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PRODUCERS, BIOLOGY, SELECTION, AND GENE ENGINEERING

Biosynthesis of Amylase Inhibitor by Streptomycete Cultures

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Abstract—We studied the effects of different sources of carbon, nitrogen, and dietary elements on the biosynthesis of amylase inhibitor produced by two strains from the Russian National Collection of Industrial Microorganisms: *Streptomyces violaceus* (code: Ac-1734) and *Streptomyces lucensis* (Ac-1743). We found that the biosynthesis of amylase inhibitor can be regulated by limiting carbon and oxygen concentrations, maintaining constant a C : N ratio and a stable proportion of carbohydrates (dextrins, maltose and glucose) in the medium, and by adding an extra organic source of nitrogen to the medium. The inhibitors produced by the cultures are pseudo-polysaccharides.

Keywords: biosynthesis, amylase inhibitor, *Streptomyces violaceus* Ac-1734, *Streptomyces lucensis* Ac-1743

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Polysaccharides consumed by humans are first exposed to salivary amylase; as a result, a small amount of them are transformed into glucose and oligosaccharides, which are then hydrolyzed by glycosidases in the small intestine. Amylase inhibitors are potentially useful biologically active substances that decelerate the digestion of carbohydrates and the formation of glucose and its intake in the blood, thus decreasing the risk of diabetes mellitus and comorbid carbohydrate metabolism disorders. Several studies have proved that amylase inhibitors are a promising material for the development of antidiabetic drugs [1–3].

The majority of known amylase inhibitors are the products of microbial synthesis; they are thermolabile polypeptides that are poorly dialyzable or nondialyzable and are deactivated by protease treatment, which makes them difficult to isolate and store. A group of amylase inhibitors called "pseudo-polysaccharides" have been used as the active ingredients in drugs. Apart from glucose residues, pseudo-polysaccharides contain other subunits, such as aminosaccharides and cyclitol [4]. These amylase inhibitors are stable in a wide range of pH and temperature values and are partially dialyzable. Examples of pseudo-polysaccharides are acarbose (the active ingredient of Glucobay) and nojirimycin/1-desoxynojirimycin (the active ingredients of Miglitol and Emiglitat, Bayer AG, Germany). A number of other drugs are based on the derivatives of listed inhibitors: Voglibose, Glyset, Xenical, Glucophage XR, and thiazolidinediones. Amylase inhibitors have been used in combination with the addition of high-calorie products to the diet (sweetened beverages with nojirimycin, wheat bread and confectionery with statins). All of the listed drugs mainly inhibit the enzymes of the second stage of carbohydrate digestion, i.e. glycosidases in the small intestine. However, the use of inhibitors with high affinity not only to the glycosidases of the small intestine but also to the salivary and pancreatic amylases can provide a more significant decrease in glucose intake in blood. A research group from Russia has created a new drug, Hypoglukin, which inhibits α-amylase and glucoamylase in animals to a significant extent, the inhibitory activity of which is higher than that in acarbose; the drug has not yet been tested in trials [5]. Amylase inhibitors are produced by Streptomycete cultures, which are unstable and highly sensitive to external conditions [6]. The level of inhibitory activity can be increased by changing the cultivation conditions.

Streptomycetes have a complex enzyme system and metabolize organic matter easily. This is why they are wide spread in nature, and their cultivation in laboratory conditions does not take much time and effort. After screening and selection of the strains from the collection of the All-Russia Research Institute of Food Additives, the two most productive strains that synthesize pancreatic amylase inhibitor were chosen: *Streptomyces violaceus* Ac-1734 and *Streptomyces lucensis* Ac-1743.

The purpose of this study is to determine ways to regulate the biosynthesis of amylase inhibitor by these strains.

Abbreviations: AA—amylolytic activity, IU—inhibitory unit, CMC—carboxymethylcellulolytic activity, MW—molecular weight, DE—dextrose equivalent.

MATERIALS AND METHODS

We studied two strains from the Russian National Collection of Industrial Microorganisms: *Streptomyces violaceus* Ac-1734 [5] and *Streptomyces lucensis* Ac-1743 [6]. The mycelial inoculum was cultivated on a medium with the following composition (g/L): soy flour (low-fat deodorized soft flour, Tagris Moloko, Russia), 10.0; glucose (Dneprovsky Krakhmalopatochny kombinat, Ukraine), 10.0; sodium chloride (Khloren-Khima, Russia), 5.0; calcium carbonate (Khimfarminvest, Russia), 1.0; pH = 7.0. The mycelium was then inoculated on a fermentation medium of the following composition (g/L): starch or flour hydrolysate (see the next paragraph), 20.0; soy flour, 5.0; sodium chloride, 3.0; potassium monophosphate (Komponent-Reactiv, Russia), 1.0; magnesium sulfate heptahydrate (Komponent-Reactiv) 0.5; pH = 7.0. The cultures were placed in 750-mL flasks of a Multitron shaker incubator (INFORS, Switzerland) [5, 6] with 100 mL of the medium and kept at a speed of 200 rpm at a temperature of 28°C for 96 h. A Biostat CPlus bioreactor (Sartorius, Germany) with a volume of 30 L was used for fermentation.

A number of starch or flour hydrolysates were used as carbon sources (GOST R 52672-2006): corn starch (Yarsnab, Russia), native (Altika-Praim, Russia) and soluble (Avebe, Germany) potato starch, wheat starch (Roquette, France), rye starch (from the collection of the All-Russia Research Institute of Starch Products), wholemeal rye flour (Richlan, Russia), and rice flour (Baltiyskaya mel'nitsa, Russia). As alternative sources of carbon, we used glucose (Dneprovsky Krakhmalopatochny kombinat, Ukraine), maltose (Anhui Elite Industrial Co., Ltd., China), and sucrose (Advanced Technology & Industrial Co., Ltd., China), and dextrins (Dekstrinozavod, Russia).

Hydrolysates of starch and rice flour were obtained by enzymatic hydrolysis with the use of Amylosubtilin G3x (Vostok, Russia). Rye flour hydrolysate was obtained by consequent enzymatic hydrolysis with the use of Celloviridin G3x and Amylosubtilin G3x (Sibbiofarm, Russia) [7]. The weight of carbohydrates in the hydrolysates was calculated by the Zieherd-Bleyer method as modified by Smirnov; the value of the dextrose equivalent (DE) was determined by the total weight of glucose and maltose on a dry basis [8]. Apart from low-fat deodorized soft soy flour (Tagris), ammonium nitrate (Khloren-Khima, Russia), dry enzymatic peptone (State Research Center of Applied Microbiology) and yeast extract (Khelikon, Russia) were used as the nitrogen sources.

The activity of the obtained inhibitors against pancreatic α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase, Sigma, United States) was measured by the spectrophotometric method described in [9]; the dehydrogenase activity was determined by the method described in [10]. The activity against glucoamylase of *Aspergillus awamori* (EC 3.2.1.3; 1,4-α-D-glucan glucohydrolase, Enzim, Belarus) was measured by glucose oxidase test [11].

The activity against a bacterial amylase (α-amylase of *Bacillus subtilis*, Sibbiofarm, Russia) was measured by the method described in [9].

The inhibitor preparation was obtained by the method described in patents [5, 6].

The amount of inhibitor that decreases the activity of pancreatic (α-amylase at a temperature of 37°C and a pH of 7.0 by 50% was taken as the unit of inhibitory activity (IU).

The molecular weight of the inhibitor was determined by gel-filtration chromatography with the use of sephadex G-25 (Pharmacia, Sweden) in a 2.2 × 65 cm column. The markers used for gel-filtration were N-α-benzoyl-arginine (MW = 439.95 kDa; Sigma-Aldrich GmbH, Germany), NADF-Na (MW = 765.4 kDa; Sigma-Aldrich GmbH), vitamin B12 (MW = 1579.6; Verofarm, Russia), and polyethylene glycol (MW = 2000 kDa; RusKhimTrade, Russia). Distilled water was used as the eluent; the rate of elution was 6 mL/h/cm^2.

In order to determine the pH-stability, 0.1% inhibitor solution was thermostated in 0.1 M universal buffer solution (pH = 1.68–10.01; HANNA Instruments, Germany) at a temperature of 25 ± 1°C for 3 h; the inhibitory activity was then measured.

In order to determine the temperature stability, 0.1% inhibitor solution was kept in distilled water at a temperature of 25–200°C for at least 3 h; the inhibitory activity was then measured again.

The IR spectra of the inhibitors were recorded by a Specord 75 R spectrometer (Germany) in transmission mode (resolution: 4.000; amplification: 8.0; mirror speed: 0.6329; diaphragm: 100.00; detector: deuterated triglycine sulfate/KBr; beamsplitter: KBr).

RESULTS AND DISCUSSION

Effect of Carbon Sources on the Biosynthesis of Amylase Inhibitors

Actinomycetales use amylase inhibitors (α-amylases and glucoamylases) for the bioconversion of polysaccharides. In this study, we used porcine pancreatic α-amylase, which catalyzes the hydrolysis of polysaccharides, including starch, and has a specificity similar to that of human pancreatic amylase. Starch and the products of its hydrolysis are carbon sources that are widely used in the biosynthesis of amylase inhibitors [12]. We used soluble starch, enzymatic starch hydrolysates of various origins, hydrolysates of rice and rye flours, and low-molecular-weight carbohydrates, including glucose, maltose and dextrins. The proportion of mono-, di- and polysaccharides in the hydrolysates varied depending on the dose of enzyme preparation (Table 1). The composition of soluble starch is similar to that of the hydrolysates of native

Table 1. Starch-containing hydrolysates used in the media

The dose of enzyme preparation, AA units/g*; (AA+CMC units)/g**	The dose of carbohydrates in hydrolysates, %			DE, %
	Glucose	Maltose	Dextrins	
Hydrolysates of native potato starch, corm starch, wheat starch, rye starch and rice flour*/rye flour**				
0.5*;	1.8 ± 0.1	10.3 ± 0.1	87.9 ± 0.1	12.5 ± 0.1
(0.5+0.5)**	17.1 ± 0.1	18.1 ± 0.1	80.2 ± 0.1	21.1 ± 0.1
1.0*	3.1 ± 0.1	19.8 ± 0.1	77.1 ± 0.1	20.9 ± 0.5
1.5*	3.3 ± 0.1	24.2 ± 0.1	72.5 ± 0.1	25.1 ± 0.1
(1.5 – 1.5)**	3.7 ± 0.1	20.5 ± 0.1	75.8 ± 0.1	27.5 ± 0.1
2.0*	4.4 ± 0.1	27.4 ± 0.1	68.2 ± 0.1	31.8 ± 0.1
(2.0 + 2.0)**	5.6 ± 0.1	21.8 ± 0.1	72.8 ± 0.1	32.2 ± 0.1
2.5*	5.5 ± 0.1	31.9 ± 0.1	62.6 ± 0.1	35.5 ± 0.1
3.0*	6.3 ± 0.2	39.5 ± 0.3	54.2 ± 0.3	42.1 ± 0.1
(3.0 + 3.0)	7.7 ± 0.1	42.1 ± 0.1	50.3 ± 0.1	44.5 ± 0.1
Soluble potato starch				
–	2.7 ± 0.1	23.5 ± 0.1	73.8 ± 0.1	24.3 ± 0.1

* The dose of enzyme preparations is calculated for 1 g of dry starch basing on the amylolytic activity (AA) against the hydrolysates of starch and rice flour.

** The dose of enzyme preparations is calculated for 1 g of dry starch basing on the amylolytic and carboxymethylcellulolytic (CMC) activity against rye flour hydrolysate.

Table 2. Production of α-amylase inhibitors by the cultures of Streptomycetes based on starch-containing substrates

Strain	DE, %	Inhibitory activity in different substrates, IU/mL							
		Corn starch hydrolysate		Potato starch hydrolysate, soluble potato starch		Wheat starch hydrolysate		Rice flour hydrolysate	
		Pancreatic amylase	Bacterial amylase	Pancreatic amylase	Bacterial amylase	Pancreatic amylase	Bacterial amylase	Pancreatic amylase	Bacterial amylase
S. lucensis	14.5 ± 1.5	1620 ± 140	184 ± 10	825 ± 70	125 ± 6	210 ± 4	192 ± 2	280 ± 10	165 ± 5
Ac-1743	22.5 ± 2.5	3564 ± 50	356 ± 25	1200 ± 100	185 ± 10	310 ± 8	205 ± 7	650 ± 10	245 ± 7
	33.5 ± 1.5	2336 ± 120	402 ± 12	1700 ± 50	240 ± 12	490 ± 10	258 ± 9	780 ± 10	350 ± 10
	38.5 ± 1.5	1960 ± 80	257 ± 13	1536 ± 70	185 ± 8	510 ± 14	200 ± 8	320 ± 10	254 ± 5
S. violaceus	14.5 ± 0.5	820 ± 50	120 ± 5	860 ± 40	85 ± 5	180 ± 5	92 ± 5	410 ± 10	100 ± 5
Ac-1734	22.5 ± 2.5	2020 ± 110	250 ± 7	1750 ± 120	110 ± 8	310 ± 5	128 ± 6	820 ± 10	145 ± 8
	33.5 ± 1.5	3000 ± 20	354 ± 15	2250 ± 120	130 ± 6	510 ± 5	142 ± 5	1000 ± 10	162 ± 8
	38.5 ± 1.5	2310 ± 56	250 ± 12	2500 ± 50	110 ± 5	560 ± 12	135 ± 5	500 ± 10	145 ± 5

potato starch obtained with the use of Amylosubtilin G3x at a dose of 1–1.5 units/g.

Flour has a more complex composition than starch: it contains monosaccharides (glucose, fructose, arabinose, and galactose), disaccharides (sucrose, maltose and raffinose), and polysaccharides, including starch, cellulose and hemicellulose. Hemicelluloses are hexosans consisting of mannose, galactose, and fructose or pentosans consisting of xylose and arabinose; in water, they swell up and form a viscous mucous mass. All of these saccharides interact with starch, thus preventing its interaction with the amylases contained in the flour or added to the media. Hydrolysis by cellulases and amylases causes the destruction of flour polysaccharides (including those that form mucous masses); thus, hydrolysates similar in their viscosity to starch hydrolysates can be obtained (0.88 ± 0.01—1.69 ± 0.03 mPa s).

Our experiments showed that the productivity of the biosynthesis of amylase inhibitor is highest in cultures based on corn starch hydrolysates; *S. lucensis* was most productive on hydrolysate with a DE of 22–33%, while *S. violaceus* was most productive at a DE of 32–35%. The productivity of strains cultivated on soluble potato starch and hydrolysates of native potato starch, wheat starch, and rice flour was inversely proportional to the degree of destruction (Table 2).

The inhibitor activity in the hydrolysate of native potato starch was lower than that in corn starch hydro-

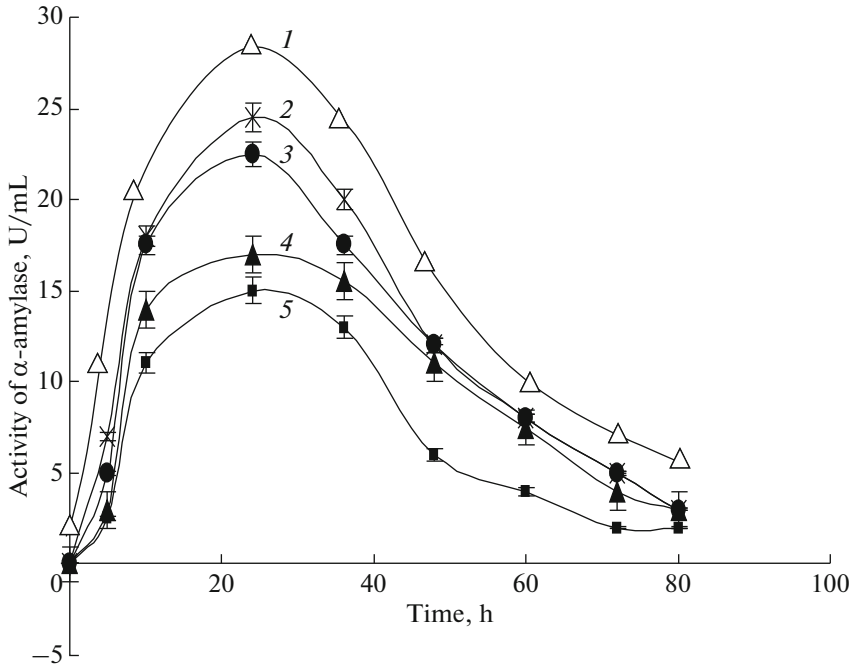

Fig. 1. Amylolytic activity of cultures after fermentation of *S. lucensis* Ac-1743 in starch-containing hydrolysates: (*1*) rice flour, (*2*) rye flour, (*3*) wheat starch, (*4*) potato starch, (*5*) corn starch.

lysates by 1.2–2 times (Table 2). This may result from differences in the structure of the polysaccharides. Potato starch contained 20% of amylose, while corn starch contained only 15%; thus, potato starch contains more branched dextrins, which are more suitable primary substrates for amylases (Fig. 1). The rate of pancreatic α-amylase inhibitor synthesis in cultures based on wheat flour and rice flour hydrolysates was low (3–7 times lower than on corn starch) (Table 2), which provided higher amylolytic activity (AA) in these cultures (Fig. 1). This may result from a higher concentration of amino acids, which promote the synthesis of enzymes, including amylases. Although the content of glucose, maltose, and dextrins in rye flour hydrolysates was similar to that in rice flour and starch hydrolysates (Table 2), no inhibitory activity against pancreatic α-amylase was detected in the cultures on rye flour hydrolysates (no data are included in Tables). One of the possible causes is the fact that rye flour contains hemicellulose, while starch does not. The products of hemicellulose hydrolysis induce the synthesis of cellulose and xylanase in Streptomycetes, thus decelerating the synthesis of amylase inhibitors. A high phosphorus concentration (0.49% in rye flour, 0.305% in rice flour, and 0.045–0.176% in starch) can also have some effect, since phosphorus stimulates the biosynthesis of amylases and proteases. The same tendencies have been observed in the activity of bacterial α-amylase (Table 2).

When starch hydrolysates were replaced by low-molecular-weight carbohydrates (glucose, maltose, sucrose or dextrins), Streptomycetes synthesized an inhibitor that was active against glucoamylase of *A. awamori* but not active against α-amylases (see data below). The productivity was highest in cultures with maltose as the carbon source, although the consumption rate of dextrins by producers was higher (Fig. 2). Dextrins may be required not only for biomass gain but also for the formation of the carbon skeleton of amylase inhibitors [13]. The activity of inhibitors synthesized by *S. lucensis* and *S. violaceus* cultivated on maltose was 2000 ± 100 IU/mL 1500 ± 800 IU/mL, respectively. The activity of inhibitors was lower in cultures based on dextrins (800–1000 IU/mL), glucose (500–600 IU/mL), and sucrose (250–300 IU/mL). Accordingly, the presence of oligo- and polysaccharides, in addition to monosaccharides, is preferable for the biosynthesis of pancreatic α-amylase inhibitors by Streptomycetes (Table 1). We suppose that the studied strains of Streptomycetes tend to produce amylase inhibitors in the presence of the products of hydrolysis by these amylases or specific substrates for them. For example, acarbose obtained in the culture of *Actinoplanes sp.* on sucrose-containing medium is an aminopseudo-tetrasaccharide, which inhibits the activity of invertase of *Saccharomyces cerevisiae* (EC 3.2.1.26; β-fructofuranosidase) and maltase of *A. awamori* (EC 3.2.1.20; α-glucosidase) but does not inhibit the activity of dextranase of *Aspergillus insuetus* (EC 2.4.1.19; 1,6-alpha-D-glucan 6-glucanohydrolase) [12].

According to our results, active biosynthesis of amylase inhibitors occurs in the stationary phase of culture growth, when the carbohydrate concentration

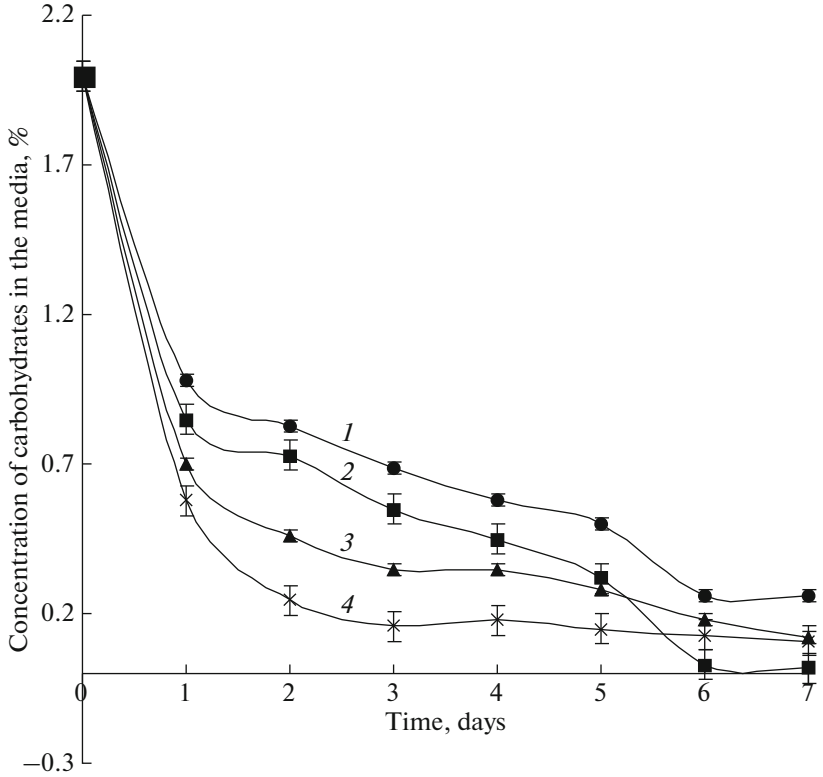

Fig. 2. Consumption of low-molecular-weight carbohydrates by *S. lucensis* Ac-1743 and *S. violaceus* Ac-1734 (the dynamics of consumption in these strains is similar): (*1*) glucose, (*2*) saccharose, (*3*) maltose, (*4*) dextrins.

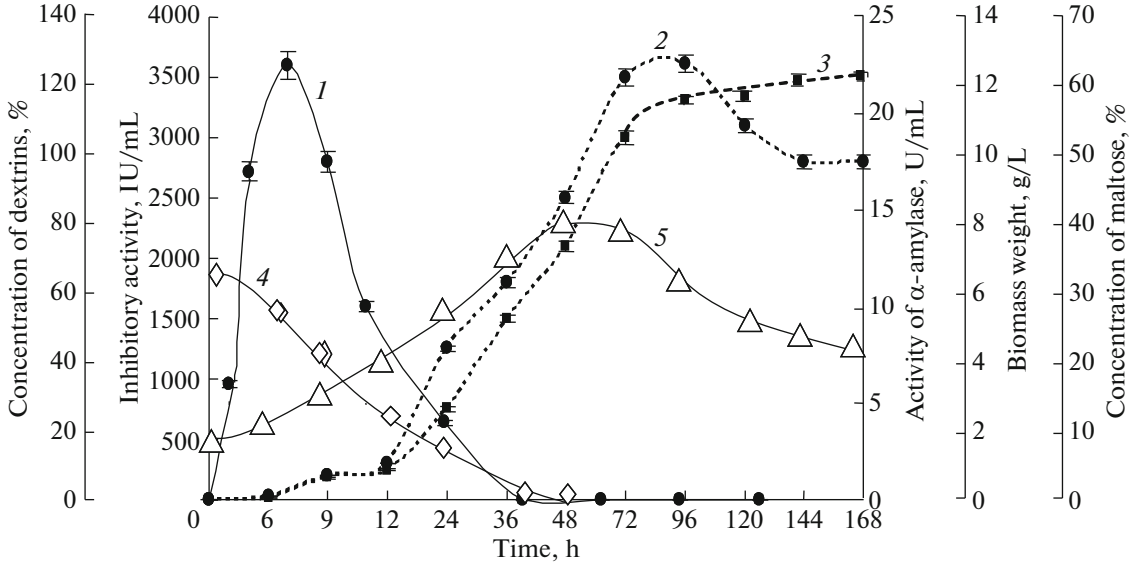

Fig. 3. Amylolytic (*1*) and inhibitory (*2*) activity, the rate of biomass accumulation (*3*) and the concentration of dextrins (*4*) and maltose (*5*) in the culture fluid and in the medium of fermentation of corn starch (20 g/L) by *S. lucensis* Ac-1743.

is decreased (Fig. 3). Obviously, the amount of carbohydrates is the limiting factor for the synthesis of amylase inhibitors. The synthesis productivity is highest when the concentration of carbohydrates is 20–40%, which is 5–10 times lower than the concentration needed for effective amylase synthesis. This means that the direction of synthetic processes depends on changes in the external conditions, which results in the adaptation of physiological parameters of producers. *S. violaceus* Ac-1734 needs more carbohydrates for the

Table 3. Effect of organic sources of nitrogen on the activity of amylase inhibitors

Source of nitrogen	Concentration of the source in the medium, g/L	Inhibitory activity, IU/mL	
		S. lucensis Ac-1743	*S. violaceus* Ac-1734
Soy flour (control)	1.0	1500 ± 30	1100 ± 50
	2.5	3200 ± 40	3100 ± 50
	5.0	3700 ± 50	3500 ± 20
	10.0	3700 ± 50	3500 ± 20
Yeast extract	1.0	300 ± 10	220 ± 40
	2.5	540 ± 20	600 ± 20
	5.0	740 ± 10	700 ± 30
	10.0	616 ± 20	580 ± 30
Dry enzymatic peptone	1.0	480 ± 20	170 ± 20
	2.5	500 ± 10	525 ± 10
	5.0	680 ± 30	710 ± 30
	10.0	530 ± 10	590 ± 30

accumulation of amylase inhibitors than *S. lucensis* Ac-1743 (Fig. 4).

Effect of the Sources of Nitrogen and Dietary Elements on the Biosynthesis of Amylase Inhibitors

Sources of nitrogen commonly used for the biosynthesis of amylase inhibitors are protein-containing products, such as corn flour, yeast extract, soy flour, fish meal, and skimmed milk [12]. Protein nitrogen, together with the carbohydrates in the medium, seems to induce the synthesis of amylase inhibitors. Our experiments proved this suggestion. We found that, when soy flour was replaced by ammonium nitrate with equal amount of nitrogen, both *S. lucensis* Ac-1743 and *S. violaceus* Ac-1734 produced amylases but not amylase inhibitors. The activity rate of amylases was 20-25 IU/mL; the value was constant during the entire period of fermentation (7 days). When soy flour was replaced by yeast extract or peptone, the rate of synthesis of amylase inhibitor was decreased by 5–6 times (Table 3).

The optimal ratio of carbon and nitrogen for the biosynthesis of amylase inhibitors by *S. lucensis* and *S. violaceus* was 20–22 and 42–44, respectively (Fig. 4).

The addition of sodium, potassium, and magnesium to the media increased the synthesis rate by 1.2–1.4 times as compared with the control cultures (without dietary elements). The addition of copper and zinc had no effect on the synthesis of pancreatic α-amylase inhibitor (no data are included in tables).

The productivity of the synthesis of amylase inhibitors was affected by the concentrations of sodium (or potassium) and calcium added to the culture in the form of chlorides and carbonates. The productivity of fermentation of starch hydrolysates was highest after the addition of sodium (or potassium) and magnesium ions. Apparently, these salts increase the permeability of cell walls of Streptomycetes, which is indicated by slightly higher concentrations of reducing compounds and proteins in the culture fluid (125 ± 3 and 0.42 ± 0.02 mg/mL, respectively) as compared with control cultures (120 ± 3 and 0.22 ± 0.01 mg/mL, respectively). Potassium and magnesium ions seem to act as stabilizers of the activity of enzyme system.

Based on these results, we propose the following composition for nutrient media for the biosynthesis of pancreatic α-amylase inhibitor:

(1) The medium for mycelial inoculum (g/L): soy flour, 10.0; glucose, 10.0; sodium chloride, 5.0; calcium carbonate, 1.0; water, to the volume of 1 L; pH 7.0.

(2) The medium for fermentation (g/L): corn starch hydrolysate, 20.0 (DE = 20–25% for *S. lucensis* Ac-1743) or 40 (DE = 30–35% for *S. violaceus* Ac-1734); soy flour, 5.0; sodium chloride, 3.0; potassium monophosphate, 1.0; magnesium sulfate heptahydrate, 0.5; water, to the volume of 1 L; pH 7.0.

The inhibitory activity of inhibitors obtained after the fermentation of the proposed media in a shaker incubator (at a speed of 160 ± 20 rpm) at a temperature of 30 ± 2°C for 5 days was 3700 ± 50 IU/mL (*S. lucensis*) and 3500 ± 20 IU/mL (*S. violaceus*).

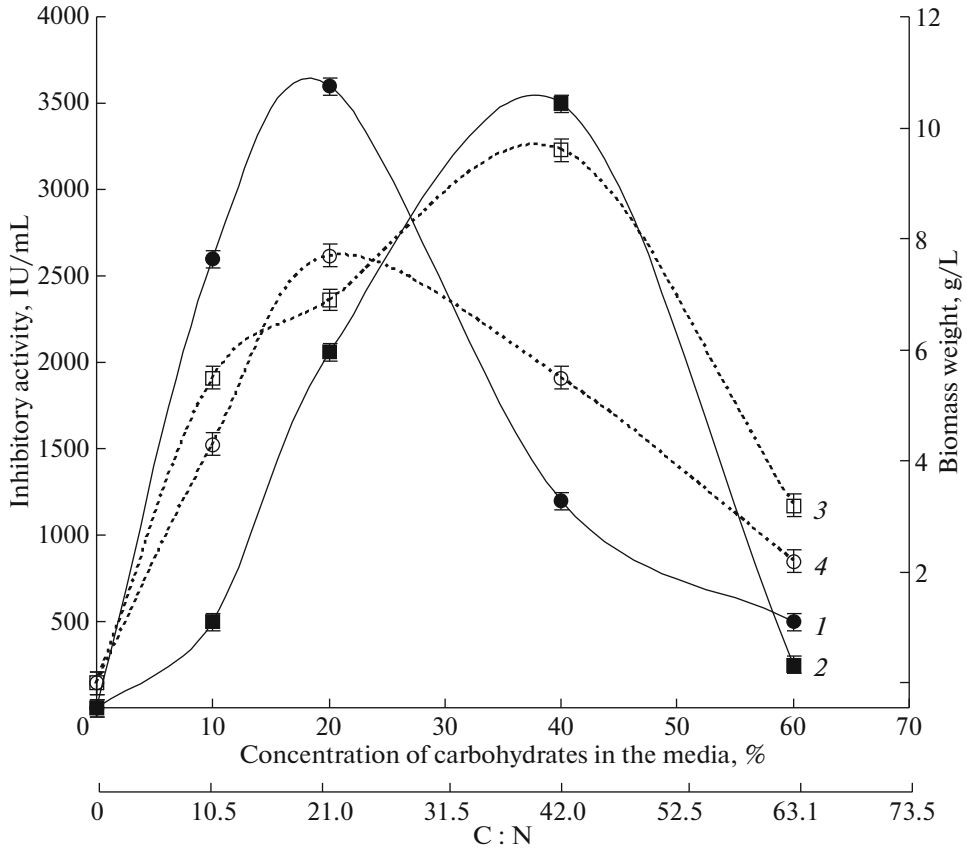

Fig. 4. Effects of the C : N ratio and the carbohydrate concentrations in Streptomycetes cultures on the inhibitory activity and biomass accumulation: (*1*) inhibitory activity of *S. lucensis* Ac-1743, (*2*) inhibitory activity of *S. violaceus* Ac-1734, (*3*) biomass accumulation of *S. violaceus* Ac-1734, (*4*) biomass accumulation of *S. lucensis* Ac-1743.

Effect of Technological Conditions on the Biosynthesis of Amylase Inhibitors

The optimal temperature for normal growth and development of Streptomycetes is 26–29°C. The producers of amylase inhibitors synthesize them in a wide range of temperature (24–40°C) [14, 15]. We studied the effect of the temperature on the productivity of biosynthesis in chosen strains and found that the most intense production of amylase inhibitors occurs at a temperature of 28 ± 1 to 32 ± 1°C. The productivity decreased by 35 ± 5% in the temperature range from 35 ± 1 to 38 ± 1°C; no culture growth was observed at a temperature of 24 ± 1°C.

Aerobes are producers of amylase inhibitors. We measured the productivity of biosynthesis at different levels of aeration and found that the speed range from 160 ± 5 to 195 ± 5 rpm provided highest inhibitory activity, while the speed of 120 rpm decreased this value by two times.

The optimal volume of medium for efficient biosynthesis of amylase inhibitors is 200 mL or less (in a 750 mL flask). The inhibitory activity in the volumes of 300 mL and 50 mL was lower by 50–80%.

Another important parameter of the inoculum is its physiological activity. For example, the dehydrogenase activity of cultures indicates the intensity of respiration and can be measured by the time that vegetative mycelium needs for destaining methylene blue. V.A. Kolyadina [13] found that the the mycelium activity of *Streptomyces sp*. K-20, which is a producer of pancreatic α-amylase inhibitor, is maximal after 48 h of cultivation: the time of methylene blue destaining was 3 minutes, while this value was 23 and 17 minutes, respectively, after 24 and 72 h of cultivation. In this study, we found that the activity of both *S. lucensis* Ac-1743 and *S. violaceus* Ac-1734 was maximal after 43–48 h of cultivation (the time of methylene blue destaining was 2–3 minutes). In the *S. violaceus* cultures, no destaining was observed after 24 and 72 h; the time of destaining in the cultures of *S. lucensis* after 24 and 72 h of cultivation was 12 and 4 minutes, respectively.

The inoculum mass determines the direction and productivity of biosynthesis. For example, the volume of inoculum needed for the synthesis of amylase inhibitor by *Actinoplanaceen S/E* 50/13 is only 1.2% of the medium volume [4]. According to our data, the opti-

Table 4. Parameters of fermentation of the cultures

Parameter	Mode of fermentation	Strain	
		S. violaceus Ac-1734	*S. lucensis* Ac-1743
Inhibitory activity, IU/mL	B	2500 ± 300	2800 ± 200
	S	3375 ± 500	3600 ± 500
	SB	2000 ± 100	2200 ± 150
Inhibitory activity per cycle, IU/mL	B	$(40.0 \pm 4.8) \times 10^6$	$(48.0 \pm 3.2) \times 10^6$
	S	$(54.0 \pm 8.0) \times 10^6$	$(56.0 \pm 4.8) \times 10^6$
	SB	$(32.0 \pm 1.6) \times 10^6$	$(35.2 \pm 2.4) \times 10^6$
Biosynthesis rate, IU/L/day	B	$(4.4–5.6) \times 10^5$	$(5.2–6.0) \times 10^5$
	S	$(4.8–5.5) \times 10^5$	$(4.4–5.9) \times 10^5$
	SB	$(2.7–3.0) \times 10^5$	$(2.9–3.4) \times 10^5$
Biomass weight, g/L	B	10–11.7	8.2–9.3
	S	6.0–6.9	5.8–6.2
	SB	6.0–6.8	5.7–6.1
Productivity rate, IU/g	B	191.7–225.0	320.0–365.7
	S	372.1–496.1	532.3–634.4
	SB	262.5–316.7	347.5–398.3
Duration, days	B	5	5
	S	7	7
	SB	7	7

* B—batch mode; S—sequencing-batch mode; SB—semibatch mode.

mal amount of mycelium for the active synthesis of amylase inhibitor is 10% of the volume of fermentation medium.

The results of cultivation with the use of Biostat CPlus bioreactor with a volume of 30 L proved that the activity rate of obtained inhibitors depends on the mode of fermentation. The use of sequencing-batch mode provides the highest productivity: the biosynthesis rate of pancreatic α-amylase inhibitor is almost the same as that in batch mode, but the productivity of 1 g of biomass and the total value of inhibitory activity are higher by 1.5 and 1.3 times, respectively (Table 4). The biosynthesis rate in semibatch mode was relatively low. The sequencing-batch mode was started on the second and third day of fermentation, when an intense increase in inhibitory activity was observed in the culture fluid. The samples containing 10% of the total volume of culture fluid were collected every day; the removed volume was replaced by the same volume of corn starch hydrolysate, such that the mass concentration of saccharides in the medium was 9 ± 1 g/L (*S. lucensis*) or 18-20 g/L (*S. violaceus*). According to the literature data, the production of secondary metabolites in Actinomycetales starts when one of the medium components is completely consumed or when the level of aeration is changed [16]. The majority of Actinomycetales have a relatively low growth rate and high endogenous respiration rate. However, our data showed that the biosynthesis rate in the cultures of studied strains is highest when the concentration of oxygen is limited. When the culture fluid was stirred intensely and saturated with oxygen, the inhibitory activity decreased by 1.5–10 times.

Thus, the optimal technological conditions for fermentation are the following:

(1) The volume of mycelial inoculum is 10% of the total volume of the medium.

(2) The inoculum is cultivated for 43–48 h before inoculation.

(3) The temperature is 28–30°C.

(4) The concentration of carbohydrates is 20–40 g/L.

(5) The sequencing-batch mode is used.

(6) The culture is stirred at 80–150 rpm with air inflow of 290–580 L/h.

The advantage of new strains is that the activity of biosynthesis of amylase inhibitors is equal to or even higher than that of other known producers (Table 5).

Table 5. Activity of amylase inhibitors during cultivation of different actinomycetes

Strain	Inhibitory activity in culture fluid, IU/mL	Information source
S. lucensis Ac-1743	3600 ± 140	Data of this study
S. violaceus Ac-1734	3500 ± 50	
Streptomyces species 1328-D	2300 ± 120	[12]
Actinoplanaceen S/E 50/13	2800 ± 280*	[17]
Streptomyces dimorphogenes nov. sp. NR-320-OM7HB	1240 ± 120*	[18]

* Inhibitory activity is represented in IU as a result of recalculation from AIU [9, 19].

Chemical Structure of Produced Amylase Inhibitors

According to the literature data, the amylase inhibitors produced by the cultures of Actinomycetales can be proteins, saccharides, or glycopeptides, depending on the medium composition. Streptomycetes are the most widely used producers of pseudo-saccharides, which contain sugar derivatives in addition to glucose residues [13–15]. The amylase inhibitors produced by *S. lucensis* Ac-1743 and *S. violaceus* Ac-1734 belong to the group of pseudo-saccharides or glycopeptides. They consist of saccharide residues (85–88% of total mass); 7–8% of these residues contain amino groups. Two active fractions of inhibitors were obtained by gel filtration: the first had a MW from 900 to 1200 kDa; the second had a MW from 1800 to 2300 kDa. According to literature data, Actinomycetales cultivated on media containing poly- and oligosaccharides produce glycosidase inhibitors with small MWs (1000–2000 kDa) [12].

Analysis of the inhibitors obtained by IR spectroscopy showed that they contain α-1,2- and α-1,4-glycoside bonds, double bonds and aldehyde, hydroxyl, and amino and imino groups.

The inhibitory activity of cultures is equal to or even higher than that of other known producers of amylase and sucrose inhibitors (500–700 IU/mg); the activity remains constant after long-term storage (12 months) and with changes in the pH (2–8) and temperature (20–200°C). These findings are generally consistent with the data on microbial pseudo-saccharides active against glucosidases.

Thus, we conclude that the biosynthesis of amylase inhibitors by the studied strains can be regulated in the following ways: (1) limitation of carbon and oxygen concentrations; (2) maintenance of the constant proportion of carbohydrates, including dextrins, glucose, and maltose (which results in maintenance of the maximal rate of saccharide bioconversion); (3) optimal C : N ratio (which promotes the biosynthesis of target products); (4) additional organic sources of nitrogen (which results in the balance of carbon and nitrogen consumption and increases the activity of obtained inhibitors).

These conclusions suggest that Actinomycetales cultures can be successfully used for the biosynthesis of amylase inhibitors, which can then be used for the production of biologically active dietary supplements and active ingredients for the prevention of diabetes mellitus and comorbid carbohydrate metabolism disorders.

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