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ORIGINAL ARTICLE

Quantification of the Genetic Expression of *bgl-A*, *bgl*, and *CspA* and Enzymatic Characterization of β-Glucosidases from *Shewanella* sp. G5

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Abstract *Shewanella* sp. G5, a psychrotolerant marine bacterium, has a cold-shock protein (*CspA*) and three β-glucosidases, two of which were classified in the glycosyl hydrolase families 1 and 3 and are encoded by *bgl-A* and *bgl* genes, respectively. *Shewanella* sp. G5 was cultured on Luria-Bertani (LB) and Mineral Medium Brunner (MMB) media with glucose and cellobiose at various temperatures and pH 6 and 8. Relative quantification of the expression levels of all three genes was studied by real-time PCR with the comparative Ct method ($2^{-\Delta\Delta C_t}$) using the *gyrB* housekeeping gene as a normalizer. Results showed that the genes had remarkably different genetic expression levels under the conditions evaluated, with increased expression of all genes obtained on MMB with cellobiose at 30 °C. Specific growth rate and specific β-glucosidase activity were also determined for all the culture conditions. *Shewanella* sp. G5 was able to grow on both media at 4 °C, showing the maximum specific growth rate on LB with cellobiose at 37 °C. The specific β-glucosidase activity obtained on MMB with cellobiose at 30 °C was 25 to 50 % higher than for all other conditions. At pH 8, relative activity was 34, 60, and 63 % higher at 30 °C than at 10 °C, with three peaks at 10, 25, and 37 °C on both media. Enzyme activity increased by 61 and 47 % in the presence of Ca^{2+} and by 24 and 31 % in the presence of Mg^{2+} on LB and MMB at 30 °C, respectively, but it was totally inhibited by Hg^{2+}, Cu^{2+}, and EDTA. Moreover, this activity was slightly decreased by SDS, Zn^{2+}, and DTT, all at 5 mM. Ethanol (14 % *v*/*v*) and glucose (100 mM) also reduced the activity by 63 and 60 %, respectively.

Keywords β-glucosidases · Marine bacteria · Biotechnology industry · Real-time PCR · *Shewanella*

Carlos Mauricio Abate passed away during the preparation of the study.

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Introduction

Marine bacteria exhibit a wide range of enzymes with great potential as novel biocatalysts for many biotechnological processes (Kennedy et al. 2008; Cristóbal et al. 2013). In that sense, food industry has shown increasing interest in enzymes, and in particular, the enological sector has focused its attention on pectinases and glycosidases. Glycosidases promote the release of wine aroma via a hydrolysis mechanism of glycosidic aroma precursors (e.g. terpene glycosides) that are responsible for the varietal character of many grapes (Barbagallo et al. 2004). The enzymatic hydrolysis of glycosides occurs sequentially in two stages. In the first one, the intersugar bond is cleaved by a rhamnosidase (Rha, EC 3.2.1.40) and an arabinosidase (Ara, EC 3.2.1.55), and in the second, the β-glucosidase (βG, EC 3.2.1.21) releases aglycone, which is responsible for wine aroma intensification (Barbagallo et al. 2004). The Ara and βG combination can also be utilized in citrus juice technology, particularly in debittering by naringin hydrolysis (Spagna 2000), and in food processing for the

aromatization of fruit juices extracted from passion fruit, apple, apricot, peach, tomato, pineapple, cherry, pear, papaya, and banana (Iwashita et al. 1999; Spagna et al. 1998). βGs are a homogeneous constitutive group of enzymes found in several organisms able to perform different functions. In bacteria and fungi, βGs are part of the cellulase system catalyzing the final degradation step of cellulose and xylan hydrolysis, both plant polymers (Faure 2002; Murray et al. 2004). Based on amino acid similarity, βGs have been divided into two glycosyl hydrolase families (GHFs): 1 and 3 (Bourne and Henrissat 2001; Tsukada et al. 2006). Both of them hydrolyze their substrate with a net retention of the anomeric carbon configuration (Murray et al. 2004). Due to their great hydrolytic activity, βGs are important for the applications in medicine: They are used in general biomedical research as antitumor agents and to display transglycosylase activity (Bhatia et al. 2002; Cournoyer and Faure 2003; Murray et al. 2004). Cournoyer and Faure (2003) remarked that the prevalence of open reading frames (ORFs) homologous to genes encoding GHF 1 and 3 enzymes in the majority of genomes analyzed makes biochemical and physiological studies of microbial βGs even more necessary.

In the last years, a great interest on the investigation of several cold-adapted organisms was focused on the synthesis of high levels of different specific proteins that are induced at low temperature, called cold-shock proteins (CSPs) (Gao et al. 2006). In cold-adapted microorganisms, this family of bacterial major CSPs is characterized by a conserved sequence of 65–75 amino acid residues long (Lopez and Makhatadze 2000). Therefore, the observed roles of CSPs are different, such as in the process of translation at low temperature and as regulatory proteins of other cold-induced proteins. This shows the importance of studying them in these novel aspects: the functions and potential applications in food industry (D'Amico et al. 2006). In psychrophilic microorganisms, a cold-shock protein A (*CspA*) gene encodes a 70-kDa protein which is expressed at 10 °C and repressed at 30 °C (Gao et al. 2006).

Real-time quantitative reverse transcription PCR (RT-qPCR) has been accepted as one of the most powerful and sensitive techniques to analyze gene expression, since it allows the accurate quantitative detection of mRNA levels (Botteldoorn et al. 2006; Sellars et al. 2007). Usually, in RT-qPCR assays, one of two standard procedures is followed: absolute or relative quantification (Sellars et al. 2007; Liu et al. 2009). The latter is better to evaluate gene expression under different conditions since it allows the comparison of mRNA transcription levels in a test sample to the equivalent in a calibrator sample (Nolan et al. 2006; Livak and Schmittgen 2001; Strube et al. 2008). In order to perform accurate gene quantification analysis, the normalization of qPCR data is essential to eliminate template variations between samples derived from differences in initial sample amounts (Ellefsen et al. 2008; Theis et al. 2007). To date, normalization is most frequently achieved by the use of internal controls, often referred to as housekeeping genes (Theis et al. 2007; Nielsen and Boye 2005; Vandesompele et al. 2002).

Shewanella sp. G5, a psychrotolerant bacterium used in this study, was isolated from the intestines of benthonic organisms (*Munida subrrugosa*) from Beagle Channel, Argentina. The production of two cold-active βGs (EF141823 and DQ136044), coming from this strain, was partially characterized in previous work (Cristóbal et al. 2008, 2009). The aims of this study were (i) to detect the highest gene expression level of *bglA*, *bgl*, and *CspA* genes in response to different culture conditions using RT-qPCR method in order to select the optimal ones for the production of β-glucosidase and (ii) to complete the characterization the β-glucosidase activity produced under those conditions in the presence of different chemical species and reagents. This information becomes of essential importance, and in fact, it is the initial step when attempting to optimize enzymatic production and reaction at an industrial scale.

Materials and Methods

Culture Conditions

Shewanella sp. G5 was cultured in liquid Luria-Bertani medium (LB) supplemented with 20 g/l of sodium chloride and 10 g/l of cellobiose (LBC) or glucose (LBG) as carbon source and on liquid Mineral Medium Brunner (MMB), DSMZ (2012), modified by the addition of 20 g/l of sodium chloride and 10 g/l of cellobiose (MMBC) or glucose (MMBG).

One-liter flasks containing 300 ml of the aforementioned media were inoculated with 100 μl of fresh cultures obtained overnight on the same media and incubated in an orbital shaker (150 rpm) at 10 and 30 °C respectively (Table 1). Growth was monitored with a Beckman (DU®640) spectrophotometer until an approximate 0.8 absorbance was obtained. *Shewanella* sp. G5 specific growth rate (μ, in h^{-1}) was determined on liquid LBC, MMBC, LBG, and MMBG media incubated at 4, 10, 15, 20, 25, and 37 °C. Absorbance values were transformed to Ln(absorbance) so that specific growth rates could finally be calculated by fitting the growth curves with the equation described by Nerbrink et al. (1999).

From the LBC10, MMBC10, LBG30, and MMBG30 cultures, 2 ml (in triplicate) was extracted to isolate the RNA that was later used for gene expression quantification. From the remaining volume, biomass was separated from the supernatant, which was discarded, and the cells were used to measure the enzymatic activity. Once the best conditions were determined, they were replicated to produce the enzymes that were later characterized in the presence of different chemical agents.

Table 1 Conditions for the growth of *Shewanella* sp. G5 in an orbital shaker at 150 rpm, on Luria-Bertani medium (LB) using cellobiose (LBC) or glucose (LBG) as carbon source, and on liquid Mineral Medium Brunner (MMB) using cellobiose (MMBC) or glucose (MMBG) as carbon source

Conditions	Culture Media	Carbon source	Temperature (°C)
LBC10	LB	Cellobiose	10
LBG10	LB	Glucose	
MMBC10	MMB	Cellobiose	
MMBG10	MMB	Glucose	
LBC30	LB	Cellobiose	30
LBG30	LB	Glucose	
MMBC30	MMB	Cellobiose	
MMBG30	MMB	Glucose	

The number 10 or 30 in Conditions nomenclature is indicative of the temperature

Enzyme Activity Assay

Shewanella sp. G5 cultures previously obtained were centrifuged for 15 min at 33,000×*g* to collect cells. Pellets were suspended in 3 ml of distilled water and disrupted in a French Press (SLM Instruments) at 25,000 psi. Intracellular protein (IP) was separated from the cell debris by centrifugation for 15 min at 30,000×*g* and used to measure β-glucosidase activity. The activity was assayed with 100 μl of IP, 15 μl of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, 0.1 M), and 900 μl of 0.1 M potassium phosphate buffer at pH 6 and 8 and incubated at 37 °C for 1 h (Cristóbal et al. 2008). Absorbance of *p*-nitrophenol released during the reaction was monitored spectrophotometrically at 420 nm. Enzymatic activity was calculated using the extinction coefficient of *p*-nitrophenol ($\varepsilon_{420nm} = 1.6 \times 10^4$ M/cm) and one enzyme unit was defined as *the amount of enzyme required for the hydrolysis of 1 μmol of substrate per minute under experimental conditions* (Cristóbal et al. 2009). Specific activity was calculated as enzyme activity per mass unit of protein, which was determined using the Bradford method (Bradford 1976). Relative activity was calculated as the percentage of the maximum values obtained at 37 °C. All analyses were performed in triplicate.

Effect of Metals, Reagents, Glucose, and Ethanol on Enzyme Activity

We also assessed how βG activity was affected by various metallic monovalent and divalent ions of: $HgCl_2$, $CuCl_2$, NaCl, KCl, $CaCl_2$, $MgCl_2$, and $ZnCl_2$ and by reagents: sodium dodecyl-sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT), at 5 mM. Similarly, we studied the effect of different concentrations of glucose (5, 10, 15, 25, 50, and 100 mM) and ethanol (5, 7, 9, 11, 12, and 15 % *v*/*v*) on enzymatic activity. For all the assays, the reaction mixture was prepared in 0.1 M Tris–HCl buffer at pH 8 and incubated at 37 °C for 1 h. The reaction was interrupted with the addition of 100 μl of $CaCO_3$ 1 M, and absorbance was measured at 420 nm. Enzymatic activity was expressed as relative activity, thus as a percentage of the value determined in the absence of metal ions, reagents, glucose, and ethanol, used as a reference. All analyses were performed in triplicate.

RNA Extraction and cDNA Synthesis

Total RNA was extracted, in triplicate, from the biomass of each 2 ml sample of *Shewanella* sp. G5 liquid cultures (with absorbance being approximately 0.8) using the RNA Isolation System Kit (Promega), according to the manufacturer's protocol. From each sample, 50 μl of total RNA solution was obtained and treated by DNase provided by the manufacturer. The integrity of total RNA was verified by electrophoresis in a 1 % (*w*/*v*) agarose gel (data not shown), and RNA concentration was determined by spectrophotometry at 260 nm. Reverse-transcription (RT) was performed in a 24-μl reaction volume using 10 μl of RNA and 2 μl of random primers. The preparation was incubated at 70 °C for 5 min and cooled down rapidly to 4 °C, allowing the primers to anneal. Then, 5 μl of 5× buffer (Promega), 4 μl of dNTP (10 μM), and 3 μl of enzyme (AMV 10 U, Promega) were added, and the reaction mixture was homogenized and incubated at 37 °C for 1 h. This prolonged incubation was critical for cDNA to be successfully synthesized from *Shewanella* sp. G5 cultures. This final step allowed the primers to anneal, and the prolonged incubation was indeed critical for cDNA to be successfully synthesized from *Shewanella* sp. G5 cultures. To minimize potential effects of differential synthesis efficiency during RT reaction, three separate RT assays were carried out and pooled for each analyzed RNA sample. All cDNA solutions were stored at −20 °C until use for qPCR.

Design of Oligonucleotide Primers for qPCR

Optimal primers are essential to ensure that only a single specific PCR product is amplified. Primer sequences for *Shewanella* sp. G5 were designed for qPCR based on the sequences of the *bgl-A* (DQ136044), *bgl* (EF141823), and *gyrB* (DQ268831) genes, which encode for β-glucosidase A, β-glucosidase, and gyrase subunit B, respectively. For *CspA*, primer sequences were designed for *Shewanella baltica* OS155 (CP001252). Universal primers for 16S rDNA, selected from the literature, were also used (Muyzer et al. 1993). The 16S rDNA and *gyrB* genes were used as internal controls of the constitutive expression. All primers (Table 2) were designed using DNA-MAN software version 4.03 (Lynnon BioSoft, Vandreuil, QB, Canada) and evaluated *on-line* with the in silico simulation of the molecular biology experiments

Table 2 Specific oligonucleotide primers used to amplify *blg-A*, *bgl*, *CspA*, *gyrB*, and 16S rDNA genes from *Shewanella* sp. G5

Target gene	Locus access (NCBI)[a]	Primer name	5′ to 3′ sequence	Tm (GC+AT) (°C)[b]	PCR product (pb)[c]	Reference
bgl-A	DQ136044	F-gh1 R-gh1	GCATTAGCGCCAGAAGACAGA ATAGGTTTGATTTAAAGAAAC	64	218	This study
bgl	EF141823	F-gh3 R-gh3	ATCACGGTAATCCTTATTTATT CTTGCGCGATAGTGTTTTCATA	62	204	
CspA	CP000961	F-cspA R-cspA	TTACTGGTGTTGTTAAGTGGTTCA TTACGTTTTCAGCTTGTGGACC	64	186	
gyrB	DQ268831	F-gyrB R-gyrB	TTTCCGTAGTGCGTTGACACGT GGTTTTCCAGCAGATAATCGTTC	66	242	
16S rDNA	AY398666	F357 R518	ACTCCTACGGAGGGCAGCAG ACGTATTACCGCGGCTGCTGG	57	200	(Muyzer et al. 1993)

[a] NCBI database: National Center for Biotechnology Information (Web site, http://www.ncbi.nlm.nih.gov/)

[b] Melting temperature (Tm) of the primers according to DNA-MAN (version 4.03) software

[c] PCR product tested on-line with the in silico PCR program for *Shewanella baltica* OS155 (CP001252)

program (http://insilico.ehu.es/). The predicted primer melting temperatures (*Tm*) ranged from 55 to 60 °C. The ideal amplicon sizes were about 200 bp, with upper limit of 250 bp.

Evaluation of All Primers Designed with ConvSentional PCR

Extraction of genomic DNA (gDNA) from *Shewanella* sp. G5 was carried out according to a protocol previously described (Cristóbal et al. 2008). The *bgl-A*, *bgl*, *CspA*, *gyrB*, and 16S rDNA genes were amplified by conventional PCR using specific primers (Table 2). Amplification of each gene was performed in 25 μl of reaction mixture which contained 1 μl of gDNA, 5 μl of GoTaq 5× buffer (Promega), 0.5 μl of the corresponding forward and reverse primers (33 μM), 0.4 μl of bovine serum albumin (BSA, 10 mg/ml), 0.2 μl of Go-Taq (Promega), and water to complete the volume. PCR conditions for all genes consisted of an initial denaturation step of 4 min at 94 °C, followed by 30 amplification cycles, which comprised a denaturation step of 1 min at 94 °C, annealing at 56 °C for 1 min, and extension at 72 °C for 30 s. Reactions were completed in additional 5 min at 72 °C, followed by cooling down to 4 °C. Amplification reactions were carried out in an automated thermal cycler (Perkin-Elmer, model 9700). PCR products were separated by 1.0 % (*w*/*v*) agarose gel electrophoresis, stained with ethidium bromide (10 mg/ml), and then visualized using an Image Analyzer Gel Doc *BIO RAD*.

Real-Time PCR

Real-time PCR (qPCR) was performed in a thermal cycler (GeneAmp® 5700 Sequence Detection System Applied Biosystems, USA) using SYBR Green stain that binds double-stranded DNA. The qPCR reactions were carried out in a total volume of 25 μl that contained 5 μl of cDNA, 1 μl of each forward and reverse primers (33 μM), 12.5 μl of SYBR Green ER™ master mix (qPCR SuperMix kit for ABI PRISM, Invitrogen), and water to complete the volume. The qPCR amplification was performed under the following conditions: incubation at 50 °C for 2 min (uracil-*N*-glycosylase activation) and at 95 °C for 10 min (polymerase activation), and 40 cycles at 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min. After the last amplification, a dissociation step from 60 to 95 °C (0.3 °C/s) was performed to obtain the melting curves. Each sample was analyzed by triplicate; negative controls with water instead of cDNA templates were included in all qPCR plates. The threshold cycle value (*Ct*), which is the number of cycles required to reach the threshold fluorescence value, was employed to calculate the expression level of each gene under study. The data expressed as *Ct* were exported to a Microsoft Excel data sheet for subsequent analysis.

Previous to the relative quantification, standard curves for each amplification system were generated, using 10-fold serial dilutions of the cDNA pool, in order to evaluate the efficiency of amplification and the dynamic range and sensitivity of the systems. The amplification efficiency (*E,* in percentage) was calculated as:

$$E = \left[10\left(-1/\text{slope}\right)-1\right] \times 100 \quad (1)$$

The standard curves were used to quantify the genes analyzed in all the samples of *Shewanella* sp. G5 grown under different culture conditions.

Relative Quantification and Data Analysis

The 16S rDNA and *gyrB* genes were used as endogenous references and simultaneously amplified with the target genes

in order to quantify and normalize gene expression. Target concentrations were adjusted and normalized by dividing the average concentration of the target gene by the average concentration of the reference DNA. The average *Ct* values were used to calculate relative transcript quantification using the comparative *Ct* method ($2^{-\Delta\Delta Ct}$) described by Livak and Schmittgen (2001). This was possible since the amplification efficiencies of both the reference and target genes were similar. Each target was normalized against the *gyrB* gene. Relative quantification was expressed as the $2^{-\Delta\Delta Ct}$ value (Livak and Schmittgen 2001; Nielsen and Boye 2005).

Results and Discussion

Specific Growth Rate of *Shewanella* sp. G5 after Physiological Changes

Within the temperature range assayed, *Shewanella* sp. G5 specific growth rates (μ, in h^{-1}) varied significantly depending on the culture media: Luria-Bertani (LB) or Mineral Medium Brunner (MMB), and on the carbon source: cellobiose or glucose (Fig. 1). *Shewanella* sp. G5 showed the highest μ at 37 °C on both culture media (LBC: 0.48 h^{-1} and MMBC: 0.38 h^{-1}; LBG: 0.41 h^{-1} and MMBG: 0,31 h^{-1}) (Fig. 1), having recorded the highest value for LB at all temperatures assayed, especially with cellobiose as carbon source.

Pakchung et al. (2006) reported typical growth curves of cold-adapted *Shewanella* species when grown at 4 °C. They observed an increase in cell density in *Shewanella benthica* and *Shewanella gelidimarina* cultures, but when the strains were grown at 20 or 37 °C, such increase was not evident. *Shewanella* sp. G5 presented higher μ values with cellobiose than with glucose as a carbon source when grown on LB medium, with the highest values being also found at 37 °C (Cristóbal et al. 2008). Microbial growth reached the stationary phase after 9 and 39 h of incubation at 37 and 4 °C, respectively, in LBC medium. However, the final cell density (OD_{540nm}) obtained were 0.73 and 0.95 at 37 and 4 °C, respectively. On MMBC medium, the G5 strain reached the stationary phase after 22 and 59 h at 37 and 4 °C, respectively, and cell density (OD_{540nm}) was again higher at 4 °C (1.5) than at 37 °C (1.3). Our studies revealed that *Shewanella* sp. G5 was able to grow within a wide temperature range (4 to 37 °C) on both rich (LB) and saline (MMB) media through assimilation of cellobiose or glucose as carbon sources (Cristóbal et al. 2009).

Influence of Culture Medium on Enzyme Production

Shewanella sp. G5 was grown on different culture media and conditions (Table 1). The intracellular protein (IP) was then extracted from each culture and β-glucosidase activity was measured. Enzyme production obtained under MMBC30 conditions (MMB medium, with cellobiose as carbon source, at 30 °C) was 25 to 50 % higher than under all the other culture conditions (Fig. 2a).

In this work, temperature did not show a significant effect on enzyme production, whereas carbon source did have a major impact. The presence of cellobiose inducing the enzyme synthesis increased the production. However, although it was lower, enzymatic activity was also observed when glucose was used as a sole carbon source, showing that βG is a constitutive enzyme. Villas-Bôas et al. (2006) and Yang et al. (2008) reported that different inducers, such as carbon source or metabolites, led to the production of several glycosidases.

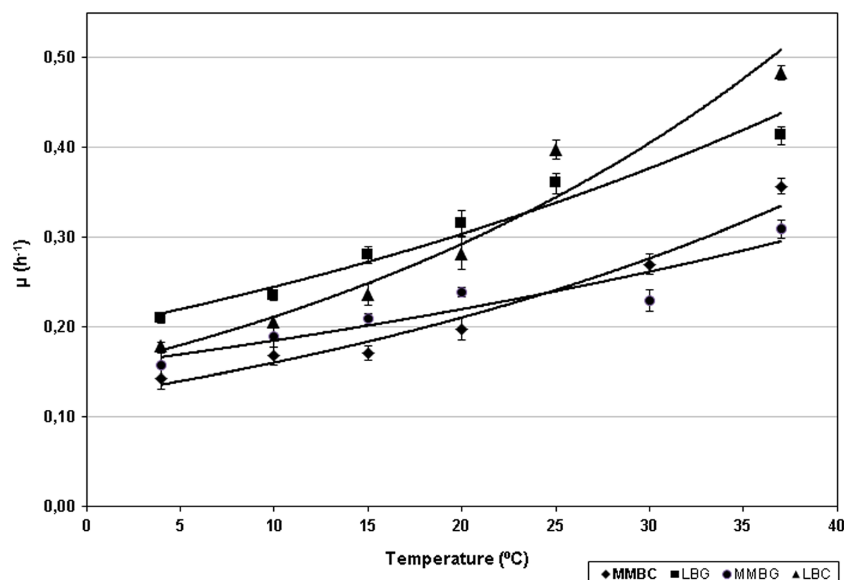
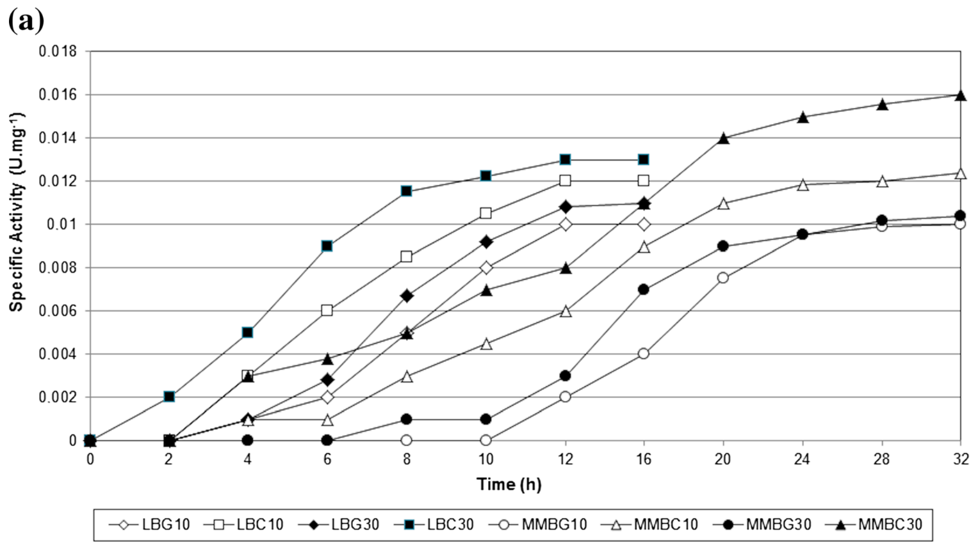
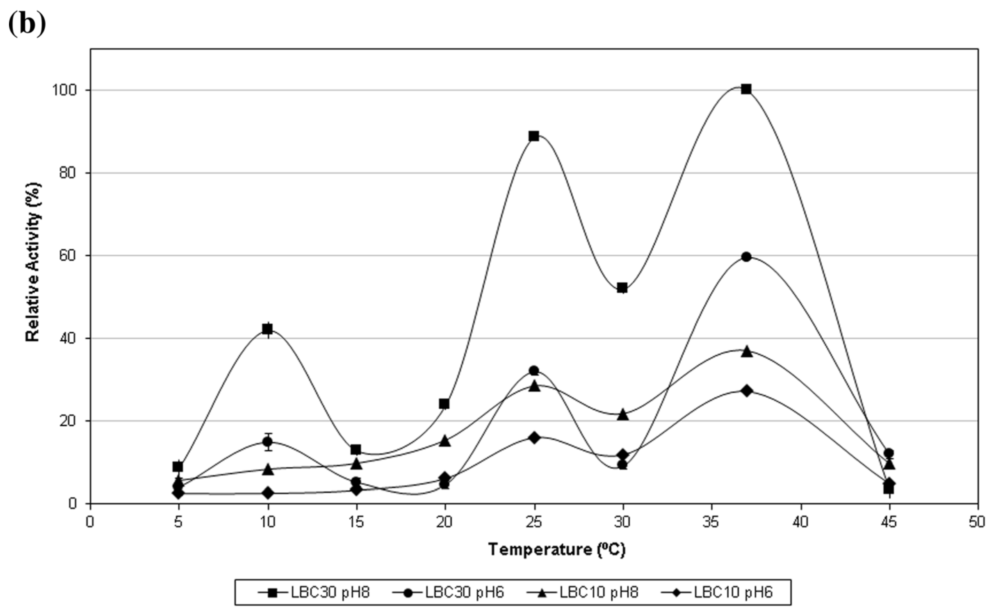


Fig. 1 Specific growth rate (μ, in h^{-1}) of *Shewanella* sp. G5 cultured on LBC, MMBC, LBG, and MMBG at pH 7 and different temperatures

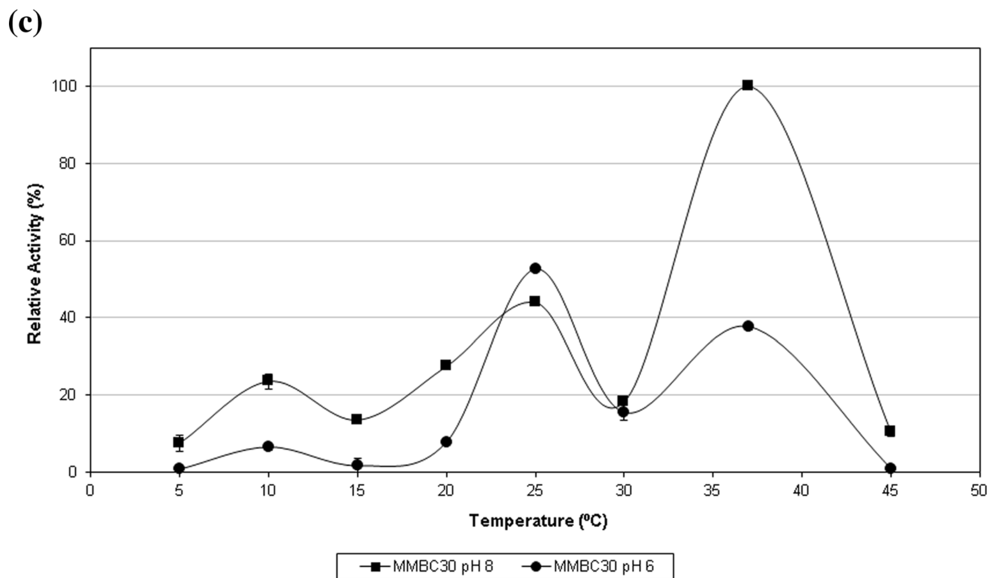
(a)



(b)



(c)



◀ **Fig. 2** β-glucosidase activity in *Shewanella* sp. G5: **a** specific enzyme activity produced under different culture conditions (see Table 1). Relative activity when grown on **b** LBC and **c** MMBC media at 10 and 30 °C measured at different temperatures (5–45 °C) and pHs (6 and 8). Experiments were carried out in duplicate; the deviation bars are too small to be seen

Effect of Temperature on ß-Glucosidase Activity

The highest activity of the IP from all the culture conditions studied was obtained at 37 °C; therefore, that value was used as a reference for calculating relative activity. In general, βG production was remarkably higher on LBC (Fig. 2b) than on MMBC (Fig. 2c) at 30 °C, at both pH studied (6 and 8), even though the curve patterns were the same, showing three peaks at 10, 25, and 37 °C. Instead, only the last two ones were found when the bacteria were cultured on LBC at 10 °C (Fig. 2b). At this temperature, the enzyme production on MMBC was so low that it could not be appreciated at same scale as displayed in Fig. 2c. On both media (LBC and MMBC), relative activity was higher at pH 8.

This study confirmed what was suggested in previous works, abased on zymogram assays performed after growing *Shewanella* sp. G5 on cellobiose (Fig. 2b, c): the possibility that three isoenzymes could be produced (Botteldoorn et al. 2006; Cristóbal et al. 2009). It is remarkable that the peak at 37 °C (isoenzyme 1) on LBC30 showed increases in activity of 63 and 32 % as compared to LBC10 at pH 8 and 6, respectively. The peak at 25 °C (isoenzyme 2) also showed a 60 and 16 % increase in activity on LBC30 compared to LBC10 at pH 8 and 6, respectively. In addition, the activity peak at 10 °C (isoenzyme 3) showed a 34 and 12 % increase on LBC30 compared to LCB10 at pH 8 and 6, respectively (Fig. 2b). Similar effects were observed by Liu et al. (2009), who reported a new *bgl1B* (ACY09072) gene, which had been obtained from a marine microbial metagenomic library, as belonging to GH1family of βG. In other study, both the expression and repression of two distinct βG from *Phanerochaete chrysosporium* were observed when the fungus was grown on a medium supplemented with cellobiose or glucose (Tsukada et al. 2006). Riou et al. (1998) reported that *Aspergillus oryzae* secreted two different βG when it was grown on a medium with quercetin; the enzyme with lowest activity exhibited a high tolerance to glucose inhibition. In addition, Cai et al. (1998) reported the expression of two βG isoenzymes in *Volvariella volvacea*; genetic expression level was determined by the presence of different substrates, such as salicin and cellobiose. The βG activities obtained in the *Shewanella* sp. G5 culture with glucose as a sole carbon source (LBG30, LBG10, MMBG30, and MMBG10) were very low compared to those obtained with cellobiose (Cristóbal et al. 2009). Under all these conditions, βG appeared to be a constitutive enzyme, since it was produced in the absence of an inducer and in the presence of the most widely known catabolic repressor. Several bacteria and fungi exhibit the property of expressing different βG isoenzymes, depending on culture conditions or carbon sources employed in the assays (Singhania et al. 2011; Werbrouck et al. 2006). For instance, endoglucanase and βG were reported to be expressed as response to carbon sources in *Aspergillus terreus* (Nazir et al. 2010). The sequential induction of isoenzymes has been associated to the presence of distinct metabolites (Villas-Bôas et al. 2006).

Effect of Metals, Reagents, Glucose, and Ethanol on Enzyme Activity

The effect of metal ions, reagents, glucose, and ethanol on βG activity was determined for the IP from *Shewanella* sp. G5 cultured on LBC30 and MMBC30 conditions (Table 1). Enzyme activity was increased by 61 and 47 % in the presence of Ca^{2+} and 24 and 31 % in the presence of Mg^{2+}, when cultured on LBC30 and MMBC30, respectively (Fig. 3a). Conversely, activity was totally inhibited by the presence of Hg^{2+}, Cu^{2+}, and the chelating agent EDTA at 5 mM, indicating that this βG could be a metalloprotein (Karnchanatat et al. 2007). The activity was strongly decreased by SDS on both media and by Zn^{2+} on MMBC30 (but not on LBC30), as well as by DTT (Fig. 3a), suggesting that disulfide bonds are not essential for enzyme activity (Yang et al. 2008). The monocations Na^+ and K^+ did not affect the enzyme activity, but probably contributed to stabilizing the enzyme structure. Riou et al. (1998) reported 77 % activity increase in the presence of Mn^{2+} (5 mM) and suggested that the enzyme did not require Ca^{2+}, Mg^{2+}, or Co^{2+}; moreover, these authors informed that the enzyme activity was inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+}. On the other hand, Karnchanatat et al. (2007) confirmed an enzymatic activity increase in the presence of Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, glycerol, and dimethyl sulfoxide. They also reported that EDTA did not affect it and that, conversely, Hg^{2+} ions completely inactivated the enzyme. Total inactivation by the latter ion and Cu^{2+} was also found by Yang et al. (2008). In addition, Painbeni et al. (1992) reported enzyme inactivation by Hg^{2+} and only activity reduction triggered by DTT and SDS (5 mM): a 30 and 85 % decrease caused by these two reagents, respectively.

On the other hand, the effect of different ethanol concentrations on enzyme activity was evaluated. A 63 % activity reduction was observed with a 14 % (*v*/*v*) ethanol concentration (Fig. 3b). It is noteworthy that these enzymes remained active in the presence of alcohol, in spite of the fact that the tests were performed with crude extracts. Consequently, these enzymes could be used as biocatalysts for testing wines. Painbeni et al. (1992)

reported that certain range of ethanol concentration has a stimulating effect on the activity of some βG. Riou et al. (1998) verified that enzyme activity increased by 30 % in the presence of ethanol 15 % (*v*/*v*). Saha et al. (1994) also reported a similar effect on the activity, which was increased by up to 15 % with ethanol 7.5 % (*v*/*v*); nonetheless, it was also observed that the activity dropped at higher ethanol concentrations.

In biotechnological processes, the application of highly glucose-tolerant β-glucosidases could increase the efficiency of cellulose degradation and, in some cases, lead to the production of 20–30 % glucose syrup (Liu et al. 2011). Although some exceptions were reported, glucose is an inhibitor of several β-glucosidases produced by different microorganisms (Saha et al. 1994; Yang et al. 2008), which is an important drawback for the industrial use of these enzymes (Karnchanatat et al. 2007; Painbeni et al. 1992; Riou et al. 1998). In this study, increasing glucose concentrations led to the reduction of enzyme activity so that a 60 % decrease was obtained with 100 mM glucose (Fig. 3c). It can thus be said that glucose inhibited *p*NPG hydrolysis only partially. These results showed that enzymes can be used in winemaking processes, which is restricted in most of the other cases. A remarkable effect was described by Saha et al. (1994), who reported that the presence of 6 % (*v*/*v*) ethanol and glucose (56 mM) did not inhibit the enzyme activity. On the other hand, Liu et al. (2011) showed that the activity of recombinant *bgl1B* was competitively inhibited by 30 mM of glucose.

Conventional PCR and Real-Time PCR Analysis

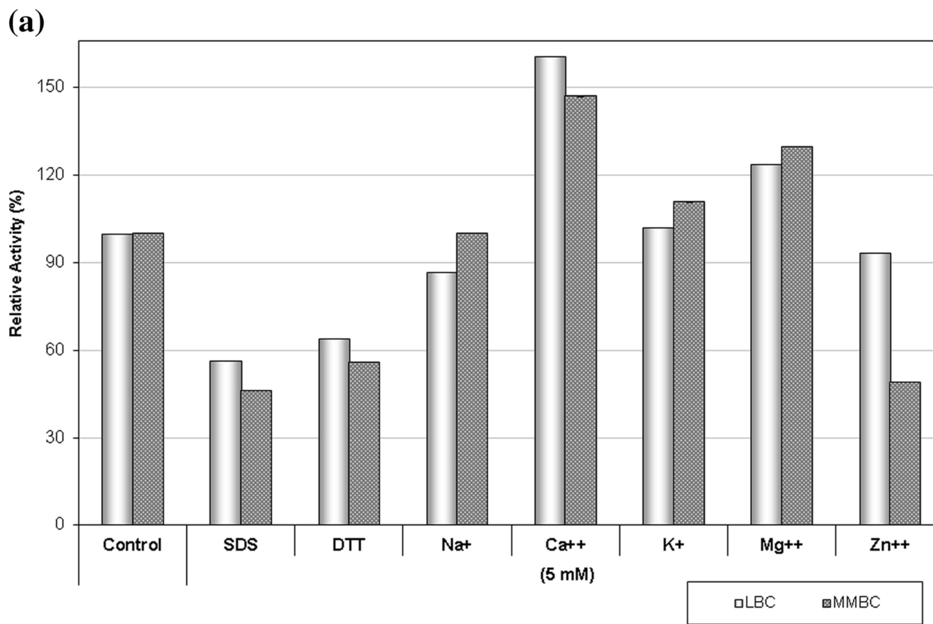
Agarose gel electrophoresis showed positive specific amplifications (Fig. S1a) of the expected size (Table 2) in all genes by conventional PCR. With these results, we proceeded to optimize the RT-qPCR reactions for the *bgl-A*, *bgl*, and *CspA* genes using SYBR Green I in order to minimize dimer formation (Sellars et al. 2007; Wang et al. 2006). The cDNA obtained under LBC10 condition was used for that purpose. The absence of non-specific qPCR products and primer-dimer artifacts was verified by the dissociation analysis. In all cases, the melting curves showed a single peak for each gene, indicative of the specific amplification (Fig. S1b, c, d, e, and f). Three amplification conditions, with differences in the annealing and extension steps, were considered for qPCR optimization (Table S1). Positive *Ct* values were obtained for all the genes under all the conditions studied, with the exception of *CspA* gene, which was not amplified under Condition 2. The expression of 16S rDNA gene was remarkably higher than those for the other genes in all the cases considered (*Ct* values were about half). Instead, *gyrB* was expressed at the same level as the other genes, and for that reason, it was selected

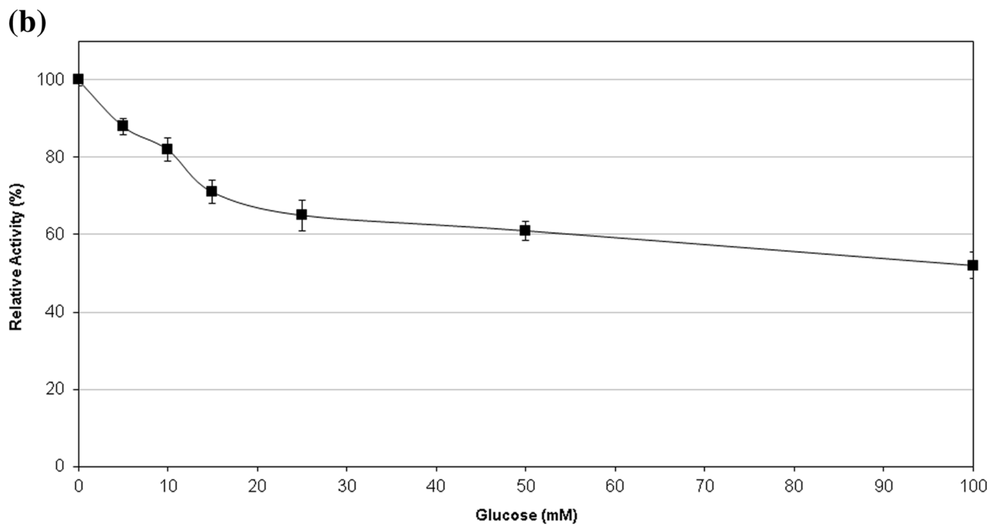
Fig. 3 Several effects on β-glucosidase activity: **a** effect of metal ions and other reagents on β-glucosidase activities under LBC30 and MMBC30 conditions, **b** effect of ethanol (%) on β-glucosidase activity under MMBC30 condition, and **c** effect of glucose concentration (5 mM) on β-glucosidase activity under MMBC30 condition

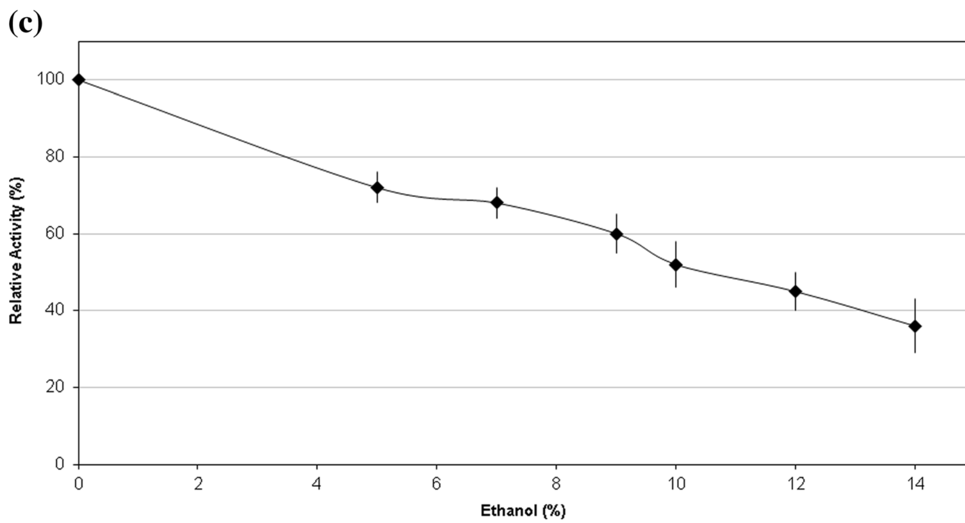
as a reference housekeeping gene and normalizer, and Condition 3 was found to be the optimum.

Construction of Standard Curves

Standard curves for each gene under study were built using the optimum conditions found for qPCR (Condition 3). The square of Pearson correlation coefficient (r^2) was used to assess linearity for which r^2 should be very close to 1 (≥0.995) (Botteldoorn et al. 2006; Ellefsen et al. 2008). Linearity was verified in all the amplified systems, and the slopes allowed calculating the amplification efficiency (Eq. 1), with values ranging between 99 and 116 % (Fig. S2). The fact that these systems amplified so efficiently also indicated that there was no inhibition affecting the reaction. According to other authors (Spagna 2000; Theis et al. 2007), the efficiency of the standard curve should be between 90 and 110 % (corresponding to −3.6 and −3.1 slopes, respectively), as a 100 % efficiency indicates a perfect replication of the amplicon in each cycle. An efficiency which is significantly lower than 100 % implies a slow reaction at some stage, which may result from the presence of inhibitors in the reaction mixture, or from unsuitable set of primers or reaction conditions. Efficiencies significantly higher than 100 % indicate typical experimental errors: wrongly calibrated pipettes, probe degradation, or formation of non-specific products or primer dimers. Efficiency deviations may also be due to poor preparation of serial dilutions, or concentrations that can inhibit qPCR (high concentrations of primers or of target) or exceed the particular sensitivity of the assay (low amounts of template). It is important for this type of quantification to run the standard curve on the same plate as that used for the unknown samples. Test repetitions may lead to *Ct* variations when run at different times or on different plates. Consequently, they would not allow for direct comparison with other tests, as variations would amount from 5 to 10 % (Ali-Benali et al. 2005; Botteldoorn et al. 2006). This work allowed us to find an optimal condition for enzyme production by *Shewanella* sp. G5, which will lead to further studies for large-scale production. Previous studies showed that *Shewanella* sp. G5 exhibited different βG isoenzymes, induced by cellobiose and encoded by two genes, *bgl-A* and *bgl* (Cristóbal et al. 2008). In a proteome assay for these marine bacteria, re-arrangement of proteins was observed after growth on cellobiose and glucose as sole carbon source. Therefore, *Shewanella* sp. G5 provides an optimal system for studies of genetic variation levels in *bgls* genes that encode for different βG isoenzymes (Cristóbal et al. 2009).


(a)
Relative Activity (%)
0
30
60
90
120
150
Control
SDS
DTT
Na+
Ca++
K+
Mg++
Zn++
(5 mM)
LBC
MMBC


(b)
Relative Activity (%)
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90
100
Glucose (mM)


(c)
Relative Activity (%)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
Ethanol (%)

Normalization and Relative Quantification Analyses

Relative quantification is the most commonly used method, whereby gene expression level is quantified and normalized to against of an internal reference gene. Theis et al. (2007) and Ellefsen et al. (2008) have explained that normalization is an essential process to compensate for errors in qPCR experimental procedure. They studied several housekeeping genes (e.g., 16S rDNA, *gyrB*, *recA,* and *rho*), for which they determined the variation of the expression using the comparative *Ct* method and selected genes with similar *Ct*s. In the current study, two housekeeping genes were initially considered: *gyrB* and 16S rDNA. Of the two, *gyrB* was finally selected for normalization because of its better performance, as explained before. Genes are considered to be up- or downregulated if their relative expression levels are at least 2-fold higher or lower, respectively, than those of the control sample or the calibrator. In this work, the *Ct* values obtained by qPCR for all the evaluated genes under each condition considered were used for relative quantification of gene expression, which was accomplished by the application of the $2^{-\Delta\Delta Ct}$ method. Relative quantification is represented in Fig. 4, which should be interpreted as follows: under MMBC30 condition, *bgl-A* gene expression as $2^{-\Delta\Delta Ct}$ values was 6, 15, 6, 14, and 28 times higher than under LBC10, LBG10, MMBC10, LBC30, and LBG30 conditions, respectively. The expression of *bgl* gene as $2^{-\Delta\Delta Ct}$ values was 4, 12, 10, and 30 times higher than under LBC10, LBG10, LBC30, and LBG30 conditions, respectively, and so forth for the expression of the same genes under different conditions. It can be observed that gene expression levels were variable and strongly affected by culture conditions (Fig. 4). In most of these conditions, *bglA* and *bgl* genes showed a high genetic expression level. The highest $2^{-\Delta\Delta Ct}$ values of relative quantification for *bgl-A* and *bgl* were 14, 15, and 28, and 10, 12, and 30, respectively, obtained under MMBC30 conditions (Fig. 4). Thus, this condition was the optimum for the expression of both genes, which is in agreement with the optimum conditions found when determining the enzyme activity (Fig. 2a). Although cellobiose was not used as an inducer when *Shewanella* sp. G5 was cultured under MMBG30 and MMBG10 conditions, *bglA* and *bgl* genes were also expressed at high levels, which allowed the enzyme synthesis (Fig. 2a). In fact, these expression levels were 11 and 19 times, and 9 and 19 times higher under MMBG30 and MMBG10 conditions, respectively, than under LBG30 (Fig. 4).

Constitutive enzymatic activity was also reported in previous studies of cellular fractions of *Shewanella* sp. G5 grown on glucose as a sole carbon source (Cristóbal et al. 2009). Similar results were also found by Tsukada et al. (2006) who reported the expression of two genes, *bgl1A* and *bgl1B*, which encode for two family 1 βG. Both enzymes were produced by *P. chrysosporium*, and their expression was monitored by RT-PCR with cellobiose and glucose supplementing the culture media. Gene *bgl1A* was expressed constitutively on both

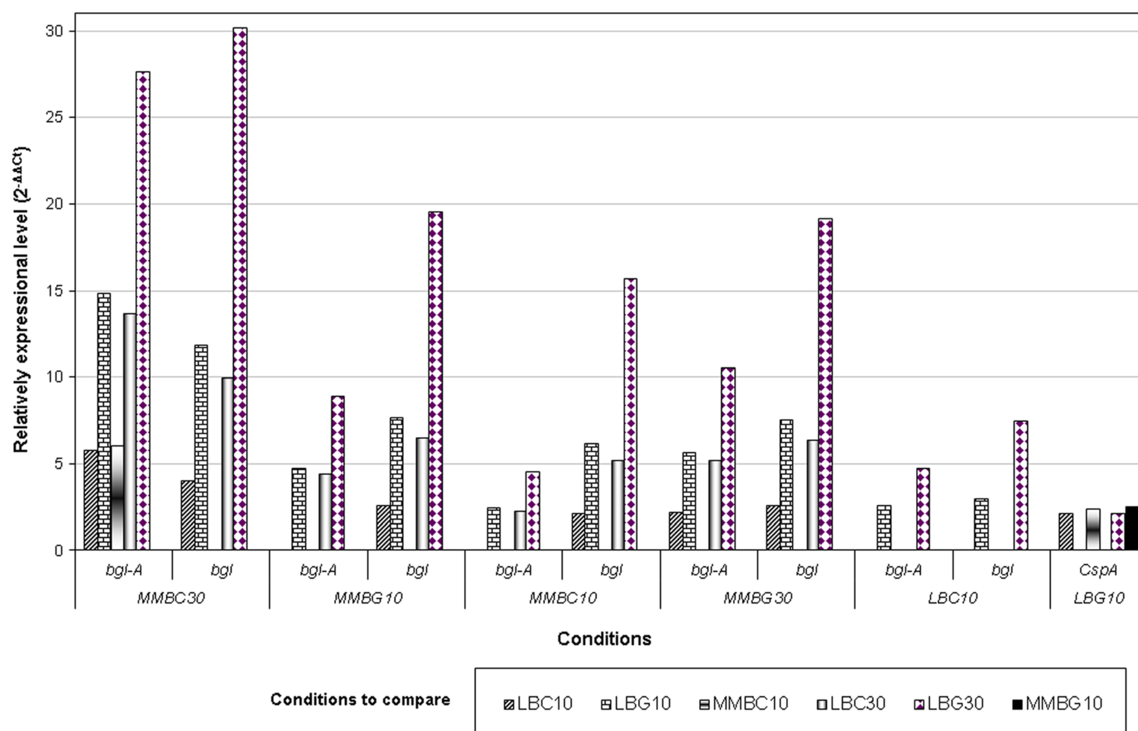


Fig. 4 Relative quantification of *bgl-A*, *bgl*, and *CspA* genetic expression levels obtained with the comparative $2^{-\Delta\Delta Ct}$ method. All values were normalized against *gyrB* gene expression levels. The expression levels of *bgl-A* and *bgl* genes increased under the following conditions: MMBC30, MMBG10, MMBC10, MMBG30, and LBC10

media, whereas *bgl1B* was only expressed on the medium with cellobiose, while glucose actually repressed it. Karnchanatat et al. (2007) reported that β-glucosidase produced by *Daldinia eschscholzii* belongs to family 3 of glucosyl hydrolase, and glucose competitively inhibited this enzyme. Yoshida et al. (2004) reported similar effects regarding cellobiose metabolism in the basidiomycete *P. chrysosporium*, based on the transcriptional analysis of βG (*bgl*) and cellobiose dehydrogenase (*cdh*) genes with qPCR. The results showed that the addition of glucose to the cellulose-degrading culture significantly decreased the amount of both transcripts. In contrast, the addition of cellobiose only repressed *bgl* gene transcription, but did not affect that of the *cdh* gene. Induction assays of the two genes showed that in a medium with cellobiose the level of *bgl* transcripts was considerably lower than with glucose, whereas that of *cdh* transcripts was 2.3-fold higher than that with glucose. .

Other effects of carbon source on the variation of the gene expression level were observed by Ohnishi et al. (2007). These authors found that in *Polyporus arcularius*, the *cel1* and *cel2* genes encoding cellobiohydrolases were induced by microcrystalline cellulose and cellopentaose, whereas they were repressed by glucose, cellobiose, cellotriose, and cellotetraose. These results suggested that *P. arcularius* cells constitutively synthesized a very low level of cellulase that is capable of degrading insoluble crystalline cellulose and that transcription of *cel1* and *cel2* in the cells is induced by products generated by cellooligosaccharides.

On the other hand, in this work, gene expression was studied following the growth by analyzing it every 30 min, starting from a cell density of $\pm 7 \times 10^6$ CFU/ml onwards, with the standard RT-qPCR procedure previously described by Werbrouck et al. (2006).

The cold-active enzymes offer potential economic benefits, as they play an important role in modern biotechnology, e.g., through saving energy substantially in large-scale processes which would otherwise require expensive heating of reactors (Jones et al. 1996; Cristóbal et al. 2011). The cold-shock protein (CspA), synthesized in *Escherichia coli* when temperature is reduced from 37 to 15 °C, has the hypothetical role of RNA chaperone (Jones et al. 1996). In our studies, LBG10 condition was the best for *CspA* gene expression level. In this work, no relationship was observed between *bgl, bgl-A,* and *CspA* genes, where the latest encode for the cold-shock protein A. This protein is present in psychrophilic bacteria, and it is synthesized when temperature decreases from 37 to 10 °C. During the cold-shock response, several cold-shock proteins are induced including CspA, which accounts for 10 % or more of the total cellular protein. It is remarkable that this protein binds to single stranded RNA of 74 base-pairs or greater with very low sequence specificity. These proteins have an effect on the translation of mRNA of both, the transcriptional regulation and of other proteins in the cell at low temperatures.

Conclusion

Shewanella sp. G5 was able to grow within a wide temperature range (4 to 37 °C) on both rich (LB) and saline (MMB) media through assimilation of cellobiose or glucose as carbon sources. Its highest specific growth rate was recorded when the bacterium was grown on LB medium at 37 °C, using cellobiose as sole carbon source. However, biomass production was higher on MMB medium.

Regarding βG production, growth temperature did not have any effects, while carbon source did play an important role. Although cellobiose induced higher enzyme levels, certain enzymatic activity was also recorded when the microorganism was cultured with glucose as the only carbon source, thus showing that the enzyme is constitutive. Three peaks of βG activity were found at 10, 25, and 37 °C (corresponding to three isoenzymes), and the highest relative activity in the culture conditions studied was obtained at 37 °C and pH 8.

The monocations Na^+ and K^+ did not affect enzyme activity, but probably contributed to stabilizing enzyme structure. Enzyme activity was increased in the presence of Ca^{2+} and Mg^{2+}, was strongly affected by SDS, Zn^{2+} (only under MMBC30 conditions) and DTT, and was totally inhibited by the presence of Hg^{2+}, Cu^{2+}, and EDTA at 5 mM. Although ethanol and glucose partially affected βG activity, the enzyme itself was still active at concentrations in which these substances are found in wine, so it can be deduced that these βG can be used in the winemaking industry.

The genetic expression of *bgl-A*, *bgl*, *CspA,* 16S rDNA, and *gyrB* genes was evaluated with RT-qPCR after optimizing the detection system. Their quantitative relative expression, attained by using the $2^{-\Delta\Delta Ct}$ method and *gyrB* gene as a normalizer, showed that the gene expression levels were variable and closely dependent on culture conditions. The highest expression levels for *bgl-A* and *bgl* genes were obtained on mineral medium culture at 30 °C and with cellobiose as sole carbon source, in agreement with the optimum conditions found when determining the enzyme activity.

To conclude, this work contributes to the knowledge of gene expression in *Shewanella* sp. G5 under optimal conditions for producing βG, which could be used in the winemaking industry. Winemaking and citrus juice industries indeed have their attention focused on glycosidases to optimize industrial processes using commercial exogenous enzymes. In particular, winemaking is a biotechnological process in which the use of exogenous enzyme preparations isolated from yeasts or bacteria helps to overcome the problem of insufficient endogenous activity in grapes (Palmeri and Spagna 2007). Consequently, commercial preparations of *Aspergillus niger* glycosidases are used to enhance wine aroma (Spagna 2000), and the techniques that allow identifying these enzymes have been of

fundamental importance in oenology, as they are used in the enzymatic treatment of commercially prepared wine (Palmeri and Spagna 2007).

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