Int.J.Curr.Microbiol.App.Sci (2022) *11*(04): 6-12

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 11 Number 04 (2022)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2022.1104.002

Biocatalytic Preparation of Chiral Alcohols with Micro Green Algae: Bio-reduction of Carbonyl Compounds by *Chlorogonium* Strains as a Novel Biocatalyst

Kohji Ishihara[1*], S. Oka[1], Y. Hayashi[1], Y. Kondo[1], Y. Kitagawa[1], K. Shimoda[2], N. Masuoka[3] and N. Nakajima[4]

[1]*Department of Life Science, Facalty of Science, Okayama University of Science, Okayama, Japan*
[2]*Department of Biomedical Chemistry, Faculty of Medicine, Oita University, Hasama-machi, Japan*
[3]*Department of Research and Development, Institute for Fruit Juice Research in Tsudaka, Co., Ltd., Okayama, Japan*
[4]*Department of Nutritional Science, Okayama Prefectural University, Soja, Okayama, Japan*

**Corresponding author*

Keywords

Micro green algae, Chlorogonium, Biocatalyst, Stereoselective reduction

Article Info

Received:
05 March 2022
Accepted:
28 March 2022
Available Online:
10 April 2022

A B S T R A C T

Chlorogonium strains were screened to examine the potential ability of microgreen algae to act as biocatalysts. Two culture media (National Institute for Environmental Studies (NIES) recommended AF-6 medium and a synthetic AY medium) were tested for the use in the liquid culture of three algal strains. When static cultured in the AY medium containing acetate and yeast extract under continuous light conditions, these strains showed promising growth. Thus, the stereoselective reduction of various carbonyl compounds using these strains was investigated. This study discovered that these strains could reduce aliphatic and aromatic α-keto esters, α-substituted *β*-keto ester, and aromatic α-keto amide. Based on the conversion ratio and stereoselectivity of the yielded alcohols, *C. elongatum* NIES-1357 strains cultured in the AY medium is a potential biocatalyst for the stereoselective reduction of aliphatic α-keto esters, 2-chlorobenzoylformamide, and ethyl 2-methyl-3-oxobutanoate to the corresponding chiral alcohols in the presence of L-alanine.

Introduction

Chlorogonium (Heamatococcaceae family, Volvocales order, Chlorophytaphylum) distributed worldwide is a genus of unicellular, biflagellate micro green algae (Ettl, 1980). Biochemical investigations for the microgreen algae about the metabolic pathway of the starch fermentation through a formate production (Kreuzberg, 1984)and the effects of light and acetate in cells enzyme regulation (Struck *et al.*, 1987) have been reported. Additionally, Nakada *et al.*, (2005, 2010) have

studied green algae's morphological and taxonomic investigations. Therefore, various microbiological and phycological studies of the genus *Chlorogonium* have been reported.

In contrast, there have been limited studies on the use of the green algae. For example, to cultivate *Paramecium bursaria*, *Chlorogonium elongatum*has been used as the sole food (Omura *et al.*, 2004). Furthermore, concerning the usage of other green algae, stereoselective reduction of numerous carbonyl compounds using *Chlorella sorokiniana* has been studied (Ishihara *et al.*, 2000, 2011). Nevertheless, the potential biocatalyst activity of *Chlorogonium* strains has not been reported.

This study investigated the stereoselective reduction of various carbonyl compounds by microgreen algae, *Chlorogonium*, in order to identify potential novel biocatalysts (Figure 1).

Materials and Methods

Instruments and chemicals

Gas chromatography (GC) was performed using a GL Science GC-353 gas chromatograph (GL Science Inc., Japan) equipped with capillary columns (DB-WAX, 0.25 µm, 0.25 mm x 30 m, Agilent Technologies, USA; TC-1,0.25µm, 0.25 mm x 30 m, GL Science Inc.; CP-Chirasil-DEX CB, 0.25 µm, 0.25 mm x 25 m, Varian Inc., USA; Gamma DEX 225, 0.25 µm, 0.25 mm x 30 m, Sigma-Aldrich Inc., USA). Ethyl pyruvate (Figure 1, **1a**), diatomaceous earth (granular), and sodium acetate were purchased from Wako Pure Chemical Industries Ltd. (Japan). Bacto™ yeast extract was purchased from Becton Dickinson and Co. (USA). Ethyl lactate (**2a**), ethyl 3-methyl-2-oxobutanoate (**1f**), ethyl 2-oxo-4-phenylbutanoate (**1h**), ethyl 2-hydroxy-4-phenylbutanoate (**2h**), and beef extract were purchased from Sigma-Aldrich Inc. Ethyl benzoylformate (**1g**), ethyl 2-methyl-3-oxobutanoate (**1j**), and ethyl mandelate (**2g**) were obtained from Tokyo Chemical Industry, Co., Ltd. (Japan). Ethyl 2-oxobutanoate (**1b**), ethyl 2-oxopentanoate (**1c**), ethyl 2-oxohexanoate (**1d**), ethyl 2-oxoheptanoate (**1e**), 2-chlorobenzoylformamide (**1i**), 2-chloromandelamide (2i), α-hydroxy esters (**2b–f**), and ethyl 3-hydroxy-2-methylbutanoate (**2j**) were prepared as described previously (Mitsuhashi and Yamamoto, 2005; Kawai *et al.*, 1995; Nakamura *et al.*, 1988). All the other chemicals used in this study were of analytical grade and commercially available.

Microorganisms and culture

The Microbial Culture Collection at the National Institute for Environmental Studies (NIES Collection, Tsukuba, Japan) is where *Chlorogonium euchlorum* NIES-760 and *Chlorogonium elongatum* NIES-1357 were purchased. Associated Professor, T. Suzaki, Graduate School of Science, Kobe University, Japan, donated *Chlorogonium capillatum* (same as NIES-3374 strain). Three *Chlorogonium* strains were kept in an NBRC recommended AF-6 liquid medium and a synthetic AY medium at 20°C under light conditions (2,000–3,000 lx) for 12 h daily. The AF-6 medium was composed of 140 mg of $NaNO_3$, 22.0 mg of NH_4NO_3, 30.0 mg of $MgSO_4•7H_2O$, 10.0 mg of KH_2PO_4, 5.0 mg of KH_2SO_4, 2.0 mg of Fe-citrate, 2.0 mg of citric acid, 2.0µg of biotin, 10.0 µg of thiamine hydrochloride salt, 1.0 µg of vitamin B_6, 1.0 µg of vitamin B_{12}, 5.0 mL of PIV metals solution, per 1 L of distilled water (pH 6.6).The PIV metals solution was 100 mg of $Na_2EDTA•2H_2O$, 19.6 mg of $FeCl_3•6H_2O$, 3.6 mg of $MnCl_2•4H_2O$, 2.2 mg of $ZnSO_4•7H_2O$, 0.4 mg of $CoCl_2•6H_2O$,0.25 mg of $NaMoO_4•2H_2O$ per 100 mL of distilled water. The AY medium consisted of 1.0 g of sodium acetate and 5.0 g of Bacto™ yeast extract, per 1 L of distilled water (pH 7.0). The strains were grown instatic liquid culture with the synthetic media (AF-6 or AY medium) for 4weeks at 20°C under continuous light (2,000–3,000 lx) or in the dark conditions at the time of large-scale culture. Then, at 5,000 x*g* for 10 min of centrifugation, the algal cells were harvested and washed with a saline (0.85% NaCl aq.). After washing with the saline, the harvested cells were immediately used for the algal reduction.

Reduction of α- and β-keto esters, and an aromatic α-keto amide using resting actinomycete cells

Using a large test tube (ϕ30 mm x 200 mm) containing 20mL of saline, saline-washed wet algal cells (0.5 g, dry weight approximately 0.13 g) were resuspended. Then, the substrate (0.15 mmol; 7.5 mM) was then added, and the reaction mixture was incubated aerobically (with reciprocated shaking at 120 strokes/min) at 25°C for 48 h. Next, a portion (0.5 mL) of the mixture was added to a short diatomaceous earth column (ϕ 10 mm x 30 mm), extracted with diethyl ether (5.0 mL), and then concentrated under reduced pressure.

Analysis

The production of alcohols (Figure 1, **2a**–**j**) was measured using a GC with a DB-WAX capillary column (100 kPa He at 110°C: **1a**, 3.78 min; **2a**, 4.75 min; **1b**, 4.73 min; **2b**, 5.92 min; **1f**, 4.54 min; **2f**, 6.41 min; 120°C:**1c**, 4.84 min; **2c**, 6.45 min;**1j**, 5.54 min; **2j**-*anti*, 7.62 min;**2j**-*syn*, 8.13 min; 150°C: **1d**, 3.83 min; **2d**, 4.68 min; **1e**, 4.78 min; **2e**, 6.07 min; 180°C: **1g**, 9.01 min; **2g**, 12.08 min) or a TC-1 capillary column (100 kPa He at 140°C: **1h**, 10.02 min; **2h**, 10.96 min; 170°C: **1i**, 6.85 min; **2i**, 8.34 min).

Using a GC instrument equipped with an optically active CP-Chirasil-DEX CB (**2a–e, 2g–h,** and **2j**) or a Gamma DEX 225 capillary column (**2f** and **2i**), the enantiomeric excess (e.e.) of the product was measured. The following formula was used to calculate the e.e.: e.e.(%) = {(*R*-*S*)/(*R*+*S*)} x 100, where *R* and *S*are the respective peak areas of the isomer in GC analyses.

By comparing their retention times as determined by the GC analyses with those of authentic samples, the absolute configurations of the α- and β-hydroxy esters (**2a**–**h** and **2j**), and the α-hydroxy amide (**2i**) were identified (Mitsuhashi and Yamamoto, 2005; Kawai *et al.*, 1995; Nakamura *et al.*, 1988).

Results and Discussion

Screening of *Chlorogonium* strains

In comparing the large-scale culture of three *Chlorogonium* green algae in two types of liquid media under continuous light conditions, the amounts of wet cells obtained in the AF-6 medium were 0.4 g or less per 5-L of culture (see Table 1). In contract, more than 1.1 g of wet cells were obtained for three cultures with AY medium containing acetate ion and yeast extract. NIES-1357 strain grew faster at the start of culture, whereas NIES-3374 and NIES-760 strains grew slowly. Nonetheless, 4 weeks after culturing, the NIES-760 strain indicated the highest number of wet cells (1.3 g/5-L of culture). Like general bacterial culture, heterotrophic cultivation was attempted for three *Chlorogonium* strains under dark conditions to investigate the effect of light irradiation on the growth of green algae during liquid culture. In the static culture in the dark condition, the three strains' growth rate was slower than the static culture under continuous light conditions (Table 1). Furthermore, the amounts of wet cells obtained by culturing in the dark condition were less than half that of culturing under continuous light conditions.

Thus, the potential ability of three *Chlorogonium* strains cultured in AY medium under the continuous light conditions to act as biocatalysts for the asymmetric reduction of carbonyl compounds was investigated.

Reduction of α-keto esters and aromatic α-keto amide by *Chlorogonium* wet cells

Three algal strains (*C. euchlorum*NIES-760, *C. elongatum* NIES-1357, and *C. capillatum* NIES-3374) were tested for their ability to reduce various carbonyl compounds (Figure 1). The results of the algal reductions are presented in Table 2. Three strains could reduce aliphatic and aromatic α-ketoesters (**1a–h**) and an aromatic α-ketoamide (**1i**). Compared with NIES-760 or NIES-3374 wet cells, the reduction rate of substrates by the NIES-1357

wet cells tended to be higher. Among the reduction of nine substrates tested by the NIES-1357 strain, there were compounds in which the conversion rate was over 99% (**2a, 2c, 2f**, and **2i**); nevertheless, the reduction of other substrates indicated only low values to moderate conversion rates. Additionally, the stereoselectivity of the product in the reduction of each substrate also showed low values except for the reduction of ethyl pyruvate (**1a**) and ethyl benzoylformate (**1g**). Hence, by introducing additives (D-glucose, sodium hydrogen L-glutamate, and L-alanine), we attempted to improve the conversion rate and the stereoselectivity of the alcohols produced by *C. elongatum* NIES-1357 strain, as shown in Table 3.

Table.1 The cultivation of *Chlorogonium* strains under several conditions

Scientific name	NIES number	Wet cells (g/5-L of culture)[1]		
		AF-6 medium	AY medium	
		Light	Light	Dark
C. euchlorum	760	0.2	1.3	0.4
C. elongatum	1357	0.4	1.1	0.5
C. capillatum	3374	0.3	1.1	0.4

[1]The green algae were grown in static culture with the liquid medium at 20°C for 4 weeks under continuous light (2.000 – 3,000 lx) or dark conditions.

Table.2 Stereoselective reduction of α-keto esters (**1a-h**) and aromatic α-keto amide (**1i**) by *Chlorogonium* strains[1-4]

Product	*C. euchlorum* NIES-760			*C. elongatum* NIES-1357			*C. capillatum* NIES-3374		
	Conv. (%)	e.e. (%)	*R*/*S*	Conv. (%)	e.e. (%)	*R*/*S*	Conv. (%)	e.e. (%)	*R*/*S*
2a	48	>99	*S*	>99	>99	*S*	42	41	*S*
2b	29	90	*S*	95	94	*S*	38	91	*S*
2c	10	87	*S*	>99	84	*S*	3	>99	*S*
2d	11	73	*S*	90	88	*S*	5	73	*S*
2e	<1	N.D.[5]	N.D.[5]	54	72	*S*	15	>99	*S*
2f	34	76	*S*	>99	70	*S*	6	91	*S*
2g	27	9	*S*	64	>99	*S*	18	6	*S*
2h	12	53	*S*	38	88	*S*	6	39	*S*
2i	50	80	*R*	>99	85	*R*	>99	42	*R*

[1]The green algae were grown in AY medium at 20°C for 4 weeks under continuous light conditions.
[2]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g), and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) at 25 °C for 48 h.
[3]Conversion was measured by a GLC analysis.
[4]Enantiomeric excess (e.e.) and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.
[5]Not determined.

Table.3 Effect of additive on the reduction of α-keto esters and aromatic α-keto amide by *C. elongatum* NIES-1357[1-4]

Product	D-Glucose			Sodium hydrogen L-glutamate			L-Alanine		
	Conv. (%)	e.e. (%)	*R*/*S*	Conv. (%)	e.e. (%)	*R*/*S*	Conv. (%)	e.e. (%)	*R*/*S*
2a	>99	83	*S*	>99	79	*S*	>99	>99	*S*
2b	96	81	*S*	>99	84	*S*	>99	99	*S*
2c	>99	62	*S*	>99	>99	*S*	>99	>99	*S*
2d	89	67	*S*	90	72	*S*	97	96	*S*
2e	66	68	*S*	98	69	*S*	98	>99	*S*
2f	>99	72	*S*	>99	68	*S*	>99	98	*S*
2g	68	8	*S*	87	16	*S*	91	68	*S*
2h	38	67	*S*	57	71	*S*	78	86	*S*
2i	>99	86	*R*	>99	83	*R*	>99	95	*R*

[1]The green algae were grown inAYmedium at 20°C for 4 weeks under continuous light conditions.
[2]Substrate (0.15 mmol), additive (5.0 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g), and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) at 25 °C for 48 h.
[3]Conversion was measured by a GLC analysis.
[4] Enantiomeric excess (e.e.) and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.

Table.4 Stereoselective reduction of ethyl 2-methyl-3-oxobutanoate (**1j**) to ethyl 2-methyl-3-hydroxybutanoate (**2j**) by *Chlorogium* strains[1-4]

Scientific name	NIES No.	Conv. (%)	*Syn* / *Anti* ratio	enantiomeric excess (%)	
				Syn-(2*R*, 3*S*)	*Anti*-(2S, 3*S*)
C. euchlorum	760	13	60 / 40	>99	>99
C. elongatum	1357	48	56 / 44	>99	>99
C. capillatum	3374	6	57 / 43	>99	>99

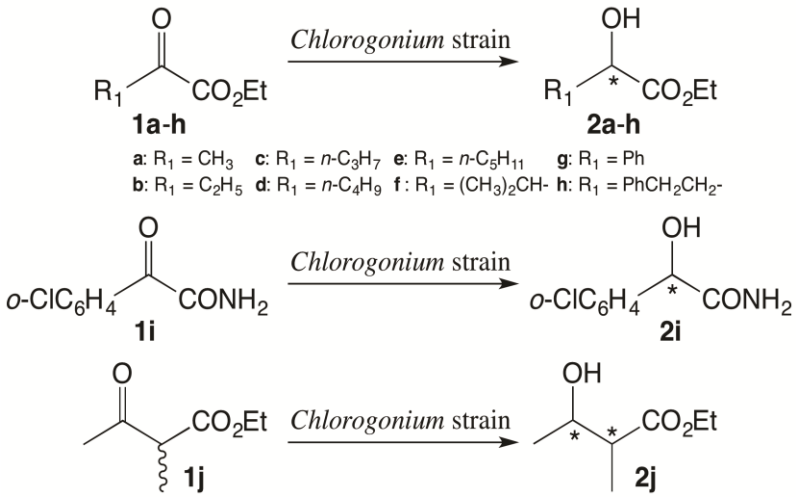
[1]The green algae were grown in AY medium at 20°C for 4 weeks under continuous light conditions.
[2]Substrate (0.15 mmol)and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g), and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) at 25 °C for 48 h.
[3]Conversion and *syn* / *anti* ratio were measured by a GLC analysis.
[4]Enantiomeric excess and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.

Table.5 Effect of additive on the reduction of **1j** by *C. elongatum* NIES-1357[1-4]

Additive	Conv. (%)	*Syn* / *Anti* ration	Enantiomeric excess (%)	
			Syn-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)
D-Glucose	55	51 / 49	>99	>99
L-Glutamate	40	54 / 46	>99	>99
L-Alanine	75	82 / 18	>99	>99

[1]The green algae were grown in AY medium at 20°C for 4 weeks under continuous light conditions.
[2]Substrate (0.15 mmol), additive (5.0 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g), and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) at 25 °C for 48 h.
[3]Conversion and *syn* / *anti* ratio were measured by a GLC analysis.
[4] Enantiomeric excess and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.

Fig.1 The reduction of various carbonyl compounds (**1a-j**) to the corresponding alcohols (**2a-j**) by *Chlorogonium* strains



However, the conversion rate and the stereoselectivity of the produced alcohols (**2a–i**) did not improve by adding D-glucose in the reductions of substrates (**1a–i**) by *C. elongatum* NIES-1357 strain. Furthermore, in the reduction in the presence of L-glutamate, the conversion rate increased to>87% except for 1h, but the stereoselectivity of products hardly changed. In contract, the introduction of L-alanine to the reaction mixture significantly improved the conversion rate of the substrate. Further, the substrates having an aliphatic alkyl group side chain (**1a–f**) were reduced stereo specifically to (*S*)-alcohols.

These results indicated that the NIES-1357 strain cultured in the AY medium is a useful and valuable biocatalyst for the asymmetric reduction of carbonyl compounds like α-keto esters and aromatic α-keto amides.

Reduction of substituted *β*-keto ester by *Chlorogonium* wet cells

Ethyl 2-methyl-3-oxobutanoate (**1j**) was reduced by three *Chlorogonium* strains to the corresponding *β*-hydroxy ester (**2j**) with a low conversion rate and a low *syn*/*anti* ratio (see Table 4).

However, the carbonyl group at the 3-position of the substrate was reduced stereoselectively, and its enantioselectivity of the 3-hydroxy group was excellently high (>99% e.e.). Hence, the substrate was reduced by *C. elongatum* NIES-1357 strain in the presence of additives to improve the low conversion rate of the product (see Table 5).

When L-alanine was added to the reaction mixture, the conversion rate increased to 75% from 48%, and the *syn*/*anti* ratio improved to 82/18, while maintaining excellent enantioselectivity of the 3-position hydroxy group.

Summarily, three *Chlorogonium* strains from the Heamatococcaceae family converted different α-keto esters and an aromatic α-keto amide to the corresponding hydroxy esters and hydroxy amide. Based on the conversion ratios and the stereoselectivity of the products, we suggested *C. elongatum* NIES-1357 strain cultured in the AY medium under light conditions for potential use as a biocatalyst for the stereoselective reduction of various carbonyl compounds (aliphatic and aromatic α-keto esters,2-chlorobenzoylformamide, and ethyl 2-methyl-3-oxobutanoate) to produce the corresponding chiral alcohols.

Acknowledgement

This study was supported by a grant from the UESCO Scientific Promotion Foundation (2021). Furthermore, we grateful to associate professor Toshinobu Suzaki (Graduate School of Science, Kobe University, Japan) for kind gift of the *C. capillatum* NIES-1357 strain and for his useful suggestion.

References

Ettl, H. 1980. Die taxonomische abgrenzung für gattung *Chlorogonium* ehrenberg (Chlamydomonadales, Chlorophyta). Nova Hedwigia 33: 709-722.

Ishihara, K., Iwai, R., Yoshida, M., Morishita, M., Yamaguchi, H. and Nakajima, N. 2011. Purification and characterization of a novel keto ester reductases from green algae, *Chlorella sorokiniana*: Comparison of enzymological properties with other microbial keto ester reductases. World J. Microbiol. Biotechnol. 27(1): 17-24.

Ishihara, K., Yamaguchi, H., Adachi, N., Hamada, H. and Nakajima, N. 2000. Stereocontrolled reduction of α- and *β*-keto esters with micro green algae, *Chlorella* strains. Biosci. Biotechnol. Biochem. 64(19): 2099-2103.

Kawai, Y., Takanobe, K. and Ohno, A. 1995. Stereochemical control in microbial reduction. XXV. Additives controlling diastereoselectivity in a microbial reduction of ethyl 2-methyl-3-oxobutanoate. Bull. Chem. Soc. Jpn. 68(1): 285-288.

Kreuzberg, K. 1984. StarchFermentation via formate producing pathway in *Chlamydomonas reinhardtii*, *Chlorogonium elongatum* and *Chlorella fusca*. Physiol. Plantarum 61(1): 87-94.

Mitsuhashi, K. and Yamamoto, H. 2005. Method for producing optically active mandelic acid derivative. Jpn. Kokai Tokkyo Koho 2005-295817 (Oct. 27).

Nakada, T., Nakazawa, A. and Nozaki, H. 2005. Two species of *Chlorogonium* (Volvocales, Chlorophyceae) from Japan. J. Jpn. Bot. 80(4): 197-207.

Nakada, T., Soga, T., Tomita, M., and Nozaki, H. 2010. *Chlorogonium complexum* sp. nov. (Volvocales, Chlorophyceae), and morphological evolution of *Chlorogonium*. Eur. J. Phycol. 45(1): 97-106.

Nakamura, K., Inoue, K., Ushio, K., Oka, S. and Ohno, A. 1988. Stereochemical control on yeast reduction of α-keto esters. Reduction by immobilized bakers' yeast in hexane. J. Org. Chem. 53(11): 2589-2593.

Omura, G., Ishida, M., Arikawa, M., Khan Mostafa Kamal, S. M., Suemoto, Y., Kakuta, S., Yoshimura, C. and Suzaki, T. 2004. A bacteria-free monoxenic culture of *Paramesium bursaria*: its growth characteristics and the re-establishment of symbiosis with *Chlorella* in bacteria-free conditions. J. J. Protozool. 37(2): 139-150.

Struck, F., Groelz-Krug, S., Boschek, B., and Zetsche, K. 1987. Light and acetate regulate a mitochondrial malate dehydrogenase. Plant Physiol. 85(1): 124-130.

How to cite this article:

Kohji Ishihara, S. Oka, Y. Hayashi, Y. Kondo, Y. Kitagawa, K. Shimoda, N. Masuoka and Nakajima, N. 2022. Biocatalytic Preparation of Chiral Alcohols with Micro Green Algae: Bio-reduction of Carbonyl Compounds by *Chlorogonium* Strains as a Novel Biocatalyst. *Int.J.Curr.Microbiol.App.Sci.* 11(04): 6-12. doi: https://doi.org/10.20546/ijcmas.2022.1104.002