African Journal of Biotechnology Vol. 10(80), pp. 18532-18538, 14 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2837
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Lectin from the sea mussel *Crenomytilus grayanus* and its effects on *Saccharomyces cerevisiae*

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Accepted 11 November, 2011

In this study, the properties of a GalNAc/Gal-specific lectin from the sea mussel *Crenomytilus grayanus* (CGL) and its effects on *Saccharomyces cerevisiae* were studied. The heat stability of CGL was determined and the free energy change (ΔG') of hemagglutination activity of the lectin in denaturation process was evaluated by Van't Hoff plot. The amino acid sequences about six segments of CGL were acquired by mass spectrum (MS) and the sequences have no homology with other lectins. In the effect of CGL, it can be observed that CGL can promote the yeast cells growth and ethanol production by yeast cells. In addition, the nitric oxide (NO) production decreases with increasing CGL concentration, except for the concentration of 3.25 mg/l. The results show that CGL is the potential yeast stimulators in fermentation process.

Key words: Lectin, sea mussel, *Saccharomyces cerevisiae*, ethanol, nitric oxide.

INTRODUCTION

Lectins are carbohydrate-binding proteins with carbohydrate-specific binding properties. They have been widely expressed in marine invertebrates, including bivalves. In the last decade, a large number of researchers have intensively investigated the effects of lectins on microorganisms (Takahashi et al., 2006; Zhu et al., 2008; Dresch et al., 2009; Xu et al., 2010; Yakovleva et al., 2011). In these studies, several lectins from bivalves showed binding activities to some micro-organisms (Zhang et al., 2009; Adhya et al., 2008; Tsutsui et al., 2007; Kim et al., 2008). The results suggest that exogenously added lectins could control some biological processes through interactions between lectins and microorganism surface glycoconjugates.

Saccharomyces cerevisiae is traditional yeast which affect for the industrial-scale production of ethanol. And numerous studies have focused on the development of engineered yeast strains with bigger efficiency of ethanol production (Ho et al., 1998; Eliasson et al., 2000; Jin et al., 2004; Edgardo et al., 2008). Stimuli with biological molecules can cause metabolic changes in an organism involved in the synthesis of specific compounds (Banat et al., 1998). In the practical technology, the most common stimuli were applied for fungi growth, such as superphosphate, ammonium sulphate, urea, magnesium sulphate, terpenes, amines antibiotics, alkaloids and so on (Govind et al., 1982; Kotarska et al., 2006). But few studies about the functions of lectins on fungi were reported. The only lectin from windowpane flounder *Lophopsetta maculata* whose agglutination activity against fungi and the C-type lectin from conger eel (*Conger myriaster*) (conCLs) whose agglutination activity

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Abbreviations: CGL, lectin from the sea mussel *Crenomytilus grayanus*; **MS,** mass spectrum; **NO,** nitric oxide.

against a yeast *S. cerevisiae* have been characterized (Kamiya and Shimizu, 1980; Tsutsui et al., 2007).

A GalNAc/Gal-specific lectin from the sea mussel *Crenomytilus grayanus* (CGL) was isolated and characterized (Belogortseva et al., 1998). Unfortunately there are limited data about CGL with those of other marine invertebrates and only a few studies are reported. Thus, CGL possessing anti-HIV activity have been described (Luk'yanov et al., 2007). In addition, the other biological activity of CGL was still not clear.

In this study, the physicochemical properties of CGL, identification and the effects of this lectin on the *S. cerevisiae* were investigated. The results showed that CGL can induce the yeast growth and increase their ethanol production.

MATERIALS AND METHODS

CGL was isolated from the sea mussel *C. grayanus* and purified by affinity chromatography on acid-treated Sepharose 6B followed by gel filtration on Sephacryl S-200 (Belogortseva et al., 1998). Human erythrocytes were obtained as outdate red cell concentrates from the Center of Blood Transfusion (Dalian).

The yeast *S. cerevisiae* was obtained in a commercial store (Angel Yeast Co., Ltd., China). PSM, porcine stomach mucin (type III) was purchased from Sigma Chemical (USA).

Digestion of erythrocytes with trypsin

1% suspension of erythrocytes in 0.01 M PBS (10 ml) was treated with trypsin (2 mg) for 2 h at 37°C. The material obtained was washed three times with a buffer used for titration. The trypsinized erythrocytes were suspended at concentration of 2% in the same buffer.

Hemagglutination assay

The solution of CGL was serially diluted 2-fold with PBS (25 µl) in the microtiter U-plates. An equal volume of 2% suspension of human erythrocytes was added to the each well and then the mixture was agitated. The hemagglutination was visually evaluated after 30 min.

Heat stability of CGL

The heat stability of CGL was determined as described (Lima et al., 2005). The lectin solution (2 mg/ml) was incubated at 40, 50, 60, 70 and 90°C for 5, 10, 20, 30, 40, 50 and 60 min, respectively. The samples were then cooled and assayed for hemagglutinating assay. The free energy change (ΔG') of activation of the lectin denaturation was determined using the formula of Arrhenius (Leite et al., 2005). The velocity constant of the reaction (k_1) was firstly determined as the slope of the curve obtained by the equation $k_1 t = -\ln A/A_o$, where A = residual hemagglutinating activity after heat treatment, A_o = initial hemagglutinating activity before heat treatment, and t = time of heat treatment (in seconds). The velocity constant k_1 is related to the standard free energy change by the formula: $\Delta G' = RT \ln(kT/k_1 h)$, were R is the gas constant (1.987 cal/mol·K), T is the absolute temperature (K), k is the Boltzmann constant (1.37×10^{-16} ergs/K), k_1 is the velocity constant and h is Planck's constant (6.25×10^{-27} ergs/s).

Protein identification by mass spectrometry

CGL sequencing was performed by digestion of the lectin and sequencing of the different peptides using nano ESI-MS/MS (Q-TOF2, Micromass, UK) at the National Center of Biomedical Analysis, Chinese Academy of Military Medical Sciences. The obtained peptide sequences were analyzed by BlastX (http://www.ncbi.nlm.nih.gov/BLAST/).

Cell surface binding assay

The FITC-conjugated CGL was chosen as probe for labeling cell surface. The CGL was labeled with FITC according to the protocol described by Sun et al. (2002). In the determination of the binding of CGL to yeast cells, 500 µl of 50 µg/ml of FITC-conjugated CGL in PBS was added to 500 ml of cell suspension (6×10^6 cells/ml) and mixture was incubated for 2 h at 4°C. The cells were washed with PBS and centrifugated at 500 g for 10 min at 4°C. The fluorescence at 517 nm with excitation at 493 nm was observed using a fluorescent microscope (Novel XYL-1, Ningbo Yongxin Optics Co., Ltd., China).

Effect of CGL on growth of yeast

Cultivation media: D-glucose (150 g/L), yeast extract (5 g/L) and peptone (10 g/L). Cell growth was determined by plate counting in some cases. Samples were withdrawn throughout fermentation and diluted appropriately in dilution medium (Bely et al., 2008).

Yeast cells grew in 100 ml cultivation medium to an exponential phase for 2 h after the number of cells had reached approximately 6×10^6 cells/ml and was used to initiate growth in the other media used in this study using 1% v/v inoculum. All ethanol fermentation studies were performed at 150 rpm in rotary agitated 250 ml Erlenmeyer flasks containing 100 ml of cultivation medium at 29°C for 36 h. The effects of lectin were studied in cultivation medium by adding different concentration (3.25, 9.75, 16.25, 22.75 mg/L (w/v)) of CGL. Samples were withdrawn at 36 h.

The concentration of soluble solids (degrees Brix, °Bx) was determined with a portable refractometer (Nikon Corporation, Japan). The pH of the samples was determined using a pH-meter (pHS-3C, Shanghai Leichi Instruments, China). The percentage of ethanol (v/v) was determined by chemical oxidation method (Caputi and Wright, 1969). The method is based on the complete oxidation of ethanol by dichromate in the presence of sulfuric acid with the formation of acetic acid. Dichromate ($Cr_2O_7^{-2}$) is yellowish in color and the reduced chromic product (Cr^{+3}) is intensely green. A 5 ml sample was steam distillated into acidified potassium dichromate solution. The concentration of unreacted dichromate was determined by titration with ferrous ammonium sulphate solution using phenanthroline as an indicator.

Nitrite production was used as an indicator of nitric oxide (NO) production of sample. It was measured as previously described (Kim et al., 2005). The supernatants of sample were mixed with an equal volume of Gries reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 10 min. Nitrite production was determined by OD 550 nm.

Statistical analysis

Each experiment was carried out in triplicate. Values are given as

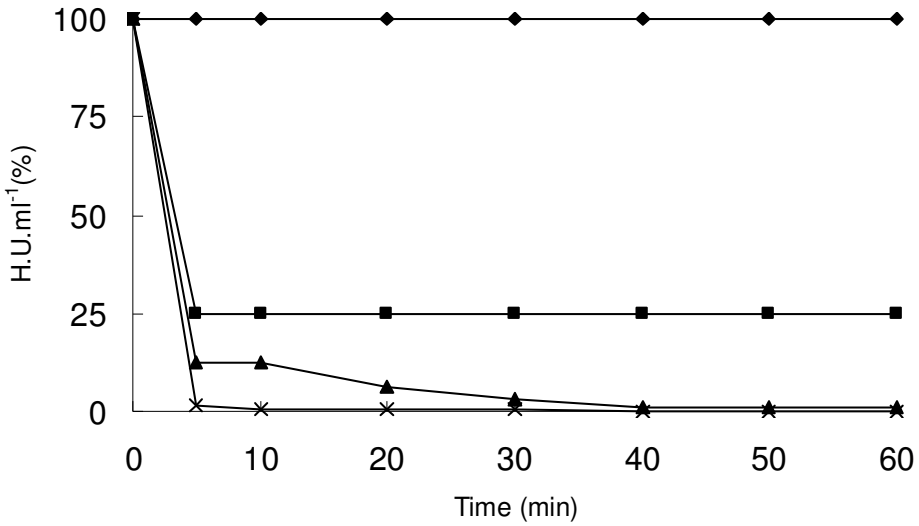

Figure 1. Effect of temperature on the hemagglutinating activity of CGL: (◆) 40°C; (■) 50°C; (▲) 60°C; (*) 70°C. The residual lectin activity along heat treatments is shown as the hemagglutinating units (H.U.) at each time in relation to the hemagglutinating titer (H.U. /ml) of the native protein.

means ± standard deviation.

RESULTS

Heat stability of CGL

Five minutes after heat stability of CGL, the purified CGL retained 25% of its original activity during the heat treatment at 50°C. However, its retained hemagglutinating activity was entirely stable after 5 min at 50°C (Figure 1). The free energy change (ΔG') of activation of the denaturation process was evaluated to be 18.917 kcal/mol.

CGL identification by mass spectrometry

Edman degradation of CGL was unsuccessful which suggested its N-terminus is blocked. The CGL was digested with trypsin and analyzed with nano ESI-MS/MS. Six peptide sequences (FAMDFFNDNLMHK, HAAMEFLFVSPK, LQGLVSWGSGGSGTK, MYFQFD-VVDER, PPNETNMVIHQDR and ALFAMDFFNDNLMHK) were obtained and subjected to the protein BlastX (http://www.ncbi.nlm.nih.gov/BLAST/). After the protein sequences in the protein BlastX were compared, the six peptides showed no significant homology with other lectins listed in the BLAST.

Effect of CGL on the *S. cerevisiae*

We investigated the binding of CGL-FITC to the surface of yeast cells by fluorescence detection. Incubation of CGL-FITC with yeast cells resulted in uniform staining of cell surface at 4°C (Figure 2A). The addition of CGL-FITC with PSM to yeast cells abolished the cell binding fluorescence incompletely. It indicated that the binding of CGL to yeast cells is not just the carbohydrate-protein or protein-protein interaction (Figure 2B).

In order to investigate the activity of CGL, its stimulative effect on the growth of yeast cells was investigated. OD_{600} of yeast suspension in different concentration (3.25, 9.75, 16.25, 22.75 mg/L (w/v)) of CGL was obviously different from that of the control (Figure 3). The results of ethanol production in the series of experiments with different concentration of CGL are shown in Figure 4. It can be seen that ethanol production increased from 11.05 to 19.96 g/L and pH values decreased from 3.81 to 3.44, when CGL concentration was increased from 0 to 22.75 mg/L (w/v) at 36 h (Figures 4 and 5). The residual D-glucose after 36 h of fermentation was also analyzed in the solutions with different concentrations of CGL (Figure 6). It seemed that the incubation of yeast utilized D-glucose increasingly with adding to incremental concentrations of CGL, but showed no significant differences.

The effects of CGL on NO production of fermentation are shown in Figure 7. When CGL were at the concentration of 3.25 mg/L, the NO production was weakly increased, but at the following concentrations the NO production was significantly decreased.

DISCUSSION

The free energy change (ΔG') of activation of the

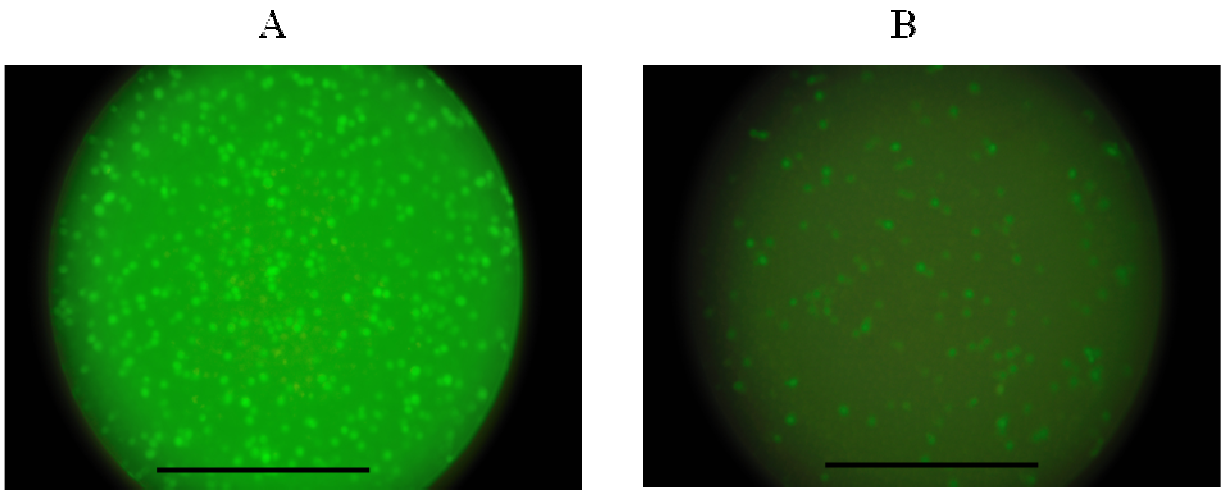

Figure 2. CGL-FITC staining of yeast cells. Yeast cells were incubated for 2 h at 4°C with CGL-FITC (a); with CGL-FITC at presence of PSM (b). Scale bars are 50 μm.

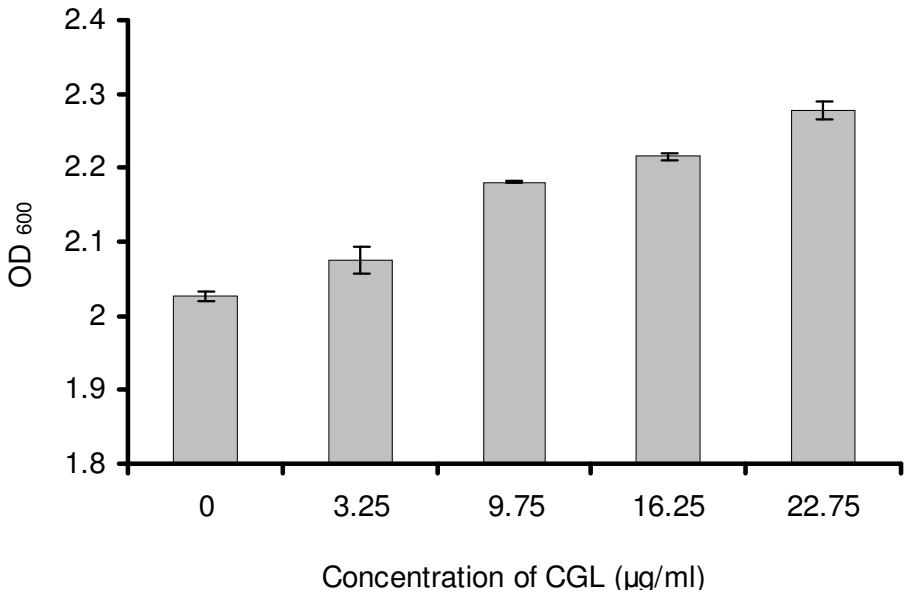

Figure 3. Growth test of CGL with *S. cerevisiae* at 36 h.

denaturation process for CGL is lower than the values found for the lectins from the algae *Enantiocladia duperreyi* (Benevides et al., 1998), *Amansia multifida* (Sampaio et al., 1998) and *Gracilaria ornate* (Leite et al., 2005). This suggests that CGL structure is not stable as the structure of seaweed lectins characterized. Since many lectins exert varied effects on cultivated cells including microorganisms, the determination of the free energy of activation of the lectin denaturation process is an important physicochemical parameter for the increasing applications of lectins in cell cultures and related areas.

Since CGL was found in the sea mussel *C. grayanus*, its amino acid sequence was studied. But Edman degradation of CGL was unsuccessful. It suggested that its N-terminus is the blockade. The six segments of CGL obtained using nanoESI-MS/MS will be useful to understand the primary structure of CGL. In connection with the fact that only the guache-gauche-trans configuration of CGL C-C-S-S-C-C linkage have been observed by laser Raman spectra nowadays (Li and Kobelev, 2003).

Tsutsui et al. (2007) reported that C-type lectin (conCLs) agglutinated a yeast *S. cerevisiae*. The

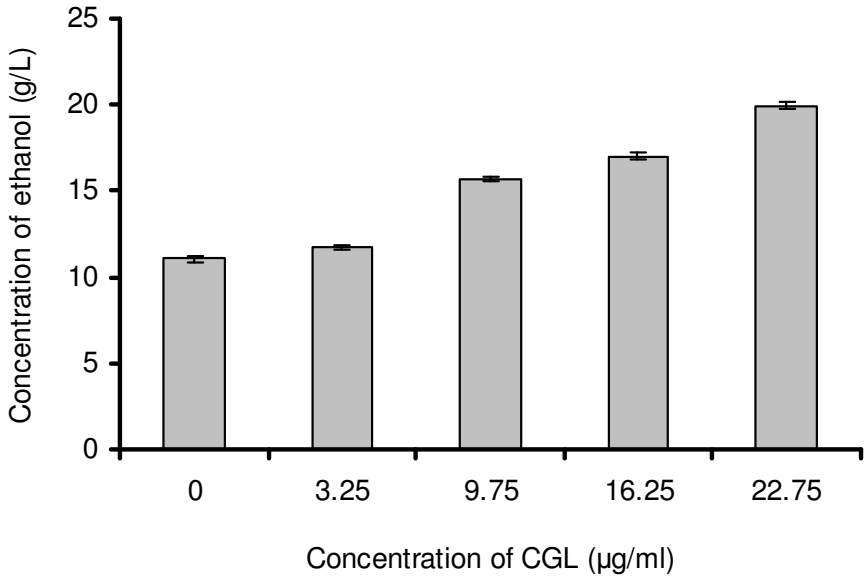

Figure 4. The concentrations of ethanol in cultivation medium of yeast cells with different concentrations of CGL at 36 h.

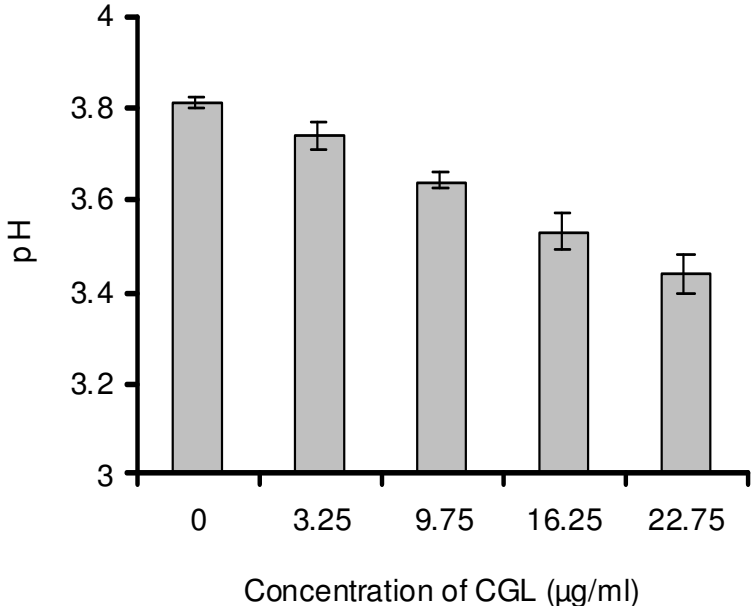

Figure 5. pH in cultivation medium of yeast cells with different concentrations of CGL at 36 h.

carbohydrate-recognition domain of conCLs contained the EPN (Glu-Pro-Asn) motif. The conCLs could not suppress the growth of yeast cells. So we examined the effects of CGL on yeast cells. As a result, the binding of CGL-FITC to the surface of yeast cells was observed. The binding of CGL to yeast cells was inhibited by PSM. It is possible, that the PSM-binding site could be important for adhesive activity of CGL to yeast. The number of yeast and ethanol production increased with increasing concentration of CGL. The reason remains unclear, but it suggested that CGL has a stimulating effect on the yeast and contributes to an increase in the efficiency of fermentation.

Recently, Almeida et al. (2007) reported that NO signaling is linked with H_2O_2 or age-associated apoptotic yeast cell death. NO levels are mediating the apoptotic

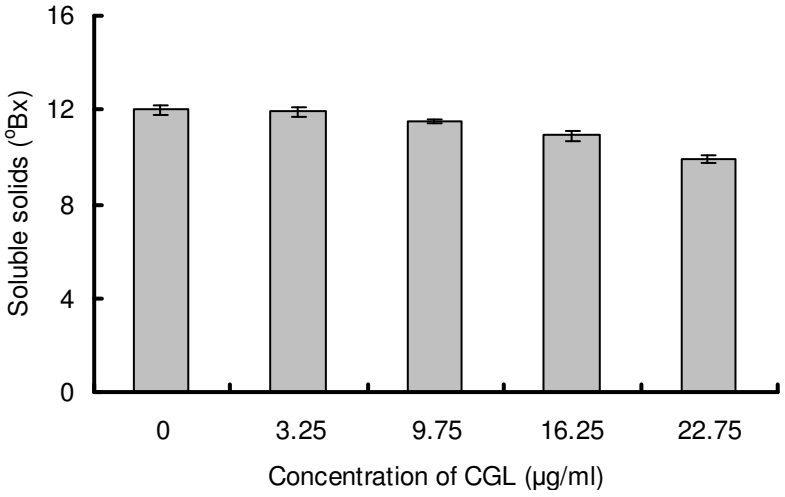

Figure 6. The residual D-glucose in cultivation medium of yeast cells with different concentrations of CGL at 36 h.

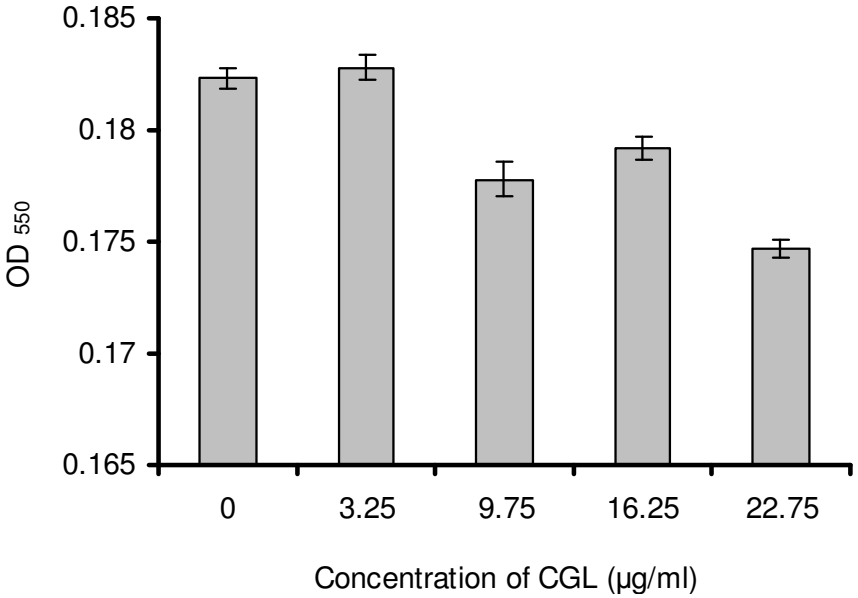

Figure 7. The production of NO in cultivation medium of yeast cells with different concentrations of CGL at 36 h.

production (Ho et al., 1998; Eliasson et al., 2000; Jin et cell death occurring during chronological life span. In this study, we investigated the NO production of yeast cells with CGL. The NO production decreased with increasing CGL concentration except for the concentration of 3.25 mg/L. The effects of CGL on yeast cells seem to be of induction for the yeast growth.

Ethanol is one of the most important fuels from renewable sources that are currently produced at competitive prices. *S. cerevisiae* is effective for the industrial-scale production of ethanol. Accordingly, many researchers have engaged in the development of engineered yeast strains with higher efficiency of ethanol production (Ho et al., 1998; Eliasson et al.,2000; Jin et al.,al., 2004; Edgardo et al., 2008). Stimuli with biological molecules can cause metabolic changes in an organism involved in the synthesis of specific compounds (Banat et al., 1998). Our study indicates that CGL is potential yeast stimulators in fermentation process.

ACKNOWLEDGEMENTS

We thank Prof. Dr. Thomas Haertle for his assistance.

The work was supported by grants from Natural Science Foundation of China (31071612), Natural Science Foundation of China (21075012) and Education Department Project from Liaoning Province (No. 2009A169).

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