OPTIMIZATION OF THE *NOS*Z, *NIF*H, AND *PHA*C POLYMERASE CHAIN REACTION PRIMER SETS FOR TERMINAL RESTRICTION FRAGMENT PATTERN ANALYSIS

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Abstract

Attempts were made to optimize polymerase chain reaction primer sets for the genes nitrous oxide reductase (*nosZ*), nitrogenase (*nifH)*, and polyhydroxyalkanoate synthase (*phaC*). These genes correspond to two processes of the nitrogen cycle and a process for carbon storage, respectively. Since the primers were optimized for terminal restriction fragment pattern analysis, they had to amplify the target fragment with no amplification of nonspecific fragments. The optimization attempts included adjusting thermal cycling temperatures and times, adjusting reagent concentrations and adding the PCR enhancing reagents betaine and DMSO. The *nosZ* primers were optimized for a single organism but the *nifH* and *phaC* primer sets consistently amplified the wrong fragments or didn't amplify at all. Further work will be continued to optimize *nosZ* on soil DNA and to attempt optimization of the other two primers on DNA from a single organism.

Introduction

The Nitrogen cycle, which is carried out by microorganisms in aquatic and terrestrial habitats plays a major role in the environment and is responsible for the production of many important nitrogen-containing molecules. One process in this cycle is denitrification in which organisms use nitrogen compounds such as NO_3^- and NO_2^- as electron acceptors rather than O_2 for metabolic processes (2). This process converts these oxidized forms of nitrogen to gases such as NO, N_2O and N_2, all of which are gaseous products and are emitted from the environment to the surrounding atmosphere. Not only does this lead to a loss of fixed nitrogen in the soil or aquatic environment, but the emitted products are also greenhouse gases and play a major role in O_3 destruction (2). Denitrification occurs in two steps. The first step is nitrite reduction. Two different enzymes that are functionally identical but differ in structure carry out this process. One of these enzymes contains a copper catalyzing site while the other contains a cytochrome cd_1. These enzymes are encoded by the genes *nirK* and *nirS*, respectively (5). Primer sets have been developed and used for these two enzymes, but using these two primer sets together to determine the diversity of the denitrifying community would require twice as much labor and analysis as using a process for which only one known gene was responsible.

The final step in denitrification is the reduction of nitrous oxide to N_2 by the nitrous oxide reductase enzyme. There is only one known gene that codes for this functional enzyme, and a primer set has already been developed for it. These primers amplify an 1100 base pair fragment of the *nosZ* gene, and this experiment attempted to optimize those primers for amplification of genomic DNA from a soil community.

Nitrogen is incorporated into organic molecules in the environment only after it is converted to ammonia. This process is carried out by the enzyme nitrogenase and the gene *nifH*

encodes an iron-containing protein subunit of this enzyme (9). Primers for this gene were developed by Zehr et al.(9) and attempts were made to optimize amplification of the *nifH* gene from soil communities.

Since carbon cycling and nitrogen cycling are related, it would be useful to study the storage of carbon in a soil environment along with the nitrogen cycle genes. When carbon stores are in excess, prokaryotic cells produce molecules called polyhydroxyalkanoates, which can serve as carbon and energy stores (12). Many pathways have been discovered for the formation of these molecules, but all include the crucial enzyme Polyhydroxyalkanoate Synthase (*pha*C) (12). Three primers for the *pha*C gene have been synthesized to amplify this gene by semi-nested PCR, and attempts were made to optimize these primers for amplification of extracted soil genomic DNA.

All three primer sets (*nos*Z, *nifH*, and *pha*C) will be optimized for analysis by terminal restriction pattern (TRFP) analysis. TRFP analyzes a gene fragment that has been copied and amplified by Polymerase Chain Reaction then cut by a restriction enzyme. Since the gene is copied with terminally labeled primers, the length difference (in base pairs) of the restricted fragment will account for the different organisms that carry the gene. Essentially, it shows how many different versions of a certain gene are present in a bacterial community (13). Knowing the diversity of a bacterial community that is carrying out the nitrogen and carbon cycling process as described above can be of very high interest in the environmental and agricultural communities.

Optimizing a primer set for TRFP is more stringent than merely getting the primers to amplify the gene of choice. In this case the primers must only amplify the specific fragment for which they were designed. If other fragments are amplified, smaller or larger, they will only

give false peaks when analyzed with TRFP. Extra peaks will not pose a large problem when comparing TRF patterns, but when further analysis is attempted, the false peaks can pose a major problem. When analyzing a restriction fragment by TRF, the number of peaks can tell you the minimum number of different organisms carrying the functional gene of interest (13). This assumes that each peak represents a different organism or group of organisms. However, if the primers are not optimized properly and extra bands occur in PCR reaction, one organism can account for more than one peak (13). This can make data correlation very difficult and shows the importance of good primer optimization.

Extraction of DNA

Organisms known to carry the *nos*Z gene were grown on Trypticase Soy Agar medium for 24 hours, then removed from the medium and taken through the MoBio soil DNA extraction protocol (MoBio Inc., Solera Beach, CA). The extracted DNA was quantified by spectrophotometry at 260 and 280nm.

DNA was extracted from soil samples with the MoBio Soil DNA extraction kit as well. One-quarter gram of soil was used for each extraction and three extractions were performed per soil sample. The three extractions were compared on a 1.2% agarose gel to ensure uniformity for the three extractions, then combined and quantified by spectrophotometry.

Optimization of nosZ Primers

The *nos*Z primers were created and published by David Scala and Lee Kerkhof in their 1998 FEMS Microbiology Letter (1). The sequences were ordered as published in this paper and contained the following sequences.

Nos661F: 5’CGGCTGGGGGCTGACCAA

Nos1773R: 5’ATRTCGATCARCTGBTCGTT

These primers were ordered from Sigma-Genosys and were received in a lyophilized state. They were immediately diluted to 10 μM in preparation for Polymerase Chain Reaction Amplification. The first PCR amplification run with the *nosZ* primers was run on extracted *Pseudomonas oleovorans* genomic DNA extracted from pure culture using the MoBio Soil DNA extraction protocol. The primers were used in a reaction with 26.3 ng, 2.63 ng and .263 ng of pure *P. oleovorans* DNA. Other specifications for the reaction were as follows: 1X PCR Gold Buffer (Applied Bio Systems), 150 μM each dNTP, .8 ng/μL Bovine Serum Albumin, 3.5 mM $MgCl_2$, 10 pmol each primer, and 1.5 units of Taq Gold Polymerase (Applied Bio Systems). The reagents were combined in these concentrations into 50 μL reaction volumes then subjected to the following thermal cycles: 94^oC for 10 min. to activate the methylated Taq Gold Polymerase, then 35 cycles of 95^o for 0.5 min. to separate the double stranded DNA, 55^o for 0.5 min. to allow the *nosZ* primers to anneal to the template DNA, and 72^o for 1.5 min. to allow the Taq polymerase to extend the new DNA fragment. After these cycles were completed, the reactions were held at 72^o for 10 min. to allow any incomplete strands to be elongated.

The 1,100 base pair product was amplified successfully under these conditions when 26.3 ng DNA was used and it was amplified very slightly when 2.63 ng of DNA was used. No amplification occurred when 0.263 ng DNA was used in the PCR reaction. With these results, soil was collected from a residential backyard and DNA was extracted using the MoBio protocol. These DNA samples were then used as template for the *nosZ* primers and subjected to the PCR conditions shown above. These DNA templates yielded three unsuccessful PCR attempts with the *nosZ* primers.

After these unsuccessful attempts, a series of nine thermal cycling and reagent changes were made during successive PCR attempts. Table 1 shows the different conditions in which the

reactions were run with the *nosZ* primers. The first two reactions in this series determined the amount of template to be used in the reaction. Then melting, annealing and extension temperatures and times were adjusted in an attempt to optimize the primers (See table 1, run 3).

In run 4, attempts were made to eliminate nonspecific fragments by decreasing the concentration of $MgCl_2$ in the reaction mixture. A cationic salt such as Mg^{2+} in solution shields the negatively charged phosphate molecules that reside on the outside of the DNA molecule. This allows a complementary strand of DNA to bind to a template more easily. By decreasing the salt concentration, it creates more stringent conditions, only allowing DNA fragments that are more exactly complementary to bind to each other. By lowering the $MgCl_2$ concentration in run four, it was hoped that the primers would bind only to the *nosZ* fragment, which their sequence is specific for.

After more unsuccessful attempts at eliminating nonspecific bands (see runs 5-7), it was decided to begin using PCR enhancing reagents. Researchers have had success in primer optimization when using these chemicals in the past. The two reagents that seemed promising in this case were betaine and Dimethyl Sulfoxide (DMSO).

Betaine, shown in figure 4, is the amino acid glycine with three methyl groups attached to the amino end. This molecule is an isostabilizing agent, equalizing the contribution of GC- and AT- base pairing to the stability of the DNA molecule (7). This facilitates strand separation, which could be an issue with the *nosZ* primers considering the heavy GC content on the 5' end of the forward primer. DMSO also facilitates strand separation by disrupting base pairing (8). Researchers have had success in the past using these two reagents to help optimize their PCR reactions (8). Runs eight and nine incorporated betaine and DMSO and they were unable to amplify the target fragment strongly with no nonspecific amplification.

The final attempt to optimize the primers came after Lee Kerkhof was contacted and it was found that the primers had produced single bands when the reaction mixture contained 0.5mM $MgCl_2$. It was also noted that different brands of Taq polymerase produced different results. This led to a reaction containing 0.5mM $MgCl_2$ and two different master mixes were used. One master mix used the standard Taq Gold Polymerase and the other used Fisher Scientific Brand Taq polymerase. When run on a 1.2% agarose gel, the products appeared as shown in figure 7. These results led to the use of Fisher Taq on the following reactions. The next reaction will be run on soil samples from a Morro Bay estuary and on soil samples received from Los Alamos National Laboratory (LANL).

Optimization of nifH Primer Sets

The primers for the gene which encodes the iron-containing portion of nitrogenase, *nifH*, were adopted from a paper by Zehr and McReynolds (9). These primers were designed to amplify to a 359 base pair fragment of the *nifH* gene. The primer sequences were as follows:

*NifH*1 (forward): 5'-TGYGAYCCNAARGCNGA

*NifH*2 (reverse): 5'-ADNGCCATCATYTCNCC

The primers were used in a reaction under the following conditions. Some of these conditions were not specified by Zehr in his paper. The reaction mixture contained 150 μM each dNTP, 0.8 ng/μL BSA, 3.5 mM $MgCl_2$, 10 pmol of each primer, and 1.5 units of Taq Gold Polymerase. Thermal cycling conditions were an initial 95^o for 10 minutes followed by 35 cycles of 94^o for 1 min, 50^o for 1 min and 72^o for 1.5 min. Then a final extension step of 72^o for 10 minutes was used to finish off the amplification. Reaction with these primers resulted in amplification of multiple gene fragments. The fragments that amplified best consisted of 2000 bp, 850 bp and 400 bp. The 359 bp segment was not amplified.

After referring to a paper by Chelius and Lepo (10), who used a 6.7 mM concentration of $MgCl_2$, the effect of $MgCl_2$ concentration was tested by doubling the concentration and halving the concentration. The results of this test eliminated the larger fragment, but the 359 bp fragment still showed no signs of being amplified.

After other tests showed no signs of amplifying the desired fragment, Zehr was contacted personally and it was found that the primers only worked when purified by High Pressure Liquid Chromatography (HPLC). The HPLC-purified primers were ordered and used in a reaction under the following conditions: 200 pmol each dNTP, 2 ng/µl BSA, 6.7 mM $MgCl_2$, 10 pmol each primer, and 2.5 units of Taq Gold polymerase. The reaction amplified an 850 bp fragment brilliantly and slightly amplified what could be the 359 bp target fragment. These results are shown in figure 8. These primer sets seem to be amplifying non-specific fragments too much for TRF analysis.

Pha primer optimization

The polyhydroxyalkanoate synthase gene primer sequences (*phaC*) were adopted from Sheu et. al. who had been using the primers in a semi-nested colony PCR reaction (12). The sequences are shown below:

PhaCF1: 5'-ATCAACAA(GGG/A)T(TT/A)CTAC(AA/G)TC(CC/T)T(CC/G)GACCT-3'

PhaCF2: 5'-GT(CCC/GG)TTC(GGG/AA)T(GGG/CC)(AAA/GG)T(CC/G)(TT/A)(CCC-/GG)CTGGCGCAACCC-3'

PhaCR4: 5'-AGGTAGTTGT(TT/C)GAC(CC/G)(AAA/CC)(AAA/CC)(GGG/A)TAG-(TTT/G)TCCA-3'

It should be noted that the primers were not ordered in this fashion exactly. Rather than ordering the primers with the exact ratios of basepairs as shown above, the primers were ordered

with degeneracies instead. It was not specified at what ratio to have each base in the degenerate position.

Since the products were to be used for community TRF analysis, they were used with genomic DNA that had been extracted from pure colonies of *P. oleovorans* and *Azotobacter chroococcum*. The reaction conditions were as follows. 1X PCR amplification buffer, 2.5 mM MgCl2, 200 μM each dNTP, 100 μg/mL BSA, 10 pmol each primer, and 2.5 units of Taq Gold Polymerase. 10 ng of DNA from *P. aerigunosa* and *A. chroococcum* were used as template DNA and this reaction mixture was then subjected to a cycle of 94^{o} for 10 min, 51^{o} for 2 min, 72^{o} for 2 min, and 35 cycles of 94^{o} for 20 s, 57^{o} for 45 s (decreased by 1 s per cycle), 72^{o} for 1 min, and a final incubation at 72^{o} for 5 min. This reaction yielded no amplification of any DNA fragment whatsoever.

The developers of the primer set had used 3% DMSO and 1M betaine as additives in the PCR reaction mixture so these reagents were obtained and used in a new reaction. The conditions of this new reaction were the same as described above, except the 57o anneal was not decreased by 1 s per cycle. This reaction also yielded no product and use of these primers has been put on hold for the time being.

Results and Recommendations

Of the three primer sets that were worked on, the *nosZ* primers are the most promising. They have been optimized for a single organism and optimization from soil DNA should not be too difficult to achieve. The next step for these primers should be attempts to amplify the fragment from extracted soil DNA. A ramp should be run with increasing concentrations of soil DNA to see the minimum amount of DNA required for amplification of the *nosZ* fragment from soil. This value may vary depending on the environment from which the soil is extracted.

The *nifH* primers are amplifying the fragment of interest, but the strong amplification of the large nonspecific fragment poses a major problem. The next best step to take would be to order the exact primers used by Zehr. These primers were HPLC-purified and Polyacrylamide Gel Electrophoresis purified as well. It would also be wise to order these primers from the same company that Zehr obtained his primers from.

Although the *phaC* primers produced no results to date, work should still be attempted on them. Since the additives DMSO and Betaine have been incorporated, it may be wise to try changing brands of Taq. The Fisher Taq seems to do better in more stringent conditions so it may work better with DMSO and betaine than Taq Gold does. A ramp should also be run with these primers that progressively increases the amount of template DNA used in the reaction mixture. These primers were used in colony PCR in the reference paper so a larger amount of soil DNA may be required to amplify the target fragment.

Table 1- Summary of experiments run to optimize *nos*Z primers.

			Thermal Cycling Information										
			Denature		Anneal		Extension		Reagent Concentrations				
Run	Template DNA source	Mass of Template (ng)	Temp (oC)	Time (min)	Temp (oC)	Time (min)	Temp (oC)	Time (min)	$[MgCl_2]$ (mM)	[Betaine] (M)	%DMSO (w/v)	[Primer] (pmol)	Result
1	*P. oleovorans;* DNAG*	132.5, 13.25 133.5, 13.35	95	1	55	1	72	2	3.5	0	0	10	Best amplification to date for *P.oleo.* DNA, but no amplification for soil DNA (See fig. 1).
2	*P. oleovorans;* DNAT*	10 10	95	1	55	1	72	2	3.5	0	0	20	Successful amplification for *P.oleo.* DNA, but no amplification for soil DNA (See fig. 2).
3	*P. oleovorans*; GSDNA*; GSDNA spikes w/ *P.oleo* DNA	10 50 50 w/ 10	95	1	Ramp: 56, 57, and 58	1	72	2	3.5	0	0	10	All anneal temps amplified DNA from *P. oleo.* and soil DNA. However, some nonspecific banding occurring. Results led to change in anneal temp to 58oC (See Fig. 3).
4	*P. oleo* DNA; Est DNA*	10 10	95	0.5	58	1	72	1.5	2.5	0	0	10	Eliminated smallest nonspecific fragment but not all nonspecific fragments.
5	*P. oleo* DNA; Est DNA	10 10	95	0.5	59	1	72	1.5	2.5	0	0	10	Decreased amplification of non-specific fragments
6	*P. oleo* DNA; Est DNA	10 10	95	0.5	59	1	72	1.5	1.75	0	0	10	No amplification occurred
7	*P. oleo* DNA	10	95	0.5	57.5	1	72	1.5	2.5	0	0	10	Nonspecific fragments were still present, but much more faint than ever before
8	*P. oleo;* Est DNA	10 10	95	0.5	57.5	1	72	1.5	2.5	1	3	10	Amplified the target fragment slightly and eliminated all nonspecific fragments.
9	*P. oleo;* Est DNA	10 10	95	0.5	57.5	1	72	1.5	2.5	0.5 0.75	1.5 2.25	10	Nonspecific bands returned with decreased levels of betaine and DMSO (See Fig. 6).

* DNAG, DNA extracted from soil under a residential lawn; DNAT, DNA extracted from soil near a tree; GSDNA, DNA from a garden soil and Est DNA, DNA extracted from Morro Bay estuary sediment. Reagent concentrations are shown only for the reagents that were changed. Reagents not shown in the table were kept in the same concentrations as described in the text (See pg. 5).

Figure 1- 1.2% agarose gel electrophoresis image of PCR on soil and control samples. DNA obtained from soil underneath grass will be referred to as DNAG. Lane assignments are as follows. **Row 1:** a) $1Kb^+$ standard c) – open control e) Amplified *P. oleovorans* DNA (132.5ng) g) Amplified *P.oleovorans* DNA (13.25ng) i) $1kb^+$ standard **Row 2:** a) $1kb^+$ standard c) Amplified DNAG (133.5ng) e) Amplified DNAG (13.35ng) g) – Closed Control i) $1kb^+$ size standard

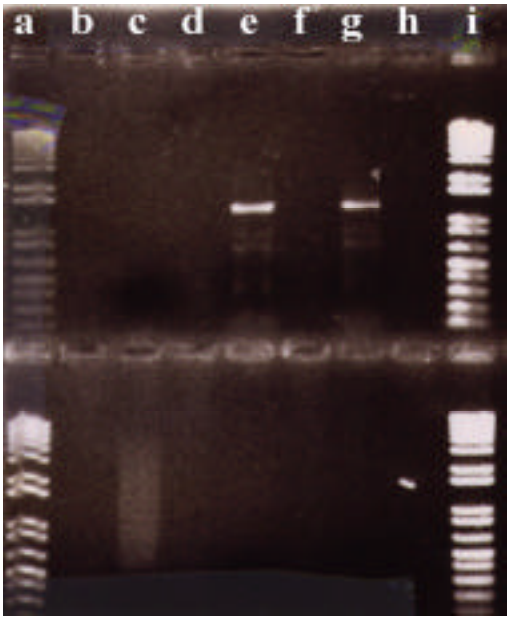


Figure 2- 1.2% agarose gel image of *nosZ* PCR products. Lane assignments are as follows. Lane a) $1Kb^+$ ladder. b) – open control d)Amplified *P. oleovorans* genomic DNA (10ng) f) Amplified soil DNA i)1Kb+ ladder. All other lanes were left empty during the electrophoresis.

Figure 3- Results from *nosZ* temperature ramp. Lane assignments are as follows. **Row 1:** Lane a) $1Kb^{+}$ standard. b) – Open control d) 10 ng *P. oleovorans* DNA amplified with a 56^{o} anneal. f) 50 ng soil DNA amplified w/ 56^{o} anneal. h) 50 ng soil DNA spiked w/ 10ng *P. Oleovorans* DNA w/ 56^{o} anneal. j) 10 ng *P.oleovorans* DNA w/57^{o} anneal. **Row2:** Lane a) $1Kb^{+}$ size standard. b) 50 ng soil DNA w/ 57^{o} anneal. d) 50 ng soil DNA spiked w/ 10 ng *P. oleovorans* DNA w/57^{o} anneal. f) 10 ng *P. oleovorans* DNA w/ 58^{o} anneal. h) 50 ng soil DNA w/ 58^{o} anneal. j) 50 ng soil DNA spiked w/ 10ng *P. oleovorans* DNA w/58^{o} anneal.

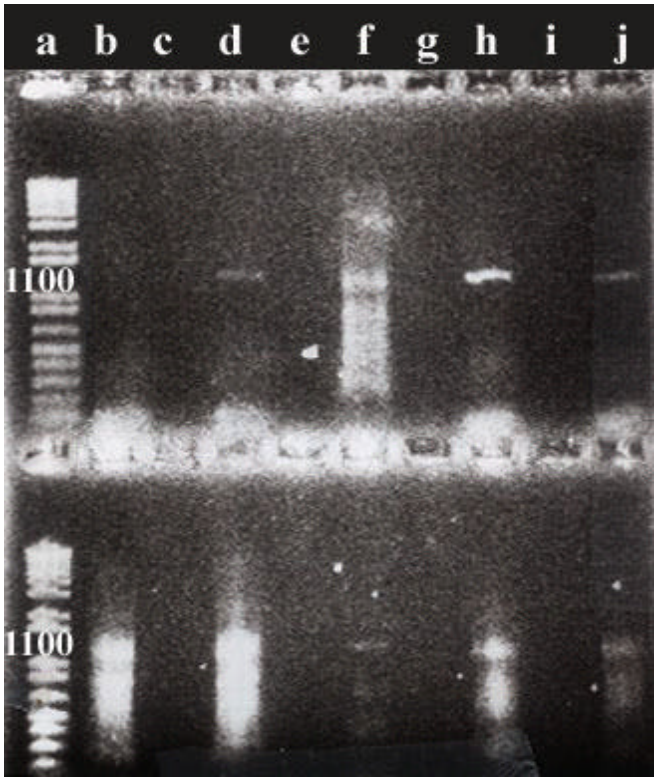


Figure 4- Chemical Structure of the PCR additive Betaine

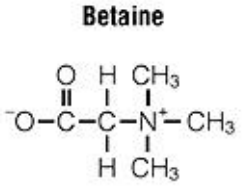


Figure 5- Chemical Structure of the PCR additive Dimethyl Sulfoxide (DMSO)

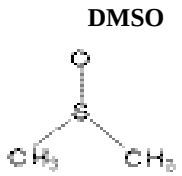


Figure 6- 1.2% Agarose gel image from the PCR with *nosZ* primers and the additives betaine and DMSO. Lane assignments are as follows. a) $1Kb^{+}$ size standard c) Amplified *P. oleovorans* DNA with 0.5M betaine and 1.5% DMSO in the reaction mixture d) Extracted DNA from soil amplified by *nosZ* primers with 0.5M betaine and 1.5% DMSO in solution e) *P. Oleovorans* DNA amplified by *nosZ* primers with 0.75M betaine and 2.25% DMSO in solution f) Extracted soil DNA with 0.75% betaine and 2.25% DMSO in the reaction solution

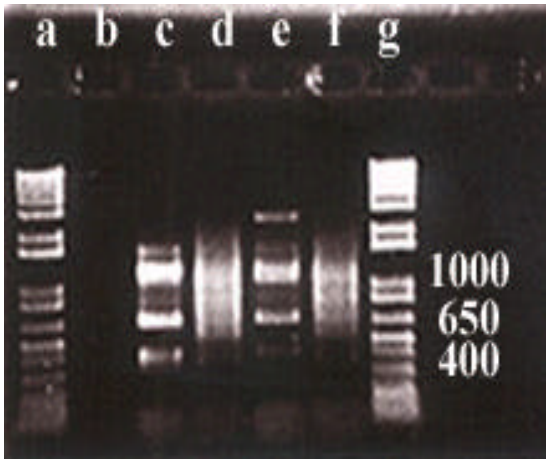


Figure 7- 1.2% agarose gel image from the *nosZ* PCR trial. All reactions used *P. aerigunosa* genomic DNA for template and contained 0.5% $MgCl_2$. The lane assignments for the gel are as follows. Lane a) 1Kb+ size standard. b-e) PCR reaction with Taq Gold polymerase. f) Blank g-j) PCR reaction with Fisher Taq polymerase.

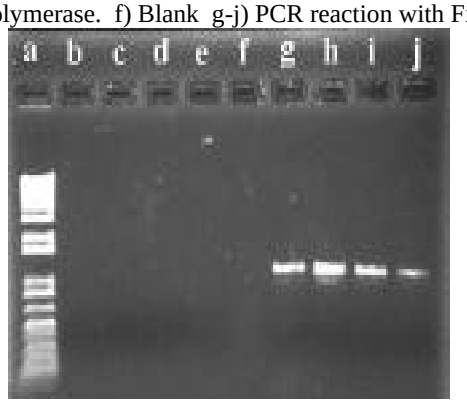
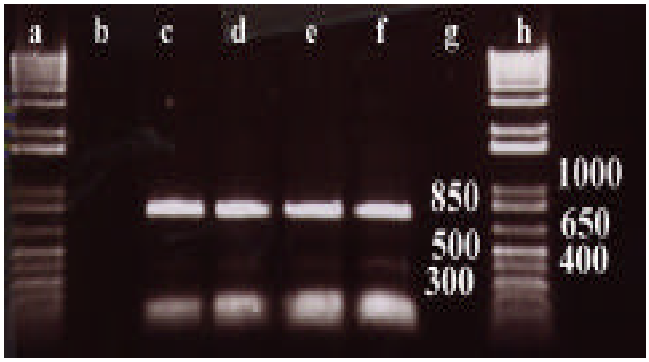


Figure 8- 1.2% agarose gel electrophoresis image of PCR results with ultrapure *nifH* primer sets. All sample lanes use *Azotobacter chroococcum* genomic DNA as template and the HPLC purified *nifH* primers. Lane assignments are as follows. Lanes a) and h) 1kb+ size standard. Lanes c)-f) *Azotobacter chroococcum* DNA amplified with *nifH*f and *nifH*r primers.



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