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EXTRACTION, PURIFICATION AND CHARACTERIZATION OF AN ANTIBACTERIAL ACTIVITY OF *Streptococcus thermophilus*

Abdel-Baky, M.A.
Food Science Dept., Fac. of Agric.,
Zagazig University, Egypt .

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ABSTRACT: Methanol-acetone (MA) extracts of lyophilized fermented skim milk of various strains of *Streptococcus thermophilus* namely ER_1, ST, a10, 106 and S4 were tested for their antibacterial activity by agar diffusion technique using *E.coli* NCTC 10418, *Protus valgaris* NCTC 1653, *Staphylococcus aureus* NCTC 6571, *Pseudomonas aerginosa* NCTC 10662, *Staphylococcus aureus* non patogenic strain, *Psudomonas flourecence, Brevibacterium spp., Crynobacterium lenus* as test microorganisms.

M.A extract of Lyophilized fermented skim milk using *Streptococcus thermophilus* (ST strain) was collected , purified and stored at –20°C then assayed for its molecular weight cut off, effect of some enzymes, heat stability as well as its minimal effective concentration.

The obtained results showed that the test organisms were inhibited with different inhibition degrees using MA extract of different strains of *S. thermophilus. S. thermophilus* ST strain was more pronounced in this respect especially against *Staphylococcus aurcus*. It has been observed that cleaning up (MA) extract using solid phase extraction columns did not affected the antibacterial activity of the purified MA extract. Ultrafiltration of the purified extract indicated that the antibacterial compound is of a molecular weight less than 3KD.

The antibacterial activity of the purified extract was supressed by proteases, while no influence of catalase or lipase was observed. Heat treatment of the purified extract cleared that the antibacterial substance is heat stable. The dilution of the purified extract up to 1/1000 did not affect the antibacterial activity of this extract whereas it inhibited the growth of *Staphylococcus aureus*.

Key words : bacteriocin, extraction, antibacterial ,lactic acid bacteria, heat stability.

INTRODUCTION

The antibacterial activity of lactic acid bacteria (LAB) against the growth of undesirable bacteria has reported by many investigators (Piard and Desmazeaud 1991). L A B bacteriocin could be widely used as a food preservative (Stiles & Hastings 1991, Daeschel 1992 and Ray & Daeschel 1992).

Bacteriocin is an antibacterial substance which a protein portion is involved antibacterial activity. However, the antibacterial activity of this product is narrow and Limited to bacteria that can produce it. (Harris et al., 1988 and Yann et al., 1992 and PCTEP 1994).

Very Limited *Streptococcus thermophilus* bacteriocin is Known. Smaczny (1984) reported on *Streptococcus thermophilus* bacteriocin which was characterized by a molecular weigh of 10-20 K.D, exhibits thermolability at 90°C and sensitive to pepsin. Pulusani et al., (1979) found that *S.thermophilus* bact-eriocin which was strongly inhibit *Pseudomonas* and was not sensitive to pepsin and contain sugar residues.

Abdel-Baky (1989) described antibacterial substance produced by *S.thermophilus* CH1 strain and it was mainly described by its bacterial inhibition spectrum and has especial capacity to inhibit the growth of genus *Staphylococcus* and *Pseudomonas*.

Gilano, et al.(1990) showed that bacteriocin of *S. thermophilus* was not sensitive to pepsin and contain suger residues and did not pass across a membrane with porosity of 100 K.D. Finally the (PCTEP 1994) mentioneda patent concerned with two bacteriocin from *S. thermophilus* (a strain producing those bacteriocins) .This process of producing as well as uses of bacteriocin in the preparation of food products.

There is a need for more bacteriocin which have antibacterial activity against undesirable and pathogenic bacteria. *S. thermophilus* could be considered as an important source for such products. Therefore the present investigation was carried out as an attempt to obtain *S. thermophilus* preparations with antibacterial activity.

MATERIALS AND METHODS

Microorganisms :

This work was carried out in Micrbiological Institute Germany, Kiel, using available *Streptococcus thermophilus* strains namely ST, ERI a 10, 106 and 54. These strains were tested

for their antibacterial activity against *E coli* NCTC 10418, Protusualgaris NCTC 1953, *Staphylococcus aureus* NCTC 6571 and *Pseudomonas aerginosa* NCTC 10662 which were obtained from the Mycological Ref. Lab. London, England and *Staphylococcus aureus* non pathogenic Strain, *Pseudomonas flourecence, Brevobacterium sub.sp. and Crynobacterium Lenus, E.coli* which were obtained from the Microbiological Institute,Kiel, Germany.

Streptococcus thermophilus strains were maintained in Sterilized skim milk stored at 5°C and transferred biweekely during this work. The test organisms were maintained on nutrient agar slopes at 5°C.

Extraction of antibacterial substance:

Sterilized skim milk fermented with different strains of *S. thermophilus* was lyophilized using freeze dryer. The powder residue was dispersed in methanol and stirred for 30 min. The methanolic dispersion was centrifuged at 3000 rpm for 15 min. to remove solid materials. The supe-rnatant was collected for further processing.

The pooled methanol extracts were concentrated under vacum yielding yellow liquid residue, which was yieldingfurther extracted with acetone and centrifuged to remove solids. The supernatant of every strain was concentrated until no further reduction in volum occurred. The volum of every product was adjusted to 10 ml using ultra clean water (special ultra clean water used in Kiel institute for microbiological purposes) and was termed methanol acetone (MA) extract.

Purification of MA extract:

MA extract obtained from *Streptococcus thermophilus* ST strain was cleaned up and discoloured using Solid Phase Extraction (SPE) Columns. The obtained extract was termed purified MA extract.

Estimation of molecular weight of the purified MA extract:

The purified MA extract obtained using *S.thermophilus* ST strain was ultrafiltrated through a membrane with 3 KD molecular mass cut off.

Examination of antibacterial substance:

The antibecterial substances produced during this work (MA extracts) of different strains of *S.thermophilus*, purified MA extract of *S.thermophilus* Strain ST, enzymes treated purified MA extract of ST strain were tested for their antibacterial activity against the test organisms using disc assay technique. Melted nutrient agar fortified with 0.3% yeast extract

was inoculated with 0.5% of an over night old broth culture of the tested organisms. Ten ml of this seeded agar were poured into sterile petri dishes and allowed to solidify. A sterile filter puper disc 6mm diameter was placed on the agar plates and incorporated with 30 µL of the tested substances and left at room temperature for 1h to make the tested materials diffuse into the agar plates then incubated at the aptimum growth temp. for each test microorganism for 24 hr. The agar plates were examined for zone of growth inhibition (clear zone around the disc) the degree of inhibition was measured by: Inhibition zone of 15mm diam. and above (strong), inhibition zone of 12-14 mm (moderate) and inhibition zone of 11 m.m diameter or less (weak).

Influence of some enzymes on the antibacterial activity of the purified MA extract:

Pepsin, proteinase K, pronase, trypsin, catalase and lipase enzymes were obtained from the Microbiological Institut, kiel, Germany. These enzymes were used to evaluate effect of its on the antibacterial activity of the above mentioned extract using the disc assay method. For all the enzymes used 1 µg/ml is added to the extract diluted 3x in buffer then allawed to act for 30 min at the temperature recomanded by the enzyme supplier. The diameter of the inhibition zone is compared with the control diameter of the inibition zone without addition of enzymes.

Examination of heat stability of the extract :

The effect of temperature was tested by incubation 500 µL of the purified extract in sterilized Ependorf tubes placed in a water bath. The incubation temperature were room temperature (about 20°C), 50°C and 90°C. The incubation time was 30 and 60min. The treated extract was tested for antibacterial activity using disc assay technique.

Determination of minimal effective concentration:

The purified extract obtained from strain of *Streptococcus thermophilus* was examined to assess information on the minimal effective concentration. Serial dilutions namely 0.1, 0.05, 0.01, 0.001, 0.0005, 0.0001 were prepared from the extract to select the minimal effective concentration and examined for their antibacterial activity.

RESULTS AND DISCUSSION

Antibacterial activity:

Table (1) show the inhibition zone of antibacterial extracts obtained from the different strains of *Streptococcus*

Table (1): Antibactaerial activity of methanol acetone extracts obtained from different strains of *S.thermophilus.*

Test organism	*S. thermophilus* strains				
	ERI	ST	alo	106	54
	Diameter of the inhibition zone (mm)				
Ecoli NCTC 10418	0	0	0	0	0
Protus valgaris NCtC 1653	12	14	13	12	10
Staphylococcus arueus NCTC 6571	12	15	12	11	9
Pseudomanas aerginosa nctc 10662	13	15	12	11	9
Staph. aureus non pathogenic	14	16	13	15	7
Pseudanonas flourecence	12	17	15	11	7
Brevibacterium sp.	12	18	13	11	8
Crynobacterium lenus	12	17	13	12	7

Table (2) : Antibacterial activity of purified methanol acetone extract produced by ST strain of *S. thermophilus* as affected by different enzymes.

Enzymes	pH of the suitable buffer	Incubation temperature	Inhibition zone m.m diameter
Pepsin	2.00	37	-
Proteinas K	4.00	37	-
Pronase	7.5	37	-
Trypsin	7.5	37	-
Catalase	7.5	25	15
Lipase	7.5	25	15

thermopilus namely ERI, ST, alo, 106 and S4.

From these results it could be noticed that the prepared extracts showed antibacterial activity against the majority of the tested bacterial strains.

In the screening for the antibacterial production by different studied strains of *Streptococcus thermephilus* namely ER_I, ST. alo, 106 and S4, 4 strains out of the studied 5 strains showed inhibitory effect against the majority of the tested bacterial cultures.

It can be also observed that ERI, ST, a10 and 106 strains have strong antibacterial activity against all tested bacteria except *E. coli* while S4 strain showed week inhibition effect against the same tested bacterial cultures. Also, *Streptococcus thermophilus* (St) strain showed the strongest antibacterial activity against the tested bacteria compared with the other studied strains of *Streptococcus thermophilus*.

The most sensitive tested bacteria with ST strain was *Staphylococcus aureus* non pathogenic strain.

Pulusani et al.,(1979) reported similar broad spectrum antibacterial activity for different strains *of S. thermophilus*. In the light of the attained results it could be seen that strain ST of *S. thermophilus* showed the widest and strongest antibacterial activity against the majority of the tested bacteria especially *Staphylococcus aurous* non pathogenic strain. Therefore, the extract of this strain was selected to carry out other examinations to evaluate its stability against the activity of some enzymes and heat at 50 and 90°C. Also, the minimal effective concentration was determined.

Purification and molecular weight of antibacterial extract.

Cleaning up as well as discoloration of the extract using solid phase extraction (SPE) columns to clean up MA extract indicated that the discoloration of the extract did not affect the antibacterial activity of the extract.

The ultrafiltration of the purified extract through a membrane with a 3 KD molecular mass cut off in microconcentrators (MWCO Moleular weight cut off in Daltons) indicated that the antibacterial purified extract produced by ST strain of *S. thermophilus* has a molecular weight less than 3KD.

Table (2) showed that protease namely pepsin, proteinase k, pronase, trypsin supress the antibacterial activity of the extract of *S. thermophilus* ST strain against *Staphylococcus aureus* non pathagenic strain. This result demonstrate that the antibacterial activity could be attributed to protein substances and it

complied with a number of criteria for bacteriocin. These results agree with EL Sayed et al., (1996) who sugested that the antibacterial activity produced by *E.facaum* is protein and could be considered as bacteriocin. Pulusani et al., (1979) also found that the antibacterial substance produced by *S. thermaphilus* complied with number of the criteria for bacteriocin.

No influence of catalase was observed on the antibacterial activity of the tested extract indicating that the inhibition is not due to H_2O_2 since H_2O_2 would have been degraded by catalase. Meanwhile, lipase has no influence on the antibacterial activity of the studied extract. These results are in agreement with El Sayed et al., (1996) since they found that antibacterial agents were sensitive to proteolytic enzymes.

Heat stability:

Table (3) showed the effect of various temperature (room temperature, 50°C and 90°C) on the antibacterial activity of the purified extract obtained from *Streptococcus thermophilus* ST strain when the incubation time was 30 and 60 min . It could be concluded that the antibacterial activity of this extract was heat stable over a wide range of temperature even when the extract was exposed to 90°C for 60 min . These results agreed with Pulusaini, et al. (1979) and EL Sayed et al., (1996).

Minimal effective concentration:

Table (4) showes that *Staphylococcus aurous* non pathogenic strain was affected with the dilution of 1/10 from the purified extract of *Streptococcus thermophilus* ST strain up to $\frac{1}{1000}$ whereas the dilutions of $\frac{1}{10}$, $\frac{5}{100}$, $\frac{1}{100}$, $\frac{5}{1000}$, $\frac{1}{1000}$ were effective against the test organism when the purified extract was diluted up to $\frac{5}{10000}$ the inhibition activity has decreased to be 7mm diameter of the inhibition zone. This result indicates that the dilution of $\frac{1}{1000}$ could be recommended for use in controlling the activity of some undesirable bacteria in milk and milk products.

On the light of the above mentioned results, *Streptococcus thermophilus* ST strain can produce antibacterial substance with wide range of antibacterial activity against pathogenic and undesirable bacteria in milk and milk products.

Table (3) : Effect of heat treatment on the antibacterial activity of the purified extract.

Temperature °c	Incubation time (min)	
	30	60
	Inhibition zone (m m)	
Room temperature	14	14
50	15	16
90	15	16

Table (4): Effect of serial dilution of purified extract of *S. thermophilus* ST strain on the antibacterial activity.

Serial dilution	Inhibition zone
1/10	16
5/100	15
1/100	14
5/1000	13
1/1000	11
5/10000	7
1/10000	-

The effective substances was found to be protein in nature, heat stable, and with molecular weight of less than 3KD. The highest dilution which showed antibacterial activity was found to be $\frac{1}{1000}$.

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أستخلاص وتنقية ودراسة المواد ذات التأثير المثبط لميكروب *Streptococcus thermophilus*

محمد عبد المعطى عبد الباقى

قسم علوم الأغذية – كلية الزراعة – جامعة الزقازيق

تـم اختيـار مجموعـة من السـلالات مـن نـوع بكتـريا ***Streptococcus thermophilus*** هى(ST و 54 و alo و 106 و ER_1) وذلك لمقدرتها على إنتاج مواد مثبطة لنمو العديد من الميكروبات المرضية غيرالمرغوب فيها فى اللبن ومنتجاته.

وقد تم الحصول على هذه المواد المختبرة باستخلاصها بالمذيبات العضوية (الميثانول – الأسيتون) من اللبن الفرز المعقم المتخمر باستخدام السلالات التابعة لـ ***S.thermophilus*** ثم تجفيد كل سلالة على حدة وقد كان لكل السلالات المختبرة من النوع ***S.thermaphilus*** تأثير مثبط على السلالات المدروسة بدرجات مختلفة ومؤثرة كان أعلاها قوة فى التأثير سلالة (ST) وكما لوحظ أن ميكروب ***Staphylococcus aureus*** أكثر الميكروبات تأثراً بعملية التثبيط ، لذا تم استخدام *الميكروب* ***S.thermophilus*** سلالة (S.T) لاستكمال الدراسة ، كما تم استخدام ميكروب ***Stuphylococcus aureus*** كميكروب مختبر لدراسة التأثير وباستخدام بعض التطبيقات الحديثة وجد أن هذه المواد هدمت بتأثير الإنزيمات المحللة للبروتين وكانت ذات وزن جزيئى أقل من ٣ كيلو دالتون كماكان لهذه المواد ثبات حرارى وكان أكبر تخفيف مؤثر هو ١/١٠٠٠.