

Characterization of Novel Alkaliphilic Isolate of *Bacillus mannanilyticus*, Strain IB-OR17, Displaying Chitinolytic and Antifungal Activities

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Abstract

The novel alkaliphilic strain, designated as *Bacillus* sp. IB-OR17 and isolated from soda lake sediments, was identified and characterized. Isolated strain demonstrated slight antifungal activity against some plant pathogen fungi that are capable to grow under alkaline conditions. Based on its morphological, physiological and biochemical characteristics as well as on 16S rRNA gene analysis data, *Bacillus* sp. IB-OR17 were related to alkaliphilic species *B. mannanilyticus*. Such as type species, *Bacillus* sp. IB-OR17 produced extracellular β -mannanase but additionally it displayed also chitinolytic activity which is a new property reported for this species. *Bacillus* sp. IB-OR17 grew in pH range 8.0 - 11.0 with maximal intensity under 9.0 - 10.0 but not showed halophilic properties (growth limit under NaCl concentrations < 5%). Maximal production of chitinase is observed at the same pH interval after 96 h of submerged cultivation of the strain. *Bacillus* sp. IB-OR17 produced chitinase(s) in presence of colloidal chitin as main carbon source and sodium carbonate (0.25% - 1.0%) demonstrating high enzyme yield under enough low concentrations of the substrate (0.20%). Unlike chitinase, β -mannanase was constitutively produced by *Bacillus* sp. IB-OR17 in presence of various substrates including crab shell chitin. Probable involvement of the enzymes in antifungal activity of *Bacillus* sp. IB-OR17 is discussed shortly in terms of further researches and application of this strain.

Keywords

Bacillus mannanilyticus, Alkaliphilic Bacteria, Bacilli, 16S rRNA, Chitinase, β -Mannanase, Antifungal Activity, Microbial Antagonism

1. Introduction

Alkaliphilic microorganisms attract increased attention during last decades in the context of their great potential for biotechnological applications and research of ecological diversity, physiologic, biochemical and molecular specifics of extremophiles in general [1]. Species belonging to genus *Bacillus* are one of most studied and industrially significant groups among alkaliphilic microorganisms in terms of their ability to produce various valuable compounds, primarily, extracellular enzymes [2]. As against alkaline proteases, amylases, lipases and cellulases, production of chitin-degrading enzymes by alkaliphilic bacilli are reported more scarcely [3] [4], although this group together with other extremophiles can be considered as a promising source of chitinases and chitosanases characterized with new properties interesting for their biotechnological application. Chitinases being active and stable in alkaline interval of pH values are applicable in composting process of chitin-containing wastes in sea-food industry; especially, the enzymes combining alkaline pH-optima and high thermostability are most suitable for these purposes [5]-[7]. Some alkaliphilic species of bacilli are capable to produce also thermostable chitinases [8] [9]. Furthermore, chitinases effectively functioning under alkaline pH interval are very promising as biological control agents of agricultural pests [4] [6]. Chitin-degrading activity is also an important indicator in ecological researches of alkaliphilic bacilli isolated from various habitats. The strain *Bacillus* sp., IB-OR17, was isolated originally as antagonistic alkaliphile from sediments of Orongoyskoe (White) alkaline lake (Buryatia, Russia). The isolate displayed slight growth-inhibiting activity against some soil-borne fungi under alkaline conditions. Later, it demonstrated capability of visible hydrolysis of colloidal chitin when it cultivated on agar alkaline media containing this substrate.

Present research was aimed to characterize and identify alkaliphilic bacterium of *Bacillus* sp. IB-OR17 and evaluate its potential in the production of chitinases.

2. Materials and Methods

2.1. Isolation and Identification of Alkaliphilic Bacterium

Bacterial culture was isolated as a result of screening of alkaliphilic/alkalitolerant bacteria antagonistic to soil-borne plant pathogenic fungi. The small samples of various soil grounds including alkaline environments were applied on surface of alkaline agar medium I [10] plates that were inoculated previously with spore suspension of plant pathogens *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur) and *Fusarium oxysporum* (Schlecht.) Snyder & Hansen No. F-137 from own microbial collection and from All-Russian Collection of Microorganisms (VKM, Pushchino-Moscow, Russia), respectively. The plates were incubated for 4 days at 28°C after that the bacterial consortium growing around applied soil samples and forming sterile zones in fungal growth lawn was collected, suspended in sterile physiological solution and seeded on the same medium by serial dilution method. After 48 - 72 h various bacterial colonies grown discretely were individually tested against fungi at the same conditions. Fungal spore suspensions were inoculated on agar plates of Horikoshi medium II containing 1% (w/v) D-glucose and 1% sodium carbonate (w/v). After 1 h of pre-incubation bacteria were seeded on the plate by stab-inoculation. Active isolates, including strain IB-OR17, forming sterile zones around colonies were selected for further study (Figure 1).

Morphological and physiological-biochemical properties of the isolate IB-OR17 were studied according to Bergey's Manual of Systematic Bacteriology [11]. Amplification and sequencing of 16S rDNA gene from *Bacillus* sp. IB-OR17 was carried out by using of universal bacterial primers 16SF27 (5'-AGAGTTTG-ATCMTG GCTCAG-3'), 16SF515 (5'-GTGCCAGCAGCCGCGGTAA-3'), 16SF930 (5'-CCCGCACAAGCGGTGGA-3') and 16SR1512 (5'-ACGGCTACCTTGTTACGACTT-3'). The gene sequence was deposited in GenBank database (Accession No. HE663240.1) and analyzed using BLAST tool and RDP Sequence Match program (RDP-II database); multiple alignment of related reference sequences was performed using ClustalW software [12] [13] succeeded by manual update. Phylogenetic tree was constructed using TREECON program editor [14].

2.2. Culture Growth Conditions and Maintenance

Bacillus sp. IB-OR17 was maintained by quarterly passages on starch-containing alkaline agar medium II [10] either on its modified variant (g/l): K₂HPO₄·3H₂O, 1.0, KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.2, CaCl₂, 0.1, peptone, 4.0; yeast extract, 1.0, colloidal chitin, 5.0, agar, 16.0, Na₂CO₃ (sterilized separately), 5.0. The culture was grown at 37°C during 2 - 3 days and at room temperature during 3 - 4 days after that it was kept at 4°C. Influences of

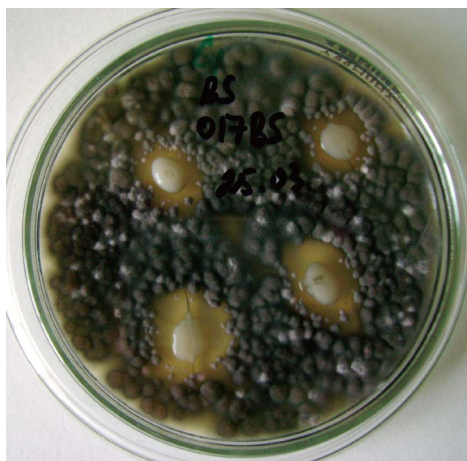


Figure 1. Growth-inhibiting effect of *Bacillus* sp. IB-OR17 against *Bipolaris sorokiniana* after 96 h of simultaneous incubation under 28°C.

initial medium pH and sodium chloride concentration on culture growth were studied in liquid medium analogous in main composition to medium II described by Horikoshi and Akiba (1983) [10] with small modifications. Sodium bicarbonate was not added into the medium with different initial pH values; in this case, pHs (7.50 - 11.0) were adjusted by sterile 2 M NaOH solution after autoclaving of the medium. In all other cases nutritional media included 0.5% (w/v) of Na₂CO₃. The bacteria were cultivated on orbital shaker-incubator at 250 rpm and 36°C during 72 h. Bacterial biomass growth intensity in liquid culture of *Bacillus* sp. IB-OR17 was evaluated on optical density at 630 nm.

2.3. Preparation of Colloidal Chitin

Colloidal chitin was prepared according to the modified method of Rodriguez-Kabana *et al.* (1983) [15]. Commercial purified flaked chitin from crab shells (ZAO “BioProgress”, Russia) was preliminary ground in electric mill and sieved through screens to particle size 0.25 - 0.50 mm. Grounded chitin (10 g) was soaked by acetone (high grade) to dense paste and dissolved in 200 ml of concentrated hydrochloric acid (high grade) under continuous stirring and 55°C - 60°C during 15 - 20 min. Dissolved chitin was precipitated by pouring of the solution in cold distilled water (4.8 l). In further colloidal chitin was successively washed several times with tap and distilled water until pH 6.0 - 6.5. After decantation of scouring water chitin was finally washed and collected by filtration on support in Buchner funnel *in vacuo*. Washed solid colloidal chitin was suspended in 250 - 300 ml of distilled water and stored at 5°C until immediate use. The dry weight chitin content in 1 ml of obtained suspension was determined after its drying at 50°C to constant weight.

2.4. Enzyme Assay

The chitinase activity was measured using colloidal chitin as substrate. Reaction mixture including 1 ml of culture supernatant appropriately diluted with phosphate citrate buffer (50 mM, pH 6.0) and 0.5% (w/v) suspension of colloidal chitin in the same buffer was incubated for 60 min under 55°C. Reducing sugars liberated in reaction supernatant were quantified by Schale’s method [16] on calibrate curve with N-acetyl-D-glucosamine (GlcNAc) as standard. One unit of chitinase was defined as 1 μM of GlcNAc released for 1 min in 1 ml of reaction mixture under described conditions.

β-Mannanase activity was determined using D-galactomannan (“Sigma”) as substrate. Conditions of incubation and measurement protocol were same that described for chitinase except that time of incubation was 30 min and D-mannose was used as standard for calibrate curve building.

2.5. Chitinase and β-Mannanase Production

Production of these enzymes by *Bacillus* sp. IB-OR-17 was monitored during its 5-days old cultivation in mod-

ified liquid medium containing (g/l): KH_2PO_4 , 1.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2, bactopectone, 5.0; yeast extract, 1.0, colloidal chitin, 4.0, Na_2CO_3 , 2.5. Other conditions were same that mentioned above. The same medium base was used for study of the influence of different concentrations of colloidal chitin (0.20% - 1.60% $\text{dw} \cdot \text{v}^{-1}$) and sodium carbonate (0.10% - 1.00% $\text{dw} \cdot \text{v}^{-1}$) on the enzyme production. In both last cases cultivation time was 72 h, 1-ml culture aliquots were taken every 24 h, centrifuged and resulting supernatants were used for enzyme assay.

2.6. Statistical Analysis

The experiments were carried out in triplicates, and the main data are presented as the averages of obtained values including standard errors as confidential ranges (Origin 7.0 SR0 software, version 7.0220 (B220), OriginLab Corp., USA).

3. Results

3.1. Isolation and Identification of Alkaliphilic Bacterial Culture

Bacillus sp., strain IB-OR17, isolated from alkaline lake sediments displays a slight growth-inhibiting effect against some plant pathogenic fungi under testing on the nutritional agar medium containing 1% of D-glucose and 1% of sodium carbonate (Ph ~ 9.5 - 10.0). Although these conditions are not very favorable for typically neutrophilic soil fungi such as *Bipolaris sorokiniana* and *Fusarium oxysporum* growing here as separate small colonies rather than mycelial lawn, production of antifungal compounds by IB-OR17 was confirmed visibly (Figure 1).

Phylogenetic analysis of sequenced 16S rRNA gene (~1490 b.p.) from *Bacillus* sp. IB-OR17 demonstrated its highest similarity to *B. mannanilyticus* A-001 and *B. horti* A62 species among eighteen of reference type-strains (gene homology 99.9% and 95%, respectively). *Bacillus* sp. IB-OR17 was combined with type strain of *B. mannanilyticus* A-001 in single sub-cluster within cluster of *B. horti-mannanilyticus* with authentically high bootstrap value 100 (Figure 2). Authenticity of clustering and high homology of 16S rRNA genes (99.9%) were confirmed with similarity of some phenotypic features of *Bacillus* sp. IB-OR17 and *B. mannanilyticus* A-001 (Table 1). However, specific difference of IB-OR17 was manifested in its ability to partial nitrate reduction and

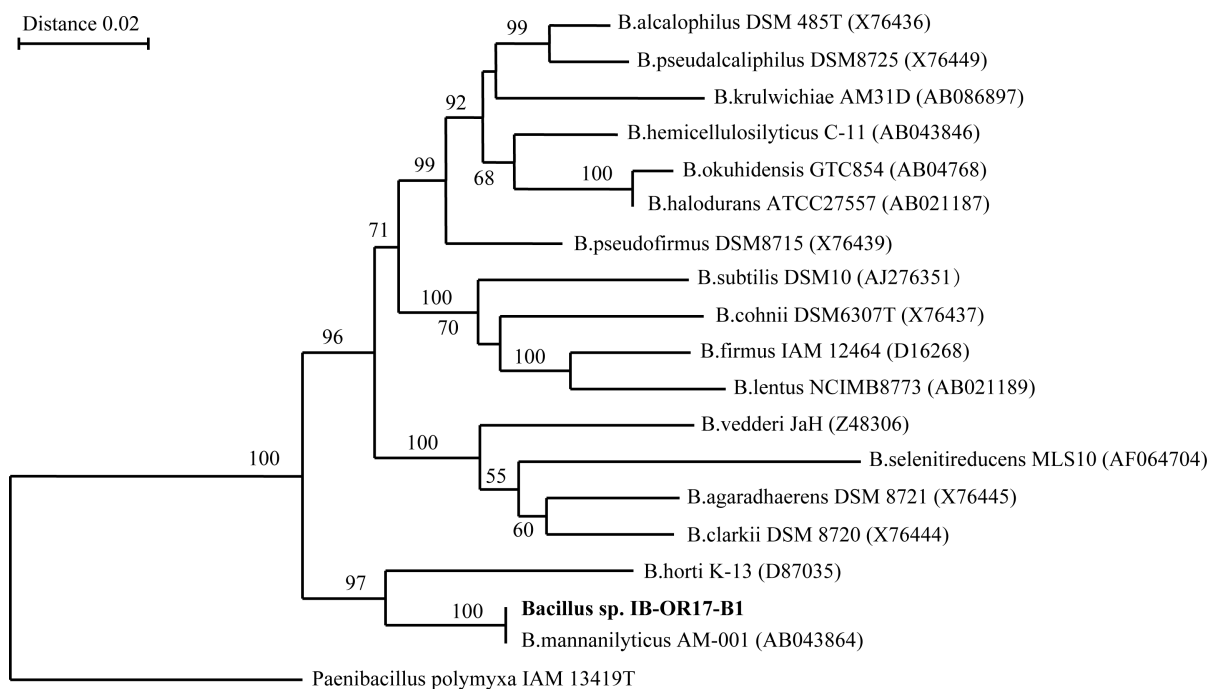


Figure 2. Phylogenetic tree showing the relationships of *Bacillus* sp. IB-OR17 and the type strains of closely related *Bacillus* spp., constructed using the neighbor-joining method and based on 16S rRNA gene sequences. GenBank accession numbers are shown in parentheses. *Paenibacillus polymyxa* IAM 13419T is taken as out group.

Table 1. Comparative physiological and biochemical characteristics of isolate *Bacillus* sp. IB-OR17 and type strain *Bacillus mannanilyticus* AM-001.

Property	Strain IB-OR17	<i>B. mannanilyticus</i> AM-001 (Nogi et al., 2005)
Cell size, µm (length × width)	2 - 3 × 0.4 - 0.6*	3 - 6 × 0.6 - 0.8
Gram reaction	+ (12 - 24 h culture)	Variable
Spore location	Terminal	Terminal
Swollen sporangium	+	+
Catalase	+	+
Oxidase	+	-
Anaerobic growth	+ facultative anaerobe	ND
Voges-Proskauer test	-	ND
O/F test	+/+	ND
Acid from		
D-glucose/gas	+/-	+/-
L-arabinose	+	ND
D-xylose	+	+
D-mannitole	+	+
Ammonia production from amino acids	+	ND
Indole production from tryptophan	-	-
H ₂ S production from sulfur-containing amino acids	-	-
Urease production	+	ND
Nitrate reduction	+	-
Nitrite reduction to N ₂	-	ND
Utilization of NH ₄ Cl as nitrogen source	-	ND
Utilization of KNO ₃ as nitrogen source	+	+
Starch hydrolysis	+	+
Gelatin hydrolysis	+	+
Casein hydrolysis	+	+
Lecitinase	+	ND
Hydrolysis of Tween-60	+	+
Hydrolysis of Tween-80	+	ND
Utilization of citrate	-	ND
Utilization of propionate	-	ND
Tyrosine degradation	-	ND
Phenylalanine deamination	-	ND
Growth under pH 6.8	-	ND
Growth under pH 9	+	+
Growth under 2% of NaCl	+	+
Growth under 5% of NaCl	-	-
Growth under 13°C	+	ND
Growth under 20°C - 45°C	+	+
Growth under 50°C	-	-

Note: (+)—positive reaction; (-)—negative reaction; ND—not of data.

oxidase positive reaction. The bacteria form circular opaque colonies with relatively constant characteristics under cultivation on various media, such as meat-peptone agar and potato-dextrose agar supplemented with sodium carbonate either modified medium of Horikoshi for alkaliphiles [10], containing glucose or colloidal chitin. Common cultural features of the strain's colonies include also cream-white to whitish color, smooth and glossy surface, straight edge, convex profile, homogenous structure and semisolid consistence (Figure 3). Growing culture consist single gram-positive rod-shaped cells and chains including two or more cells. Mature culture forms swelling ellipsoid endospores locating terminally in mother cells.

Physiologically the strain IB-OR17 is characterized as typical alkaliphile [2] growing only in range of pH 8.0 - 11.0 with optimum in pH ~ 9.5 - 10.0 (Figure 4(a)). At the same time, it not showed halophilic or halotolerant properties and grew only at concentrations of NaCl less than 5% (Figure 4(b)). Further cultivation of *Bacillus* sp. IB-OR17 resulted to increase of its growth under higher initial pH values (9.5 - 11.0), while under lower pHs (8.0 - 9.0) bacterial population density decreased in course of time (Figure 4(c)).

3.2. Production of Chitinase and β -Mannanase by *Bacillus* sp. IB-OR17 under Submerged Cultivation

Bacillus sp. IB-OR-17 produces large amounts of chitinase in chitin-amended media in pH range 8.0 - 11.0 with optimal values at pH 9.0 - 10.0 (Figure 5(a)). Although chitinase(s) of *Bacillus* sp. IB-OR-17 is inducible, its production slightly and nonlinearly changed in range of 0.2% - 1.6% (w/v) of substrate concentrations (Figure 5(b)). The substrate content influence on chitinase production exhibited more distinctly after 24 h of growth cycle than at the end of cultivation; maximal increase of the enzyme production reached about 100% in this step (Figure 5(b)). Maximal increase both of β -mannanase and chitinase production by *Bacillus* sp. IB-OR-17 was recorded after 96 h of submerged cultivation, however further incubation resulted to enough sharp decline in β -mannanase activity while chitinase remained at the same level (Figure 5(c)).

4. Discussion

Phylogenetic and partially phenotypic analyses confirm the closest match of *Bacillus* sp. IB-OR-17 to reclassified relatively recent alkaliphilic species *Bacillus mannanilyticus* sp. nov. Nogi, deposited in several international microbial collections under numbers AM-001, CIP 109019, DSM 16130 and JSM 10596 [17]. Before the strain, it was isolated and characterized as alkaliphilic producer of β -mannanase and β -mannosidase enzymes [18]. Our finding of constitutive production of β -mannanase (EC 3.2.1.78) by *Bacillus* sp. IB-OR-17 is additional characteristic closing it together with *B. mannanilyticus* A-001 but chitinolytic activity displayed by the strain in solid

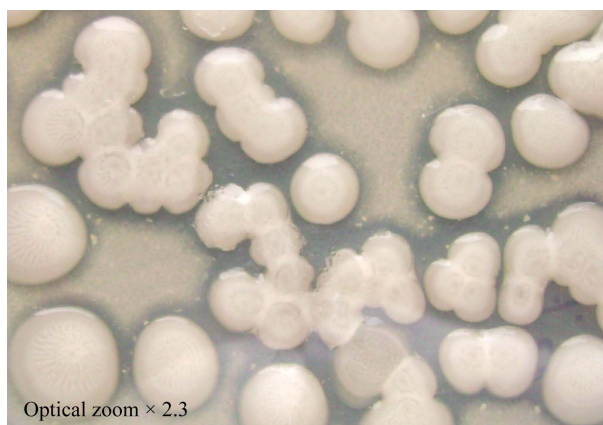


Figure 3. Chitinolytic activity of *Bacillus* sp. IB-OR17 visualized after 72 h of bacterial growth under 37°C. Bacteria were inoculated with serial dilution method on modified Horikoshi medium containing 0.5% (w/v) of colloidal chitin from crab shells and 0.5% (w/v) of sodium carbonate. Activity is detected as clearing zones around single colonies as result of hydrolysis of colloidal chitin.

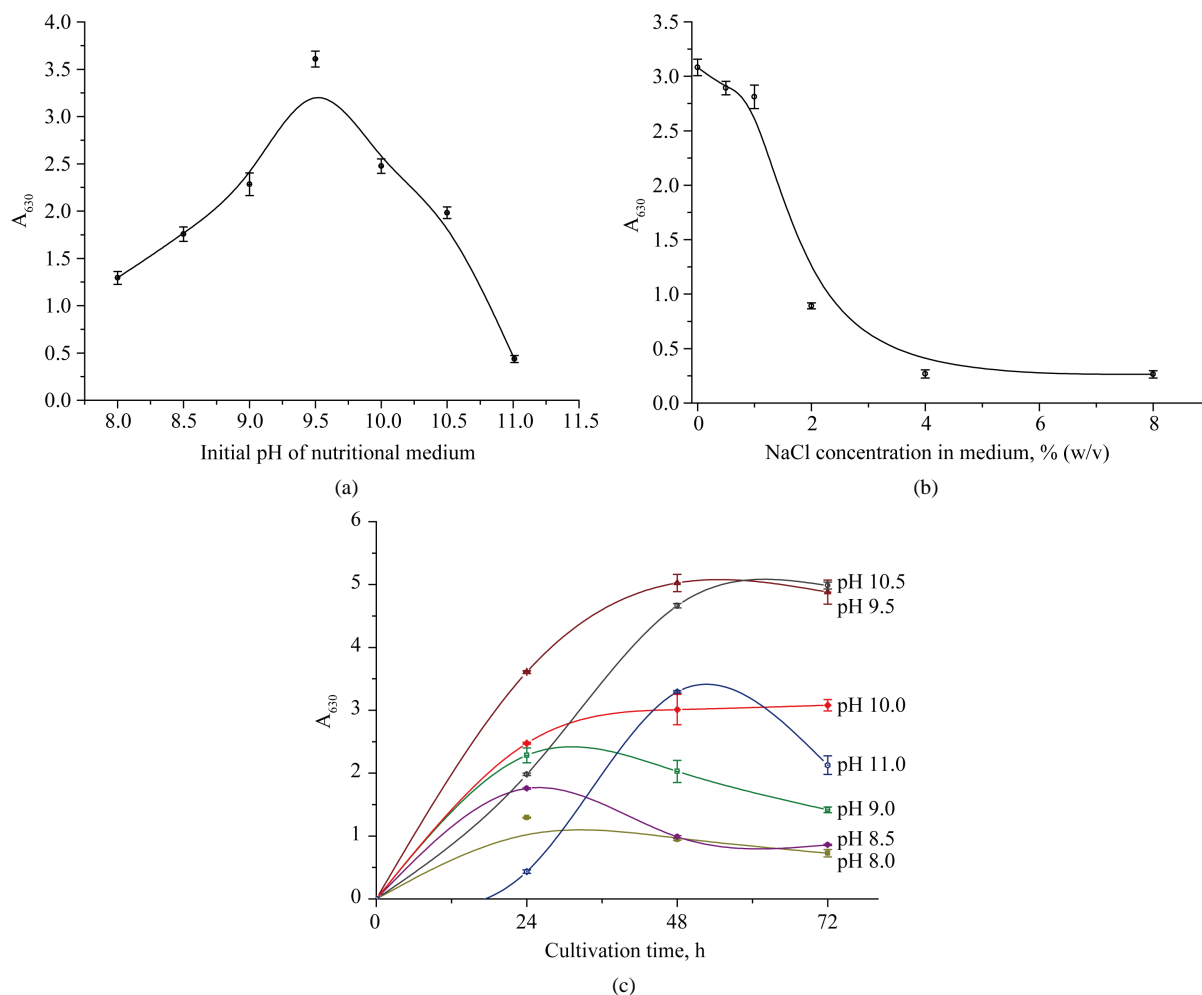


Figure 4. Influence of initial pH (a), (c) and salt concentration (b) in liquid medium on growth of *Bacillus* sp. IB-OR17. (c) growth kinetic curves of the bacterial culture under different initial pH values of nutritional medium (the conditions are same that for experiment in (a)).

medium (Figure 3) is firstly reported for this species in general terms. Our further research was focused mainly on some specifics and potential of chitinase production by *Bacillus* sp. IB-OR-17 at batch submerged cultivation.

Kinetic growth changes of *Bacillus* sp. IB-OR-17 under different initial pHs were coincidental with changes of pH values of culture media after 72 h of cultivation (Figure 4(c)). In case of media having initial pHs 9.5 - 11.0 final pH decreased differently but stabilized equally at pH 9.0 - 9.05, whereas it arrived about pH 7.50 for media with lower initial pH (8.0 - 9.0). Ability of alkaliphilic bacilli to decrease sharply pH values of culture media, mainly, due to production of organic acids in presence of carbohydrates as carbon source is well known [19]. However, it not clarifies completely different degrees of pH decrease (0.5 - 2 pH units) in media having different initial pHs but characterized with same composition and conditions of cultivation. Therefore, this fact may suppose existence of possible non-regulated external pH-lowering mechanism in *Bacillus* sp. IB-OR-17 within local alkaline environment. Nevertheless, so far it was not supported by verified data, and physiological specifics of alkaliphilic *Bacillus* spp. are attributed mainly to mechanisms of maintenance of inner pH including cytoplasmic pH regulation by cell wall composition and membrane transport system of Na^+ ions [2].

Maximal production of the chitinase by *Bacillus* sp. IB-OR-17 is observed under 0.25% - 0.50% of sodium carbonate and agrees with culture growth maximum in these conditions (pH ~ 9.0 - 10.0) (Figure 5(a)). Similar results were reported for production of other enzymes by alkaliphilic bacilli, for example, alkaline protease by *B. circulans* MTCC 7942 [20]. Data of chitinase production by alkaliphilic bacilli are scanty. Bhusan (2000)

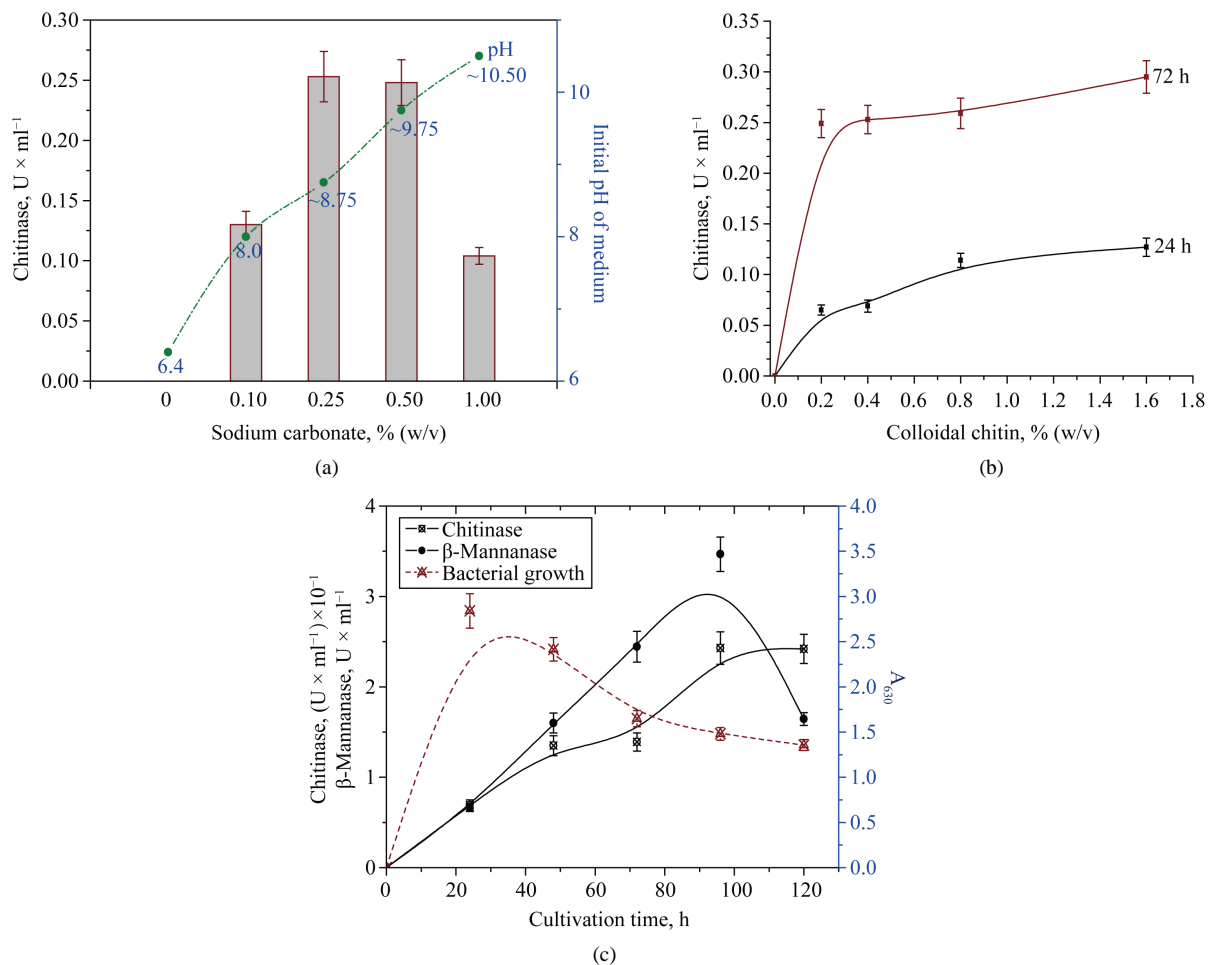


Figure 5. Influence of sodium carbonate (a) and substrate (b) concentration on chitinase production by *Bacillus* sp. IB-OR17; and kinetics of chitinase and β -mannanase production and bacterial growth during submerged cultivation (c). Chitinase production values in (a) are given for 72-h-old culture.

described thermostable chitinase produced by alkaliphilic *Bacillus* sp. BG-11 under initial pH 8.5 in nutritional medium [9], however, as known, it is not enough suitable for cultivation of true alkaliphiles because a lot of neutrophilic and alkali-tolerant microorganisms are actively capable to grow at this pH value. Other authors report on chitinase produced by *B. firmus* SBPL-05 under initial pH 10.5 in chitin-containing broth [7]. Our data suppose that chitinolytic enzymes produced by *Bacillus* sp. IB-OR-17 are stable under optimal conditions for the strain's growth (pH 9.0 - 10.0) but it should be checked by further characterization of purified enzyme(s).

The substrate content influence on chitinase production exhibited more distinctly after 24 h of growth cycle than that at the end of cultivation; maximal increase of the enzyme production reached about 100% in this step (Figure 5(b)).

So, it is evident that lower concentrations of colloidal chitin are more preferable for enough high production of chitinase(s) by *Bacillus* sp. IB-OR-17. The strain produces comparable and even higher amounts of chitinase using other chitin substrates for growth, in particular, partially purified chitin from dead bees (data are not shown).

Unlike chitinase, β -mannanase is produced constitutively by *Bacillus* sp. IB-OR-17 on media including various carbon sources. Heretofore, production of β -mannanases by *Bacillus* spp., *Paenibacillus* spp. and other bacteria was reported using such substrates as copra meal, locust bean gum, galactomannan, guar gum, konjac mannan, konjac powder, wheat bran, potato peel, palm kernel cake etc. [21]. It is the first report on microbial production of β -mannanase in presence of crab chitin as carbon source.

Biosynthesis of chitinase and β -mannanase by *Bacillus* sp. IB-OR-17 could be probably attributed to its anti-

fungal activity. At the same time, under conditions of antifungal assay test medium composition (see above) rather not favored to induction of chitinase of *Bacillus* sp. IB-OR-17. Production of bacterial glycosyl hydrolases as rule is repressed partially or completely by various sugars including both monomers of their substrates and some others. Considering that chitinase of *Bacillus* sp. IB-OR-17 is also inducible enzyme and its production in presence of 1% D-glucose as main carbon source is limited or repressed. Nevertheless, this fact still requires further evidence. So far, production of chitinases displaying antifungal activity among alkaliphilic microorganisms was evidently reported only for actinomycete, *Nocardiopsis prasina* OPC-131 [22]. On the other hand, it is not improbable that antibiotic-like compounds are also involved in antifungal activity of *Bacillus* sp. IB-OR-17 in addition to its extracellular hydrolases, remarkable potential of *Bacillus* sp. IB-OR-17 both in though, in general, production of antibiotics by alkaliphilic bacilli is still reported scarcely. In particular, finding of two-peptide lantibiotic produced by alkaliphile *Bacillus halodurans* C-125 is just singularly reported by Lawton *et al.* (2007) [23]. The findings demonstrate production of chitinase(s) and β -mannanase(s) stable under alkaline conditions and probably as perspective source of novel antifungal compounds.

So, present research firstly reports on antifungal and chitinase activity of novel alkaliphilic strain of *Bacillus* sp. IB-OR17 isolated from soda lake sediments and belonging to species of *Bacillus mannanilyticus* is based on phenotypic features and analysis of 16S rRNA gene. Isolated strain demonstrates high similarity with singular described type alkaliphilic species *B. mannanilyticus* A-001 including ability to production of β -mannanase. Identified and characterized strain is a perspective producer of novel chitinase(s), β -mannanase and antifungal compounds stable under alkaline pH values. Detailed characterization of the enzymes and study of antifungal mechanism of *Bacillus* sp. IB-OR17 are in contemplation to be done in further researches.

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