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Bioprospection of cellulolytic and lipolytic South Atlantic deep-sea bacteria

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Abstract

Background: Cellulases and lipases have broad industrial application, which calls for an urgent exploration of microorganisms from extreme environments as valuable source of commercial enzyme. In this context, the present work describes the bioprospection and identification of deep-sea bacteria that produce cellulases and lipases, as well their optimal temperature of activity. **Results:** The first step of this study was the screening of cellulolytic and lipolytic deep-sea bacteria from sediment and water column, which was conducted with substrates linked with 4-Methylumbelliferyl. Among the 161 strains evaluated, 40 were cellulolytic, 23 were lipolytic and 5 exhibited both activities. Cellulolytic and lipolytic bacteria are more common in sediment than at the water column. Based on the ability to produce cellulases and lipases three isolates were selected and identified (16S rRNA sequencing) as *Bacillus stratosphericus*, *B. aerophilus* and *B. pumilus*. Lipases of strain *B. aerophilus* LAMA 582 exhibited activity at a wide temperature range (4º to 37ºC) and include psychrophilic behaviour. Strain *Bacillus stratosphericus* LAMA 585 can growth in a rich (Luria Bertani) and minimal (Marine Minimal) medium, and does not need an inducer to produce its mesophilic cellulases and lipases. **Conclusions:** Deep-sea sediments have great potential for bioprospection of cellulase and lipase-producing bacteria. The strains LAMA 582 and LAMA 585 with their special features, exhibit a great potential to application at many biotechnology process.

Keywords: bacteria, cellulases, deep-sea, lipases

INTRODUCTION

Deep-sea sediments (water depth ≥ 1000 m) represent one of the planet's largest ecosystems, covering about 95% of the total oceanic bottom and 67% of the Earth's surface (Jørgensen and Boetius, 2007; Kouridaki et al. 2010). In this depth the light intensity is too low to sustain photosynthetic production, the temperatures are close to freezing (-1ºC to 4ºC), the hydrostatic pressure is too high, and the availability of organic matter controls benthic productivity and biomass (Jørgensen and Boetius, 2007; Dang et al. 2009; Polymenakou et al. 2009; Kouridaki et al. 2010). In generally, the organic material produced by phytoplankton is recycled in the upper 1000 m of the water column, but a significant fraction of the primary production also reaches the seafloor, where it is either remineralized or permanently buried in the sediment (Wenzhöfer and Glud, 2002). The sedimentation of this organic materials and phytodetritus triggers the production of hydrolytic enzymes and, subsequently, the growth of the community, on similar timescales to those found in shallow-water sediments (Jørgensen and Boetius, 2007). To survive in this environment the organisms have to adapt to the physical, chemical and biological conditions found, which is reflected in their physiology and biochemical properties (De Carvalho and Fernandes, 2010).

Among the hydrolytic enzymes, lipases and cellulases have broad industrial application. Lipases (glycerol-ester-hydrolases, E.C.3.1.1.3.) are carboxylesterases that are capable to catalyze the hydrolysis (and synthesis) of long-chain acylglycerols (Paiva et al. 2000; Lee et al. 2003). Lipases have many applications, such as in organic syntheses, hydrolysis of fats and oils, modification of fats, flavour enhancement in food processing, resolution of racemic mixtures and chemical analyses (Aravindan et al. 2007). Endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) are cellulases, which act synergistically to hydrolyze cellulose (Lynd et al. 2002; Percival Zhang et al. 2006). Cellulases have applications in brewing, paper pulp, textile industry, and detergent industries (Bhat, 2000; Anish et al. 2007).

The expanding application of the cellulases and lipases calls for an urgent exploration of microorganisms from extreme environments as valuable source of commercial enzyme (Shanmughapriya et al. 2010). In the present study, we report the bioprospection of deep-sea cellulolytic and lipolytic bacteria, as well the study of their characteristics for further biotechnological exploration (identification, enzyme localization, optima temperature and growth).

MATERIALS AND METHODS

Isolation of the microorganisms

Deep-sea microorganisms used in this work were obtained from a collection of the Laboratory of Applied Microbiology (LAMA) from Universidade do Vale do Itajaí (Itajaí, SC, Brazil). The organisms were previously isolated from the samples of sediment and water collected (500-5000 m depth) in the Mid-oceanic of South Atlantic and Rio Grande Rise by the project South Atlantic Mar-Eco - Patterns and Processes of the Ecosystems of the Southern Mid-Atlantic. A hundred and sixty one bacteria were replicated for a 96-well plate with 100 µL of marine broth 2216 (HiMedia). The plates were incubated at 14ºC for six days and stored at -20ºC in glycerol (20% final concentration).

Enzymatic assay

Enzymatic assay in solid medium was conducted in Petri dishes containing Marine Agar (2216) supplemented with tween (0,1%) for lipases and carboxymethylcellulose (0,1%) for cellulases evaluation. Also, fluorogenic substrate (4-Methylumbelliferyl - MU; 0,1 mM - final) linked to fat acids and sugars were added to the plate, for instance was used MU-palmitate (MUP), MU-oleate (MUO) and MU-butyrate (MUB) for lipases and MU-β-D-glucopyranoside (MUG), MU-β-D-cellobioside (MUC) and MU-β-D-xylopyranoside (MUX) for cellulases. After the period of incubation, the plates were exposed to Ultraviolet Light (excitation 312 nm), the fluorescence recorded by digital camera (CANON Rebel X, 12 megapixels) and the images were analyzed on Image-Pro Plus software. The enzyme unit (U) for the MU substrates was defined as Fluorescence Intensity Units. Moreover, enzymatic reaction were conducted with 4-Methylumbelliferyl (MU) and/or 4-Nitrophenyl (PNP) linked substrates. Similarly, for MU assays the same substrates used in solid medium. For PNP tests PNP-butyrate (PNPB) and PNP-palmitate (PNPP) were used for lipases, and PNP-β-D-glucopyranoside (PNPG), PNP-β-D-cellobioside (PNPC) and PNP-β-D-xylopyranoside (PNPX) for cellulases. The reaction with MU, was conducted using 50 µL of culture added of 50 µL of MU (0,1 mM - final). After incubation the relative fluorescence activity was recorded and calculated as described before. PNP reactions were conducted in a total volume of 100 µL, constituted of 50 µL of culture and 50 µL of PNP (2 mM, final concentration). After the incubation the reaction was stopped using Ca_2Na_3 (1 M), and the absorbance was measured on spectrophotometer (λ = 405 nm). An enzyme unit (U) for the PNP substrates was defined as the enzyme activity that catalyses the conversion of 1 µmol of PNP in one hr.

Phylogeny of the bacterial strain

The molecular identification of the selected strain was carried out by sequencing and analysis of the 16S rRNA gene. Therefore, genomic DNA was extracted with the commercial kit DNeasy Blood & Tissue kit (Qiagen) and then the partial 16S gene was amplified as described by Hiraishi (1992) using the pair of primers 16S-F: AGAGTTTGATCCTGGCTCAG and 16S-R: AAGGAGGTGATCCAGCCGCA. The reaction consisted of 50 µL containing 1x buffer of Taq DNA polymerase (Invitrogen), 0,2 mM of each dNTP, 0,5 µM of each primer, 2 mM of magnesium chloride, 1 U of Taq DNA polymerase (Invitrogen), 50 ng of genomic DNA, and the final volume adjusted with

ultrapure water. The program used for PCR was 94ºC for 240 sec, followed by 35 cycles of 94ºC for 30 sec, 50ºC for 30 sec and 72ºC for 30 sec and finally, extension at 72ºC for 360 sec. Subsequently, the amplification product was cleaned (QIAquick PCR Purification Kit, Qiagen) and analyzed on capillary sequencer (ABI3730XL, Applied Biosystems, CA). For the sequence, a query was made by the online BLAST program to the NCBI GenBank database for an initial determination of the nearest neighbour sequences (Altschul et al. 1997).

Growth of the microorganism and enzyme production

Specific growth rate was determined in two different medium, in a rich medium Luria Bertani (LB - 1.0% Tryptone, 0.5% Yeast Extract and 1.0% Sodium Chloride (NaCl) at pH 7.0) and Marine Minimum (MM) medium described for (Baumann et al. 1971). The cultivation was performed for 30 hrs at 37ºC and 2,5 Hz of agitation. The optical density (λ = 600 nm) was measured periodically (1-6 hrs intervals) for 30 hrs cultivation time and data was converted to cell concentration (g/L) using a specific linear regression for LB ($y = 2.87x - 0.0686$; $R^2 = 0.999$; data not shown) and MM ($y = 1.3269x + 0.0327$; $R^2 = 0.998$; data not shown). Finally, the specific growth rate (μ) was calculated considering the angular coefficient obtained from a linear regression between log of cell concentration and cultivation time. Together, with the same fraction were measured the enzyme production with the PNPG and PNPP assay, previously described.

RESULTS AND DISCUSSION

Screening of cellulolytic and lipolytic bacteria

One hundred and sixty one deep-sea bacteria (500-5000 depth) were evaluated for their ability to produce cellulases and lipases. For the evaluation on solid medium, the substrates MUC, MUG, MUX, MUO and MUP were used, and the bacteria were cultivated for 48 hrs at 37ºC. Among the 161 bacteria, 68 strains (42.24%) showed activity in at least one of the evaluated substrates (Table 1). Among these, 40 produced only cellulases, 23 only lipases and 5 strains (LAMA 580, LAMA 582, LAMA 585, LAMA 610, LAMA 743) were able to produce both enzymes. The ability of the deep-sea bacteria to produce hydrolytic enzymes has been report by Yang and Dang (2011). According to a review of Jørgensen and Boetius (2007), part of phytodetritus and others labile organic materials are deposited in the sea-floor, and subsequently, enables the growth of organisms that possess hydrolytic enzymes. In this case bacteria which possess cellulases and/or lipases.

Among the 161 strains that were evaluated, 52.7% were isolated from sediments and 47.3% from the water column. Although the numbers of evaluated strains were equivalent in each type of sample, it can be seen at Figure 1 that cellulolytic and lipolytic bacteria are more common in deep-sea sediment. The presence of cellulolytic bacteria in the sediment is explained by the availability nutrients provided from phytodetritus deposited in the sea-floor (Ragukumar, 2005; Jørgensen and Boetius, 2007), that can be degraded by cellulases. The same explanation can be apply from the lipolytic bacteria from a presence of labile organic matters (which may contain fatty acids) cited from Jørgensen and Boetius (2007).

Optimum temperature and identification of the selected isolates

The five bacteria capable to produce cellulases and lipases were tested for their optimum temperature of enzymatic activity (4º to 60ºC). MUG substrate was used for evaluation of cellulases and MUO for lipases on a liquid medium. In the Figure 2 it can be seen that LAMA 580 and LAMA 582 have two peaks of activity, one at 28º to 37ºC and the other at 4ºC. Two optimal temperature peaks, may be due by the presence of more than one cellulase, what could be explained by the samples used for each strain - pool of secreted and cell attached enzymes. The existence of more than one cellulase has already been reported previously in *Bacillus pumilus* (Gioia et al. 2007), *Bacillus licheniformis* (Rey et al. 2004), *Bacillus subtilis* (Earl et al. 2012) and *Bacillus amyloliquefaciens* (Yang et al. 2011). For instance, at *Bacillus pumilus* genomic data (NCBI NC_009848) it was annotated three β-glucosidases (Gene ID: 5622859, 5620825 and 5622859) (Gioia et al. 2007). Also in Figure 2, it can be seen that cellulases presents a mesophilic behaviour, so the highest activities occurred between 28º and 37ºC for the three most active and at 50ºC for the other two. Mesophilic behavior is very common among cellulases, even in deep-sea, as is the case of Yang and Dang (2011), in which the optimum

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temperature was 30ºC. In Figure 2 it can also be seen a higher activity for lipases in a wide range of temperature (4º to 37ºC), while still maintaining psychrophilic features found in deep-sea (around 4ºC). This wide range seems to be common and was reported by Jeon et al. (2009) with deep-sea bacteria. Wide range of high activity of these lipases is presented as a major attraction for application in biotechnology processes where may be difficult or too expensive to control the temperature.

Table 2 shows the identification of three selected bacterial strains based on the 16S ribosomal RNA gene amplification and a comparison of the results with NCBI GenBank data. The segments isolated from the strains LAMA 580, LAMA 582 and LAMA 585 were compared to nucleotides accessed in GenBank. The genus *Bacillus* is common inhabitant of marine environments, according Ettoumi et al. (2009), which isolated 96 *Bacillus* from deep-sea sediments. Although we obtained the same identification for the three strains, it is clear that this species presents great diversity and plasticity concerning its metabolism, which can be exploited in several industrial processes including, more recently, the recycling waste industry.

Table 1. Qualitative evaluation of the deep-sea bacteria for a production of cellulases and lipases.

	cellulase		lipase			cellulase		lipase		
Isolate	**MUG**	**MUC**	**MUX**	**MUO**	**MUP**	**Isolate**	**MUG**	**MUC**	**MUX**	**MUO**	**MUP**
LAMA 189	-	-	+	-	-	LAMA 705	+	-	-	-	-
LAMA 571	-	+	-	-	-	LAMA 707	-	-	-	+	-
LAMA 572	+	+	-	-	-	LAMA 708	-	-	-	+	+
LAMA 573	-	-	+	-	-	LAMA 713	-	-	-	-	+
LAMA 577	+	+	-	-	-	LAMA 715	-	-	+	-	-
LAMA 580	+	+	+	+	-	LAMA 720	-	+	-	-	-
LAMA 582	+	+	+	+	+	LAMA 725	+	+	+	-	-
LAMA 583	+	-	+	-	-	LAMA 728	+	-	-	-	-
LAMA 584	-	-	+	-	-	LAMA 729	-	-	-	+	-
LAMA 585	+	+	+	+	+	LAMA 731	-	-	-	+	+
LAMA 595	+	+	+	-	-	LAMA 732	+	+	+	-	-
LAMA 604	+	-	-	-	-	LAMA 734	+	+	+	-	-
LAMA 606	+	-	+	-	-	LAMA 736	-	-	-	+	-
LAMA 607	+	+	+	-	-	LAMA 736	+	+	+	-	-
LAMA 608	+	-	+	-	-	LAMA 738	-	-	-	+	-
LAMA 610	+	+	+	+	+	LAMA 743	+	+	+	+	+
LAMA 611	-	-	-	-	+	LAMA 747	+	+	-	-	-
LAMA 612	-	-	-	+	+	LAMA 749	+	+	-	-	-
LAMA 616	-	-	-	+	-	LAMA 751	+	+	+	-	-
LAMA 618	+	+	-	-	-	LAMA 755	-	-	+	-	-
LAMA 644	+	-	-	-	-	LAMA 757	-	-	-	+	-
LAMA 667	+	-	-	-	-	LAMA 764	-	-	-	+	-
LAMA 672	+	+	+	-	-	LAMA 766	+	+	+	-	-
LAMA 673	-	-	-	+	-	LAMA 767	+	+	+	-	-
LAMA 680	-	-	+	-	-	LAMA 768	-	-	-	-	+
LAMA 681	-	-	-	+	+	LAMA 769	-	-	-	-	+
LAMA 690	-	-	-	+	+	LAMA 771	+	-	-	-	-
LAMA 693	+	+	+	-	-	LAMA 773	-	-	-	+	-
LAMA 694	-	-	-	+	-	LAMA 775	+	+	+	-	-
LAMA 695	-	-	-	+	-	LAMA 778	-	+	-	-	-
LAMA 699	-	-	-	+	-	LAMA 779	+	-	-	-	-
LAMA 701	-	-	-	+	-	LAMA 781	+	+	+	-	-
LAMA 702	-	-	-	+	-	LAMA 782	-	+	-	-	-
LAMA 703	+	+	-	-	-	LAMA 893	+	-	-	-	-

(+) Presence of the enzyme to degrade the substrate.
(-) Absence of the enzyme to degrade the substrate.

Characterization of *Bacillus stratosphericus* - LAMA 585

Among the five isolates, LAMA 585 - *Bacillus stratosphericus* was selected due to its capacity to present both activities (lipase and cellulase) at high level when compared to the other isolates for further characterization: i) location of the enzymes (extracellular or intracellular); ii) optimum temperature of β-glucosidase, exoglucanase, endoxylanase, esterase and lipase; and iii) specific growth and enzyme production (rich and minimal medium).

Table 2. Identification, percentage of similarity and strains of the isolates selected.

Strains	Specie based on BLAST analysis	Identity	Access
LAMA 580	*Bacillus pumilus*	100	JQ428828
LAMA 582	*Bacillus aerophilus*	100	JQ342857
LAMA 585	*Bacillus stratosphericus*	100	JQ818357

[a]percentage based in number of identical nucleotides in relation to the total number of compared nucleotides. Genbank (NCBI) access number with higher local BLAST bit score.

Table 3. Enzymatic activity (U/ml) for lipases and cellulases from intracellular and extracellular fractions of the strain LAMA 585 (*Bacillus stratosphericus*).

	lipases		cellulases		
Fraction	**MUO**	**MUP**	**PNP-G**	**PNP-C**	**PNP-X**
Extracellular	2.4	1.9	1.4	0.9	0.3
Intracellular	42.2	25.1	30.1	12.5	10.7

A quantitative assay for enzymes localization was performed with the substrates MUO, MUP, PNPG, PNPC and PNPX on liquid medium. For all substrates two fractions were evaluated, one containing the extracellular culture supernatant after centrifuging (14,000 G for 5 min) the cells, and another with the intracellular, remaining cells resuspended in buffer (Tris-HCl, 100 mM, pH 7.2). It can be seen in Table 3 that the extracellular (secreted and cell attached) activity was higher than intracellular activity for all the evaluated substrates. This feature can be associated with the strategic adaptation of the bacteria to the environment where they were prospected. Bacteria must cleave polymeric substrates extracellularly into small molecules to assimilate (Yu et al. 2009), thus the enzymes evaluated presented higher extracellular activity rather than the intracellular. Low temperature may cause denaturation of enzymes, in this case, according Georlette et al. (2004), intracellular enzymes has protection towards cold-denaturation. This can be achieved by the accumulation and production of compatible solutes such as potassium glutamate, trehalose and others.

Optima temperature of *Bacillus stratosphericus* enzymes was determined with specific substrates - β-glucosidase (PNPG), exoglucanase (PNPC), endoxylanase (PNPX), esterase (MUB) and lipase (MUP). The Figure 3 shows that the results were similar to another study with *Bacillus pumilus*, where cellulases such as β-glucosidase and exoglucanase have an optimum temperature at 50ºC (Lima et al. 2005) and the endoxylanase at 40ºC (Nagar et al. 2010). The lipases have an optimum temperature at 40ºC and the esterase 20ºC (Figure 4).

Finally, we performed the growth of the isolate in LB medium and MM, assessing together the production of enzymes with the substrates PNPG for cellulases and PNPP for lipases on a liquid medium (Figure 5). In LB and MM medium the specific growth rates were 0.388 h^{-1} and 0.253 h^{-1}, respectively. A higher concentration of cells was also obtained in LB medium. The production of cellulases and lipases basically followed the same pattern, indicating that the enzymes are produced constitutively and do not need an inducer for its production, which is directly related to growth.

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CONCLUDING REMARKS

The deep-sea environment is a good place to prospect cellulases and lipases. Organisms associated with deep-sea sediments have greater potential for production of cellulases and lipases than those found in the water column. Some deep-sea organisms have the potential to produce cellulases and lipases at the same time. The lipases found in strain LAMA 582 (*Bacillus aerophilus*) from deep-sea have a wide range of optimum temperature for activity (4º to 37ºC) exhibiting a psychrophilic behaviour and offer a great potential to biotechnological process. The strain LAMA 585 (*Bacillus stratosphericus*) can growth in a rich and minimal medium and does not need an inducer to produce its mesophilic cellulases and lipases.

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Figures

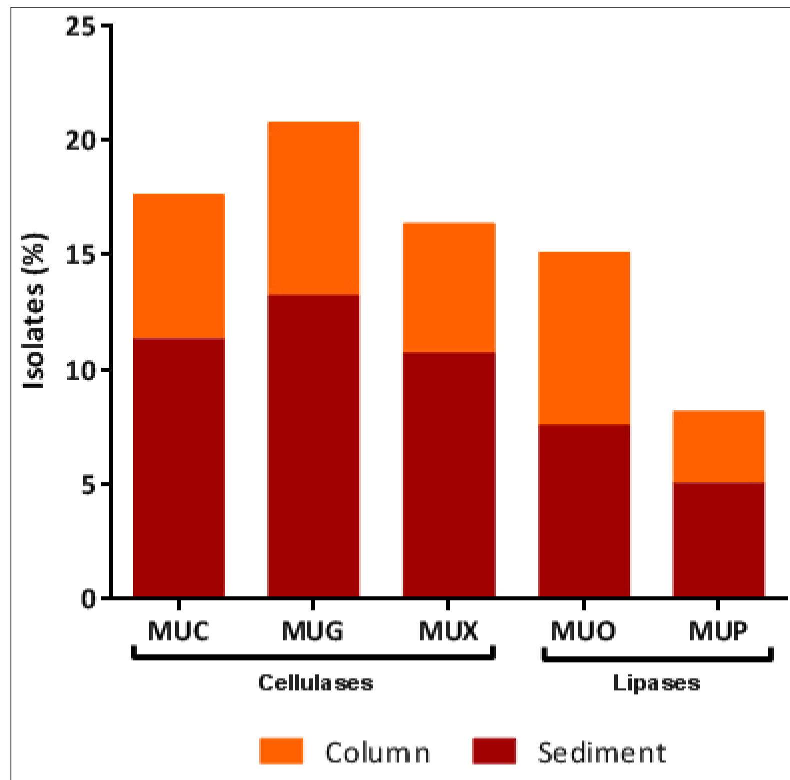

Fig. 1 Relative distribution of the cellulolytic and lipolytic bacteria isolates from deep-sea sediment and column water.

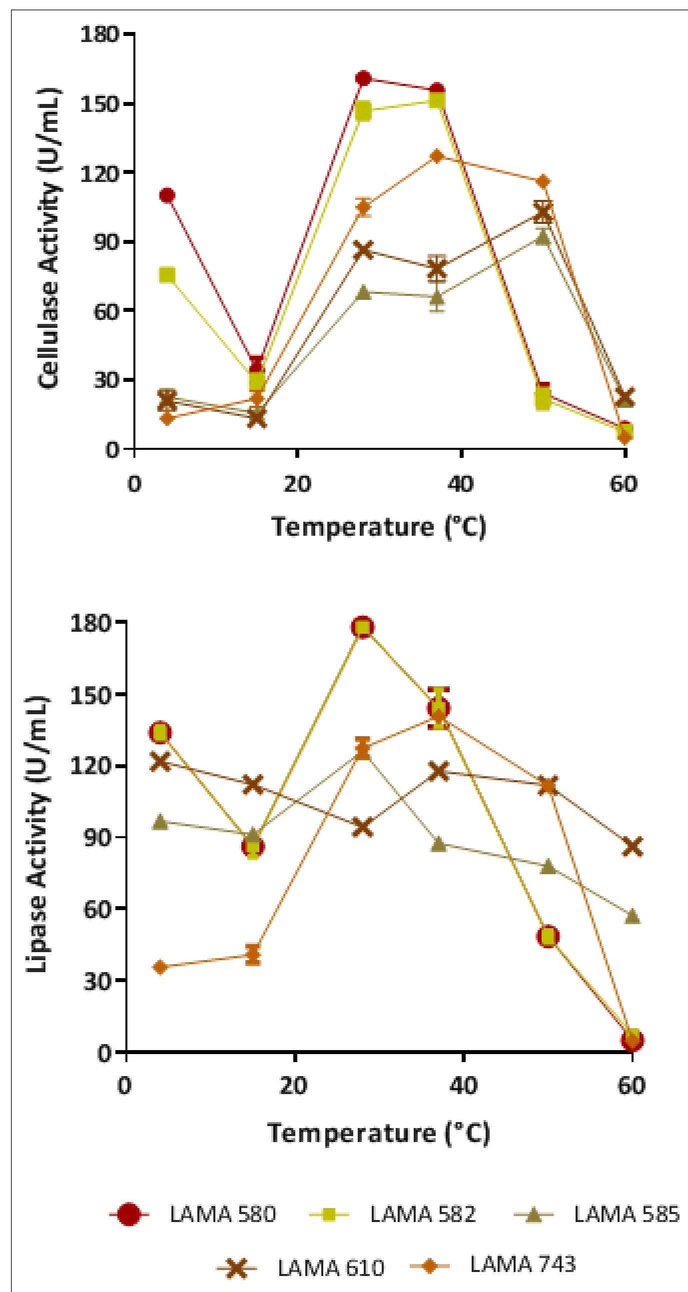

Fig. 2 Optimum temperature for cellulases and lipases of the five strains selected. Cellulases were measured with 4-Methylumbelliferyl -β-D-glucopyranoside (MUG) and lipases with 4-Methylumbelliferyl-oleate (MUO).

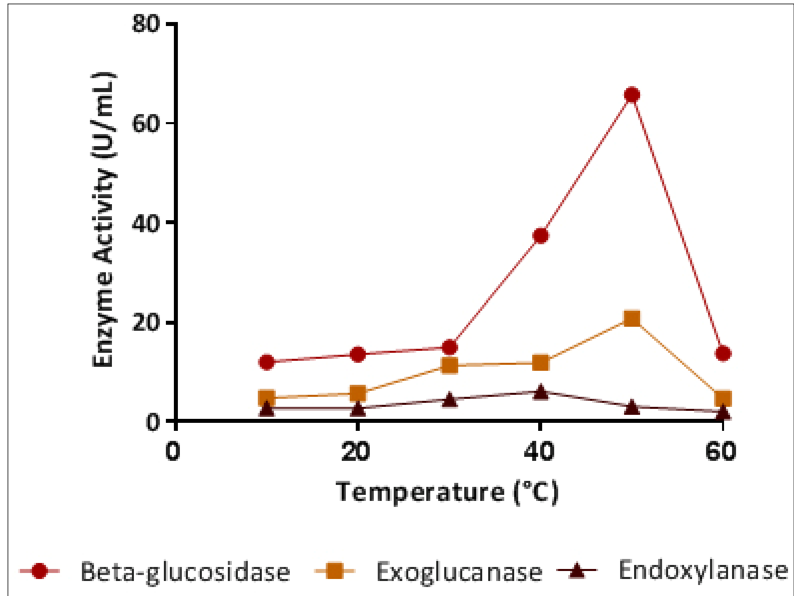

Fig. 3 Optimum temperature of the cellulases produced by *B. stratosphericus* LAMA 585.

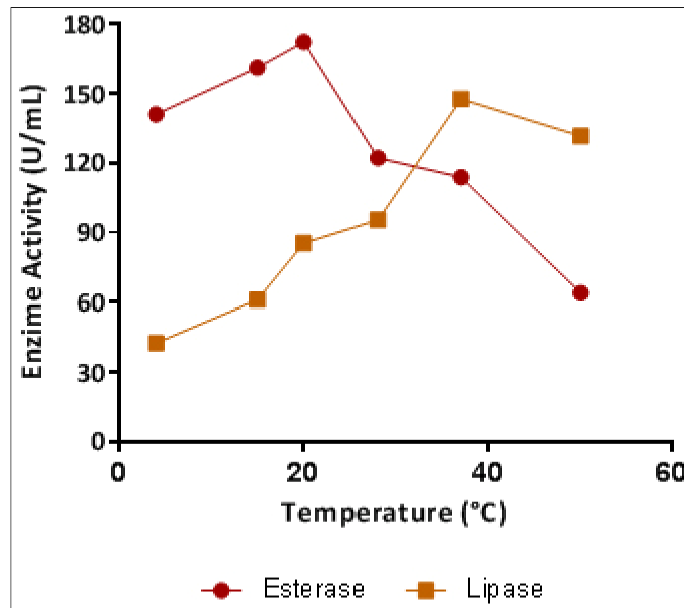

Fig. 4 Optimum temperature of the lipase and esterase produced by *B. stratosphericus* LAMA 585.

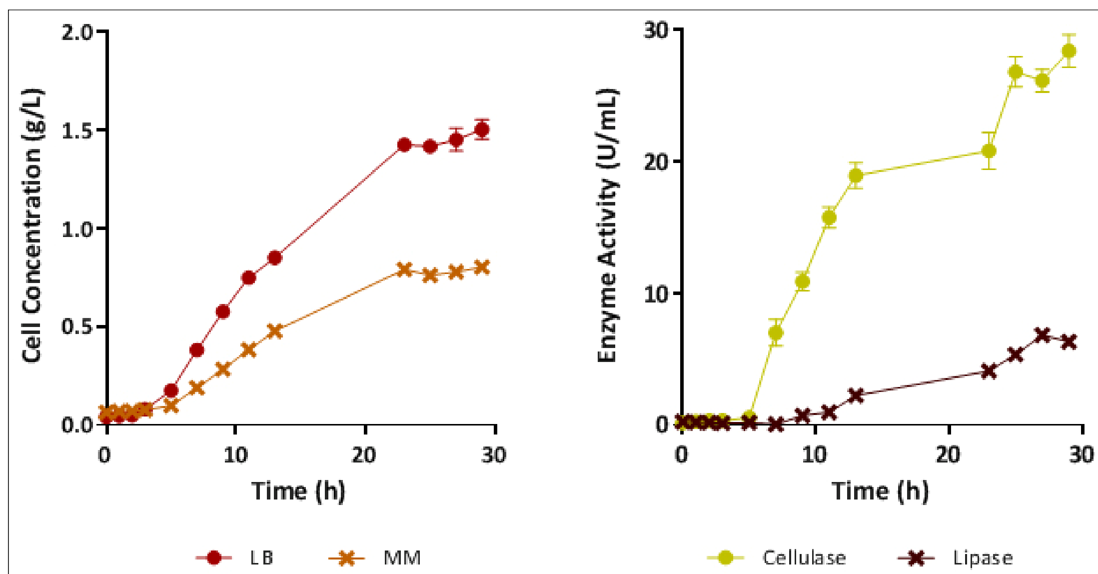

Fig. 5 Specific growth of *B. stratosphericus* LAMA 585 in a rich (LB) and minimal (MM) medium. Production of cellulases and lipases at the rich medium. Cellulases were measured with 4-Nitrophenyl-β-D-glucopyranoside (PNPG) and lipases with 4-Nitrophenyl-palmitate (PNPP).

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