海産酵母 Rhodotorulaの生長におけるビタミンB12、感受性

誌名	日本水産學會誌
ISSN	00215392
著者	河原, 孝義 高田, 英夫
巻/号	39巻9号
掲載ページ	p. 979-986
発行年月	1973年9月

Bulletin of the Japanese Society of Scientific Fisheries 39(9) 979-986 (1973)

Susceptibility to Vitamin B_{12} on Growth of Marine Yeast, *Rhodotorula**

Takayoshi KAWAHARA** and Hideo TAKADA***

(Received June 7, 1973)

This paper is concerned with the effect of vitamin B_{12} on the growth of the halophilic marine yeast, *Rhodotorula glutinis* var. *salinaria*, particularly with respect to the utilization of inorganic nitrogen sources in saline media of different hypertonicities. At a concentration of 1×10^{-5} M, vitamin B_{12} was inhibitory to growth and markedly so in salt media containing NO_3^- but no Mo ions. In non-saline media, the inhibition was less marked than in salt media and NH_4^+ was utilized more effectively than NO_3^-. Tween 80 could be substituted for glucose as the carbon source but less effectively. The significance of vitamin B_{12} for the process of inorganic nitrogen reduction to probably amino acid could be evaluated, but more precisely must await further evidence to evaluate.

Generally yeast contains vitamin B_{12} within the cell less than 0.3 μg per g dry weight, and it is not unique among the growth factors for yeast, while several bacteria and photosynthetic microorganisms such as *Euglena* require trace of it for growth. Additional roles for vitamin B_{12} have been suggested by nutrient data of growth of marine microorganisms.

In our research group, OHARA *et al.* (unpublished) indicated to stimulate the growth of a strain of *Candida* by the requirement of this vitamin in hypertonic NaCl medium supplied with oleate as a sole carbon source, and discussed the relationship of this requirement to the utilization of several fatty acid sources under the hypertonic condition. Accordingly the requirement of this vitamin for growth of the facultative halophilic yeast, *Rhodotorula glutinis* var. *salinaria* which has been isolated from salt farm and characterized by the growth at the same rate in saline as well as normal hypotonic media, has been investigated when medium was supplied with glucose and peptone. The present investigation was made to obtain the effect of vitamin B_{12} on growth of this yeast.

Materials and Methods

Rhodotorula glutinis var. *salinaria* was grown under shaking culture condition at 28°C. Culture conditions were given in detail elsewhere[1]. Glucose was used as a carbon source mainly, and Tween 80 was also used as indicated in the experiment. Suspensions contained in flasks were incubated on a rotary shaker which was operated at approximately

* This work was partly supported by the grant of the Ministry of Education to one of us (H.T.).

** Marine Development Division of Miyako Co., Ltd. Kushincho, Kitaku, Osaka, Japan (河原孝義: ミヤコ株式会社)

*** Department of Biology, Faculty of Science, Osaka City University, Sumiyoshiku, Osaka, Japan (高田英夫: 大阪市立大学)

120 oscillations per minute at a stroke length of 3.5 cm in radius.

Results

Effect of vitamin B_{12} at 1×10^{-5} M concentration on growth in salt-free, glucose medium with NO_3^- as a nitrogen source during 72 hr in the absence of Mo ions was given in Fig. 1. The onset of inhibition apparently occurred for 48 hr-incubation. As shown in Fig. 2, when 0.5 M NaCl was added to such medium, there was also effectively at 48 hr-incubation which increased the sensitivity to vitamin B_{12}. Growth was inhibited by the addition of vitamin B_{12} 29% in salt-free medium and 59% in salt medium for 72 hr.

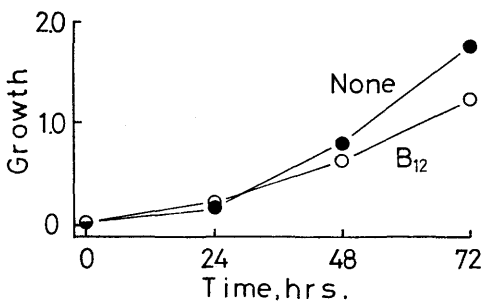

Fig. 1. The time course of growth in salt-free media with or without vitamin B_{12} at 1×10^{-5} M concentration for 72 hr. Nitrogen and carbon sources were NO_3^- and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

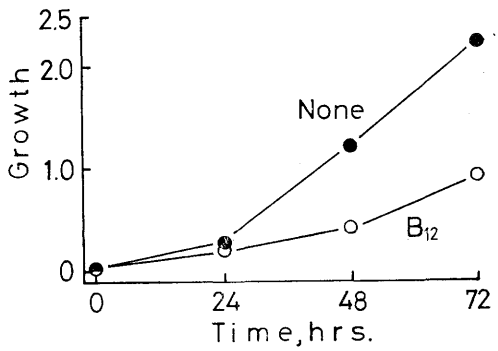

Fig. 2. The time course of growth in 0.5 M NaCl media with or without vitamin B_{12} at 1×10^{-5} M concentration for 72 hr. Nitrogen and carbon sources were NO_3^- and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

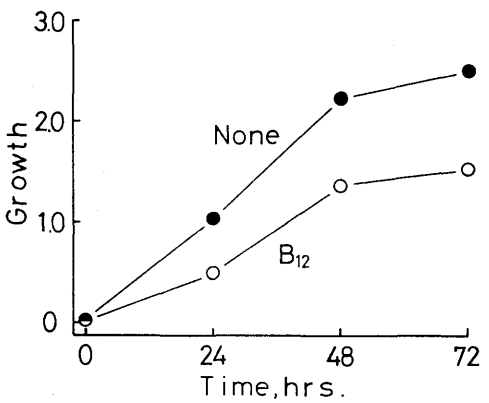

Fig. 3. The time course of growth in salt-free media with or without vitamin B_{12} at 1×10^{-5} M concentration for 72 hr. Nitrogen and carbon sources were NH_4^+ and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

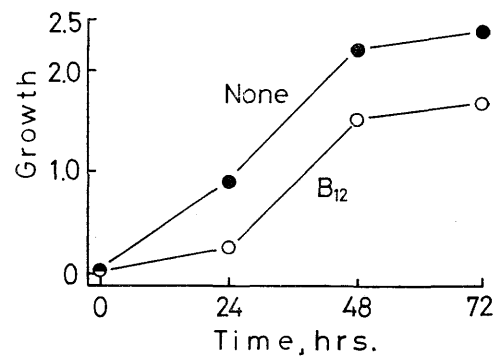

Fig. 4. The time course of growth in 0.5 M NaCl media with or without vitamin B_{12} at 1×10^{-5} M concentration for 72 hr. Nitrogen and carbon sources were NH_4^+ and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

When NO_3^- was substituted for NH_4^+ as a nitrogen source, vitamin B_{12} inhibition in salt-free and salt media became to 39% for the same culture period. These results were shown in Figs. 3 and 4, and might indicate that the site of inhibition of vitamin B_{12} in the cell grown in NO_3^- medium was not the same site with that in NH_4^+ medium under hypotonic as well as hypertonic conditions.

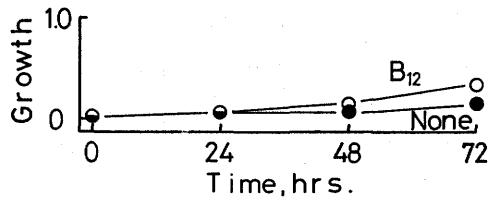

Fig. 5. The time course of growth in salt-free media with or without vitamin B_{12} at 1×10^{-5} M concentration in the further presence of molybdate at 1×10^{-4} M concentration for 72 hr. Nitrogen and carbon sources were NO_3^- and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

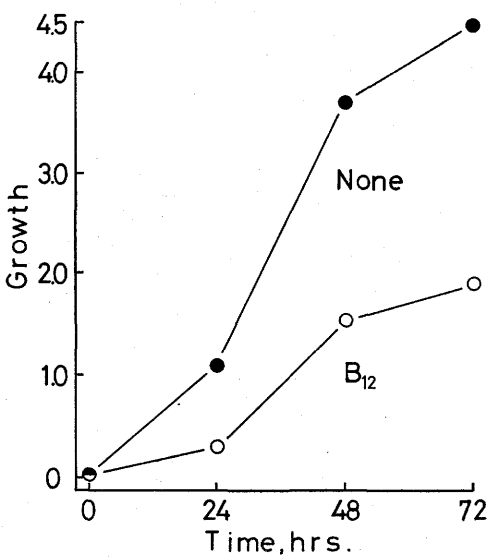

Fig. 7. The time course of growth in salt-free media with or without vitamin B_{12} at 1×10^{-5} M concentration in the further presence of molybdate at 1×10^{-4} M concentration for 72 hr. Nitrogen and carbon sources were NH_4^+ and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

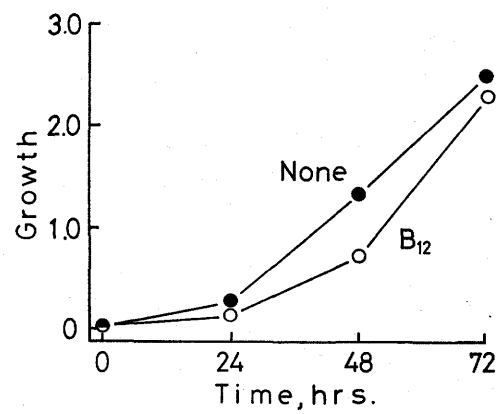

Fig. 6. The time course of growth in 0.5 M NaCl media with or without vitamin B_{12} at 1×10^{-5} M concentration in the further presence of molybdate at 1×10^{-4} M concentration for 72 hr. Nitrogen and carbon sources were NO_3^- and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

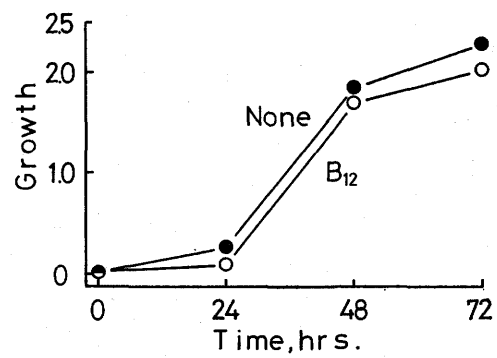

Fig. 8. The time course of growth in 0.5 M NaCl media with or without vitamin B_{12} at 1×10^{-5} M concentration in the further presence of molybdate at 1×10^{-4} M concentration for 72 hr. Nitrogen and carbon sources were NH_4^+ and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

According to the data in preliminary experiment, the growth pattern of the present material could be ascribed to different action on two separate assimilation pathways of NO_3^- and NH_4^+ between under hypotonic and hypertonic conditions with NaCl at 0.5 M by the addition of Mo ions.

In extending this work Mo ions were additively used in the present work. In salt-free and NO_3^- medium with glucose as a carbon source, the addition of Mo ions at 1×10^{-4} M resulted in significant inhibition, for growth but the antagonistic effect of Mo ions and vitamin B_{12} was observed with slight recovering effect from Mo inhibition as shown in Fig. 5. When Mo ions were added into salt medium supplied with NO_3^- as a nitrogen source together with vitamin B_{12}, the growth increased approximately with the same growth rate in two media (Fig. 6). When NO_3^- was replaced with NH_4^+ in medium as a nitrogen source in salt-free medium with vitamin B_{12}, the progressive inhibition by vitamin B_{12} increased as the incubation time increase (Fig. 7), while the inhibition of vitamin B_{12} was completely overcome by the addition of Mo ions in salt medium (Fig. 8).

To summarize the effect of an inorganic nitrogen source on growth in salt-free as well as salt media, figures which were calculated from the data of growth at 48 hr and were obtained as ratio of growth in each NO_3^- medium to that in respective NH_4^+ medium, were shown in Table 1. In all cases, NH_4^+ as a nitrogen source supported the better growth than NO_3^- did. It was somewhat less in degree in salt-free medium than that in saline

Table 1. Ratio of growth in NO_3^- medium to that in NH_4^+ medium. The data were calculated from those presented in **Figs.** from **1** to **8**. Mo: Mo ions as Na-molybdate.

Culture condition	Salt-free			0.5 M NaCl		
	Time, hr.			Time, hr.		
	24	48	72	24	48	72
None	0.15	0.36	0.70	0.29	0.55	0.94
Vitamin B_{12}	0.41	0.47	0.82	0.77	0.27	0.54
Mo	0.06	0.02	0.04	1.00	0.72	1.16
Vit. B_{12}+Mo	0.22	0.09	0.18	1.50	0.43	1.15

medium with exception of the addition of vitamin B_{12}. The inhibition of added vitamin B_{12} was relatively significant only when it was added into NO_3^--saline medium in the absence of Mo ions, as mentioned above. Reversely, the addition of Mo ions into NO_3^- medium inhibited the growth in salt-free medium and resulted relatively in the good growth in salt medium. The cells did not loss their utilizability of NO_3^- even when vitamin B_{12} was added additively into this medium.

When glucose was substituted for Tween 80 as a carbon source, the different patterns of Tween 80 response in saline medium with Mo ions for 72 hr may be discerned in the data of Table 2. The present material could grow only slowly. Similar nutritional response

for growth for 72 hr was obtained in salt-free medium with Mo ions plus vitamin B_{12}. In saline medium supplied with two compounds, the growth was inhibited significantly.

We now investigated, whether a variation in the utilization of inorganic nitrogen source might explain to relate to the utilization of organic nitrogen source by the present material. To test this, peptone was used as nitrogen source. Results were shown in Table 3. The cells were able to grow significantly in vitamin B_{12} medium. A conclusion is derived from these results to show the interference by vitamin B_{12} in mainly utilization of inorganic nitrogen at an early stage of pathway to synthesize organic nitrogen compound.

Table 2. Growth in salt-free and 0.5 M NaCl media for 72 hr-incubation period. Nitrogen and carbon sources were NO_3^- and Tween 80, respectively. Growth was expressed as mg dry weight per ml culture. Mo : Mo ions as Na-molybdate.

Culture condition	Salt-free	0.5 M NaCl
None	0.9	1.1
Vitamin B_{12}	0.7	0.7
Mo	0.3	0.6
Vit. B_{12}+Mo	0.2	0.1

Table 3. Growth in salt-free and 0.5 M NaCl media for 48 hr-incubation period. Nitrogen and carbon sources were peptone and glucose, respectively. Growth was expressed as mg dry weight per ml culture. Mo : Mo ions as Na-molybdate.

Culture condition	Salt-free	0.5 M NaCl
None	8.3	7.4
Vitamin B_{12}	8.0	7.0
Mo	7.9	7.0
Vit. B_{12}+Mo	8.1	6.1

Table 4. Growth in salt-free and 0.5 M NaCl media with $CoCl_2$ at 1×10^{-5} M concentration for 48 hr-incubation period. Nitrogen and carbon sources were NO_3^- and glucose, respectively. Growth was expressed as mg dry weight per ml culture.

Incubation time	Salt-free	0.5 M NaCl
48 hr	1.1	1.6

Furthermore, there are definite reasons to doubt whether this effect of vitamin B_{12} occurs surely to involve Co ions. Therefore it should be added that this effect did not concern with the effect of only Co ions. The data of growth as shown in Table 4 seems to show clearly that the equimolar concentration of Co ions with vitamin B_{12} did not result in any inhibitory effect on growth in salt-free as well as salt media when they were supplied with NO_3^- as a nitrogen source.

Discussion

Little is known about the toxicity of vitamin B_{12} to yeasts, although most literature deals with the effect on growth of marine autotrophic microbes as a cofactor under saline condition as well as heterotrophic ones under hypotonic condition, independently (AHEARN *et al.*[2]). In other words vitamin B_{12}-essential and vitamin B_{12}-susceptible varieties have been recognized in microorganisms, although the mechanism of essentiality has been proposed, and mechanism of suceptibility is unknown. For instance, vitamin B_{12} in culture of marine phytoplankton have been shown to promote the growth (PROVASOLI[3], PEDERSEN[4,5], LEWIN[6]). On the other hand, interaction of exogenously added vitamin B_{12} with utilization of fatty acid as well as kerosen by the another yeast, a strain of *Candida*, is supported by experiments that suggest the effect of vitamin B_{12} on methionine biosynthesis, which in turn makes avaliable acetylcholine, trimethylamine,choline and betaine. Furthermore *Candida* was above normal in the presence of vitamin B_{12} in hypotonic salt-free medium, and there was more significant in salt medium (OHARA *et al.* unpublished). The present result was counter to those mentioned above in *Candida*. A concentration of 1×10^{-5} M vitamin B_{12} in the surrounding medium created to the highest extent a decrease in growth of the present material in saline medium supplied with NO_3^- and in contrast, to higher extent in non-saline, salt-free medium than in saline medium supplied with NH_4^+ as a nitrogen source. This was somewhat referred to two findings in contrast.

Accordingly the pathway of vitamin B_{12} in a strain of *Candida* may be presented by current information, in the present material some tentative pathway may be presented. As shown in Figs. 1–4, the inferred synthetic pathway to protein was shown, first, NO_3^- reduction, second, perhaps synthesis of amino acid from NH_4^+ and third, protein synthesis from amino acid were put forward concerning the basis of growth patterns. Furthermore such pathway of inorganic nitrogen can be altered by changing the tonicity with NaCl in medium.

Another possible mechanism for the action of vitamin B_{12} on yeast, suggested by YAGI *et al.*[7] was pointed out by the changes in dimorphic growth pattern of *Endomycopsis fibuligera* between under saline as well as non-saline conditions. Even if vitamin B_{12} inhibited the total growth of *Endomycopsis* which was expressed by summation of the growth of filamentous and yeast-like formed cells this vitamin did not inhibit the growth of yeast-like cell in non-saline medium, while it inhibited the growth of filamentous cell. In contrast to this, the reversal results were obtained in saline medium. Since on the growth experiment of *Endomycopsis*, peptone was employed as nitrogen source, vitamin B_{12} must act at least on the pathway from amino acid to protein synthesis. Differential action of vitamin B_{12} on dimorphic yeast between under non-saline and saline conditions significantly was observed. The exact reverse was the cases the with growth of *Candida* and

the growth of filamentous cells of *Endomycopsis* and it is the similar case with the growth of yeast-like cells of the latter species. When peptone was used as nitrogen source in the present experiment the inhibitory effect was not detectable (Table 3). On the other hand, it is interesting to compare these results to that given by a growth of the present material in the same medium containing more than 1×10^{-5} M Co ions. The growth was not inhibited by adding Co ions into NO_3^- media with or without 0.5 M NaCl (Table 4). Therefore the effect of vitamin B_{12} can not be explained by toxic levels of Co ions.

The results of this paper suggest two possible mechanisms acting differentially on pathways of either NO_3^- reduction system to amino acid or pathway from NH_4^+ to amino acid in saline and salt-free media, respectively. Some possibilities may be concerned with the intracellular concentrations of Na ions at different levels of NaCl which were found to be approximately one-tenth of extracellular concentration in either NO_3^- or NH_4^+ medium. Therefore the pathways of NO_3^- reduction as well as that of NH_4^+ to amino acid varied in their susceptibility at the cell surface to high concentration of NaCl in external medium may be proposed. In this case different pathway must be taken into consideration when evidence for the presence of particular enzyme system which must be concerened with ion transport and modification of structural organization at cell surface is being sought.

In salt-free medium the amount of Mo ions required for 50% inhibition of growth under the similar experimental condition with the present case was less than 1×10^{-4} M concentration used here. On the contrary such inhibition on the growth of the present material entirely was recovered in salt medium. Therefore the difference in the effect of Mo ions on growth between in saline- and salt-free media can be explained in two ways: one, Mo ions affect different site, two, the changes in the state of the growing cell between in salt-free and in saline media is of important.

When Mo ions were added into hypotonic culture medium, the antagonistic effect of Mo ions and vitamin B_{12} can be ascribed to dependent action on two separate assimilation pathways of NO_3^-. When NH_4^+ was added instead of NO_3^- under the same culture condition, the postulated inhibition of growth by vitamin B_{12} would be expected by an alternate pathway by Mo ions which stimulated more significantly the growth than that in Mo ions-free hypotonic medium with NH_4^+.

Less dramatic, but neverthless highly significant the recovering effect of Mo ions on lowered growth by vitamin B_{12} in salt-free medium supplied with NO_3^- as a nitrogen source was evidenced.

On the other hand, this may be arisen by modification of structural organization including Na ion transport system by adding Mo ions into NO_3^-, salt-free medium. In salt-free as well as salt medium with NH_4^+ the regulation of amino acid formation from NH_4^+, like the case in peptone medium, could be expected to exert some crucial, indepen-

dent control of pathway of vitamin B_{12} over the growth period when Mo ions were added. This is also the case when NO_3^-, together with Mo ions was added into saline medium.

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