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Influence of Medium Components and Physical Factors on Vitamin B_{12} Production by *Pseudomonas* sp. PCSIR 99

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Abstract. Study of the influence of various carbon sources on production of vitamin B_{12} by *Pseudomonas* sp. PCSIR 99 showed that methanol, methylamine and 1, 2-propanediol promoted vitamin B_{12} production when grown on the modified basal medium emended with 0.2% 5, 6-dimethylbenzimidazole. Among the nitrogen sources tested, L-aspartate and L-methionine enhanced vitamin B_{12} production and cell mass dramatically. Any upward or downward change in pH from 6.5 and in temperature from 32 °C decreased the level of vitamin B_{12} in the medium. Whereas, 10% oxygen and 0.004 mM cobalt ion concentration increased vitamin B_{12} production significantly. It is inferred that vitamin B_{12} production can also be regulated by proper manipulation of physical parameters of the medium as well.

Keywords: vitamin B_{12}; *Pseudomonas* sp; methanol; 5, 6-dimethylbenzimidazole; L-aspartate; L-methionine; cobalt ions; propanediol

Introduction

Vitamin B_{12} (cobalamine) is one of the most alluring and fascinating molecules in the world of science and medicine. It was isolated from liver extract in 1948 and reported to control pernicious anemia (Okuda, 1999). Its structure was elucidated in 1955. Chemically, vitamin B_{12} is a molecule with the formula of $C_{63}H_{90}CoN_{14}O_{14}P$. It contains the heavy metal cobalt, which gives this water-soluble vitamin its red colour.

In humans, vitamin B_{12} is required in trace amounts (approx.1µg/day) to assist the action of two enzymes, methionine synthase and (R)-methylmalonyl-CoA mutase (Mancia *et al.,* 1996; Drennen *et al.,* 1994), yet commercially more than 10 tons of vitamin B_{12} are produced each year for its use as an essential growth factor in manufacture of important synthetic growth media (Martens *et al.,* 2002), and in modern research, as an essential component of media for cell line growth (Yamazoe *et al.,* 2006).

The chemical synthesis of vitamin B_{12} on industrial scale is in principle very complicated and expensive since it requires more than 70 steps (Eschenmoser, 1994; Woodward, 1973). Therefore, vitamin B_{12} is produced intracellularly or extracellularly on the industrial scale using the batch or fed-batch process of microbial fermentation (Yongsmith *et al.*, 1982). Several microorganisms, including those of the genera *Bacillus, Methanobacterium, Propionibacterium* and *Pseudomonas,* have been used to produce vitamin B_{12} on industrial scale. (Bykhovsky *et al.,* 1998).

Different components of fermentation media and physical factors are very critical in cell mass production and vitamin B_{12} synthesis. In the current investigation, influence of different carbon and nitrogen sources was studied on mass cell production of *Pseudomonas* sp. and its ability to synthesize vitamin B_{12} under optimized physical parameters of fermentation media.

Materials and Methods

Carbon and nitrogen sources used. ***Carbon sources.*** 1, 2 propanediol (Scharlau Chemie, S.A. Spain); lactate (Sigma-Aldrich, USA); methaneol (Scharlau Chemie, S.A. Spain); succinate (Sigma-Aldrich, USA); fumarate (Sigma-Aldrich, USA); malate (Sigma-Aldrich, USA); tartarate (Sigma-Aldrich, USA); glycerol (Sigma-Aldrich, USA); methylamine and dimethylamine (Fluka Chemie Ag and RdH, Switzerland); ethanol (Merck Chemicals Ltd., Germany); ethylene glycol (Sigma Aldrich, USA); glucose (Merck Chemicals Ltd., Germany).

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Nitrogen sources. $(NH_4)_2HPO_4$(Sigma-Aldrich, USA); NH_4Cl (Sigma-Aldrich, USA); $(NH_4)_2SO_4$ (Sigma-Aldrich, USA); $NaNO_3$ (Sigma-Aldrich, USA); urea (Sigma-Aldrich, USA) L-methionine (Merck Chemicals Ltd. Germany); L-aspartate (Merck Chemicals Ltd., Germany).

Physical factors studied. Temperature range of 25 °C to 40 °C; pH range of 6-8 and oxygen concentration range from 10% to 21% were used to observe the production of vitamin B_{12} and cell biomass of bacteria.

Microorganism. The bacterial strain of *Pseudomonas* sp. was procured from Pakistan type of culture collection (PTCC), Pakistan Council of Scientific and Industrial Research Laboratories Complex, Lahore, Pakistan and used as producer of vitamin B_{12} (Riaz *et al.*, 2007). The strain was grown at 32 °C and maintained on nutrient agar slants and was sub-cultured at four week intervals.

Inoculum preparation. Inoculum was prepared by transferring 5 ml suspension of 24 h old slant culture, to Ehrlenmeyer flask containing 45 ml of sterile inoculum medium. The composition of medium was 38 mM $(NH_4)_2HPO_4$; 0.15 mM KCl; 0.25 mM NaCl; 0.813 mM $MgSO_4.7H_2O$; 0.7 mM $CaCl_2.2H_2O$; 0.02 mM $FeSO_4.7H_2O$; 0.033 mM $MnSO_4.H_2O$; 0.004 mM $CoSO_4.7H_2O$; 0.003 mM $Na_2MoO_4.H_2O$; 150 mM CH_3OH was added as a carbon source (Toraya *et al.*, 1975).

Fermentation studies. 50 ml of 24 h old inoculum at the ratio of 10% (v/v) was added to one litre of production medium in 2 litre glass jar fermentor (Eyela, Japan) having working volume of 1litre. The composition of fermentation medium was the same as above-mentioned inoculum medium; maximum production of vitamin B_{12} was observed when 200 mM of methanol was used as carbon source. In order to test the influence of other carbon sources on vitamin B_{12} production, each carbon source was added to the medium in concentration corresponding to 200 mM carbon atom. For testing the effect of nitrogen on vitamin B_{12} production, each nitrogen source was normalized to 38mM nitrogen atoms. The pH of the culture was maintained at 6.5 with a pH controller (Metrohm AG, Herisau, Switzerland), using 1N NaOH and 1N HCl.

Growth under various oxygen concentrations was obtained by culturing the cells in one litre modified basal medium (MBM) with addition of 0.001% silicon antifoam agent (BDH Ltd., UK). Gas mixture of oxygen and nitrogen were pumped into medium through sterilized filter with gas flow rate of 0.4 l/min, controlled by gas mixture unit FM-130 Eyela,Japan. MBM was inoculated and fermentation was carried out under controlled conditions of temperature for 5 days.

Biomass estimation. An aliquot of 50 ml of culture sample was centrifuged (Backman; T2-HS Centrifuge with rotor JA-20) at 7740 x g and 4 °C for 15 min to collect the cells (Iqbal *et al.*, 1995). The cell free culture broth was stored for estimation of vitamin B_{12}. The cells were washed with sterilized distilled water. The pellet was then desiccated in an electric oven (D 06060. Model 400; Memmert) at 105 °C until constant weight was achieved.

Extraction of vitamin B_{12}. Vitamin B_{12} was extracted by harvesting the cells in fermentation broth and centrifuging the fermentation broth at 10,000 rpm. The pellets obtained were washed with 0.2 M potassium phosphate buffer (pH 5.5) and suspended in the same buffer containing 0.1% KCN. The suspension of bacterial cell biomass was autoclaved for 15 min at 121 °C. The supernatant containing extracted vitamin B_{12} was filtered through cellular acetate membrane filter 0.2 µm.

Estimation of vitamin B_{12}. Vitamin B_{12} was estimated microbiologically using *E. coli* 215 (Ikeda, 1956). This assay was also used to differentiate between physiologically active form of vitamin B_{12} that can be used by humans and analogous form of vitamin B_{12}. All vitamin B_{12} assays were carried out in triplicate at three different dilutions and each was analyzed 5 times.

Results and Discussion

Influence of precursor and growth. The cell biomass and vitamin B_{12} production of *Pseudomonas* sp. PCSIR 99 was monitored at hourly intervals. Modified Basal Medium with methanol added as carbon source (MBM-met) was initially used for analysis of cell biomass and vitamin B_{12} production. In this medium, vitamin B_{12} production started at the end of the exponential phase and kept on increasing until cell mass reached the stationary phase (Fig. 1A). During the first 24 h of stationary phase, vitamin B_{12} production remained unchanged. The maximum level of vitamin B_{12} achieved was 200 µg/g dry cell mass in MBM at 200 mM optimum carbon concentration. However, by supplementing MBM-met medium with 0.2% 5,6-dimethylbenzimidazole, the pronounced precursor of vitamin B_{12} (MBM-met-5,6-dmbm), the level of vitamin B_{12} was enhanced to 350 µg / g dry cell mass following the same growth phase(Fig. 1B) without affecting the cell mass. Thus vitamin B_{12} production was growth-phase dependent and was influenced by addition of precursor.

Influence of carbon sources. Cells of *Pseudomonas* sp. PCSIR 99 were grown in MBM-dmbm medium with equal concentrations of carbon atom such as 1,2-propanediol, lactate, methanol, succinate, fumarate, malate, tartarate, glycerol, methylamine, dimethylamine, ethanol, ethylene

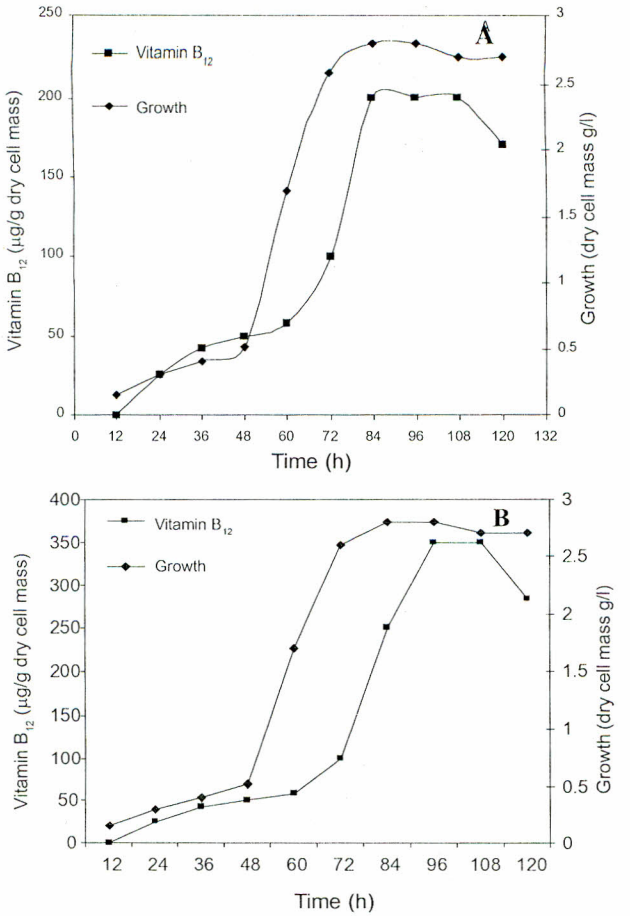

Fig. 1. Growth of and vitamin B_{12} production by *Pseudomonas* sp. PCSIR 99; **A**: on MBM-met; **B**: on MBM-met-5,6-dmbm.

glycol and glucose. Generation time, cell biomass and vitamin B_{12} production were determined at stationary phase. Short generation time (65-71 min) was required for growth on methanol, 1, 2-propanediol and methylamine, whereas, moderate generation time (90-108 min) was noted for the growth on succinate, fumarate, malate and tartrate. Growth on lactate, dimethylamine and glycerol required the longest generation time (250-540 min). However, no appreciable cell biomass and detectable vitamin B_{12} was obtained when strain PCSIR 99 was grown on carbon sources such as, ethanol, ethylene glycol, and glucose (Table 1). Bacterial growth on carbon sources like methanol, 1, 2-propanediol, and methylamine reached stationary phase with maximum growth range of 2.5 to 2.8 g dry cell mass/l of the medium. On the remaining carbon sources, early stationary phase was reached at low cell mass level varying between 2.1-2.7 g dry cell mass/l (Table 1). MBM-dmbm supplemented with methanol, 1, 2-propanediol and methylamine resulted in high vitamin B_{12} production (350 µg, 270 µg, 250 µg/g dry cell mass, respectively) as compared to all other carbon sources. High yield of vitamin B_{12} (350 µg/g dry cell mass) was achieved when methanol was used as carbon source at lower cell mass, therefore, MBM-5,6-dmbm medium supplemented with methanol (MBM-met-5,6-dmbm) was subsequently used to study the effect of various nitrogen sources, physical factors, and Co^{+2} ion concentration.

Influence of nitrogen sources. Vitamin B_{12} level was studied after replacement of nitrogen source $(NH_4)_2HPO_4$ with NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, urea, L-methionine and L-aspartate. Urea

Table.1. Influence of various carbon sources on generation time in exponential phase and cell biomass and vitamin B_{12} production at stationary phase of *Pseudomonas sp.* PCSIR 99 grown on MBM.

Carbon source (concentration)	Generation time (min)	Dry cell mass (g/l)	Vitamin B_{12} (µg/g dry cell mass)	
			with 0.2% 5, 6-dmbm	without 0.2% 5, 6-dmbm
1,2-Propanediol (66 mM)	71±25	2.5±0.2	285±23	145±25
Methylamine (200 mM)	76±28	2.7±0.3	310±35	150±32
Methanol (200 mM)	65±17	2.8±1.4	350±27	200±16
Succinate (50 mM)	90±12	2.18±0.1	150±15	80±8
Fumarate (50 mM)	97±2	2.1±0.3	154±16	70±9
Ethanol (100 mM)	500±52	0.7±0.3	-	-
Malate (50 mM)	105±7.7	2.0±0.1	164±18	72±6
Tartarate (50 mM)	108±13	1.8±0.2	178±20	85±14
Lactate (66 mM)	250±22	2.7±0.3	110±12	54±6
Dimethylamine (100 mM)	540±55	1.8±0.2	123±8	56±10
Glycerol (66 mM)	205±32	2.1±0.1	132±19	60±14
Glucose (31 mM)	400±46	1.0±0.1	-	-
Ethylene glycol (66 mM)	396±35	1.2±0.4	-	-

5, 6-dmbm = 5,6-dimethylbenzimidazol

and $NaNO_3$ resulted in decreased vitamin B_{12} production (Fig. 2A), whereas growth on 38 mM L-methionine and 38 mM L-aspartate resulted in 1 fold (670 µg/g dry cell mass) and 1.5 fold (875 µg/g dry cell mass) increase in vitamin B_{12} level, respectively, and 20% higher cell mass as compared to 19 mM $(NH_4)_2HPO_4$ (Fig. 2A). Increase in the concentration of $(NH_4)_2HPO_4$ upto 38 mM nitrogen atom resulted in increase of the vitamin B_{12} level and *vice versa* (Fig. 2B). Stimulatory effect of NH_4^+ ions and/or PO_4^{+3} ions on vitamin B_{12} level and cell mass of bacterium, were tested with equal conc. of K_2HPO_4 with $(NH_4)_2SO_4$. Both NH_4^+ ions and PO_4^{+3} ions, when used separately, resulted in one fold decrease in the vitamin B_{12} level as compared to that with standard concentration of $(NH_4)_2HPO_4$ (19 mM) (Fig. 2C). The synergistic effect was studied with addition of both the ions in different concentrations, keeping the concentration of one constant. The vitamin B_{12} level increased more than half fold when NH_4^+ ions and PO_4^{+3} ions were used at 38 mM and 19 mM concentration, respectively (Fig. 2C), as compared to the standard concentration of $(NH_4)_2HPO_4$.

The simultaneous effect of L-methionine and L- aspartate on vitamin B_{12} production, was observed with varying the concentrations of both the amino acids from 19 mM to 38 mM, keeping the concentration of any one constant. Maximum level of vitamin B_{12} (875 µg/g drycell) was achieved when the concentrations of both the amino acids were equal (19 mM each) (Fig. 2D) at 10.5% less cell biomass as compared to that of L-aspartate when used alone at 19 mM concentration.

Influence of physical factors. The cells of the strain PCSIR 99 were at the temperature range of 25-40 °C; 32 °C was found to be the optimum temperature for the growth of bacterium as well as for vitamin B_{12} production. Either increase or decrease in temperature from 32 °C had inhibitory effect on the level of vitamin B_{12}. At 37 °C there was 0.7 fold decrease in vitamin B_{12} level but growth remained constant, while at 40 °C there was 1.3 fold decrease in vitamin B_{12} production and 28% decrease in the growth as compared to that of 32 °C. However, decrease in the temperature down to 25 °C nullified the vitamin B_{12} level completely with 30% decrease in cell biomass as compared to that at 32 °C (Fig. 3A).The influence of

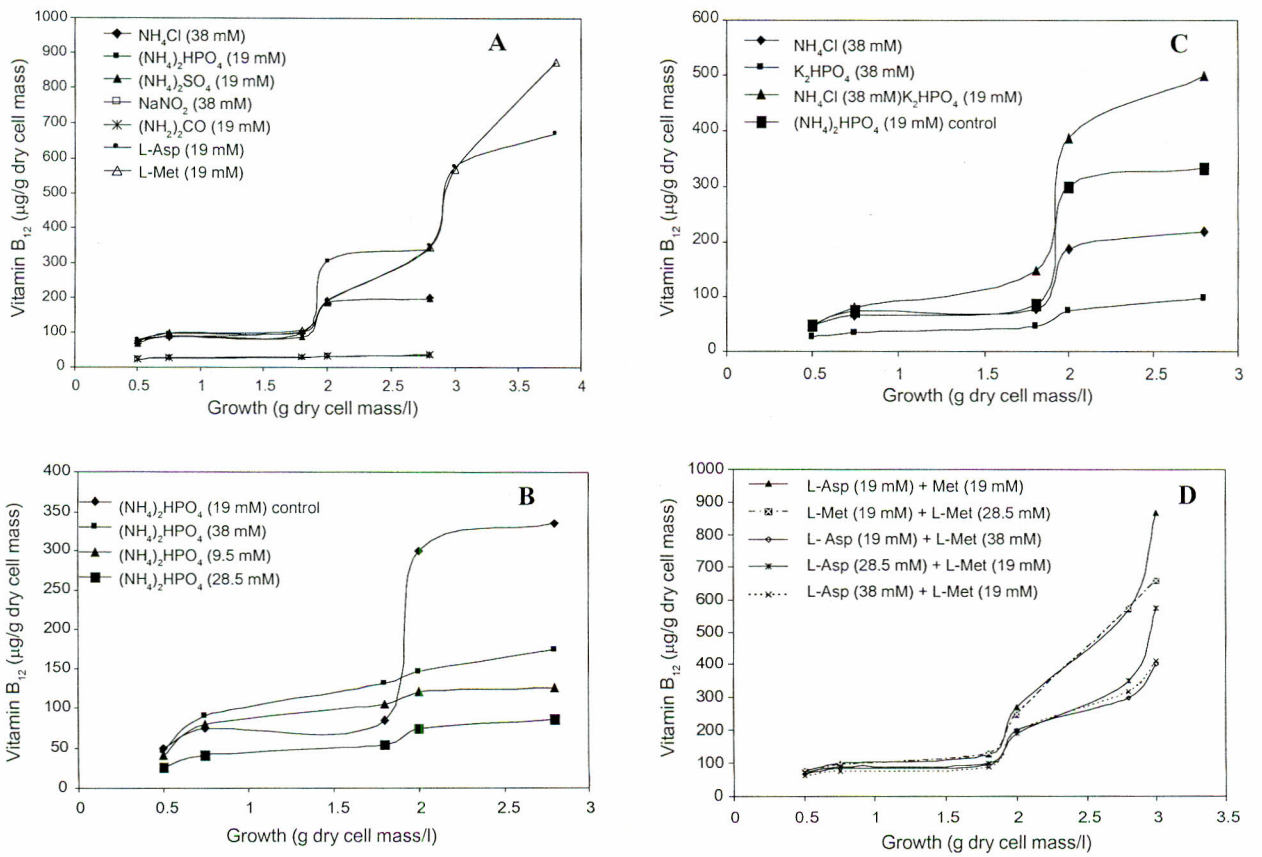

Fig. 2. Influence of various nitrogen sources on the level of vitamin B_{12} production by *Pseudomonas* sp. PCSIR 99 (on MBM-met-5,6-dmbm); **A**: grown with various nitrogen sources; **B**: various conc. of $(NH4)_2HPO_4$; **C**: synergistic influence of various conc. of NH_4^+ and PO_3^{+3} ions; **D**: effect of various conc. of L-Asp and L-Met.

the pH of the medium on vitamin B_{12} level and cell biomass, was studied by growing the strain PCSIR 99 at different pH (6-8) in MBM-met-5,6-dmbm. The maximum vitamin B_{12} level was observed at pH 6.5 (Fig. 3B).

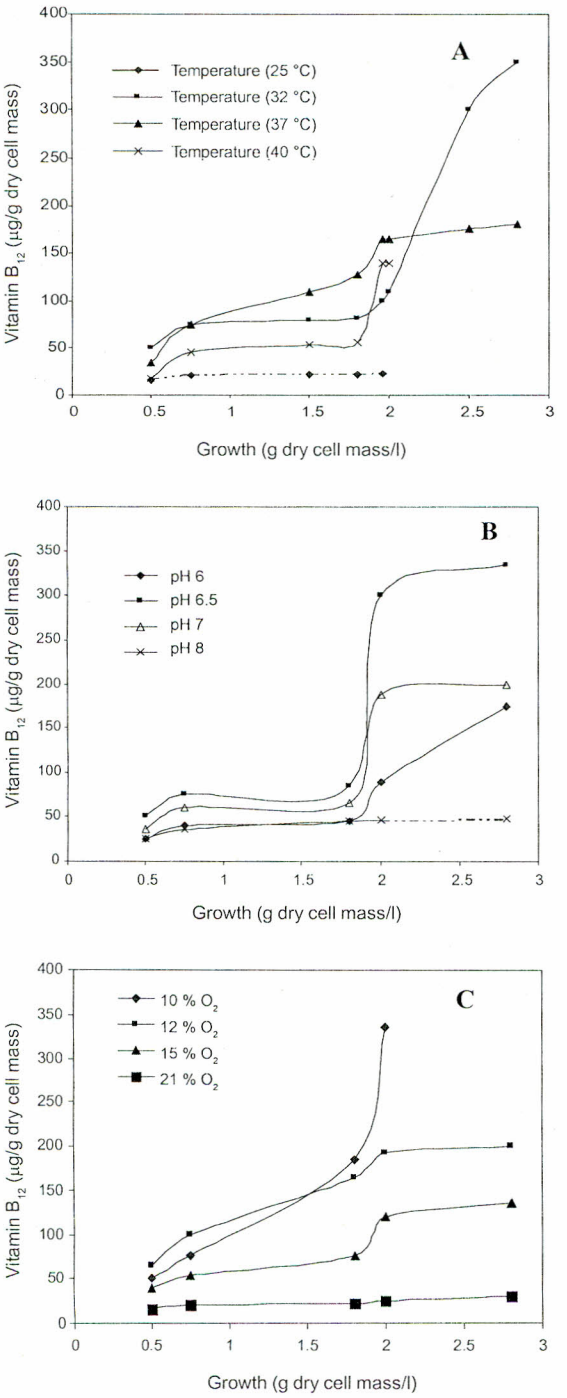

Fig. 3. Influence of physical parameters on the level of vitamin B_{12} production by *Pseudomonas* sp. PCSIR 99 grown on MBM-met-5,6-dmbm; **A**: temperature; **B**: pH values; **C**: oxygen concentration.

Influence of oxygen limitation on the level of vitamin B_{12} was investigated by aerating the medium with gas mixture containing 21%, 15%, 10% and 1% oxygen. Decreasing the oxygen concentration from 21% to 15% had little effect on vitamin B_{12} level and growth. However, aeration with 10% oxygen resulted in great increase in B_{12} level with 10% lower growth as compared to the growth and vitamin$_{12}$ production at 21% oxygen, in the similar experimental set up (Fig. 3C).

Influence of cobalt ion concentration. Among various Co^{+2} ion concentrations used, maximum vitamin B_{12} was produced (350 µg/g dry cell) at 0.004 mM Co^{+2} ion concentration using hydrated cobalt sulphate salt ($CoSO_4.7H_2O$). However, vitamin B_{12} level decreased to 0.6 fold (Fig. 4A) when Co^{+2} ion concentration was reduced to 0.003 mM without producing any significant effect on growth. Increase in Co^{+2} ions upto 0.005 mM decreased vitamin B_{12} 0.8 fold with 28 % reduction in the growth of the cells in the similar medium (Fig. 4A). Replacement of hydrated cobalt sulphate salt with anhydrous cobalt chloride ($CoCl_2$) did not have any significant effect (Fig. 4B).

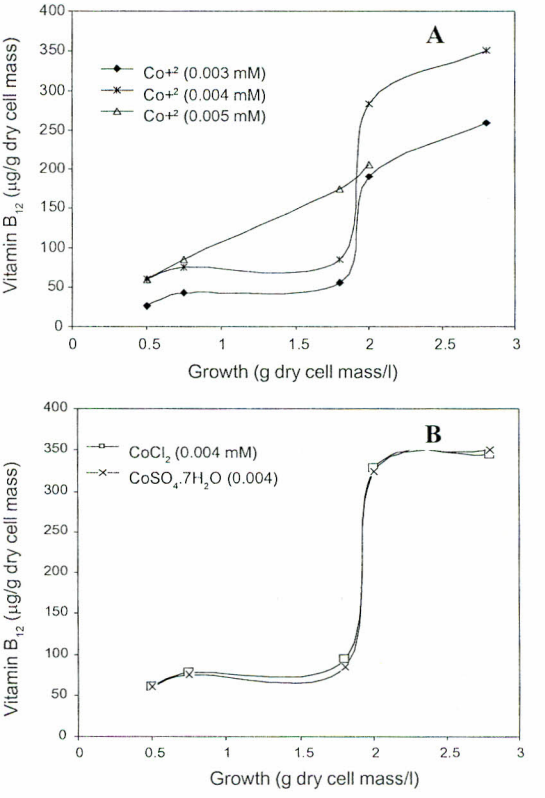

Fig. 4. Influence of various concentrations of Co^{+2} ions on the level of vitamin B_{12} production by *Pseudomonas* sp. PCSIR 99 grown on MBM-met-5, 6-dmbm., **A**: various concentrations of Co^{+2} ions; propanediol **B**: Co^{+2} ions belonging to different sources.

The work presented here is the most extensive study on the effect of different carbon sources, nitrogen sources, cobalt ions and different physical factors such as temperature, pH of the medium and oxygen supply, on microbial synthesis of vitamin B_{12}. The choice of the carbon sources studied was based on their common availability and less cost as compared to that of the end product. Among all the carbon sources, methanol, methylamine and 1, 2-propanediol yielded the highest vitamin B_{12} level in modified basal medium (MBM). The addition of 5, 6- dimethylbanzimidazole, as a precursor of vitamin B_{12}, to MBM medium enhanced the level of vitamin B_{12}. These results are in complete agreement with the previously published results (Riaz *et al.,* 2007; Kralova and Rauch,1986). Higher production of vitamin B_{12} and greater cell biomass of bacterium in MBM with 0.2% 5, 6-dimethylbanzimidazole when methanol was used as a carbon source indicated methylotrophic nature of *Pseudomonas* sp. PCSIR 99 (Martens *et al*., 2002).

Monitoring of vitamin B_{12} production as a function of the growth showed that vitamin B_{12} accumulated during the transition of the exponential to the stationary phase of cell growth (Fig. 1A, B). The comparison of vitamin B_{12} production and generation time for various carbon sources did not reveal any relationship between these two parameters (Table 1). This demonstrates that the growth does not regulate vitamin B_{12} production, at least not in a direct way.

Ammonium ions have stimulatory effect on vitamin B_{12} production (Fig. 2B) (Chou *et al.,* 1999) while nitrogen sources like $NaNO_2$ and $(NH_2)_2CO$ led to reduced vitamin B_{12} level with considerable decrease in cell biomass in the medium (Fig. 2A). However, the stimulatory effect of L-aspartate and L-methionine (Fig. 2A) on vitamin B_{12} level demonstrated that these two amino acids are excellent nitrogen sources. These results could be compared with those of Goux *et al.* (1995) and Toraya *et al.* (1975) regarding the growth of *E. coli* and *Pseudomonas* sp. Since vitamin B_{12} contains nitrogen, hence the stimulatory effect of nitrogen containing compounds on vitamin B_{12} production is observed.

Alkaline pH reduced the vitamin B_{12} production by *Pseudomonas* sp. PCSIR 99. Slightly alkaline pH, starting at pH 8 and ending at pH 7, reduced vitamin B_{12} synthesis significantly. However, pH 6.5 was found to be optimum for vitamin B_{12} production (Fig. 3B). Our results of good production of vitamin B_{12} at slightly acidic pH were consistent with the results of Tiffany *et al.* (2006) where they showed that pH remained on acidic side during the course of vitamin B_{12} production by ruminal mixed organisms grown in continued culture flow-through fermentors.

It had been reported that some bacterial strains produce comparatively higher amount of vitamin B_{12} when methanol is used as carbon source (Dumenil *et al.,* 1981). In our study, the decline in microbial growth and vitamin B_{12} production when organic acids were used as carbon source (Table 1) also indicates the methanol-assimilating nature of the *Pseudomonas* sp. PCSIR 99 which is in agreement with our previous findings (Riaz *et al.,* 2007).

Other factors which strongly influence the vitamin B_{12} production in *Psuodumonas* sp. PCSIR 99 appeared to be temperature (Fig. 3A) and O_2 concentration (Fig. 3C). The maximum growth and level of vitamin B_{12} were achieved at temperature 32 °C when 10% O_2 was supplied to the growth medium. Efficient vitamin B_{12} synthesis under 10% oxygen supply compared to 21% O_2 supply indicated microaerobic condition of the culture; these results are quite in accordance with those of Noparatnaraporn *et al.* (1986) for vitamin B_{12} production by *Rhodopseudomonas gelatinosa*.

When different concentrations of Co^{+2} ions (Fig. 4A) belonging to different salts sources (Fig. 4B) were supplied to the MBM-met-5,6-dmbm medium, significant effect was observed (Fig. 4A,B) on vitamin B_{12} production which could be elucidated on the basis of cobalt atom serving as central atom of vitamin B_{12} structure; and thence it is considered an important precursor for vitamin B_{12} synthesis (Keuth and Bisping, 1994). Similar results were also obtained by Tiffany *et al.* (2006). The present findings indicate the potential ability of *Psudomonas* sp. PCSIR 99 to produce vitamin B_{12}, when 200 mM methanol and 19 mM L-aspartate are used as carbon and nitrogen sources, respectively. However, 5, 6 dimethyl-benzimidazole being an excellent precursor of the vitamin B_{12}, had significant effect on vitamin B_{12} production. For successful biosynthesis of vitamin B_{12}, knowledge of the medium components and physical factors affecting the process of vitamin B_{12} production is necessary.

Conclusion

On the basis of the results of the study; it can be concluded that the *Psudomonas* sp. PCSIR 99 gives maximum production of vitamin B_{12}, when basal fermentation medium is supplied with 200 mM cobalt ion concentration, 0.2% 5, 6 dimethylbenzimidazole and 10% oxygen at temperature, 32 °C and optimum pH value of 6.5.

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