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Characterization of inducible cold-active β-glucosidases from the psychrotolerant bacterium *Shewanella* sp. G5 isolated from a sub-Antarctic ecosystem

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ABSTRACT

The psychrotolerant bacterium *Shewanella* sp. G5 was used to study differential protein expression on glucose and cellobiose as carbon sources in cold-adapted conditions. This strain was able to growth at 4 °C, but reached the maximal specific growth rate at 37 °C, exhibiting similar growing rates values with glucose (μ: 0.4 h^{-1}) and cellobiose (μ: 0.48 h^{-1}). However, it grew at 15 °C approximately in 30 h, with specific growing rates of 0.25 and 0.19 h^{-1} for cellobiose and glucose, respectively. Thus, this temperature was used to provide conditions related to the environment where the organism was originally isolated, the intestinal content of *Munida subrrugosa* in the Beagle Channel, Fire Land, Argentina. Cellobiose was reported as a carbon source more frequently available in marine environments close to shore, and its degradation requires the enzyme β-glucosidase. Therefore, this enzymatic activity was used as a marker of cellobiose catabolism. Zymogram analysis showed the presence of cold-adapted β-glucosidase activity bands in the cell wall as well as in the cytoplasm cell fractions. Two-dimensional gel electrophoresis of the whole protein pattern of *Shewanella* sp. G5 revealed 59 and 55 different spots induced by cellobiose and glucose, respectively. Identification of the quantitatively more relevant proteins suggested that different master regulation schemes are involved in response to glucose and cellobiose carbon sources. Both, physiological and proteomic analyses could show that *Shewanella* sp. G5 re-organizes its metabolism in response to low temperature (15 °C) with significant differences in the presence of these two carbon sources.

1. Introduction

In nature, as well as during industrial processes, bacteria are exposed to changing physico-chemical environmental parameters which may impair their growth or survival [1]. One such environmental condition rarely applied in laboratory studies is the induction of cold-responsive genes. Cold regions have been colonized by psychrophiles (0–15 °C) and psychrotolerant (15–20 °C) microorganisms which have adapted to the strong effect of low temperature on biochemical reactions that enable cell survival in cold environments [2]. A cold-active enzyme tends to have reduced activation energy at low temperature, leading to high catalytic efficiency, which may possibly be attributed to an enhanced local or overall flexibility of the protein structure [3,4]. Two glycosyl hydrolases, or β-glucosidases (EF141823 and DQ136044), were isolated from a psychrotolerant *Shewanella* sp. G5. These cold-active β-glucosidases may be of interest for biotechnology, e.g. in food processing at low temperatures and in a broad pH range [5].

The identification of proteins and protein expression patterns under different growth conditions (e.g., carbon source or temperature change) can be studied by proteome analyses which are greatly enhanced if genome sequence data are available [6]. For the genus *Shewanella*, the *S. oneidensis* MR-1 (NC_004347) genome is available with 4467 predicted genes; 1623 of which are annotated as hypothetical (36%) [7]. This assignment is given to genes that have not been characterized and whose functions cannot be deduced from simple sequence comparisons [8]. Using a closely related organism, the available genome data can be exploited to identify proteins expressed under specific conditions. Thus, it is possible to investigate a cold-tolerant relative like *Shewanella* sp. G5 for protein expression under environmentally pertinent conditions. Those approaches are now available for comprehensive measurements of gene and protein expression, a valuable tool since the lack of knowledge of the function in the protein expression in the genome limits the ability to take full advantage of capabilities for advancing in

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the compressive of its biology. Even, these genes encode several intact proteins, which is expresses in different growth conditions in a bacterium. Nonetheless, every new approach in the expression of proteins provides results in hundreds of new hypothetical genes [8]. To highlight variations in gene expression, proteome analysis of soluble and whole cell proteins represents a suitable tool. Two-dimensional gel electrophoresis (2D), which introduction of immobilized pH gradients (IPGs) for isoelectric focusing (IEF) couples in the first and SDS-PAGE in the second dimension.

It is the method of choice for resolving complex protein mixtures and reveals even very small changes in protein expression patterns [9]. It allows for quantitative and qualitative separation of complex protein mixtures typically found in cellular extracts of living cells form organisms. Both methods IEF and SDS-PAGE are critically affected by the solubility of proteins prior to electrophoresis. Proteins can only be analyzed by 2D if they are kept in solution or solubilized during the entire process [10].

The combination of 2D gel electrophoresis and mass spectrometry was used to study the differential expression of genes from the psychrotolerant *S. oneidensis*, identifying proteins by similarity to those annotated from the known genome sequence [11]. The proteomic changes of *Shewanella* sp. G5 were analyzed for cultures grown at low temperature in the presence of two alternative carbon sources, cellobiose or glucose. The enzyme β-glucosidase was used as a marker to indicate proteome re-arrangement and to elucidate environmentally relevant expression profiles.

2. Materials and methods

2.1. Cultivation

Shewanella sp. G5 isolated from the benthonic organism *Munida subrrugosa* collected on the coast of the Beagle Channel, Ushuaia, Argentina [5], was cultivated in 1 l flasks on an orbital shaker (250 rev/min), containing 300 ml liquid Luria-Bertani medium with 10 g/l cellobiose (LBC) or glucose (LBG) at 15 °C.

2.2. Basic characterization

The media were inoculated with 100 μl of culture; the growth was followed by absorbance in a Beckman (DU®640) spectrophotometer until an optical density (OD_{540}) of 0.8 at 540 nm. Specific growths were evaluated in LBC and LBG at different temperatures (15, 20, 25 and 37 °C). Absorbance values were transformed to $\log_e$ (absorbance), so specific growth rate values were calculated by fitting the growth curves with the equation described by Nerbrink et al. [12].

The *Shewanella* sp. G5 tested were grown in LBC at 15 °C and APIs (BioMérieux) strips were set up for this strain. Duplicate API ZYM, API 20E, API 20NE and API Coryne identification strips were set up as instructed by the manual and incubated at 15 °C for 1 h.

2.3. Protein extraction of Shewanella sp. G5

Sub-cellular protein fractions were obtained by differential centrifugation Kong et al. [13]. After obtaining proteins in the supernatant (15 min at 33,000 × *g*), cells were suspended in 5 ml of distilled water and disrupted by French Press (SLM Instruments) at 25,000 psi. Cell debris was removed (15 min at 30,000 × *g*), and cytoplasmic proteins were separated by ultra-centrifugation at 144,000 × *g* into soluble cytoplasmic proteins (SCP) and membrane proteins (MP) fractions.

2.4. β-Glucosidase enzyme activity

β-Glucosidase activity was assayed with 0.1 M *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) by incubation at 37 °C for 1 h using 0.1 M potassium phosphate buffer at pH 8 [5]. Different pH conditions were used by adjusting the phosphate buffer to pH 6–8, and cellular fractions were assayed after resuspending the pellets in 0.1 M potassium phosphate buffer pH 8. Absorbance of *p*-nitrophenol released during the reaction was monitored by spectrophotometer at 420 nm. One enzyme unit was calculated using the extinction coefficient of *p*-nitrophenol ($\varepsilon_{420\,nm} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$) and was *defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate per min under the experimental conditions*. Enzymatic activity was expressed as specific activity (U/mg); all analyses were performed in triplicate.

2.5. Zymogram assay

Native PAGE from a modified procedure of Long An et al. [14] was carried out to determine isoenzymes pattern of β-glucosidases, using a Bio-Rad Mini-Protein 3 Cell electrophoresis unit (Biorad). Protein identification was performed in 0.75 mm gel in a vertical slab unit, the separating gel containing acrylamide and bisacrylamide, 10 and 0.5%, respectively. The cellular fractions previously obtained were mixed with buffer (0.35 M Tris–HCl pH 6.8, 10% glycerol, 0.0002% bromophenol blue and 0.6 M dithiothreitol, DTT), and electrophoresis was carried out at 100 V until the tracking dye migrated to the bottom of the gel at room temperature. The gel was then washed twice in 0.1 M potassium phosphate buffer pH 7 and incubated in 1 mM of 4-methylumbelliferyl-β-D-glucopyranoside (MUG) for 30 min at room temperature. Fluorescent bands indicative of β-glucosidase activity were observed under UV light, visualized and captured using an Image Analyzer Gel Doc (Biorad). After washing the gel three times with buffer potassium phosphate, Coomassie brilliant blue staining was applied to visualize the protein bands [15].

2.6. Two-dimensional electrophoresis

The cytosolic proteins were precipitated (20% trichloracetic acid, 50% acetone, 20 mM dithiothreitol, DTT) for 30 min at 20 °C, incubated for 2 h at 4 °C and centrifuged at 11,000 rpm. After a two-step acetone wash the pellet was dried (Speed Vac) and redissolved in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). After ultracentrifugation at 75,000 × *g*, proteins [16] were quantified measured used for each IEF strip in the rehydration buffer [17].

2.6.1. First dimension (IEF)

First dimension separation of proteins by isoelectric points was conduced with IPG Immobiline DryStrip pH 3–10 non-linear (NL) 18 cm (Amersham Biosciences). IEF strips were rehydrated for 12 h at 20 °C with 450 ml rehydration buffer containing 500 mg proteins and 3 ml 4% bromophenol blue for each sample. IEF was conducted in the IPGphor system (Amersham Biosciences) using the followed steps: S1 300 V (15 min), S2 500 V (30 min), S3 1000 V (1 h), S4 3000 V (1 h) and S5 8000 V (7 h). Afterward, the strip was equilibrated with 6 M Urea; 4% SDS (w/v); 30% glycerol (v/v); 1% DTT (w/v) and 3.3% bromophenol blue (w/v) at room temperature (RT) for 15 min. Subsequently, the strip was re-equilibrated with the same solution except for the addition of 4% iodoacetamide at RT for 15 min [17].

2.6.2. Second dimension

The second dimension SDS-PAGE (Ettan DALT*six* Large Vertical System, Amersham Biosciences) was performed with strips sealed with 0.8% agarose in the top 1.5 mm of a 26 cm × 20 cm vertical 10% PAGE in a SE-600 system (Hoefer SE600). Electrophoresis was performed in the presence of 181.66 g Tris–HCl pH 8.8, 30 g glycine and 4 g SDS with constant voltage (600 V) followed by constant amperage (400 mA/gel) at 5 °C for 16 h or until the bromophenol blue reached the bottom of the gel. Afterwards, gels were rinsed with distilled water for 5 min and fixed overnight in 10 ml phosphoric acid 85%, 20 ml methanol and 79 ml distilled water. The gels were stained with Coomassie brilliant blue (Roti-Blue 20%, Roth) for 12 h at RT and then discolored with glycine and methanol for 24 h [17].

2.6.3. In-gel digestion and mass spectrometry

In the present study, we used thiourea and urea as chaotropes combined with CHAPS (sulfobetine detergents) and DTT (reducing agent) to the solubilization of protein [11]. Protein spots digestion was performed in-gel [17]. Briefly, the excised spots were washed, air-dried and digested with 0.02 μg modified trypsin per spot in 50 mM ammonium bicarbonate at 37 °C overnight. Peptides were extracted with 60% (v/v) acetonitrile/0.1% (v/v) formic acid in water, thereafter dried in a SpeedVac (Thermo Savant, USA) and dissolved in 10 μl of 0.1% (v/v) formic acid.

One μl of the digested sample was added to an equal volume of matrix solution for electron spray ionization Mass Spectroscopy (ESI-MS; LCQ Deca XP, Thermo; [18] or MALDI TOF (matrix-assisted laser desorption ionization time of flight) [19]. For identification of peptides, the *S. oneidensis* MR-1 (NC_004347) genome (NCBI database) was used with Sequest 3.1 [7,17].

3. Result and discussion

3.1. Physiological characteristics

Significant differences were observed in the specific growth rate μ (h^{-1}), evaluated in LB medium with cellobiose and glucose as carbon sources. The strain presented higher μ values with cellobiose at all temperatures assayed. Nevertheless, the maxima specific growth rate in both media was reached at 37 °C (Fig. 1).

The bacterium *Shewanella gelidimarina*, which was isolated from Antarctic sea, has an optimal growth temperature at 17 °C. The related species *S. benthica* has an optimal growth temperature at 8 °C and the bacterium *Polaromonas vacuolata* a still lower (4 °C) optimal growth temperature. Pakchung et al. [20] reported the

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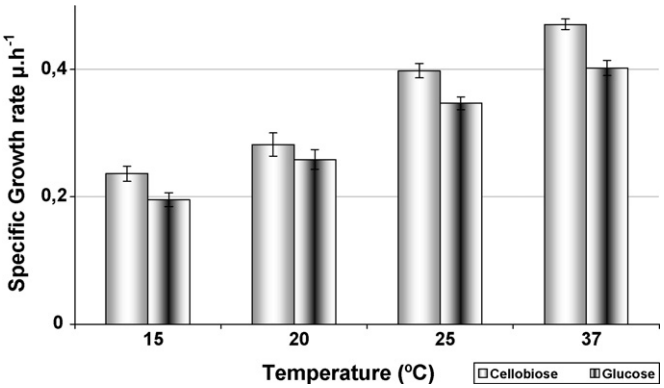

Fig. 1. Determinations of the specific growth rate μ (h^{-1}) of *Shewanella* sp. G5 in LB cultures using cellobiose (□) and glucose (◧) as a carbon source.

growth curves typical of cold-adapted *Shewanella* species when cultured at 4 °C, they observed an increase in cell density cultures of the *S. benthica* or *S. gelidimarina*; however when cultured at 20 or 37 °C, there is no observable increase in cell density determined the cold-adapted phenotype.

The results of biochemistry characteristics of *Shewanella* sp. G5 are shown in Table 1. All APIs tests were used to determine the presence of enzymes, assimilations and fermentation assay of different sugars, which were constitutively expressed during growth at low temperature in LBC medium. Vogel et al. [21] described a simple phenotypic scheme derived from various biochemical tests, which were used in the classification of several strains as belonging to the genus *Shewanella*.

Table 1
Determination of the biochemical characteristics and assimilations of carbon source from *Shewanella* sp. G5; identification of the presence of enzyme groups analyzed from API tests.

Biochemical characteristics from *Shewanella* sp. G5[a]			
Enzymes	Reaction	Enzymes	Reaction
Nitrate reductase	+	Phosphatase acid	+
Nitrate reductase	–	Naphtol-AS-BI-phosphohydrolase	+
Tryptophanase	–	α-Galactosidase	–
Production of acetone (nose)	+	β-Galactosidase	+
Production of H_2S (es aceptor)	–	β-Glucoronidase	–
Utilization of citrate (assimilation)	+	α-Glucosidase	+
Arginine dihydrolase	+	β-Glucosidase	+
Urease	+	N-Acetyl-β-glucosam inidase	–
Protease	+	α-Manosidase	–
Alkaline phosphatase	+	α-Fucosidase	–
Esterase (C4)	+	Catalase	+
Esterase lipase (C8)	+	Gelatinase (gelatin)	+
Lipase (C14)	–	Pyrazinamidase	+
Leucine arylamidase	+	Pyrolidomyl arylamidase	–
Valine arylamidase	–	Lysine decarboxylase	+
Cistine arylamidase	–	Ornithine decarboxylase	+
Trypsin	+	Triptophan desaminase	–
α-Chymotrypsin	–		

Biochemical characteristics from *Shewanella* sp. G5[a]			
Assimilations	Reaction	Fermentations	Reaction
Glucose	+	Glucose	+
Arabinose	+	Ribose	–
Manose	+	Xylose	–
Manitol	+	Manitol	+
N-acetyl-β-glucosaminidase	+	Maltose	+
Maltose	+	Lactose	+
Potassium gluconate	+	Sacarose	+
Capric acid	+	Glycogen	–
Adipic acid	–	Inositol	–
Malic acid	+	Sorbitol	+
Trisodic citrate	+	Rhamnose	+
Phenylacetic acid	–	Melibiose	+
		Amigdaline	+
		Arabinose	+

[a] Determinations from APIs® Meridian (ZYM, Coryne, 20E and 20NE).

The genus *Shewanella* has been studied since 1931 with regard to a variety of relevance topics for both applied and environmental microbiology. Recent years have witnessed the introduction of a large number of new *Shewanella*-like isolates, necessitating a coordinated review of the genus. The potential of these organisms to mediate the co-metabolic bioremediation of halogenated organic pollutants as well as the destructive souring of crude petroleum have also been considered [22,23]. *Shewanella* sp. G5 is a marine bacterium classified as psychrotolerant because it presents an optimum growth in plate at 20 °C, but the strain could grow at 4 and 37 °C. *Shewanella* sp. G5 was associated with a 99.2% identity to *Shewanella baltica* OS195, based on phylogenetics analyses of 16S rDNA and *gyrB* sequences [5,7,24].

3.2. Influence of temperature, pH and carbon source on β-glucosidase activity

We used lower temperatures as compared to earlier reports, where Cristobal et al. [5] showed the presence of two isozymes of β-glucosidases (EC 3.2.1.21) from *Shewanella* sp. G5, with optimal activity temperature at 37 and 25 °C. Here, we studied the influence of different temperatures, pH values and two different carbon sources on the growth and production of β-glucosidase from *Shewanella* sp. G5. We studied theses parameters with the hypotheses that glucose is used preferentially by most bacteria while cellobiose may represent a more likely substrate in the marine environment from which *Shewanella* sp. G5 had been isolated (Fig. 2a).

The optimum pH for production of β-glucosidase was pH 8, in further experiments; we used β-glucosidase activity at that pH value (see Fig. 2b) as a marker of protein expression. Previously we suggested the presence of 2 isoenzymes [5]. Herein, a subcellular fractionation could show that *Shewanella* sp. G5 produced three different isozymes, on both carbon sources although higher activities were detected on cellobiose. The three activities correspond to membrane protein, cell wall associated protein and intra-cellular protein (Fig. 2b), while extra-cellular β-glucosidase was not found. Our results indicate one of the possible pathways of cellobiose metabolism in *Shewanella* sp. G5; the presence of this disaccharide allow act as promoter in the synthesis of all β-glucosidases enzymes necessary for the activation of their degradation path. These enzymes are necessary for the transport of the disaccharide from extra-cellular region across the cell wall and membrane to the cytoplasm region. Thus, intra-cellular β-glucosidase is activated and degrades cellobiose in two glucose units; this last sugar is incorporated to energetic metabolism (glycolysis).

Riou et al. [25] reported that *Aspergillus oryzae* secrete two distinct β-glucosidases when it was grown in liquid culture on various substrates. The minor form, which was induced most effectively on quercetin, represented no more than 18% of total β-glucosidase activity but exhibited a high tolerance to glucose inhibition.

Cai et al. [26] reported the expression of two β-glucosidase isozymes (BGL1 and BGL2) of a molecular mass of 158 kDa and 256, respectively. Both isoenzymes are produced by *Volvariella volvacea* with a pI of 6.2 (BGL1) and 7 (BGL2), and the level of expression is determined by the presence of different substrates (salicin, cellobiose, etc.). The specific activity (5.1 U/mg of protein) was determined by substrate *p*NPG establishing as optimum inductor the salicin whit a activity temperature between 55 and 60 °C for

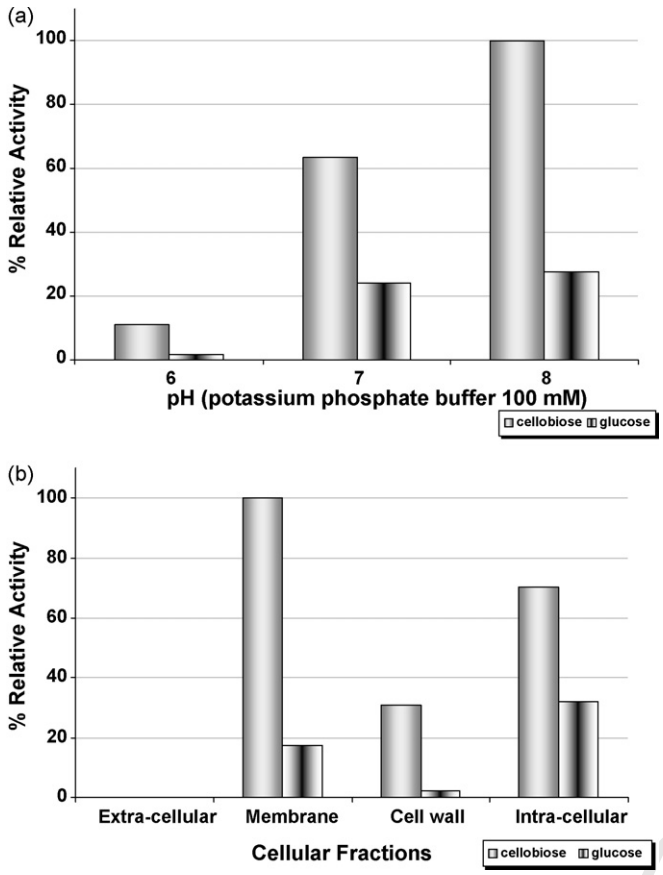

Fig. 2. Specific activity and cellular localization of β-glucosidase of *Shewanella* sp. G5 grown on glucose (□) or cellobiose (▥); experiments were performed in duplicate.

both isozymes. Therefore, in presence of glucose was not observed activity β-glucosidase.

3.3. Zymogram assay

To facilitate the localizations and characterizations of β-glucosidase isozymes, native gel electrophoresis was used for the determination of molecular mass. We used direct activity staining techniques (MUG, native PAGE) that allow rapid and specific enzyme detections with the crude cell extract in a polyacrylamide slab gel. After electrophoresis, the gel was rinsed and incubated with MUG. Two fluorescent bands were observed with high intensity in the intra-cellular region (Fig. 3a). β-Glucosidase presented an estimated molecular mass of approximately 75 kDa and a second band of approximately 115 kDa if grown on cellobiose, and little activity of about 75 kDa if grown on glucose (Fig. 3b). The membrane fractions of cells grown in presence of cellobiose contained two isozymes of 75 and 140 kDa, whereas the cell wall fraction showed only a single active band of 75 kDa. No activity was observed in the cell wall or membrane fractions from cells grown in the presence of glucose (Fig. 3b).

Riou et al. [25] described that using the technique MUG-SDS-PAGE, determined the molecular mass of two β-glucosidases; the major enzyme was of 130 kDa and highly inhibited by glucose. The other enzyme is a monomeric protein with an apparent molecular mass of 43 kDa and a pI of 4.2.

Soo-Jin et al. [27] described the molecular mass of the expressed β-glucosidase was 72 kDa, which corresponded with the predicted molecular mass of this enzyme from *Chloroflexus aurantiacus*. To characterize the β-glucosidase activities, a direct activity staining technique was used based on MUG-PAGE. A fluorescent band appeared around the expressed protein where the MUG was degraded indicating the glycosyl hydrolase activity.

This technique takes advantage of the ability of very small quantities of β-glucosidase to degrade β-sugars. As expected, cellobiose served as an inducer of β-glucosidase expression, and high levels of β-glucosidase activity were found in intra-cellular, membrane and cell wall fractions of cells grown on this β-glycosides substrate. In addition, we could show (at least) three different isozymes localized in these compartments. We found similar results in the determined specific activity of β-glucosidase from a same extracts; but with those results we could confirm the presence of several β-glucosidases in the membrane, wall and cytoplasmic regions. However, basal levels of β-glucosidase activity were observed in the extracts form cells that grew on glucose (Fig. 3a and b).

Long An et al. [14] reported a direct activity staining technique (MUG-SDS-PAGE) that allows rapid and specific detection of β-glucosidase with the crude extract of *Pectobacterium carotovorum*

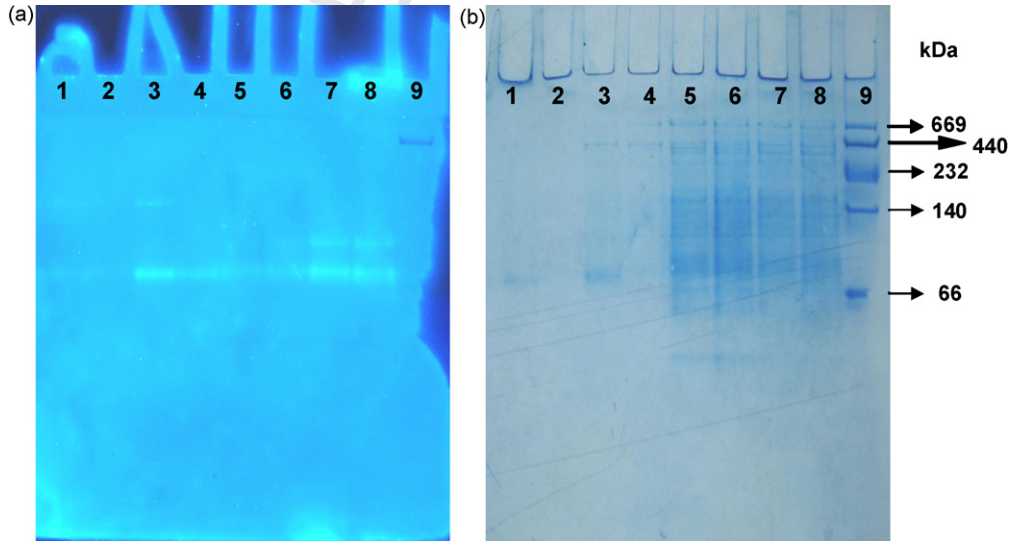

Fig. 3. Zymogram assays. β-Glucosidases isozymes pattern resolved by native gel electrophoresis analysis was carried in acrylamide gel (10%) to express β-glucosidase enzymes from various cellular regions of *Shewanella* sp. G5. (a) Gel treated with Coomassie blue staining and (b) gel treated with 100 mM phosphate potassium buffer pH 8 and 4-MuβG as specific fluorescent substrate. Protein extract from glucose 1: membrane; 2: cell wall; 5, 6: intra-cellular. Protein extract from cellobiose 3: membrane; 4: cell wall; 7, 8: intra-cellular and 9: molecular marker (Amersham).

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LY34 in a polyacrylamide gel. This technique takes advantage of detected small quantities of β-glucosidase by fluorescent band where MUG was degraded. The molecular weight of β-glucosidase was estimated to be 53 kDa by stained with Coomassie brilliant blue, this enzyme consisted in 468 amino acids and is encoding by *bglB* gene which is classified intro family 1.

3.4. Glycosyl hydrolase enzymes

The biological plasticity of glycosyl hydrolases is a consequence of the variety of β-glucosidic substrates that they can hydrolyze from disaccharides such as cellobiose and glycosides others. Three categories of cellulases; endoglucanases, exoglucanases, and β-glucosidases are produced by living organisms for the degradation of insoluble cellulose [28]. β-Glucosidases play an important role in the flavor formation of fruits, wine and sweet potato by the production of monoterpene alcohols such as linalool, alpha-terpeneol, citronellol, nerol, and geranol [29,30]. These monoterpene alcohols are non-volatile glycosides released by the action of β-glucosidases [31]. Supplementation with β-glucosidases from external sources may enhance aroma release, thus benefiting the winemaking process [32]. The hydrolytic activity of β-glucosidases permits their use in the medical field as antitumor agents, in biomedical research and in the food industry.

3.5. Proteome analyses

Performing cultures with the monosaccharide (glucose) and the disaccharide (cellobiose: glucose-β-1,4 glucose) the only difference between the cultures is the β-1,4 bound in the former carbon source. A proteomic analysis of these two culture conditions was performed.

As indicated by the different isozymes of β-glucosidase, induced by cellobiose in *Shewanella* sp. G5 are encoding by two gene *bgl-A* and *bgl* determinate previously [5], proteome re-arrangement can be expected by growth on different carbon sources.

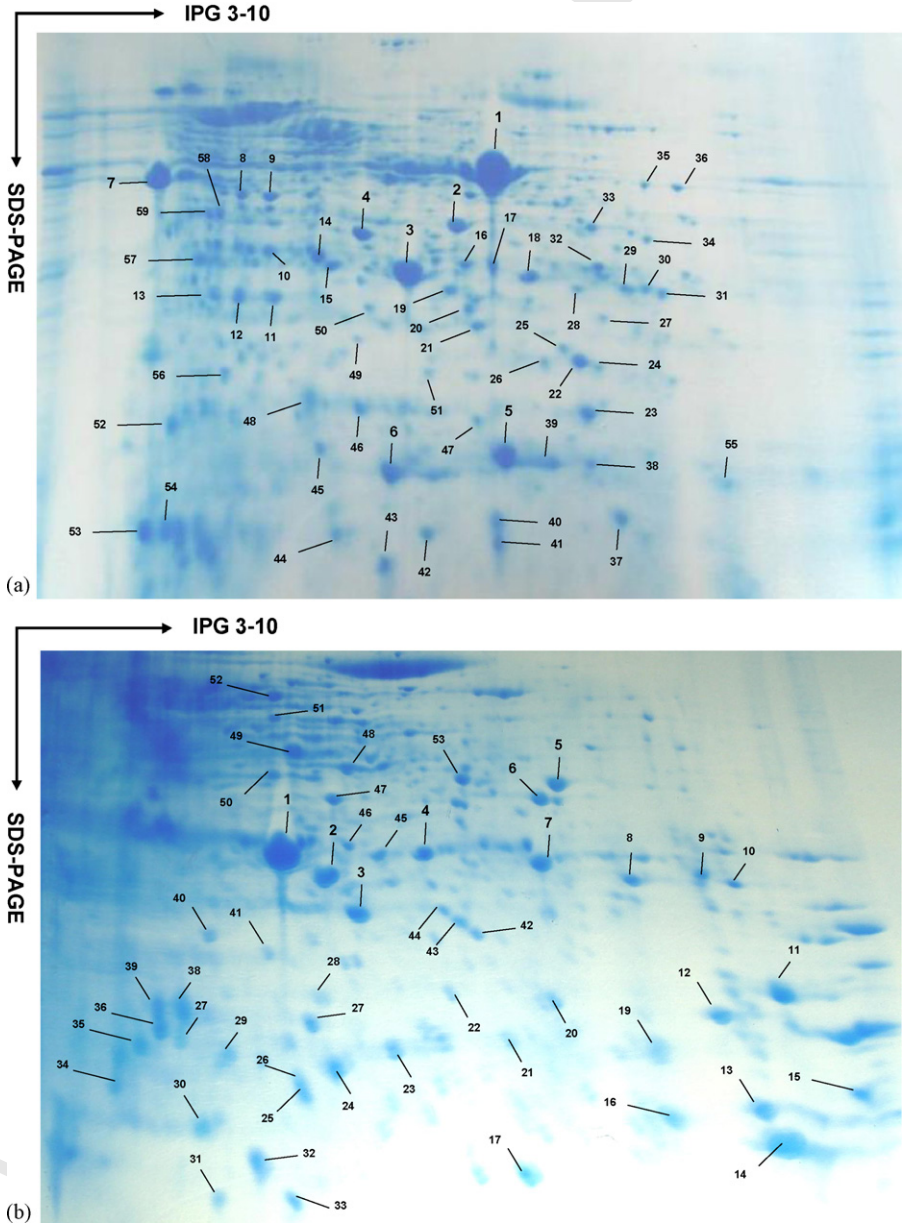

Fig. 4. Two-dimensional gel electrophoresis from *Shewanella* sp. G5. Spots numbers expressing each treatment are indicated in the gels. (a) 2D gel of intra-cellular protein grown at 15 °C on cellobiose; 1-Ce to 7-Ce spots were selected for identification. (b) 2D gel of intra-cellular protein grown at 15 °C on glucose; 1-Glu to 7-Glu spots were selected for identification.

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Table 2
Psychrophilic microorganisms, their products (cold enzymes) and their use in biotechnology and industry. Data modified from Burg [37] and Satyanarayana et al. [39].

Spot no.[a]	Protein identification on 2D from *Shewanella* sp. G5					
	Protein name[b]	No. acces[c]	Reference gi	Peptide sequences[d]	Ions[e]	XC[f]
1-Ce	L-Allo-threonine aldolase	NP_718892	24374849	K.GLC*APVGSLLLGDER.L	24/28	5,377
2-Ce	Immunogenic-related protein	NP_716093	24372051	R.QLDATLQSSGLGM#AAIR.D	27/32	5,524
3-Ce	Hypothetical protein SO4719	NP_720235	24376191	K.LGEQGDVDLVMTHAPSAEAK.F	26/38	6,640
4-Ce	Hypothetical protein SO1190	NP_716815	24372773	K.GEYLELVPITHPADIVENEPATLQFVYDGK.P	36/116	5,841
5-Ce	Aerobic respiration control protein	NP_719518	24375475	K.HFESLPDTPEIIATIHGEGYR.F	41/80	7,370
6-Ce	Ubiquinol-cytochrome c reductase	NP_716241	24372199	(R)FLTAATAVVGGAGAVAVAVPFIK	11 of 26	2.44
7-Ce	Bifunctional N-succinyldiaminopimelate-aminotransferase	NP_716250	24372208	R.VYFANSGAEANEAALK.L	27/30	6,905
1-Glu	Triosephosphate isomerase	NP_716825	24372783	NGTMAFDNAIIAYEPLWAVGTGK	20/44	4.85
2-Glu	Hypothetical protein SO_4719	NP_720235	24376191	LGEQGDVDLVMTHAPSAEAK	20/38	4.45
3-Glu	3-Oxoacyl-(acyl-carrier-protein) reductase	NP_718357	24374314	AIAETLVEAGAVVIGTATSEK	33/80	5.28
4-Glu	Thiol:disulfide interchange protein DsbA	NP_715973	24371931	ILAEKPAGVEFNQAHVDFIGK	29/80	4.54
5-Glu	Malate dehydrogenase	NP_716401	24372359	VAVLGAAGGIGQALALLLK	25/36	5.02
6-Glu	Chaperonin GroEL	NP_716337	24372295	AMLQDVAILTGGTVIAEEIGLELEK	23/48	4.37
7-Glu	Chaperone protein DnaK	NP_716751	24372709	ASSGLSEEEVAQMVR	18/28	4.14

[a] Spot name; Ce: spot from cellobiose, Glu: spot from glucose.
[b] Protein deduced from database of complete genome of *Shewanella oneidensis* obtained after MS analysis from each spot.
[c] Entry code of NCBI (National Center for Biotechnology Informations).
[d] Deduced peptide sequence obtained after MS analysis of the spots treated.
[e] Ions value tells you how many of the experimental ions matched with the theoretical ions for the peptide listed.
[f] XC value is the cross-correlation value from the search of the peptide.

Tsukada et al. [33] reported the expression of two genes, *bgl1A* and *bgl1B*, which encode for two β-glucosidase classified in the family 1. Both enzymes are produced by *Phanerochaete chrysosporium* and their expressions were monitored by RT-PCR with cellobiose and glucose in the culture medium; which *bgl1A* was expressed in both media and *bgl1B* was repressed in the presence of glucose.

To determine the expression of different enzymes, such as β-glucosidase, *Shewanella* sp. G5 was grown at 15 °C. In addition, earlier investigations failed to taken into account the induction of protein expression at moderate low temperature, which was also evident from the β-glucosidase investigation performed. Thus, we used LB medium with glucose and cellobiose to investigate proteomic changes by 2D gel electrophoresis.

The proteins of cellular extracts of *Shewanella* sp. G5 were solubilized efficiently using the re-hydratation buffer that contained two chaotropic, reducing agents and detergents (thiourea, urea, DTT and CHAPS) according to Chinnasamy et al. [11], increasing the protein spots and obtaining few horizontal streaks (Fig. 4).

Fig. 4 displays the overall 2D pattern of protein extracts of cellobiose and glucose from *Shewanella* sp. G5 cultures. The total intra-cellular proteins from *Shewanella* sp. G5 extracts were identified using combinations of the major steps of the 2D-MS workflow methods. The digestion of in-gel protein spots was performed by trypsin treatment and identify by matrix-assisted laser desorption ionization [19]. The gels resolved 59 and 55 spots induced by cellobiose (Fig. 4a) and glucose (Fig. 4b), respectively. Thus, a substantial re-arrangement of protein expression can be observed by different carbon sources at low temperature conditions. Fig. 4 displays the overall 2D pattern of protein extracts of cellobiose and glucose from cultures of *Shewanella* sp. G5. As examples for the protein spots intensity alteration in the cellobiose culture samples, a portions of the gel images is shown in Fig. 4a and b the proteins spots l to 7 showed obviously regulated over expression and were selected to identify. These results illustrated that intra-cellular proteins were significantly changed after supplementing with carbon source cell culture.

3.6. Mass spectrometry identification

Seven prominent spots differentially expressed were analyzed by mass spectrometry and identified using the *S. oneidensis* MR-1 genome data base (Table 2). In our study, were analyzed a total of 59 (Fig. 4a) and 53 (Fig. 4b) spots from cellobiose and glucose extract, Q2 respectively. L-Allo-threonine aldolase, immunogenic-related protein, hypothetical protein SO4719, hypothetical protein SO1190, Aerobic respiration control protein, ubiquinol-cytochrome c reductase, N-succinyldiaminopimelate-aminotransferase, triosephosphate isomerase, S-adenosyl-L-homocysteine hydrolase, thiol disulfide interchange protein, malate dehydrogenase, GroEL_like type I chaperonin and chaperone protein DnaK were identified using NCBI (http://www.ncbi.nlm.nih.gov/) and CMP (Comprehensive Microbial Resource, web site; http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi) databases. The main characteristics of these proteins are described in Table 3 and the possible cellular roles of these proteins identified are shown in Table 4. Gao et al. [34] reported that over 41% of the *S. oneidensis* ORFs encode hypothetical proteins. Little information is available concerning the impact of cold stress on cellular metabolism at the genomic level. Two published DNA microarray studies of two different cold-shocked *Bacillus subtilis* strains revealed a global down-regulation of metabolically relevant proteins. This concept was in agreement with earlier proteomic studies.

The importance in the determination data in the protein sequences of the genome have amplified the number and reliability of protein identifications in proteomic studies; 560 proteins have been identified from *Methanococcoides burtonii* cells growing at 4 °C, and 44 proteins have been shown to be differentially expressed from *M. burtonii* cells growing at 4 and 23 °C [35]. In addition to being a resource for identifying proteins, the physical organization of genes in the genome sequence has provided insight into associated gene partners and gene regulation and allowed inference of the function of expressed hypothetical proteins. Many findings from proteomic studies have shown that cold adaptation involves proteins that they have an important role in transcription, protein folding, protein transport and metabolism [36].

3.7. Importance of the enzymes applications in biotechnology

Driven by increasing industrial demands for biocatalysts that can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzymes. As a result, the characterizations of microorganisms able of thrive in extreme environments such as extremophiles, a valuable source of novel

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Table 3
Protein identified from intra-cellular fractions of *Shewanella* sp. G5 grown on glucose or cellobiose at 15 °C.

Putative identification[a]	Role of hypothetical proteins identified in this study by ES-MS				
	Locus name[b]	Protein length[c]	Molecular weight[d]	pI[e]	Cellular role[f]
L-Allo-threonine aldolase	SO_3338	337	36269.28	5.378	Catalyzes the conversion of L-threonine or L-allo-threonine to glycine and acetaldehyde in a secondary glycine biosynthetic pathway
Immunogenic-related protein	SO_0456	318	33532.42	8.617	Bacterial periplasmic transport systems use periplasmic binding proteins (PBPs) to transport a wide variety of substrates, such as, amino acids, peptides, sugars, vitamins and inorganic ions. PBPs have two cell-membrane translocation functions: bind subst
Conserved hypothetical protein SO4719	SO_4719	274	29366.60	6.978	Bacterial periplasmic transport systems use membrane-bound complexes to transport a wide variety of substrates, such as, amino acids, peptides, sugars, vitamins and inorganic ions
Conserved hypothetical protein SO1190	SO_1190	272	29973.11	6.964	ABC-type Co2+ transport system, periplasmic component [inorganic ion transport and metabolism]
Aerobic respiration control protein	SO_3988	238	27219.91	5.437	Bacteria use two-component signal transduction systems to detect and respond to changes in the environment. The system consists of a sensor histidine kinase and a response regulator. The former autophosphorylates in a histidine residue on detecting an ext
Ubiquinol-cytochrome c reductase	SO_0608	196	20904.93	7.965	Iron-sulfur protein (ISP) component of the bc(1) complex family, Rieske domain; This domain is a [2Fe-2S] cluster binding domain involved in electron transfer. The cytochrome bc(1) and b6f complexes are central components of the respiratory and photosynthe
Acetylornithine transaminase	SO_0617	405	43214.20	5.423	Acetyl ornithine aminotransferase family. This family belongs to pyridoxal phosphate dependent aspartate aminotransferase superfamily. The enzymes act on basic amino acids and their derivatives are involved in transamination or decarboxylation
Triosephosphate isomerase (TIM)	SO_1200	260	28072.97	5.732	TIM is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The reaction is very efficient and requires neither cofactors nor metal ions
Conserved hypothetical protein SO4719	SO_4719	274	29366.60	6.978	Bacterial periplasmic transport systems use membrane-bound complexes to transport a wide variety of substrates, such as, amino acids, peptides, sugars, vitamins and inorganic ions
S-adenosyl-L-homocysteine hydrolase	SO_2776	248	26381.21	4.904	AdoHycase catalyzes the hydrolysis of S-adenosyl-L-homocysteine to form adenosine and homocysteine. AdoHycase plays a critical role in the modulation of the activity of various methyltransferases. The enzyme forms homooligomers of 45–50 kDa subunits, each
Thiol:disulfide interchange protein (DsbA)	SO_0333	202	22303.70	8.209	DsbA is a monomeric thiol disulfide oxidoreductase protein containing a redox active CXXC motif imbedded in a TRX fold. It is involved in the oxidative protein folding pathway in prokaryotes, and is the strongest thiol oxidant known, due to the unusual s
Malate dehydrogenases (MDH)	SO_0770	311	32137.13	5.183	MDH is one of the key enzymes in the citric acid cycle, facilitating both the conversion of malate to oxaloacetate and replenishing levels of oxaloacetate by reductive carboxylation of pyruvate
GroEL_like type I chaperonin	SO_0704	545	57079.38	4.559	Chaperonins are involved in productive folding of proteins. With the aid of cochaperonin GroES, GroEL encapsulates non-native substrate proteins inside the cavity of the GroEL-ES complex and promotes folding by using energy derived from ATP hydrolysis
Chaperone protein DnaK	SO_1126	639	68861.42	4.505	Protein fate: protein folding and stabilization. Molecular chaperone DnaK; provisional

[a] Putative protein identified from the database of *S. oneidensis* MR-1 incorporated into the MS.
[b] Locus of gene encoding the hypothetical protein identified. Data from the NCBI database.
[c] Real length of the protein identified in this study, data from the CMR database.
[d] Real molecular weight of the protein identified in this study; data from the CMR database.
[e] Real isoelectric point of the protein identified in this study, data from the CMR database.
[f] Real function of protein mentioned, cellular role in the metabolism of the cell; data from the NCBI database.

enzymes, has received a great deal of attention, notwithstanding the fact that to date more than 3000 different enzymes have been identified and many of them have found their way into biotechnological and industrial applications [37,38].

If today the annual market for thermostable enzymes represents 250 millions dollars, it is likely that the potential value of cold adapted enzymes increase their search by the industry. These enzymes afford significant advantages at the level of their specific activity, lower stability and unusual specificity. Some of the more obvious applications are shown in Table 4. Cold-active enzymes are not only of extraordinary interest at the basic level to investigate the thermodynamic stability of proteins, but also to understand the relationship between stability, flexibility or plasticity and their catalytic efficiency [2,39].

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Table 4
Role cellular of protein identified from *Shewanella* sp. G5 grown on glucose or cellobiose at 15 °C.

Potential applications of psychrophilic bacteria in biotechnology[a]	
Enzymes	Use
Alkaline phosphatase	Molecular biology
Proteases, lipases, cellulases, and α-amylases	As additives in detergents
Lipases and proteases	Cheese manufacture
Proteases	Meat industry (food applications), contact-lens cleaning solutions and meat tenderizing
Amylases, proteases and xylanases	Food industry (baking processes)
Polyunsaturated fatty acids	Food additives and dietary supplements
β-Galactosidase	Food industry (lactose hydrolysis in milk products)
β-Glucosidase and α-rhamnosidase	Food additives (wine an citric juice)
Pectinases	Fruit juice industry
Cellulases	Detergents, feed and textile industry (biopolishing and stone-washing processes)
Dehydrogenases	Biosensors
Lipases	Detergents, food and cosmetics
Ice nucleating proteins	Artificial snow, ice cream, other freezing applications in the food industry
Ice minus microorganisms	Frost protectants for sensitive plants
Various enzymes (e.g. dehydrogenases)	Biotransformations
Various enzymes (e.g. oxidases)	Bioremediation, environmental biosensors
Various enzymes	Modifying flavors
Methanogens	Methane production

[a] Modified from Burg [37], Gerday et al. [2] and Satyanarayana et al. [39].

4. Conclusions

It was found that the psychrotolerant *Shewanella* sp. G5 producer of two isoenzymes β-glucosidase induced by cellobiose. A direct activity staining technique (MUG-PAGE) that allows rapid and specific detection of β-glucosidase from the crude extract of *Shewanella* sp. G5; we assay a zymogram to establish the molecular weight approximate of 75 and 140 kDa. Specific activity of β-glucosidase was tested in fractions cellular, observed that the effect of source of carbon in cultures growth, cellobiose induces the enzyme but the activity exhibit a high tolerance to glucose inhibition. Performing with these cultures, a proteomic analysis of these two culture conditions was performed. Thus, a substantial re-arrangement of protein expression can be observed by 2D gels, where the protein spots intensity alteration in the cellobiose culture samples showed obviously regulated over expression and were selected to identify.

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