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Development and Characterization of a Mixed Nutrient Base for the Culture of a Wide Range of Microorganisms

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ABSTRACT. Culture media, designed to grow as many different genera of microorganisms as possible, appear to require a nutrient base composed of polypeptides, oligopeptides and amino acids. Most modern culture media are composed by a mixture of different protein hydrolysates obtained from different proteins and enzymes in order to provide the widest spread of peptides. It seems that large peptides have a role in recovering (or resuscitating) of nutritionally fastidious organisms. The aim of this work was to develop a mixed nutrient base with local raw materials and technology. It was made physical, chemical and microbiological characterisation of three mixtures of the protein hydrolysates and it was selected the best one to promote the growth of a wide range of the microorganisms in different culture media.
Key words: protein hydrolysate, culture, fastidious microorganisms.

RESUMEN. Los medios de cultivo diseñados para el desarrollo de un gran número de géneros microbianos requieren una base nutritiva compuesta por polipéptidos, oligopéptidos y aminoácidos libres. La mayoría de los medios de cultivo modernos tienen en su composición mezclas de hidrolizados obtenidos a partir de diferentes proteínas y con ayuda de diferentes enzimas con el objetivo de garantizar una amplia distribución de péptidos de diferentes pesos moleculares. Existe la opinión de que los péptidos grandes juegan su papel en la recuperación (o resucitación) de los organismos nutricionalmente exigentes. El objetivo del trabajo fue desarrollar una base nutritiva mixta a partir de materias primas nacionales. Se realizó la evaluación fisicoquímica y microbiológica de las tres variantes de la base nutritiva y se seleccionó la variante óptima para el cultivo de la gran mayoría de los microorganismos estudiados en diferentes medios de cultivo.
Palabras clave: hidrolizado proteico, cultivo, microorganismos exigentes.

INTRODUCTION

Since the final of the century XIX when the first culture medium for microorganisms was utilised there have been developed thousands of new formulations. However, all of them have something in common: they need the nitrogen sources for the synthesis of proteins, energy sources, mineral salts and some specific growth factors for several groups of them.[14]

According to the composition of the nitrogen sources utilised, the culture media could be classificated in two groups as defined (specific aminoacid mixtures) and undefined (nutrient bases such as hydrolysates, plant and animal tissue infusions or extracts). The last group has the advantage of that bacterial growth is less affected by the presence of some toxic elements or by unappropriated incubating conditions.[4]

Culture media, designed to grow as many different genera of organisms as possible, appear to require a nutrient base composed by polypeptides, oligopeptides and aminoacids. The choice of substrate and proteases employed and the degree to which the protein is hydrolysed are factors known to affect the physicochemical and microbiological properties of resulting hydrolysates. On one hand some protein substrates lack determined aminoacids and, on the other hand, some methods of hydrolysis could provoke the destruction of aminoacids or disable their assimilation by determined microorganisms.[3,4,20]

Most of the new culture media contain peptones from mixtures of different protein/enzyme interactions, to provide the widest spread of peptides in order to achieve the best microorganism growth and recuperation rates.[1,2,6,9,10,22]

A mixed hydrolysate: tryptose, is available in the international market. It contains different peptides, including those of higher molecular weight and is very useful for growth of fastidious organisms especially when a rapid or profuse growth is required.[11,21]

The objective of this study was to develop a formulation of mixed nutrient bases, using different enzymatic hydrolysates obtained in BioCen, for the further formulation of different tryptose containing culture media, and to characterise its physicochemical and microbiological properties.

MATERIALS AND METHODS

Preparation of the mixed nutrient base. In this study to conform a mixed nutrient base the following nutrient bases were utilised: enzymatic hydrolysate of casein (BioCen), enzymatic hydrolysates of muscle tissue proteins (BioCen) and yeast extract (Biotechnica).

The powdered ingredients were thoroughly mixed in the V-form homogeniser at a pilot scale (10 l capacity) in the humidity and temperature controlled environment (relative humidity < 45 % and temperature ≅ 25 °C).

Four compositions of the mixed base were evaluated at an experimental scale (Table 1).

Three batches of the nutrient base were produced at a pilot scale, utilising the composition of the V4 variant and varying the muscle tissue protein hydrolysate obtaining methods. In the batch number 9P1 was used a papain hydrolysate, in the 9P3-hydrolysate obtained by action of the animal origin enzymes and in the 9P2 was used a mixture of both hydrolysates (1:1).

Reference materials and test strains. Microbiological performance characterisation of the mixed nutrient base was made in culture media for different purposes. All of them contain tryptose, as one of their ingredients. As a reference mixed hydrolysate was used the Tryptose (Merck, lot V202213, Germany). The test and the reference culture media (Tryptose agar, Tryptose blood agar, m-Endo medium, m-Azide medium and Tryptose phosphate broth) were prepared utilising the same raw materials, except of Tryptose.[11,12,21]

In this study were used the bacterial strains recommended for quality assurance of the tested culture media: *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 19433, *Streptococcus pyogenes* ATCC 19615, *S. pneumoniae* URSS 4/56 type B, *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Proteus mirabilis* ATCC 7002 and *Salmonella typhimurium* ATCC 14028.[11,12,21]

The strains were maintained on Brain heart infusion agar slants at 5°C and activated in tryptone soy broth for 24 h at 35 °C before use.

Testing procedure. Physicochemical characterisation of nutrient base consist on determining the lost of drying (gravimetric method),[19] the total nitrogen (Kjeldahl method, automatic Kjeltec system, TECATOR),[5] the amino nitrogen (potentiometric method),[19] the sodium chloride contents (Volhard method),[19] pH of a 2 % nutrient base solution after autoclaving at 121°C for 15 min (PHM 83 AUTOCAL, Radiometer, Denmark) and visual characterization.[12,17]

Microbiological evaluation was also carried out to detect: absence of inhibitory activity by growth curve analysis (turbidimetric method, PU 8620, PHILIPS, Netherlands),[7,13] growth promoting properties in different culture media[8,18,24] and the bacteriological reactivity tests (absence of fermentable carbohydrates, indole production by tryptophanase-forming organisms and production of sulphides by H_2S-forming organisms).[23]

Table 1. Composition of the four mixed nutrient base at experimental scale.

Nutrient bases	Contents (%)			
	V1	V2	V3	V4
Enzymatic hydrolysate of casein	50	60	50	40
Enzymatic hydrolysate of muscle tissue proteins	50	40	25	40
Yeast extract	-	-	25	20

The growth curve analysis were made using 5.0 ml of the 2 % nutrient base solution (at pH value ≅ 7.3) and 0.1 ml volumes of 50 % transmittance standardised bacterial cultures. The tubes were incubated with shaking in water bath at 37°C and the absorbance readings (at 640 nm) were made during the first seven hours.

The growth promoting properties were carried out by spread plate technique[18] using Tryptose agar, membrane filtration technique[24] using m-Endo and m-Azide media; streaking technique[8,12] using Tryptose blood agar and broth spiking technique[12,22] using Tryptose phosphate broth.

The appropriate decimal dilutions of the 50 % transmittance (at 580 nm) standardised bacterial pure culture suspensions were used for colony forming units (CFU) determination.

Statistical analysis. The assays was performed by triplicate and differences in means were calculated by multiple range test using software STATISTICA 6.0 (Statsoft. Inc., 1996). Differences in CFU counts were significant at $p < 0.05$.

RESULTS AND DISCUSSION

The variations in bacteriological performance of different hydrolysates (peptones) was explained by different peptide molecular weight distribution.[4] It was showed the effect of blending peptones to obtain the widest spectrum of peptides.[1,2,6,9,10,22]

The choice of ingredients is usually based on empirical screening or by random selection. It is known, moreover, that the yeast extract is a valuable additive to culture media, as a rich source of growth factors and either alone or in conjunction with peptone, it forms the nutrient basis for many formulations.

At experimental scale there were assayed four formulations with different contents of enzymatic hydrolysates of casein, muscle tissue proteins and yeast extract.

For the study of the absence of inhibitory activity were used nutrient bases at 2 % (w/v) solutions that contain ample amounts of amino nitrogen for optimum growth.[4]

Table 2. Physicochemical characterisation of the mixed nutrient bases at a pilot scale.

Lot	Loss on drying (%)	Total nitrogen (%)	Amino nitrogen (%)	Sodium Chloride (%)	pH	Degree of hydrolysis Na / Nt
9P1	3.30 ± 0.28	11.37 ± 0.11	4.12 ± 0.00	2.64 ± 0.08	6.78 ± 0.03	0.36
9P2	5.75 ± 0.58	10.67 ± 0.11	4.51 ± 0.19	1.79 ± 0.30	6.77 ± 0.04	0.42
9P3	6.32 ± 0.37	10.58 ± 0.14	4.71 ± 0.00	1.78 ± 0.00	6.51 ± 0.01	0.44
Tryptose	5.14 ± 0.14	9.72 ± 0.14	4.52 ± 0.00	0.27 ± 0.00	6.77 ± 0.04	0.46

According to the growth curves (absorbance vs. time, h) showed in Fig. 1, there appeared a significant differences between three groups of nutrient bases after five hours of incubation, when curves began ton plateau. The worst results offered the V1 and V2 variants of the mixed base, while the V3 and V4 variants seems to have the nutritional properties more appropriated. Taking in account that the V3 variant contains the higher level of yeast extract, the only imported nutrient base, as the best variant was selected the V4.

Visual examination of the three lots of mixed nutrient base obtained at a pilot scale confirmed that the powder colour (beige) lied within specification, were homogeneous, free flowing and have no abnormal odour.

The loss on drying, the sodium chloride contents and the appearance of the 2 % nutrient base solutions (pale yellow and transparent) corresponded to the established requirements for this type of products (Table 2).

The limiting moisture value of < 7 % (for tropical climate)[16] is an important characteristic because higher levels will reduce the stability and shelf life of peptones, causing colour changes and falling pH values.

The sodium chloride contents must be lower than 9%. The higher levels may affect osmotic pressure in culture media.

pH rate for the neutralised nutrient bases oscillates between 6.5 and 7.5.

There were no significant differences in the total nitrogen contents for the three lots of mixed base and the Tryptose, while the values of amino nitrogen contents and the degree of hydrolysis (Na/Nt) showed that the 9P1 and 9P2 variants had the higher large polypeptides contents.[4]

The bacteriological reactivity tests demonstrated that the new nutrient base did not contain fermentable carbohydrates and contain tryptophan and sulphured aminoacids.

The growth curves obtained by incubation of the lots

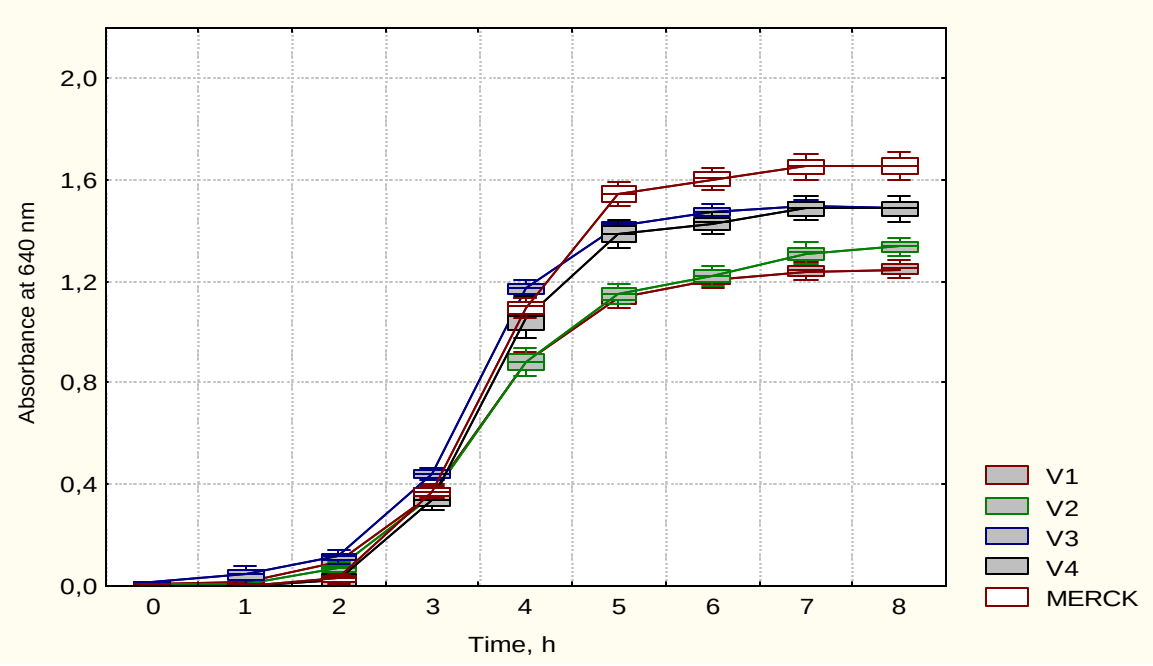

Fig. 1. Growth curves generated by *Streptococcus faecalis* ATCC 29212 in mixed nutrient bases and Tryptose at experimental scale.

produced at a pilot scale with test organisms are shown in Fig. 2 and 3. The curve of tryptose (Merck) indicated the lower nutritional properties for *S. pyogenes* ATCC 19615 strain, while there are no significative differences in absorbance values for the pilot lots of the mixed base. The curves obtained for *E. faecalis* ATCC 29212 began to plateau after 6 hours at 0.75-0.85 absorbance values for the tested bases, and after 5 hours at 0.6 absorbance value for the reference nutrient base. This results demonstrated the absence of inhibitory activity in the all variants of the new mixed base.

The microbiological evaluation (growth promoting properties) are shown in tables 3-7.

Tryptose agar is a medium for the enrichment, isolation and cultivation of streptococci, pneumococci and other pathogenic microorganisms. More colonies of *S. pyogenes, S. pneumoniae* and *E. faecalis* strains grew out on 9P1 and 9P2 variants of Tryptose agar, this difference, however, was not statistically significant. There was demonstrated by peptides molecular size distribution study (HPLC) that the hydrolysates obtained by papain digest had higher levels of polypeptides than little peptides and aminoacids.[15] So, results of this evaluation confirm that the high molecular weight peptides have a role in recovering nutritionally

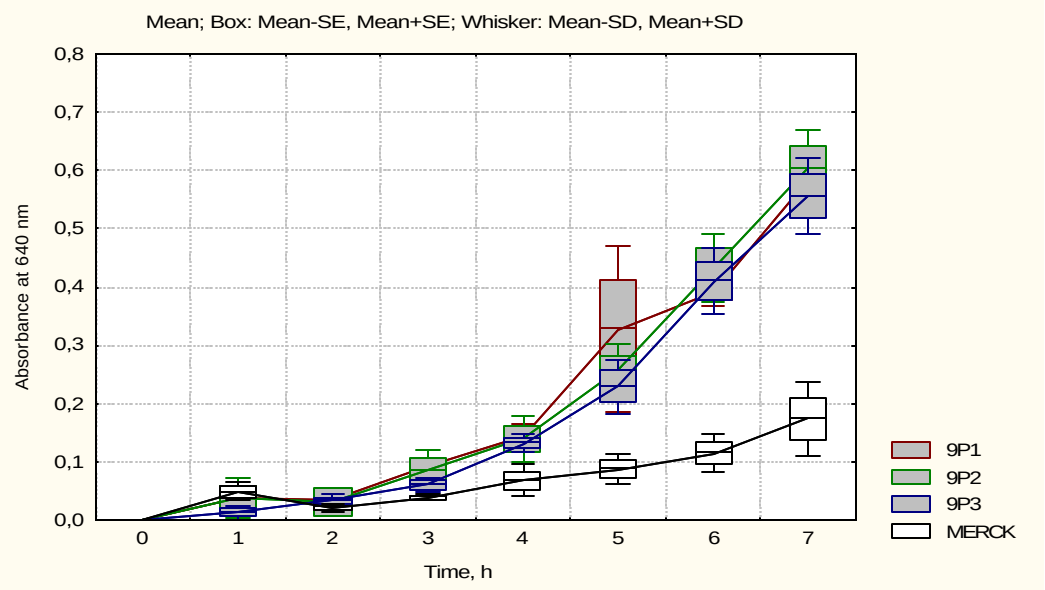

Fig.2. Growth curves generated by *Streptococcus pyogenes* ATCC 19615 in mixed nutrient base variations at a pilot scale and Tryptose.

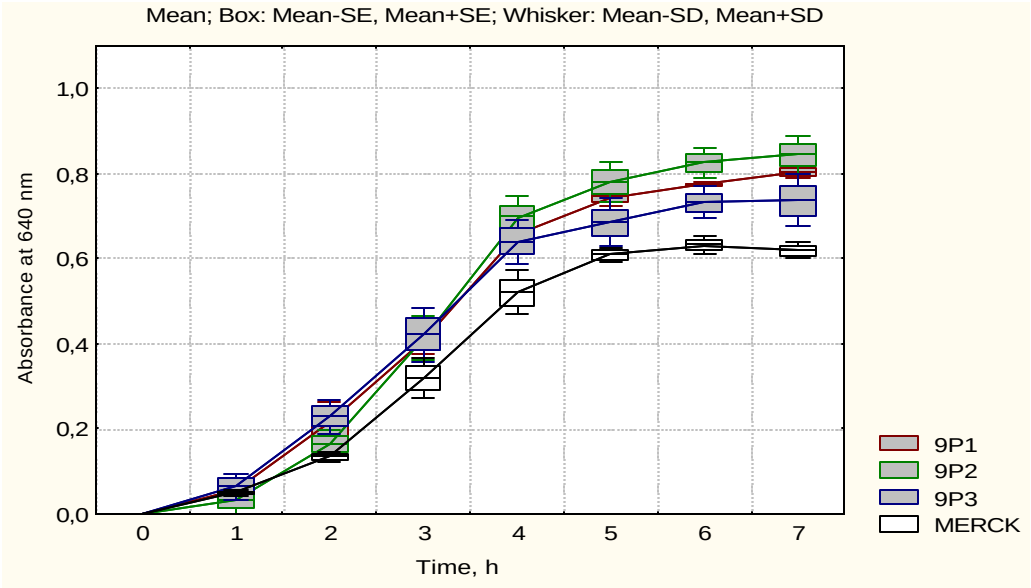

Fig. 3. Growth curves generated by *Streptococcus faecalis* ATCC 29212 in mixed nutrient base variations at a pilot scale and Tryptose.

Table 3. Microbiological evaluation in Tryptose agar.

Microorganism	Lot	Count, CFU/ml	
		Mean	Standard Deviation
Streptococcus pyogenes ATCC 19615	9P1[a]	6.13×10^6	6.40×10^5
	9P2[a]	6.05×10^6	1.80×10^5
	9P3[a]	5.77×10^6	1.16×10^6
	Tryptose[a]	6.63×10^6	4.20×10^5
Streptococcus faecalis ATCC 29212	9P1[b]	1.25×10^8	7.10×10^6
	9P2[b]	1.03×10^8	4.04×10^6
	9P3[b]	1.10×10^8	2.81×10^7
	Tryptose[b]	1.07×10^8	3.23×10^7
Streptococcus pneumoniae URSS 4/56 type B	9P1[c]	1.6×10^5	2.63×10^4
	9P2[c]	1.8×10^5	5.05×10^4
	9P3[c]	1.4×10^5	1.79×10^4
	Tryptose[c]	1.2×10^5	2.54×10^4

[a] There are no significant differences in CFU counts (p min > 0.13); [b] There are no significant differences in CFU counts (p min > 0.20); [c] There are no significant differences in CFU counts (p min > 0.27)

Table 4. Microbiological evaluation in m-Endo medium.

Microorganism	Lot	Count, CFU/ml	
		Mean	Stand. Dev.
Escherichia coli ATCC 25922	9P1[a]	9.75×10^7	1.77×10^7
	9P2[a]	5.25×10^7	3.54×10^6
	9P3[a]	14.75×10^7	1.77×10^7
	Tryptose[a]	7.25×10^7	3.54×10^6
Enterobacter aerogenes ATCC 13048	9P1[b]	7.50×10^7	7.01×10^6
	9P2[b]	6.50×10^7	4.20×10^7
	9P3[b]	1.12×10^8	3.50×10^6
	Tryptose[b]	1.00×10^8	5.60×10^7
Proteus mirabilis ATCC 7002	9P1[c]	1.52×10^8	6.70×10^7
	9P2[c]	1.38×10^8	1.10×10^7
	9P3[c]	2.05×10^8	9.20×10^7
	Tryptose[c]	1.15×10^8	1.40×10^7

[a] There are no significant differences in CFU counts (p min > 0.07); [b] There are no significant differences in CFU counts (p min > 0.12); [c] There are no significant differences in CFU counts (p min > 0.42).

Table 5. Microbiological evaluation in m-Azide medium.

Microorganism	Lot	Count, CFU/ml	
		Mean	Standard Deviation
Streptococcus faecalis ATCC 29212	9P1[a]	9.50×10^7	2.10×10^7
	9P2[a]	1.00×10^8	1.40×10^7
	9P3[a]	9.00×10^7	1.40×10^7
	Tryptose[a]	7.50×10^7	7.00×10^6
Streptococcus faecalis ATCC 19433	9P1[b]	1.52×10^8	1.70×10^7
	9P2[b]	1.85×10^8	1.06×10^7
	9P3[b]	1.45×10^8	4.20×10^7
	Tryptose[b]	1.38×10^8	1.80×10^7

[a] There are no significant differences in CFU counts (p min > 0.20); [b] There are no significant differences in CFU counts

Table 6. Microbiological evaluation in Tryptose phosphate broth.

Microorganism	Lot	Growth in spiked dilutions			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
Streptococcus faecalis ATCC 19433	9P1	+	+	+	+
	9P2	+	+	+	+
	9P3	+	+	+	+
	Tryptose	+	+	+	+
Streptococcus pneumoniae URSS 4/56 type B	9P1	+	-		
	9P2	+	-		
	9P3	+	-		
	Tryptose	+	-		
Staphylococcus epidermidis ATCC 12228	9P1	+	+	+	-
	9P2	+	+	+	+
	9P3	+	+	+	+
	Tryptose	+	+	+	-
Streptococcus pyogenes ATCC 19615	9P1	+	+		
	9P2	+	+		
	9P3	+	+		
	Tryptose	+	+		

Table 7. Microbiological evaluation in Tryptose blood agar

Microorganism	Lot	Haemolysis
Streptococcus pyogenes ATCC 19615	9P1	β
	9P2	β
	9P3	β
	Tryptose	β
Staphylococcus aureus ATCC 25923	9P1	+
	9P2	+
	9P3	+
	Tryptose	+
Streptococcus pneumoniae URSS 4/56 type B	9P1	α
	9P2	α
	9P3	α
	Tryptose	α

fastidious organisms.[4]

m-Endo is a medium for the identification and enumeration of coliform bacteria in water, milk and other liquids by membrane filtration technique. The colony characteristics of all microoganisms assayed in the media agree with the specifications (greenish metallic sheen for *E. coli*, red colonies for *E. aerogenes* and pale colonies for *P. mirabilis*). There were observed a little (no significantly) higher counts of all studied microorganisms in the media containing pancreatic hydrolysate of muscle tissue proteins (9P2 and 9P3). It may be due to the better growth promotion of this microorganisms by low molecular weight peptides.

m-Azide is a medium for the enumeration of enterococci in water and other liquids by the membrane filtration technique. On one hand, there was obtained a total inhibition of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (negative control test strains), and, on the other, there was observed a little (no significant) superiority of the m-Azide medium variant, prepared with 9P1 and 9P2 lots of the mixed nutrient base to recuperate *Enterococcus faecalis* test strains. This medium contain a high level of the selective inhibitor, sodium azide, so the large polypeptides of the mixed nutrient base may have protective antitoxic effects on the microorganisms.

Tryptose phosphate broth is used for the cultivation of nutritionally fastidious microorganisms including pneumococci, streptococci and meningococci. The final dilution in where was observed the growth recuperation of *S. pneumoniae* was the 10^{-4}, for *S. pyogenes* – 10^{-5}, for *E. faecalis* – 10^{-7} and for *S. epidermidis* – 10^{-7} for 9P2 and 9P3 variants and 10^{-6} for 9P1 and Tryptose variants. It may be explained by the better assimilation of small peptides by *Staphylococcus* strains.

Influence of the new mixed nutrient base in haemolytic characteristics of different microorganisms was studied in Tryptose blood agar. There were no differences in α- haemolysis characteristics of *S. pneumoniae* URSS 4/56 type B, while there was more clear β- haemolysis manifestation of *S. pyogenes* ATCC 19615.

Taking in account the results of the three lots of the mixed nutrient base evaluation, it may be concluded that the 9P2 variant is the best for the great majority of microorganisms studied in the several culture media for different purposes.

The physicochemical and microbiological characterisation of the new mixed nutrient base demonstrated that it can be employed in quality of fundamental ingredient of several culture media instead of Tryptose.

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