIALS AND METHODS

Bacteria and fungi:

Bacteria and fungi were isolated from 10 soil and 3 rumens each of cattle, sheep and goats, serially diluted. One gm of soil and rumen samples were dried, ground, sieved and diluted in 9 ml of sterile distilled water and inoculated onto skim milk agar media incubated at 30 ± 2C˚ for 48 h or inoculated onto potato dextrose skim milk agar media incubated at 28 ± 2C˚ for 72 hrs. Colonies producing pepsin were screened on skim milk agar medium by producing a 1 cm diameter clear zone.

Carbon sources:

We determined bacterial and fungal pepsin production in wheat and barley brans, corn caps and whey powder dried at 70˚C for 48 h, grinded and sieved to 3 gm/l aliquots. The powders were autoclaved and poured onto petri dishes (references???). We also prepared 10% liquid pasteurized whey. The metals 2 KH2Po4 , 2 CaCl2 , 2 No3NH4 , 5 MgSO4 and 2 FeSO4 were added to each media isolate cultured in the center of petri plates and incubated at 30 ± 2C˚ for 48 hours for bacteria and 28 ± 2C˚ for 72h for fungi. Pepsin activity was detected as diameter of the clear zone.

Inoculums:

Bacteria and fungi synergism for production of pepsin was determined on skim milk agar incubated at 28 ± 2C˚ for 72 hrs: Bacteria were cultured in 100 ml of nutrient broth incubated at 30 ± 2C˚ for 24 hrs and fungi on Potato dextrose broth (PDB) incubated at 28 ± 2C˚ for 72h.

Pepsin:

Clotting activity for pepsin was determined as described by Iwasaki (1967): 0.5 ml enzyme solution in placed in tube of 5 ml 10% skimmed milk and 0.01 CaCl2 is added and incubated at 35 C˚. Pepsin to clot within 1 min = 400 units activity (Iwasaki, 1967). Proteolytic activity of pepsin was determined as described by Witaker, (1958): Three gm casein is dissolved in 90 ml distilled water at 70 C˚, stirred with 5.2 ml 0.1 N NaOH dissolved and brought to 100 ml mixed with one 1/1 casein/0.2 Sodium phosphate buffer. Two ml of this solution is mixed with 0.2 ml of enzymatic crud extract incubating in water bath at 35C˚ for 20 min with 3 ml of 10% TCA??? to stop the reaction and centrifuged 5000 rpm for 20 min. Absorbance is read at 280 nm against a blank without stop buffer (2-6) for optimal pepsin production. Pepsin was then produced in a 7.0 pH media with 10% sterilized whey with 2 KH2Po4 , 2 CaCl2 , 2 No3NH4 ,5 MgSO4.7H2O and 2 FeSO4 gm/l mixed with 1ml inoculum of bacteria and fungi incubated in a shaking incubator at 150 rpm at 35 ˚C for 24 hrs.

Optimal production of pepsin:

We determined optimal clotting and proteolysis by pepsin at pH 2, 3, 5, 6 and 7 adjusted with 0.1 N HCL and 0.1 N of NaOH incubated at 35 ˚C for 24 h in temperatures of 25, 30, 40, 45 and 50 ˚C for 24 hours each. We added 0.5-3.0 ml bacterial and fungal inoculums to determine pepsin activity then tested in 10 to 30 % whey with 5% intervals for each concentration and incubated 24 to 144 h at 24h intervals.

Bacteria and fungi Isolates producing pepsin.

Bacteria morphology was determined by shape, color, appearance, size, transparency, pigmentation, form margin and elevation on colonies incubated on agar as described by Aneja, (2003). The isolates were Gram stained in accordance with the standard procedure as described by Todar K et al., (2005). Bacteria were then identified by Biomerieux VITEK 2 System. Fungii were identified by hyphae, antheridia, oospores, and sporangia as described by Middleton, (1943) and Waterhouse, (1967).

RESULTS

Table 1-2 and Figure 1 list bacteria and fungi isolated from 10 soil and rumen content that produced pepsin on skimmed milk plates or PDA medium exhibited as clear 7.0 to 8.0 cm diameter zones.

Table 1: Optimum pepsin producing bacteria by diameter (cm) of clear zone.

Bacterial isolates	Diameter (cm)
B2	8
B8	7.5
B10	7.5
B13	7.0

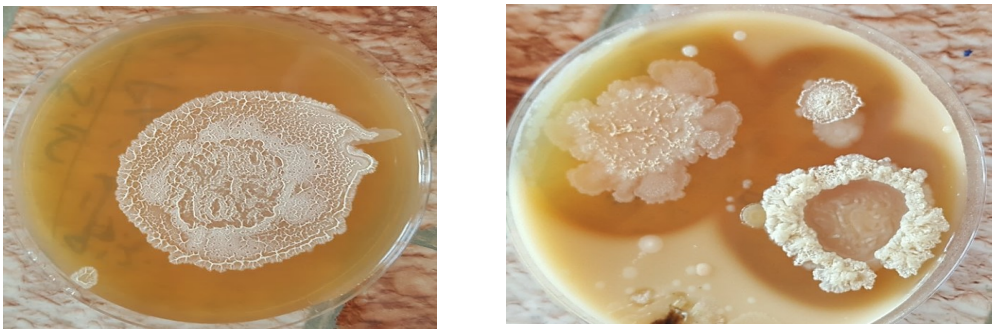
Table 2 : Optimum pepsin producing fungi by diameter (cm) of clear zone.

Fungal isolates	Diameter (cm)
F2	8
F4	7.5
F10	7.5
F11	8
F12	7.0

Table 3 : Colony morphology and microscopy of B2 isolate.

Bacteria	Gram stain	Colony Characters on Nutrient agar					
		A	B	C	D	E	F
B2	Gram-positive cocci	Medium	Dark yellow	Circular	Entire	Flat	Rough

A:Size , B:Pigment, C:Form, D:Margin, E:Elevation , F: Texture



A B

Figure 1. A: *Staphylococcus sciuri* 8.0 cm, B: *Pythium sp.* 7.5 cm.

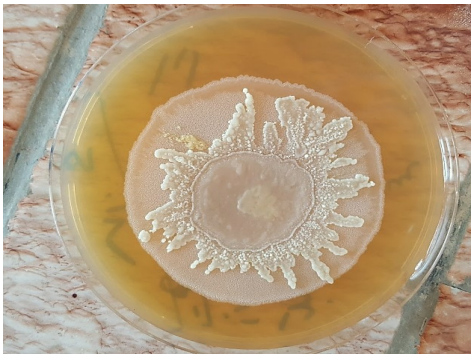


Figure 2. Synergy between bacteria B2 and fungi F10 isolate.

Table 4 : Morphological characteristics of Fungal isolate (F10)

Fungal isolate	Hyphae	Antheridia	Oogonia
F10	Coenocytic	Club-shaped	Single oospore

Table 5 : Bacteria B2 identified by BIOMERIEUX VITEK/GP Cards

Result	Test	Well	Result	Test	Well	Result	Test	Well
+	NOVO	47	-	AGAL	25	+	AMY	2
+	NC6.5	50	-	PyrA	26	-	PIPLC	4
+	dMAN	52	+	BGUR	27	-	dXYL	5
+	dMNE	53	-	AlaA	28	+	ADHI	8
+	MBdG	54	-	TyrA	29	-	BGAL	9
-	PUL	56	-	dSOR	30	+	AGLU	11
-	dRAF	57	-	URE	31	-	APPA	13
+	O129R	58	-	POLYB	32	-	CDEX	14
+	SAL	59	+	dGAL	37	-	AspA	15
+	SAC	60	+	dRIB	38	-	BGAR	16
+	dTRE	62	+	ILATK	39	-	AMAN	17
-	ADH2s	63	-	LAC	42	+	PHOS	19
+	OPTO	64	+	NAG	44	-	LeuA	20
+	dMAL	45	-	ProA	23			
+	BACI	46	-	BGURr	24			

Table 6 : Identification of B2 (*Staphylococcus sciuri*) and F10 fungi *(Pythium sp)*

Isolated	Result
Bacteria (VITEK 2 Biomerieux)	*Staphylococcus sciuri*
Fungi (by morphology)	*Pythium sp.*

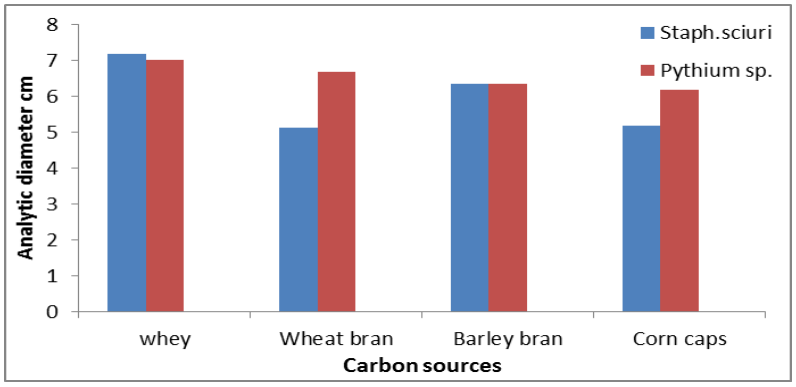


Figure 3. Growth of *Staphylococcus sciuri* and *Pythium sp.* In pasteurized whey, wheat and barley brans, and corn caps.

Table 7. Clot pepsin time (min) and pH.

pH	Clot pepsin time (min)
2	**00:48**
3	01:23
4	02:15
6	02:05
7	02:08

Table 8. Temperature (C˚) and pepsin clotting time (min).

Temperature (C˚)	Pepsin clot time (min)
25	01:31
30	01:26
40	01:15
45	01:11
50	**00:47**

Table 9. Pepsin clotting time (min) in pasteurized whey (%).

Pasteurized whey (%)	Pepsin clot time (min)
10	**00:38**
15	01:23
20	01:33
25	01:46
30	01:54

Table 10. Effect on inoculum volume (ml) on pepsin clotting time (min).

Volume (ml)	Pepsin clot time (min)
0.5	**00:38**
1.0	00:49
1.5	00:53
2.5	01:08
3.0	01:21

Table **11**: Incubation and pepsin clotting times (min)

Period time (h)	Pepsin clot time(min)
24	00:15
48	00:27
72	00:58
96	01:45
120	01:52
144	02:00

DISCUSSION

Results shown in Table 1-11 and Figures 1-3 identified bacterial isolates that produced most pepsin by clearing milk transparent yellow and fungi on P.D.A medium confirmed by Biomeriox V as Staphylococcus sciuri (B2) and Pythium sp (F10). We then determined optimal synergism between bacteria (B2) and fungi (F10) to produce most pepsin and found that whey was most suitable. Our results suggest that optimal pepsin production occurred at pH 2 with clotting at 48s with proteolytic activity of 2.5 U/ml. Higher pH's reduced clotting sharply to 02:08 min at pH 7.0. Pepsin is active at low pH (Mizote, T. *et al.,* 1997) and our finding confirms those of Caio D.R. et al., (2015) who reported high pepsin output from fish residue at PH 2.0. We found optimal volume for pepsin production to be 1.0 ml with clotting occurring by 00:38min and proteolytic activity of 1.5 U/ml. Apparently more than 1ml induces intense competition between microorganisms for nutrients in media (Purohit,1996).

Production of pepsin decreased with increased incubation time. We found maximum clot pepsin time at 24 h after incubation with coagulation occurring within 15s yielding 4.6 units/ ml. Production of pepsin decrease after 24h to 144 hours. This decline is attributed to depletion of nutrients and increased secretion of toxins and changes in pH as reported by Mobley, (1995). We found highest pepsin production at 50C as shown in table 8 with clot occurring by 47 sec and proteolytic activity of 16.6 U/ml as reported by Segal, (1976) and Whitaker and Benhard, (1972). Eskander MZ., (2017), found that 50°C was optimal for pepsin activity in rumen of sheep's.

We found that inoculum in 10% whey produced most pepsin as shown in Table 9 with clotting activity at 38s yielding 2.2 U/ml. Higher concentrations of whey decreased pepsin as apparently the higher the level of straw in whey inhibits enzyme production (Egorov NS et al., 1983). Ideal

volume for inoculum was 1.0 ml providing for pepsin clot at 00:38min yielding 1.5U/ml pepsin. Increasing the volume by more than 1 ml reduces pepsin production due to competition between microorganisms for nutrients as described by Purohit, (1996).

REFERENCES

Aneja, K.R. 2003. Experiments in Microbiology Plant Pathology and Biotechnology, 4th edition. New Age International Publishers, New Delhi, India.

Egorov , N.S. ; Yudina , Z.K. & yudina T.G. (1983) . Effect of proteins on exproteas Synthesis in Bacillus thuring iensis . Microbiol . (USSR) ; 52 : 443 – 446.

Eskander, M.Z.(2017). Utilization Chicken Gizzard Lining Immobilized Pepsin for Soft White Cheese Manufacture. Syrian journal of Agricultural Research.

Mobley, H.L.T. and Belas, R. (1995). Swarming and pathogenicity of Proteus mirabilis in the urinary tract. Trends in Microbiol. 3(7): 280-284.

Purohit,S.S. and Mathur ,S.K.(1996).Enzymes bioacceleractors In: Biotechnology ,fundamentals,and application publisher . India.

Waterhouse, G. M. 1967. Key to Pythium Pringsheim. Mycological Papers no. 109. Commonwealth Mycological Institute, Kew, England.

Al-khfaji ,Z.M.(1990).Biotechnology. Ministry of Higher education and scientific research.Iraq.Baghdad.

Benjakul, S.; Klomklao, S. and Simpson, B. K.(2010). Enzymes in fish processing. In Enzymes in Food Technology (2ndedn), Whitehurst R. J., Van Oort M., Eds. Blackwell Publishing, Chichester: 211-225.

Brandelli, A., Daroit, D.J., Riffel, A.(2010).Biochemical features of microbial keratinases and their production and applications. Appl. Microbiol. Biotechnol. 85, 1735e1750.

Gildberg A. (1992). Recovery of proteinases and protein hydrolysates from fish viscera. Bioresource Technol 39: 271-276.

Iwasaki, S., G. Tamura, and K. Arima.(1967). Milk clotting enzyme from microorganisms. II. The enzyme production and properties of crude enzyme. Agr. Biol. Chem. (Tokyo) 31:547-551.

Kageyama T, Tanabe K, Koiwai O. (1990). Structure and development of rabbit pepsinogens. Stage–specific zymogens, nucleotide sequences of cDNAs, molecular evolution, and gene expression during development. J Biol Chem 265: 17031-17038.

Kageyama T.(2002). Pepsinogens, progastricsins, and prochymosins: structure, function, evolution, and development. Cell Mol Life Sci ;59:288-306.

Kanth, S.V., Venba, R., Jayakumar, G.C., Chandrababu, N.K.(2009). Kinetics of leather dyeing pretreated with enzymes: role of acid protease. Bioresour. Technol. 100, 2430e2435

Mandujano-Gonzalez, V., Villa-Tanaca, L., Anducho-Reyes, M.A., Mercado-Flores, Y.(2016). Secreted fungal aspartic proteases: a review. Rev. Iberoam. Micol. 33, 76e82.

Middleton, J. T. 1943. The taxonomy, host range and geographic distribution of the genus Pythium. Mem. Torrey Bot. Club 20:1-171.

Mizote, T.; Yoshiyama, H. and Nakazawa, T. (1997). Urease- independent chemotactic responses of Helicobacter pulori to urea, urease inhibitors and sodium bicarbonate. Inf. Immun. 65 (4): 1519-1521.

18-Northrop JH (1930). "Crystalline pepsin". Science. 69 (1796): 580. Bibcode:1929Sci.69.580N. doi:10.1126/science.69.1796.580. PMID 17758437.

Novelli, P.K., Barros, M.M., Fleuri, L.F.,(2016). Novel inexpensive fungi proteases: production by solid state fermentation and characterization. Food Chem. 198, 119e124

Segal, I.H. (1976). Biochemical calculation. (2nd ed.) John Wiley & Sons, Inc. New York-20

Sogawa K, Fujii–Kuriyama Y, Mizukami Y, Ichihara Y, Takahashi T. (1983). Primary structure of human pepsinogen gene. J Biol Chem 258: 5306-5311.

Tavano, O.L., (2013), Protein hydrolysis using proteases: an important tool for food biotechnology. J. Mol. Catal. B Enzym 90, 1e11.

Temiz, H.; Akut; U.; Okumus, E. and Turhan, S.(2007).The partial purification and properties of pepsin obtained from turkey proventriculus. Biotechnology and Bioprocess Engineering, 12: 450- 456.).

Todar, K., Ubukata, M. and Hamada, M. 2005 .Microbiology of human Perspective. Mc Graw Hill Publisher, London.

Walsh, C.(1979). Enzymatic Reaction Mechanisms. W. H. Freeman and company. Sanfrancisco.

Whitaker, J.R. and Benhard, R.A. (1972). Experimental for an introduction to enzymology. The whiber press, Davis.

Witaker, J. R.(1958). Properties of the Proteolytic enzymes of commercial ficin. J. Food Research, 22: 483-493.