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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Detection of differentially expressed growth dependent noncoding RNAs in *Sulfolobus solfataricus*

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Noncoding RNA mediated gene regulation exists in all the three kingdoms of life including prokaryotes, eukaryotes and archaea. In prokaryotes, the ncRNAs typically bind to the 5' end of the messenger RNAs to facilitate its activation or repression. The repression of gene expression in eukaryotes by ncRNAs is regulated by its binding to the 3'-untranslated region. A significant amount of information is available on the ncRNA mediated gene regulation in eukaryotes and prokaryotes. However, the data on ncRNA interference mechanisms in archaea are largely based on bioinformatics predictions. More than 300 noncoding RNAs have been bioinformatically predicted to exist in the hyperthemophilic archeon *Sulfolobus solfataricus*. The main objective of this study was to ascertain the growth dependent differential expression of certain ncRNAs in *S. solfataricus*. The northern blot analysis confirms that certain ncRNAs are differentially expressed at particular phases of growth while others are constitutively expressed in all phases of growth. One of the three ncRNAs, RNA 22 was expressed constitutively whereas the other two ncRNAs, RNA 43 and RNA 115 was expressed at specific points of growth. In addition, we also attempt to predict the putative mRNAs that are targeted by specific ncRNAs. In conclusion, our study states that in term with the constitutive expression of ncRNAs 22, the predicted targets include a drug resistance transporter and transposase protein which should be down regulated during the normal growth of Sulfolobus. The second candidate, ncRNA 43 was specifically expressed at the late log phase with putative targets that includes critical metabolic proteins. The third RNA analyzed was ncRNA 115. The expression of ncRNA 115 was at the mid-log phase with the predicted target being the Translation recovery factor (TRF). These predictions with functional classifications of the mRNAs relevant in specific growth points out that ncRNAs plays significant role in gene regulation in *S. solfataricus*.

Key words: Noncoding RNA, Sulfolobus, Archaea, gene regulation.

INTRODUCTION

Control of RNA stability is routinely performed by cells to mount an effective regulation of gene expression, thereby modulating the synthesis of proteins in response to the physiological need. Non coding RNAs (ncRNAs) are present in all the three kingdoms of life. Apart from messenger RNAs (mRNAs), ncRNAs are not functionally

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translated to proteins and exert their activity at RNA level (Gerbi et al., 2003). In the Eukaryotic cells, microRNAs (miRNAs) and short interfering RNAs (siRNA) act as regulators of critical cellular processes. The siRNAs are usually derived from double-stranded RNA and function in cleavage of the target mRNA by RNA interference (RNAi) (Meister and Tuschl, 2004). In contrast, the miRNAs encoded from the genome are part of the ribonucleoprotein complexes. Specific binding of the ribonucleoprotein complexes to the 3′ untranslated region (UTR) of mRNAs leads to translational repression and/or mRNA decay (Filipowicz et al., 2008). At variance to eukaryotes, the bacterial regulation of mRNA is done by small regulatory RNAs (sRNAs) that predominantly bind to the 5′ UTR in mRNAs (Bell and Jackson, 1998). Although the clade of Archaea bacteria lacks specific nucleus and other organelles similar to prokaryotic bacteria, they show intense similarity to eukaryotes in molecular functioning such as transcription, translation and DNA packaging (Waters and Storz, 2009). Although bioinformatics analysis has revealed the presence of significant number of ncRNAs in archaea bacteria, it was only recently shown experimentally that these predicted ncRNAs could be involved in gene regulation. Recently, another study reported the presence of a large number of small RNAs in *S. solfataricus* P2 which are of approximately 20 nt in length based on deep sequencing (Xu et al., 2012). Nevertheless, the first experimental evidence of ncRNA mediated gene regulation was shown by Märtens et al. (2013). It was shown that a specific noncoding RNA 257 and its four orthologues (RNA-257_{1-4}) regulate the inorganic phosphate transporter protein coding mRNA (SSO1183) in the hyperthermophilic archaeon *S. solfataricus* (Märtens et al., 2013). Interestingly, the levels of RNA-257 orthologues were dependent on the amount of inorganic phosphate present in the growth media. A surplus amount of phosphate in the media leads to an increased level of RNA-257 and a reduced level of SSO1183 and vice versa in a phosphate depleted condition.

In a recent study, around 300 ncRNAs were predicted with precise Transcription Start Site (TSS) in *S. solfataricus* by the technique of whole-genome sequencing and strand-sensitive 5'-end determination (Wurtzel et al., 2010). The data obtained from the 5'-end determination approach corresponds to real start of the transcripts in the native cells. The TSS was predicted by taking into account the highest number of 5'-end reads followed by the detection of TATA motifs placed approximately 26 bp upstream of the TSS, which is a hallmark of most archaeal genes (Reiter et al., 1990; Zillig et al., 1998). Archaeal mRNAs usually have 3' UTRs of significant sizes (Brenneis et al., 2007). Interestingly, it was also reported that the ncRNA mediated gene regulation in archaea bacteria is achieved by its interaction to the 3' UTR of mRNAs, similar to eukaryotes (Tang et al., 2005). Apart from most of the available bioinformatics data on the probable interaction of ncRNAs with the 3' UTRs of mRNAs in archaea bacteria, the only study that has experimentally shown ncRNA interaction with target mRNA was the RNA-257 interaction with SSO1183 mRNA in the 3' UTR and its subsequent degradation *in vitro* (Märtens et al., 2013).

In this study we attempt to experimentally prove the existence of three ncRNAs predicted in different stages of growth of *S. solfataricus*. In addition, we also attempt to predict the putative target mRNAs of these three ncRNAs with an outlook into its possible interaction regions.

MATERIALS AND METHODS

Archaeal strains and growth conditions

The *S. solfataricus* P2 strain was used for the growth curve determination and isolation of total RNA. *S. solfataricus* P2 was grown at 75°C and pH 3.0 in Brock's medium (Brock et al., 1972). The media was supplemented with 0.2% NZamine and 0.2% D-arabinose. The culture media was inoculated with *S. solfataricus* P2 strain and was grown aerobically by shaking at 160 rpm in a rotary shaker. The samples were withdrawn for 7 days for the assessment of growth.

Isolation of total RNA and northern blotting

Total RNA from *S. solfataricus* P2 was extracted at different phases of growth corresponding to OD_{600} (0.3, 0.6, 1.0 and 1.6). The cells were pelleted at 2300 x *g* for 10 min followed by the addition of 1.0 ml Trizol (Invitrogen). The resuspended pellet in Trizol was incubated at 65°C for 10 min followed by phenol/chloroform extraction. The nucleic acids were precipitated with the addition of 96% ethanol. DNA was removed by treatment with DNase I. The concentration of RNA was determined with Nanodrop 8000. For northern blot detection of specific noncoding RNA, 1 µg of the total RNA was separated on an 8% polyacrylamide/urea gel and then transferred to nylon membranes. After UV- crosslinking, the membrane was incubated with either the [^{32}P]-5′-end labelled oligonucleotides as shown: 5′-GGCAACAACAACAGAGTGGCGGA-3′ specific for ncRNA 22, the oligonucleotide 5′-GTATGCAATAATAATAGCAGCAG-3′ specific for ncRNA 43 or the oligonucleotide 5′-GGAAAAGAAGGTGATTAGATTCAA-3′ specific for ncRNA 115. The 5S rRNA was probed with oligonucleotide 5′-CACTAACGTGAGCGGCTTAAC-3′ and served as loading control. The incubation of the membrane with the labeled primers was done at 50°C overnight. The membrane was washed following incubation and specific RNAs were detected by scanning (Typhoon 9400, Amersham) of the exposure cassette.

Target prediction of ncRNAs

Putative targets of the three ncRNAs 22, 43 and 115 (Table 1) were done by Blast analysis. The conditions for the Blast analysis is as follows: The program selection was optimized for discontinuous Mega blast. The algorithm was set for Match/Mismatch scores of 2, -3 and the Gap costs Existence: 2, Extension: 4. Following blast analysis, the predicted target sequences were scrutinized for possible interaction 'pockets' with ncRNA by IntaRNA RNA-RNA prediction tool (Busch et al., 2008). The interaction temperature was set to 75°C with a minimum 'seed pair' of 4. The best ncRNA

Table 1. Features of the studied ncRNAs.

ncRNA	Strand/orientation	Coordinates 5' end	Coordinates3' end	Length
22	+	581783	581907	124
43	+	990534	990681	147
115	-	2397330	2397165	165

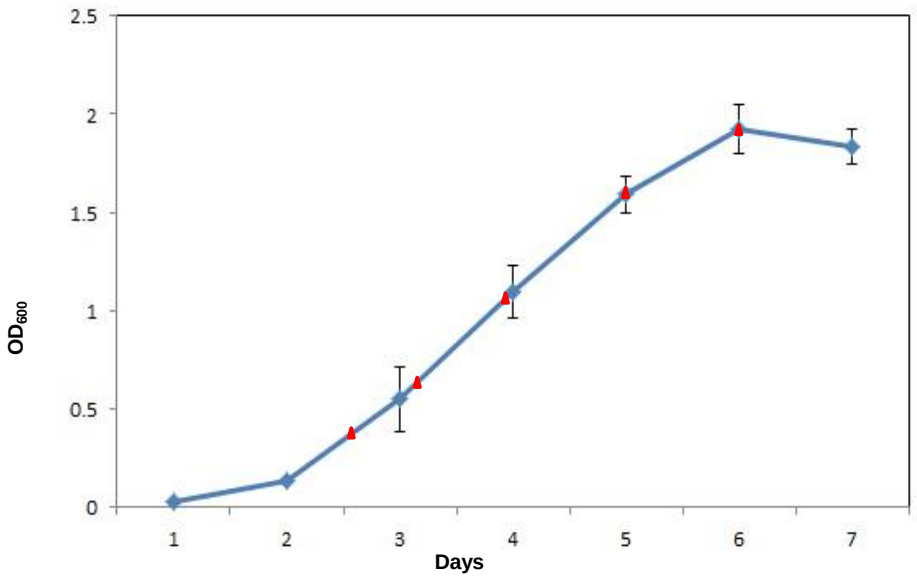


Figure 1. Growth curve of *Sulfolobus solfataricus* P2 in full growth medium. The time-points at which total RNA was extracted is shown as red triangle. The OD600 of the cultures were measured spectrophotometrically at a wavelength of 600 nm. The total RNA was extracted at OD600 of 0.3, 0.6, 1.0 and 1.6. The cell doubling time was approximately 8-10 h. The growth curve is the average of three independent experiments. The error bars shows the standard deviation.

targets were selected that had high hybridization energy between the ncRNA and the target. The interaction site of ncRNA with the target RNA was preferentially the 3' untranslated region or the internal ORF. The target genes with annotated functions were of special interest.

RESULTS AND DISCUSSION

Constitutive expression of ncRNA in *S. solfataricus* P2

In order to find the expression of specific ncRNAs predicted (Wurtzel et al., 2010), RNAs were chosen on four criteria: (a) should be of detectable length possibly larger than 100 base long, (b) ncRNAs should have a strong transcriptional start site and relative abundance as predicted by the sulfolobus transcriptome analysis software (http://www.weizmann.ac.il/molgen/Sorek/Sulfolobus_solfataricus_transcriptome/), (c) ncRNAs should not be present in multiple copies in the sulfolobus genome as seen in RNA257 (Märtens et al., 2013) and (d) should possibly interact with the 3'-UTR or internal ORF of the target RNA.

Based on these criterions we selected three potential RNAs designated as ncRNA 22, ncRNA 43 and ncRNA 115. Initially, we extracted total RNA from *S. solfataricus* P2 grown on a complete media at different phases of growth as shown in Figure 1. Total RNA was extracted until late log (OD_{600}: 1.9) phage of growth. To determine the expression of ncRNA 22, first aim was to determine the expression levels of ncRNA 22 based on the Transcripitional Start Site Mapping on the Sulfolobus transcriptome described by Wurtzel et al. (2010). A maximum read of 17915 was seen in the TSS of ncRNA 22 corresponding to the genome co-ordinate of 581783. As shown in Figure 2A, there is a strong TSS corresponding to ncRNA 22, which will be sufficient to enable its detection by northern blotting. The experimental detection of ncRNA 22 was done by the hybridization

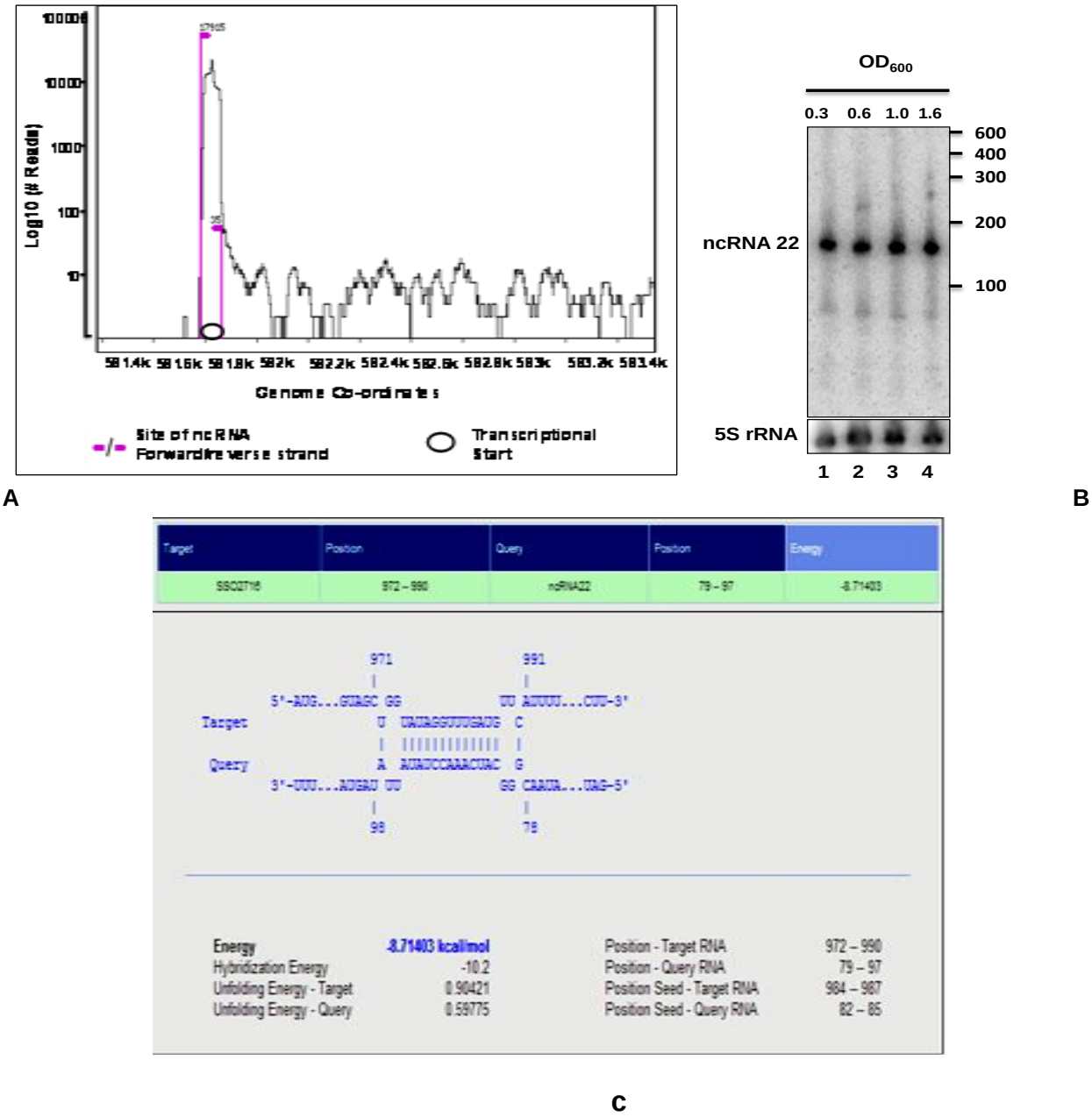


Figure 2. The transcriptional start and abundance of ncRNA22 **(A)** The transcriptional start site of ncRNA 22 is shown at position 581783 in the genome co-ordinate. The number of reads corresponding to the transcripitional start is 17915. **(B)** The constitutive expression of ncRNA in all phases of growth ranging from lag to late log phase (Lane 1-4). A strong abundant expression of the ncRNA 22 is seen. The bottom lane (5S rRNA) represents loading control. **(C)** The picture shows the hybridization of ncRNA 22 with SSO2716. SSO2716 encodes for a drug resistance transporter. The ncRNA 22 hybridizes within the ORF of SSO2716 with hybridization energy of -8.71 kcal/mol. The hybridization was done at 75°C, which is the optimal growth temperature of *Sulfolobus solfataricus* P2.

of ^{32}P labeled specific primer sequences as described above. As shown in Figure 2B, ncRNA 22 was constitutively expressed in all phases of growth ranging from early log phase to late log phase (Figure 2B: Lane 1-4). The prominent band seen on the northern blot corresponds to the predicted size of ncRNA 22. However, a lower band of around 70 nt was seen on the northern blot consistently present in all the phases of growth. These fragments to which the primer binds could be degraded fragments from the complete RNA. We cannot also exclude the possibility of a shorter transcript synthesized during transcription. Nevertheless, our

Table 2. Predicted targets of ncRNA 22.

S/N	ncRNA	ncRNA length (bases)	Target mRNA	Target mRNA length (bases)	Interaction site of ncRNA	Interaction site of Target mRNA	Hybridization energy at 75°C (kcal/mol)	Target mRNA Function
1	22	124	SSO1960	867	52-60	620-628 (internal ORF binding)	-4.4	Hypothetical Protein
2	22	124	SSO2946	726	8-14	794-800 (3' end binding)	-3.9	Unknown function
3	22	124	SSO0283	1630	49-63	1676-1690 (3' end binding)	-7.8	Putative Thermosome
4	22	124	SSO0290	1356	25-39	477-491 (internal ORF binding)	-4.6	Inorganic Phosphate Permease
5	22	124	SSO2676	765	54-62	534-542 (internal ORF binding)	-5.6	Hypothetical Protein
6	22	124	SSO0299	943	33-65	194-228 (internal ORF binding)	-9.4	Transketolase Domain Protein
7	22	124	SSO0520	315	32-60	218-242 (internal ORF binding)	-10.1	Hypothetical Protein
8	22	124	SSO1010	996	26-39	1221-1237 (3' end binding)	-6.6	FAD-dependent pyridine nucleotide-disulphide oxidoreductase
9	22	124	SSO1336	1272	29-37	255-263 (internal ORF binding)	-7.8	Transposon ISC1904
10	22	124	SSO2138	408	113-122	299-308 (internal ORF binding)	-5.0	Daunorubicin resistance ABC transporter ATPase subunit
11	22	124	SSO2209	1059	11-20	455-464 (internal ORF binding)	-6.1	Glycosyl Transferase Family 2
12	22	124	SSO2277	1486	54-63	383-392 (internal ORF binding)	-7.2	ATPase like protein
13	22	124	SSO2435	729	54-65	362-372 (internal ORF binding)	-5.1	Uroporphyrin-III C-methyltransferase
14	22	124	SSO2716	1410	79-97	972-990 (internal ORF binding)	-8.71	Drug resistance transporter, EmrB/QacA subfamily
15	22	124	SSO3153	1272	8-20	582-594 (internal ORF binding)	-8.3	DNA binding domain protein, excisionase family transposase

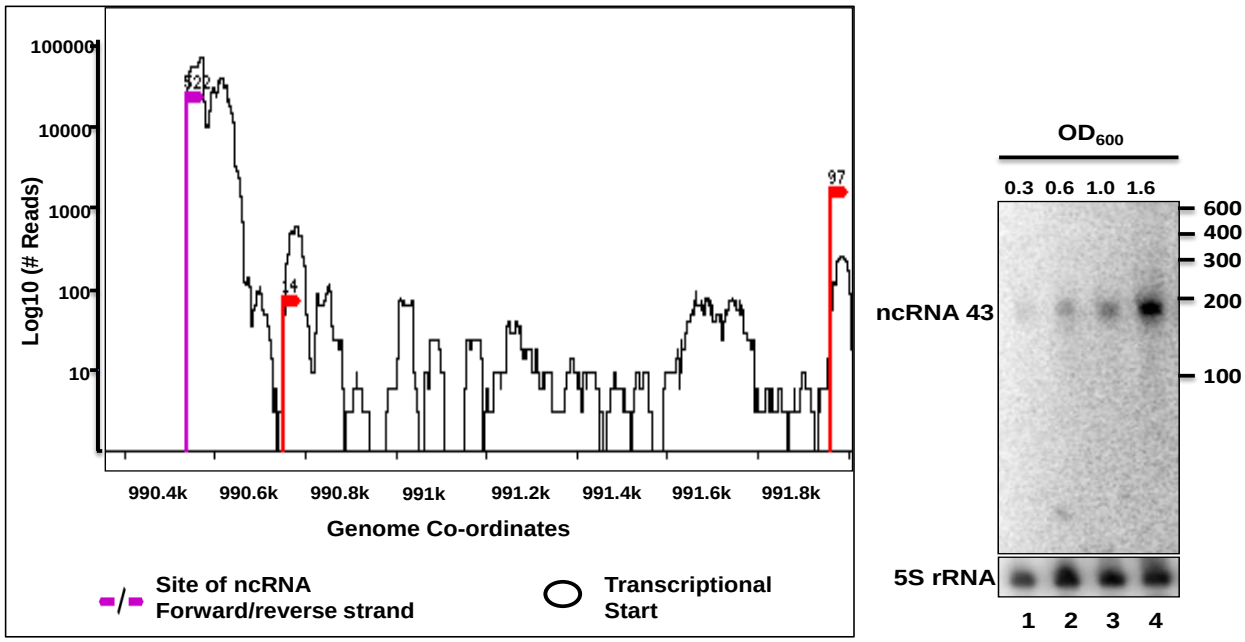
expected size of the whole ncRNA 22 corresponding to the prominent band and was only accounted for further analysis. The expression pattern of ncRNA 22 is similar even in the stationary phase with RNA extracted at OD_{600} of 1.9 (Data not Shown). The predicted mRNA targets of ncRNA 22 are shown in Table 2. A total of 15 putative target RNAs were listed out from BLAST analysis followed by IntaRNA prediction of the interaction between target mRNAs and ncRNA 22. The interaction prediction with IntaRNA was done at 75°C, the optimal growth temperature of *S. solfataricus*. Interestingly the hybridization energy of ncRNA 22 interaction with SSO2716 was -8.71 kcal/mol (Table 2, Figure 2C). SSO2716 encodes for a drug resistance transporter, which belongs to the EmrB/QacA subfamily. The Emr locus in *E. coli* encodes membrane translocases that also include multi drug resistant proteins of Gram-positive bacteria (Miyazono et al., 2007; Lomovskaya and Lewis, 1992). An archaeal homologue of EmrR, StEmrR was identified from *S. tokodaii*. Although structurally different from their bacterial counterparts, both the proteins were functionally similar in contributing to a phenotype resistant to multiple antibiotic drugs (Miyazono et al., 2007). Another interesting candidate target mRNA predicted to be down regulated by ncRNA 22 is the transposase protein encoded by SSO3153 (Table 2). All transposase enzymes possess a nuclease activity that contributes to their functionality of excising transposon DNA and subsequently integrating it into a new location. The transposase encoded by SSO3153 falls in the family of IS605. Apart from other transposon families, IS*605* elements (Kersulyte et al., 2002) do not have inverted sequences at their ends but they possess imperfect palindromic (IP) sequences located close to the

transposon ends. Another peculiar feature of IS605 family of transposases is its preference for integration in the 3′ of a specific four or five nucleotide (nt) sequence rather than random (Barabas et al., 2008). Transposon mutagenesis is a dominant mechanism of mutation in *S. solfataricus*. Spontaneous mutations that were created by IS elements arose with variable frequencies of between 10^{-4} and 10^{-5} per plated cell (Martusewitsch et al., 2000). It is speculated that even small differences in growth conditions or conditions that cause stress reactions might induce transpositions (Schleper et al., 1994). The constitutive expression of ncRNA 22 is therefore conceivable and a predicted high energy of hybridization (-8.3 kcal/mol) with SSO3153 relates to the possibility of an effective repression of the transposase by ncRNA 22. This is the first time to the best of our knowledge that the possibility of a regulation of transposase by ncRNA has been speculated in *Sulfolobus solfataricus*.

Growth phase dependent expression of ncRNAs

In contrast to ncRNA 22 which is constitutively expressed in all phases of growth, ncRNA 43 and 115 was seen to be expressed at a specific point of growth in *S. solfataricus*. Specifically, a strong Transcription Start Site was predicted for ncRNA 43 in the sulfolobus transcriptome (Figure 3A). The predominant expression of ncRNA 43 was seen in the late log phase (Figure 3B: Lane 4). Interestingly, no significant expression of the ncRNA 43 was observed in the mid-log phase or early stationary phase of growth. In order to look for the putative mRNA targets of ncRNA 43, a BLAST search followed by IntaRNA prediction was done. As shown in Table 3, 14 target RNAs were predicted. The predicted hybridization energy of the ncRNA 43 to the target RNA SSO0607 encoding for MarC protein was -8.0 kcal/mol (Figure 3C). A recent study by (McDermott et al., 2008) has pointed out that in *E. coli*, *marC* is divergently transcribed from marRAB, an operon involved in conferring resistance against multiple antibiotics (Cohen et al., 1989; George and Levy, 1983), oxidative stress (Ariza et al., 1994) and organic solvents (Asako et al., 1997). However, no function could be attributed to the MarC protein (McDermott et al., 2008). Interestingly, other potential target RNAs predicted to hybridize with ncRNA 43 were SSO2401, SSO2440 and SSO3194 coding for Ketopantoate hydroxylmethyltransferase, Glutamine synthetase and Glyceraldehyde 3-phosphate dehydrogenase, respectively. All these three proteins are involved in critical regulatory functions. Ketopantoate hydroxylmethyltransferase and Glyceraldehyde-3-phosphate are indispensable for energy metabolism. Ketopantoate hydroxylmethyltransferase is the enzyme encoded by *PanB* what catalyzes the conversion of Ketoisovalerate to Ketopantoate, which is in turn converted to Pantoate, which is a precursor in Coenzyme A biosynthesis (Hüser et al., 2005). Coenzyme A is involved in the oxidation of pyruvate in the citric acid cycle. The Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) in contrast catalyzes the conversion of Glyceraldehyde 3 phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway. Interestingly, ncRNA 43 which is predicted to target mRNAs that encode Ketopantoate hydroxylmethyltransferase and Glyceraldehyde 3 phosphate dehydrogenase is expressed at the late log phase of OD_{600} 1.6. It is understandable that the metabolic processes including energy production are kept at a minimum pace in the stationary phase of growth and targeting important enzymes in the energy production cycle is the most potent way of down regulating the metabolic pathways. In addition to these metabolic proteins, Glutamine synthetase (GS) mRNA encoded by SSO2440 is also targeted by ncRNA 43. Glutamine synthetase is an important enzyme in living cells, where it controls the use of nitrogen for synthesis of DNA or amino acids. It is known that during stationary phase, all the vital processes including protein synthesis and DNA replication are slowed down. It is therefore conceivable that the mRNA encoding for GS is targeted by ncRNA 43 and may be subsequently degraded thereby checking the production of GS.

Similar to ncRNA43, another ncRNA that was found to be expressed in a specific growth phase of *S. solfataricus* is ncRNA 115. In contrast ncRNA 22 and 43, ncRNA115 is transcribed in the reverse orientation as shown in Figure 4A. In addition, the abundance of the ncRNA 115 as judged from the transcriptome analysis was lower (3340 Reads). In order to look for the expression of ncRNA 115, northern blotting was done on total RNA extracted at different phases of growth. ncRNA 115 was specifically expressed in the mid log phase at an OD_{600} of 1.0 (Figure 4B). There appears to be two transcription products which are detected on the northern blot. Both of the transcripts appear to be in the range of the predicted ncRNA 115 with only a few base differences between the two transcripts. Interestingly, both the transcripts seem to be equally transcribed. We could not affirm if there are two copies of the gene encoding ncRNA 115 in the *Sso* genome in varying lengths. Also we cannot exclude a longer transcript being generated from the same transcriptional start site. At this point it is unknown to us why there are two transcripts in ncRNA 115. The target of ncRNA 115 was predicted to be the translation recovery factor (Trf) encoded by SSO2509. Function of the Trf protein was recently described by Märtens et al. (2014). Trf directly interacts with aIF2/aIF2γ and facilitates its release from leaderless mRNAs thereby restoring translation during outgrowth of cells from stationary phase. It was earlier shown that during stationary phase, aIF2γ was able to bind to the 5’ end of mRNAs and protect them from degradation in *S. solfataricus* (Hasenöhrl et al., 2008). In this scenario, this is evident



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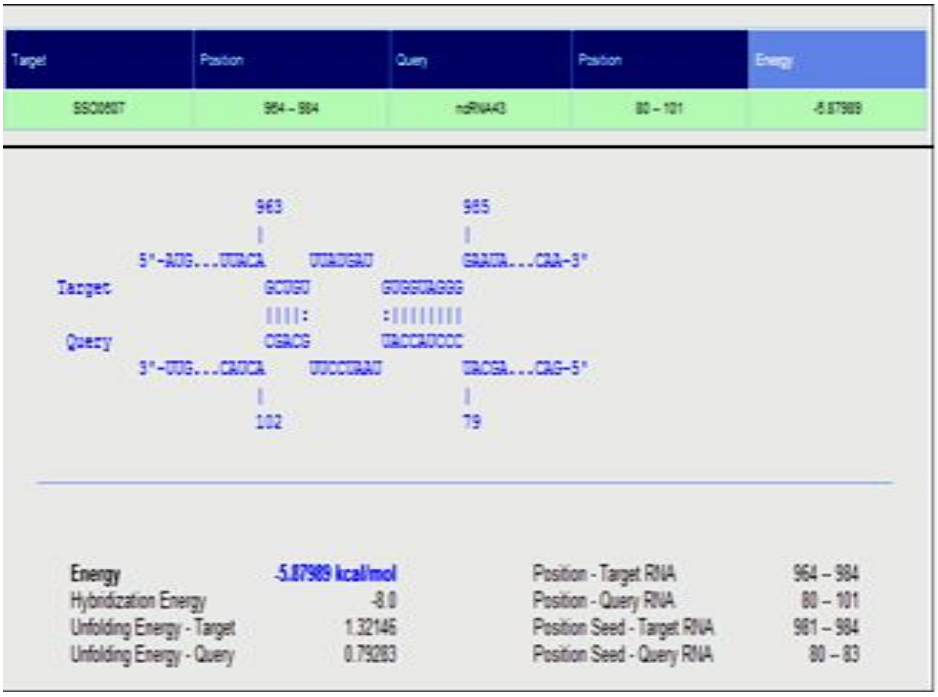

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Figure 3. The ncRNA 43 is expressed at the late log phase of growth. **(A)** The transcripition start site of ncRNA 43 is at position 990534 extending up to position 990681 in the genome co-ordinate. **(B)** The differentially expressed ncRNA 43 in different phases of growth is shown. A minimal expression of the RNA was seen in the early and mid-log phase as shown in lanes 1-3. A strong expression of ncRNA 43 was seen in the late log phase as shown in lane 4. The expression of the RNA was further subsided in the stationary phase (lane 5). The 5S rRNA represents the loading control. **(C)** The IntaRNA prediction of the interaction of ncRNA 43 with SSO0607 coding for multiple antibiotic resistance (MarC) related protein. The hybridization energy is -5.87 kcal/mol. The ncRNA 43 inteacts within the ORF of SSO0607.

Table 3. Predicted targets of ncRNA 43.

S/N	ncRNA	ncRNA length (bases)	Target mRNA	Target mRNA length (bases)	Interaction site of ncRNA	Interaction site of Target mRNA	Hybridization energy at 75°C (kcal/mol)	Target mRNA function
1	43	147	SSO2168	1033	138-150	119-131 (internal ORF binding)	-6.5	Peptide ABC Transporter
2	43	147	SSO2846	354	75-88	280-293 (internal ORF binding)	-5.1	Putative Glucosyl transferase
3	43	147	SSO0569	1446	75-88	280-293 (internal ORF binding)	-5.1	prolyl- tRNA synthetase
4	43	147	SSO0607	609	80-101	964-984 (3' end binding)	-8.0	multiple antibiotic resistance (MarC)-related protein
5	43	147	SSO3231	978	112-134	521-543 (internal ORF binding)	-5.8	Peptidase M48Ste24p
6	43	147	SSO0244	1410	1260-1273	1260-1273 (internal ORF binding)	-5.9	FAD dependent oxidoreductase
7	43	147	SSO0645	849	75-82	561-568	-5.5	Translin lysine biosynthesis enzyme LysX
8	43	147	SSO1137	516	29-37	239-247	-4.7	Hypothetical Protein
9	43	147	SSO1859	936	67-82	177-192	-6.6	peptidase M48 Ste24p
10	43	147	SSO2323	924	28-38	898-908	-6.4	Flagellin, putative
11	43	147	SSO2372	543	138-146	251-259	-4.7	Putative RNA-binding protein (contains KH domains)
12	43	147	SSO2401	684	37-50	414-427	-7.4	Ketopantoate hydroxymethyl-transferase (panB)
13	43	147	SSO2440	1278	73-98	623-647	-7.7	Glutamine synthetase (glutamate ammonia ligase)
14	43	147	SSO3194	1530	78-86	691-699	-6.9	Glyceraldehyde-3-phosphate dehydrogenase

that the Trf is expressed in the early to mid-log phase, helping in resumption of translation by removing the mRNA bound aIF2. The ncRNA 115 binds specifically to the 3' UTR of SSO2509 with hybridization energy of -8.4 kcal/mol (Figure 4C). Interestingly, ncRNA 115 was seen to be expressed at the mid-log phase. The role of Trf protein is predominantly required in the out growth phase and is least required once normal rate of translation resumes. Trf protein expression should also be regulated in the stationary phase where aIF2 should be bound to mRNAs for its protection. Hence, we speculate that the regulation of Trf protein synthesis is performed in two ways. In the first instance, immediate blockage of the Trf expression is done by ncRNA 115 by binding to the SSO2509. After the immediate cessation of translation of SSO2509, there could be another mechanism which keeps the level of Trf under check during the stationary phase. One could also not rule out the possibility of another ncRNA which may bind to SSO2509 and block the translation.

Conclusion

Here we show and discuss the expression and targets of three ncRNAs from *S. solfataricus*. The target RNAs were identified by Blast analysis followed by the interaction mapping with IntaRNA. There are open questions that are relevant to this work such as the experimental mapping of the target mRNA degradation upon interaction with ncRNA. Is there a reduction in the abundance of the target mRNA at the point of expression of ncRNAs? An experimental analysis by northern blotting or RT-PCR to detect the decrease in the amount of target RNA upon hybridization with ncRNAs will clearly prove our hypothesis that ncRNAs are effective gene regulators in the hyperthermophilic archeon *S. solfataricus*. To the best of our knowledge this is the first study that represents a divergent expression pattern of noncoding RNAs in *S. solfataricus*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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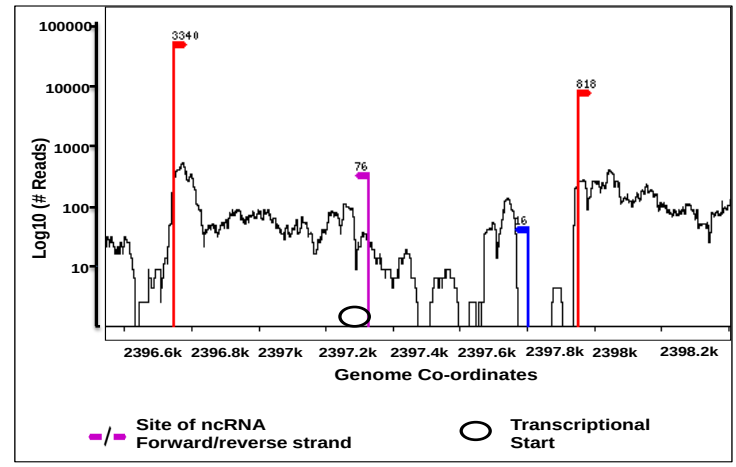

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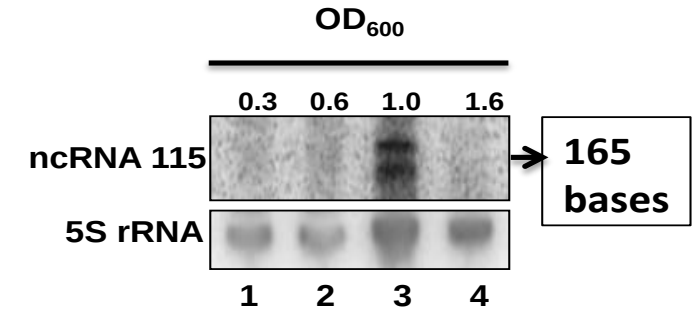

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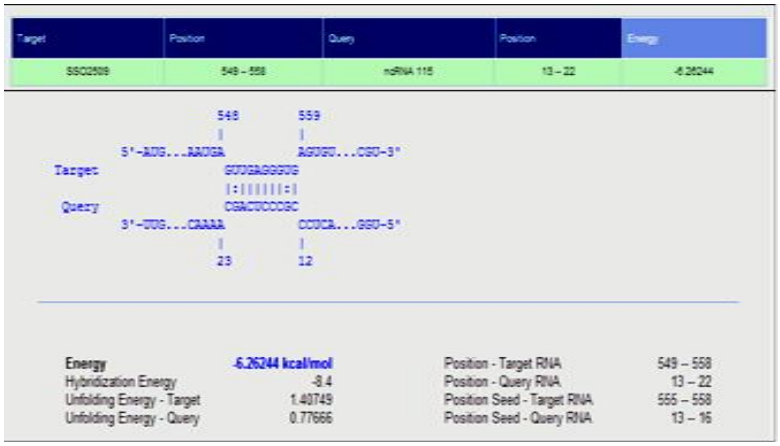

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Figure 4. The ncRNA 115 targets the mRNA coding for translation recovery factor (Trf). **(A)** The transcription start site of ncRNA 115 is shown at position 2397330 to 2397165 in the genome co-ordinates. The ncRNA 115 is encoded in the reverse orientation in the Sulfolobus genome. The reads corresponding to the transcripition start site is 76, which represents that the ncRNA 115 is not as abundantly expressed as ncRNA 22 or ncRNA 43. **(B)** The ncRNA 115 is expressed specifically at the mid-log phase of growth (Lane 3). Two bands of the RNA are seen with low expression abundance. The lower lane represents loading control (5S rRNA). A digital normalization of the bands is done and is shown as graph below. The graph represents the fold change in the intensity of ncRNA 115 in comparison to 5S rRNA. **(C)** The target of ncRNA was predicted to be the translation recovery factor (Trf) encoded by SSO2509. The IntaRNA hybridization profile of ncRNA 115 and SSO2509 is shown. The hybridization energy is -6.26 kcal/mol. The interaction with ncRNA 115 interaction occur approximately 200 bases downstream of 3'-end in SSO2509.

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Full Length Research Paper

Management of sugarcane smut (*Ustilago scitaminea)* with fungicides and bio-agents

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The efficacy of nine fungicides with different concentrations was tested against *Ustilago scitaminea*, using spore germination inhibition technique. Tilt and Emisan (50 µg/ml) are proved to be the most effective against the spore germination of *U. scitaminea,* and they completely inhibit it. These are followed Score with 100 µg/ml after 24 h of incubation. Vitavax, Dithane M-45 and Antracol (200 µg/ml) seem to be next in order of efficacy. The efficacy of two bio-agents, *Trichoderma viride* and *T. harzianum* was evaluated *in vitro* by the dual culture technique. *T. harzianum* showed mycoparasitism and completely covered the growth of *U. scitaminea* within seven days of incubation. The above fungicides and bio-agent were also tested on smut inoculated sets in the field for bud germination and disease control. There was maximum increase (21.11%) in germination when Emisan of 0.25% was used, followed by *T. viride* (20.00%, 1 × 10^6 spore/ml) and Tilt (16.40, 0.2%). Regarding smut disease control, tilt (0.2%) controlled it the most by 97.27% followed by Emisan (0.25%) by 94.96% and *T. viride* (1×10^6 spore/ml) by 9.70%.

Key words: *Ustilago scitaminea*, sugarcane, fungicides, bioagents.

INTRODUCTION

Sugarcane (*Saccharum officinarum*) is not only cash crop for the growers, but it is the main source for the production of white crystal sugar, khandsari and gur (jaggery). It as an old energetic source for humans and more recently is a replacement of fossil fuel for motor vehicles, as it is used to produce ethanol (Anonymous, 2006). Sugarcane is attacked by more than 240 diseases caused by fungi, bacteria, viruses, phytoplasmas and nematodes in India (Rott et al., 2000). Sugarcane smut (*Ustilago scitaminea* Sydow) is considered as an important disease next to red rot. As the pathogen attacks only the meristematic tissues, it is generally referred to as a primitive parasite and a main problem of tropical India. Now, it is also becoming a problem to some varieties in North India (Waraitch and Kumar, 1984). The characteristic symptoms of the smut are the dark brown, whip-like fungal sorus that develops from the apex of infected stem (Butler, 1906). Teliospores of *U. scitaminea* are shed from the whip and disseminated through the wind. The wind borne spores are spread in the standing cane fields and can infect newly planted sets in the soil. The infection takes place through the buds that may soon develop into whips; but the mycelia may remain dormant, and the use of such infected stalks as

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seed cane spread the disease. The teliospores of smut pathogen are usually present externally on the buds and may infect it when sets are planted. Moreover, the systematically and internally infected buds may give rise to infected plants. Planting of healthy sets, chemical and hot water treatment of sets, biological control, cultural control and use of resistant varieties have been found effective but there is a need to study their effectiveness in a more systematic way to manage the disease and prevent the losses inflicted by it. Keeping in view the importance of disease, and the inadequate research work carried out on the disease in the state, this study was undertaken with an objective of managing the disease through biological agents and fungicides.

MATERIALS AND METHODS

Evaluation of different fungicides and bio-agents *in vitro*

Efficacy of different fungicides in vitro

The efficacy of nine commercially available fungicides of different companies namely Tilt (propiconazole 25 EC), Score (difenoconazole 25 EC), Contaf (hexaconazole 5 EC), Vitavax (carboxin 75% WP), Bavistin (carbendazim 50WP), Raxil (tebuconazole 2DS), Dithane M-45 (mancozeb 75WP), Emisan-6 (methoxy ethyl mercuric chloride) and Antracol (propineb 70 WP), with different concentrations (0.01, 1, 10, 20, 50, 100, 200 and 500 µg/ml) was tested against the smut pathogen. Spore germination inhibition technique was used for the test, as suggested by American Phytopathological Society (Anonymous, 1947). Stock solutions of the fungicides were prepared in distilled sterilized water and the required concentrations of the fungicides were obtained by subsequent dilutions of the stock solution. Spore suspension (1×10^6 spore /ml) from freshly collected culture of teliospores, having more than 70% germination, was prepared in distilled sterilized water. Sterilized cavity slides were used to study the spore germination. Spore suspension (0.02 ml) and the same volume of fungicide suspension were placed in the cavities of slides to give the previous concentrations. Slides were kept in Petri dish containing Whatman filter paper soaked in sterile water for keeping relative humidity up to 100%. Each treatment was carried out in three replicates. Treated slides and control slides, sterilized water and spore suspension only were placed in Petri dishes and incubated at 25±1°C for 48 h. Observations for the percentage of germinated and non-germinated spores were recorded under microscope after 24 and 48 h of incubation. Percentage of spore germination inhibition was calculated according to Bliss (1934).

$$I = \frac{C - T}{C} \times 100$$

Where I = Percent inhibition of spores; C = percent spores that germinated in control; T = percent spores that germinated in treatment.

Efficacy of bio-agents in vitro

The efficacy of two bio-control agents (*Trichoderma viride* and *Trichoderma harzianum)* was evaluated by using dual culture technique. A 5 mm block of sporidia inoculum of smut pathogen was placed on one side of sterilized Petri plate containing potato dextrose agar medium. On the other side of the Petri plate, a block of bio-agent (*T. viride*) was placed. Then the Petri plate was incubated at 25±1°C and observed periodically. Final observations were taken after 7 days of incubation. Similar set of experiment was carried for the other bio-agent (*T. harzianum*). The mode of action of antagonists was observed, that is the production of inhibition zone or mycoparasitism.

Evaluation of different fungicides and bio-agents as sets treatment under field condition

Five different fungicides namely Tilt (0.1 and 0.2%), Bavistin (0.1 and 0.2%), Vitavax (0.2%), Emisan (0.25%), Raxil (0.2%) and a bio-agent, *T. viride* (1×10^6 spores/ml) were tested in a randomized block design to evaluate their efficacy on set of sugarcane germination and disease control. Three budded sets of sugarcane variety CoJ 88 were artificially inoculated by dip and out method in smut suspension (1×10^6/ml) for 30 min. Inoculated sets were incubated in moist gunny bags for 48 h at 25±2°C. Then the inoculated sets were treated with different fungicidal solutions by dip and out method before planting in the field. Similarly disease inoculated sets were dipped in *T. viride* spore suspension (1×10^6/ml) before sowing. Sugarcane bagasse was spread on sets in furrows for the multiplication of the bio-agent. Three replications were maintained for each treatment. Set inoculated with smut spore suspension served as control. For healthy control, apparently healthy sets were dipped in water only. Planting of sets was done in 3 r × 4 m × 0.75 cm plot. Forty five 3- budded sets were planted in each plot. Data on germination (%) were recorded after 30 and 45 days of planting and percent increase of germination after 45 days was calculated. Smutted clumps were recorded at fortnight intervals starting from June till harvesting of the crop. Rogueing out of smutted clump was carried out to avoid secondary infection. At the end of the season, disease incidence (%) was recorded and disease control (%) was calculated:

$$\text{Percent disease control} = \frac{\text{DI in control} - \text{DI in treatment}}{\text{DI in control}} \times 100$$

Where - DI = Disease incidence.

RESULTS AND DISCUSSION

Evaluation of different fungicides and bio-agent *in vitro*

Evaluation of different fungicides in vitro

Relative efficacy of nine fungicides namely Tilt, Score, Contaf, Vitavax, Bavistin, Raxil, Dithane M-45, Emisan and Antracol, of eight concentrations (0.01, 1, 10, 20, 50, 100, 200 and 500 µg/ml) were evaluated by spore germination inhibition technique. All the fungicides in all the concentrations tested were effective in inhibiting spore germination (Table 1). The data revealed that Tilt and Emisan (50 µg/ml), Score (100 µg/ml), Vitavax, Dithane M-45 and Antracol (200 µg/ml) completely inhibited spore germination of *Ustilago scitaminea* after 24 h of incubation. Contaf, Bavistin and Raxil were found to be least least effective, as complete spore inhibition was not observed even at 500 µg/ml. Similar types of

Table 1. *In vitro* evaluation of different fungicides on the Teliospores germination of *U. scitaminea* after 24 h of incubation.

Fungicides	Percent spore germination inhibition at different concentration (µg/ml)								
	0.01	**1**	**10**	**20**	**50**	**100**	**200**	**500**	**Mean**
Tilt (Propiconazole) 25EC	12.90* (21.03)	82.01 (64.87)	91.15 (72.69)	95.79 (78.12)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	85.23 (74.56)
Score (Difenoconazole) 25EC	14.14 (22.18)	42.40 (40.61)	81.74 (64.69)	91.63 (73.20)	96.99 (80.01)	100 (89.96)	100 (89.96)	100 (89.96)	78.36 (68.82)
Contaf (Hexaconazole) 50 WP	13.85 (21.84)	53.88 (47.20)	67.57 (55.26)	75.55 (60.34)	80.71 (63.92)	82.67 (65.38)	96.70 (79.53)	97.51 (80.92)	71.05 (59.29)
Vitavax(Carboxin) 75WP	11.28 (19.61)	56.75 (48.86)	67.21 (55.04)	78.88 (62.62)	87.29 (69.08)	98.20 (82.31)	100 (89.96)	100 (89.96)	74.95 (64.68)
Bavistin(Carbendazim) 50WP	6.87 (15.19)	22.72 (28.68)	26.40 (30.90)	38.61 (38.40)	42.80 (40.84)	44.77 (41.98)	50.95 (45.52)	52.95 (46.67)	35.75 (36.02)
Raxil (Tebuconazole) 2DS	12.22 (20.45)	14.27 (22.18)	39.86 (39.13)	45.66 (42.49)	52.61 (46.47)	69.00 (56.14)	77.64 (61.76)	86.00 (68.00)	49.65 944.57)
Dithane-M-45 (Mancozeb) 75WP	9.22 (17.66)	11.21 (19.55)	77.83 (61.89)	87.84 (69.56)	95.11 (77.20)	98.50 (82.98)	100 (89.96)	100 (89.96)	72.46 (63.59)
Emisan (Methoxy ethyl mercuric chloride) 6%	15.88 (23.47)	88.28 (69.95)	95.18 (77.30)	97.27 (80.48)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	87.07 (76.38)
Antracol (Propineb) 70 WP	15.49 (23.16)	54.00 (47.27)	67.50 (55.22)	78.10 (62.04)	89.20 (70.78)	97.70 (81.26)	100 (89.96)	100 (89.96)	75.24 (64.95)
Mean	12.42 (20.51)	47.28 (43.24)	68.27 (56.90)	76.59 (63.02)	82.91 (69.80)	86.68 (75.54)	91.69 (80.73)	92.94 (81.70)	

CD (p=0.05) level for: Fungicides = 0.29; Concentration = 0.27; Interaction (Fungicides × Concentration) = 0.83; Figure in parentheses represented arc sine transformed values and CD is applicable to these only; * Average of three replications.

results were obtained even after 48 h of incubation (Table 2).

The sporidial germination decreased with increased concentration of fungicides. Ahonsi (2003) reported that among different fungicides, Copper Oxychloride, Benomyle and Thiabendazole (TBZ) *in vitro* completely inhibited the mycelial growth of *Ustilaginoidea virens,* but in present study, it was found that among the tested fungicides, Tilt and Emisan (50 µg/ml) completely inhibited spore germination of *U. scitaminea* followed by Score (100 µg/ml), Vitavax, Antracol and Dithane M-45 (200 µg/ml). Bhuiyan et al. (2012) also evaluated fungicides for the management of sugarcane smut caused by *Sporisorium scitamineum* in seed cane. They found that Azoxystrobin, quintozene and didecyl dimethyl ammonium chloride completely stopped germination of teliospores at 2.5 mg a.i./L. Propiconazole, triadimefon, cyproconazole and acibenzolar-s-methyl significantly ($P < 0.05$) reduced spore germination at 50, 100 and 200 mg a.i./L.

Efficacy of a bio-agent in vitro

Result revealed that *T. viride* did not produce any zone of inhibition. Thus, it was not found effective in inhibiting the pathogen *in vitro*. On the other hand, *T. harzianum* showed mycoparasitism and completely covered the growth of the *U. scitaminea* within seven days of incubation.

Sinha and Singh (1983) observed that the viability of smut teliospore *U. scitaminea* was reduced when it had contact with fusarial growth of *Fusarium moniliforme* [*Gibberella fujikuroi*] var. *subglutinans,* and culture filtrate of *G. fujikuroi* var. *subglutinans* completely inhibited the germination of teliospore. The present result showed that *T. harzianum* showed mycoparasitism and completely covered the growth of *U. scitaminea*.

Evaluation of fungicides and a bioagent as set treatment under field conditions

For germination

The fungicides (Tilt, Bavistin, Vitavax, Emisan and Raxil) significantly affected the set germination when the data were recorded after 45 days of sowing (Table 3). Emisan (0.25%) and *T. viride* (1×10^6 spore/ml) led to set germination of 52.58 and 51.85%, respectively followed by Tilt (0.1%) 51.84% and Raxil (51.10%). Maximum increase (21.11%) in germination was observed in Emisan (0.25%) followed by 1×10^6 spore/ml *T. viride* (20.00%) and 0.1% Tilt (19.98 percent). The minimum increase in germination was recorded in Tilt (0.2%).

Table 2. *In vitro* evaluation of different fungicides on the Teliospores germination of *U. scitaminea* after 48 h of incubation.

Fungicides	Percent spore germination inhibition at different concentration (µg/ml)								
	0.01	1	10	20	50	100	200	500	Mean
Tilt(Propiconazole) 25EC	12.30* (20.51)	81.89 (64.79)	90.39 (71.92)	94.88 (77.09)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	84.93 (74.26)
Score(Difenoconazole) 25EC	13.94 (21.58)	41.70 (40.20)	80.63 (64.65)	90.60 (71.97)	95.20 (77.31)	100 (89.96)	100 (89.96)	100 (89.96)	77.75 (68.19)
Contaf (Hexaconazole) 50WP	12.56 (20.75)	52.04 (46.15)	66.42 (54.56)	74.02 (59.33)	78.97 (62.68)	81.48 (64.48)	95.37 (77.55)	96.00 (78.44)	69.60 (57.99)
Vitavax(Carboxin) 75WP	10.57 (18.96)	55.07 (47.89)	66.11 (54.37)	76.93 (61.26)	86.48 (68.40)	97.31 (80.54)	100 (89.96)	100 (89.96)	74.05 (63.91)
Bavistin(Carbendazim) 50WP	5.37 (13.38)	21.50 (27.61)	25.29 (30.18)	37.07 (37.49)	41.56 (40.12)	43.52 (41.25)	50.05 (44.41)	51.00 (45.55)	34.42 (34.99)
Raxil (Tebuconazole) 2DS	11.01 (19.37)	13.03 (21.15)	38.07 (38.08)	44.15 (41.62)	51.03 (45.57)	67.96 (55.50)	76.00 (60.64)	84.99 (67.18)	48.28 (43.63)
Dithane-M-45 (Mancozeb) 75WP	8.29 (16.32)	10.65 (19.03)	76.37 (60.86)	86.36 (68.30)	94.02 (75.82)	96.72 (79.54)	98.89 (83.93)	100 (89.96)	71.41 (61.72)
Emisan (Methoxy ethyl mercuric chloride)6%	14.08 (22.02)	86.88 (68.73)	94.00 (75.79)	95.91 (78.30)	100 (89.96)	100 (89.96)	100 (89.96)	100 (89.96)	86.35 (75.58)
Antracol (Propineb) 70WP	14.67 (22.50)	53.02 (46.71)	66.07 (54.35)	77.10 (61.38)	88.00 (69.70)	95.99 (78.42)	100 (89.96)	100 (89.96)	74.35 (64.12)
Mean	11.42 (19.48)	46.19 (42.47)	67.03 (56.08)	75.22 (61.86)	81.69 (68.83)	86.99 (74.40)	91.03 (79.59)	92.44 (81.21)	

CD (p=0.05) level for: Fungicides = 0.31; Concentration = 0.29; Interaction (Fungicides × Concentration) = 0.88; Figure in parentheses represented arc sine transformed values and CD is applicable to these only. *Average of three replications.

Table 3. Efficacy of different fungicides and a bioagent on germination of sugarcane sets inoculated with *U. scitaminea* under field condition.

Fungicides/bioagents	Concentration (%)	Percent germination		Germination increase over inoculated control (%)
		After 30 day	After 45 day	
Tilt (Propiconazole)	0.1	52.58(46.46)	51.84(46.04)	19.98
"	0.2	48.14(43.91)	49.62(44.76)	16.40
Bavistin (Carbendazim)	0.1	47.40(43.49)	50.36(45.19)	17.63
"	0.2	51.10(45.61)	51.10(45.61)	18.82
Vitavax (Carboxin)	0.2	48.88(44.34)	50.36(45.19)	17.63
Emisan (Methoxy ethyl mercuric chloride)	0.25	50.36(45.19)	52.58(46.46)	21.11
Raxil (Tebuconazole)	0.2	52.58(46.46)	51.10(45.61)	18.82
Trichoderma viride (Bioagent)	(1×10^6/ml)	51.84(46.04)	51.85(46.04)	20.00
Inoculated check (Control)	(1×10^6/ml)	45.18(42.21)	41.48(40.07)	-
Un-inoculated check (Healthy sets)	-	53.33(46.89)	54.81(47.74)	-
Mean		50.13(45.06)	50.51(45.27)	
CD (p=0.05)		2.83	2.67	

Variety – CoJ 88 (Three budded sets); *Average sets germination of three replications; Figure within parentheses represent arc sine transformed values and CD is applicable to these only.

Disease incidence

Out of the tested fungicides, 0.2% Tilt controlled disease (97.27%) greatly followed by 0.25% Emisan (94.96%); a bio-agent, *T. viride* had minimum (9.70%) control (Table 4). No significant difference was observed in two

Table 4. Efficacy of different fungicides and a bioagent on smut incidence under field conditions.

Fungicides/bioagents	Concentration (%)	Percent disease incidence	Percent disease control
Tilt (Propiconazole)	0.1	4.31*(11.97)	92.21
"	0.2	1.51(4.09)	97.27
Bavistin (Carbendazim)	0.1	38.12(38.10)	31.14
"	0.2	29.15(32.34)	47.34
Vitavax (Carboxin)	0.2	21.61(27.50)	60.96
Emisan (Methoxy ethyl mercuric chloride)	0.25	2.79(7.86)	94.96
Raxil (Tebuconazole)	0.2	20.19(26.61)	63.52
Trichoderma viride (Bioagent)	(1×10^6/ml)	49.99(44.98)	9.70
Inoculated check (control)	(1×10^6/ml)	55.36(48.06)	-
Un-inoculated check (healthy sets)	-	5.44(13.27)	-
Mean		17.22(21.72)	
CD (p=0.05)		11.79	

*Average disease incidence from 1st June to 17th February, 2007. Figure within parentheses represent arc sine transformed values and CD is applicable to these only.

concentrations of Tilt (0.1 and 0.2%) and between Tilt and Emisan for disease incidence (%). Similarly, no significant difference was observed in inoculated check (55.36%) and a bio-agent treatment (49.99%) for smut incidence.

In the present study, the maximum percent set germination was observed with 0.25% Emisan followed by a bio-agent (*T. viride*). Whereas Tilt at 0.2% gave maximum disease control of 97.27% followed by 0.25% Emisan (94.96%).

Similarly, Propiconazole (Tilt) has also been found to have complete control of smut when sets were dipped in 0.25% solution (Waraitch and Kumar, 1999). Waraitch (1986) reported that smut disease was controlled by treating the sets with 0.5% Vitavax (Carboxin) for 1 h, and surface infection of inoculated material was controlled by dipping treatment for 10 min in Carboxin, Bavistin and Dithane M-45. Agnihotri and Sinha (1996) observed that Dithane Z-78, Benomyle, Oxycarboxin and organo mercurials were most effective as set treatment for the control of sugarcane smut. Natrajan and Muthusamy (1981) observed that germination was highest (74.1%) in set treatment with Dithane R-24 at 1.4 ml/L and smut incidence was lowest (6.6%) after treating with Bayleton (Triadimefon) at 1 g/L. Wada et al. (1999) in Nigeria reported that maximum disease control was in the sets treated with Mancozeb followed by Chlorothalonil and Benomyl.

Bharathi (2009) found that set treatment with Triademifon (0.1%) followed by Propiconazole (0.1%) had shown radical reduction in smut incidence. There was slight smut incidence with Triademifon or Propiconazole for 2 h dip, but for 4 h, there was no smut incidence. Set treatment with fungicide did not exhibit any influence on germination and shoot production. Bhuiyan et al. (2012) also reported that Cyproconazole, Propiconazole, Triadimefon and Azoxystrobin significantly ($P < 0.05$) suppressed disease expression for up to 6 months in a summer experiment and 9 months in an autumn experiment, which is in line with our study.

Meena and Ramyabharathi (2012) found that set treatment and foliar spray with Triademefon (0.1%) effect-tively reduced smut infection followed by set treatment and foliar spray with Propiconazole (0.1%). The biocontrol agents were less effective in reducing the smut infection.

Hence, set dip with Propiconazole (0.2%) or Emisan (0.25%) can be recommended for an effective management of set of transmitted sugarcane smut.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Effect of some culture extracts of *Aspergillus oryzae* on dehulling properties of pigeon pea (*Cajanus cajan* L.)

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Pre-dehulling treatments with some culture extracts of *Aspergillus oryzae* of various incubation periods played an important role in improving the dehulling properties of pigeon pea (*Cajanus cajan* L.). Yield percent of dehulled grains and dehulling efficiency increased concurrently with increase of incubation period of culture extracts. Maximum dehulled grains were achieved by 12-day old culture extract to the tune of 73% with least amount of undehulled kernels and fines (6.6 and 6.5%, respectively). Pre-dehulling trials conducted on pigeon pea grain employing wheat bran and pigeon pea husk based culture extracts of *A. oryzae* showed dehulling efficiency of 73% for wheat bran (12-day incubation period) and pigeon pea husk (9-day incubation period) in comparison with uninoculated extract (control) in the range 62.2-64.4%. Based on the results obtained, dehulling properties affected by pigeon pea husk based culture extract proved better than wheat bran culture extract.

Key words: Dehulling, pigeon pea, culture extracts, incubation periods, pre-dehulling treatment.

INTRODUCTION

Pigeon pea (*Cajanus cajan* L.) is a tropical grain legume grown mainly in India. The crop represents about 5% of world legume production with more than 70% being produced in India (Odeny, 2007). The high nutritive value of pigeon pea is perhaps the most important reason why it finds an important place among the smallholder poor farmers in India. Pigeon pea is abundant in protein, making it an ideal supplement to traditional cereal-based diets of most Indians which are generally protein-deficient. Generally, pigeon pea contains 20–25% protein and is consumed after suitable processing (Tiwari et al., 2008). Researchers at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India have developed high protein lines (HPL) with up to 32.5% protein content and significantly higher sulphur-containing amino acids (cysteine and methionine) (Singh et al., 1990; Saxena et al., 2002). Pigeon pea is therefore a good source of amino acids (Berrios et al., 1999).

Pigeon pea seeds are mainly eaten as dry decorticated split cotyledons by a milling process called dehulling. Dehulling is defined as the removal of the outer hull (fibrous seed coat or testa) which is tightly attached to the

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cotyledons (Kurien and Ramakrishnaiah, 1985). Dehulling is one of the most important processes applied to pigeon pea and is practiced widely in Asia and Africa on either a home or cottage industry scale (Tiwari et al., 2010). Dehulled seeds take less time to cook and have acceptable appearance, texture, palatability, digestibility and overall nutritional quality. There are two approaches to remove hulls, namely wet and dry milling. Generally, the dry method of milling is used throughout the Indian subcontinent for milling of pigeon pea because the quality of splits obtained from wet milling is poor (Kurien and Parpia, 1968; Kulkarni, 1991). The maximum theoretical recovery of dehulled pigeon pea is around 87-89%, whereas traditional dehulling recovery is only about 65-75% (Singh, 1995). Pigeon pea is recognized as hard-to-dehull pulse because of the presence of strong bond between the hulls and cotyledons usually via a thin layer of gums and mucilages along with uronic acids in the form of calcium pectate (Kurien and Ramakrishnaiah, 1985). Dehulling of pigeon pea incurs loss to the tune of 10 - 12% (broken grain and powder) edible cotyledon invariably (Mangaraj and Singh, 2011). Pre-treatment is required prior to removal of the hull to: (a) loosen the hull, (b) ease milling, (c) reduce breakage and (d) improve the quality of splits (Tiwari et al., 2007). Pigeon pea hulls can be loosened with pre-treatments of oil, heat or chemicals (Saxena et al., 1990). But importantly all these methods confront limitations of shape deformation or poor cooking quality of dehulled split and are time consuming even (Phirke and Bhole, 2000). A novel pre-dehulling technique involving enzyme is prospective to improve dehulling efficiency upon reducing the dehulling loss and improving cooking quality of pigeon pea (Deshpande et al., 2007; Sreerama et al., 2009; Bhowmik, 2012; Sangani et al., 2014). Partial hydrolysis of the mucilaginous bonds (in the interface of hull and cotyledon) by enzymatic reactions facilitates the easy dehulling of legumes (Verma et al., 1993). Enzyme mediated degradation of cell wall polysaccharides of horse gram and pigeon pea resulted in expansion of the grain with improved nutritional and functional properties upon thermal treatment as documented by Sreerama et al. (2008a, b). Enzymes are also reported to be utilized in rice polishing in a more selective way through hydrolysis of cell wall polysaccharides (Arora et al., 2007; Das et al., 2008) and aqueous extraction of vegetable oil by rupturing of oil-seed liposomes (Tano-Debrah and Ohta, 1999; Vierhuis et al., 2001; Lamsal et al., 2006). However, high cost of pure enzyme may be disadvantageous for its commercial application in dehulling venture. One of the alternative approaches to overcome this obstacle is to make continuous search for microorganism(s) and/ or their consortium for production of desired enzyme(s) in copious amounts and to optimize enzyme(s) production with them under submerged culturing condition for desired dehulling effect on application to pulses ahead. Optimization of enzyme production upon manipulation of fungal growth substrate, fermentation period are proven (Narasimha et al., 2006; Acharya et al., 2008). However, research in manipulation of microbial growth substrate and fermentation period for optimization of dehulling properties of pulses is meagre. Generally, hydrolytic enzymes, e.g. cellulases, xylanases, pectinases, etc. are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth. *Trichoderma* spp. and *Aspergillus* spp. have most widely been used for these enzymes. *Aspergillus oryzae,* a multicellular fungus producing multiplicity of hydrolytic enzymes (namely cellulases, *β*-glucosidase, protease, lipase, *α*-galactosidase, *β*-galactosidase, *α*-amylase, glucoamylase etc) on several agro-substrates is well reviewed by Pandey et al. (1999). Enzymes of *A. oryzae* have been used for several years in food processing, feed preparation, waste-water treatment, detergent formulation, textile production and other areas. Moreover, *A. oryzae* is considered to be a safe organism for production of food enzymes because it lacks expressed sequence tags for the genes responsible for aflatoxin production (Machida et al., 2005). In view of biotechnological importance of the *A. oryzae*, the present study emphasizes and evaluates the dehulling properties of pigeon pea using wheat bran and pigeon pea husk based culture extracts of *A. oryzae* developed under different incubation periods in order to optimize enzyme fermentation in different culture extracts for effective dehulling of pigeon pea. The cellulase activity and protein content in the culture media in relation to growth of *A. oryzae* for all treatments under study are also assessed.

MATERIALS AND METHODS

Raw material

Pigeon pea (of a white variety) was purchased from a local market in Ludhiana, India. The grains were thoroughly cleaned and were passed through 5.0 and 4.5 mm round holed sieve. The overflow of 5.0 mm sieve and underflow of 4.5 mm sieve was rejected and grain size between 5.0 and 4.5 mm was used for conducting the experiments.

Population density and culture extract

Aspergillus oryzae a local isolate from ITCC, IARI, New Delhi was grown in submerged culture of wheat bran media (Zambre, 1994). 100 ml of water deionised was amended individually with 4% wheat bran and sterilized (15 psi for 15 min) in Erlenmeyer flask of 500 ml capacity. Pigeon pea husk based media was prepared in similar fashion in parallel. The flasks were inoculated with 1% spore suspension of 6-day old *A. oryzae* grown on Potato Dextrose Agar (PDA) with 2 x 10^6 spores and incubated at 28±2°C on rotary shaker (180 rpm) for 3, 6, 9 and 12 days. Uninoculated media as corresponding controls were however incubated for 3 days only (on obtaining negative microbial growth results in test of sterility in Petri plates). The fungal population density at 3, 6, 9 and 12 days of incubation along with control was examined by most-probable-number (MPN) method upto 1:10^6 dilution in 0.9% (w/v) NaCl water and was expressed as colony-forming units (CFU)/ ml from separate

set (Bhowmik et al., 2013). Flasks were withdrawn at 3 days interval over a period of 12 days and filtered through Whatman No.1 filter paper to separate the mycelial mat of fungi with other coarse particles. The liquid filtrates were centrifuged at 10000 rpm at 10°C for 10 min. The supernatant solutions, thus obtained are natural multi-enzyme laden culture extracts (CEs) of *A. oryzae* of 3, 6, 9 and 12-day old incubation periods. The uninoculated media received similar treatments for control extracts production. These CEs were stored below 4°C and later employed for pre-treatment trials and enzyme analysis tests in this investigation.

Pre-dehulling treatment

CEs of 3, 6, 9 and 12-day old were added to pigeon pea (200 g) individually at optimized ratio of 0.5 (v/w) in sterile 1 L Erlenmeyer flask. The flasks were plugged, shaken manually and equilibrated at 7°C for 8 h. The CE treated kernels were later incubated at optimized conditions of 35°C for 3 h. The optimum ratio, incubation period and temperature were deduced from culture extract-to-pigeon pea ratio, incubation period, incubation temperature in the range of 0.16 - 1.84 (v/w), 0.95 - 11.05 h, 31.6 - 48.4°C (Zambre, 1994; Sarkar et al., 1995; Deshpande et al., 2007; Sreerama et al., 2009) respectively. Finally, the seeds were heated in a recirculatory air drier at 70°C for 15 min to inactivate crude enzymes of CE and dried (50°C for 4-6 h) to minimal moisture of 9-10%. Control seeds were also subjected to similar processing conditions except with extracts of uninoculated media.

Dehulling process

Moisture content of pigeon pea at the time of dehulling was measured according to the method of AACC (1995) and expressed as an average of three determinations. A batch-type laboratory mill (Model No. TM 05 Satake Grain Testing Mill Satake Engineering Co. Ltd, Tokyo, Japan) fitted with abrasive wheel at 4 mm exit clearance was used for dehulling of pigeon pea. The samples (100 g) were dehulled by maintaining abrasive wheel speed 400 rpm for 40 s. After dehulling, hulls were collected by aspiration. The abraded fractions were sieved through 2 mm sieve to collect fines and powder. The material remained in the sieve was manually separated as dehulled and undehulled kernels (Figure 1). All fractions were weighed and then expressed as proportion of the total original sample weight.

Dehulling data analysis

Dehulling index (DI) was calculated using the following equation (Sreerama et al., 2009):

$$DI = \frac{(W_2 + W_h) - (W_3 + W_b)}{W_1}$$

where, W_1 is the initial weight of sample taken for dehulling (g), W_2 is the weight of dehulled grains (g), W_3 is the weight of undehulled grains (g), W_h is the weight of hulls (g), W_b is the weight of brokens and powder (g).

The degree of hull removal is the percentage of dehulled kernels to the initial weight of sample taken for dehulling. The degree of dehulling (DD) was defined using the following equation (Sreerama et al., 2009):

$$DD(\%) = \frac{W_1 - W_3}{W_1} \times 100$$

Dehulling efficiency (DE) is an estimate of the efficiency of producing the major product, dehulled kernels. It was calculated using the following equation (Sreerama et al., 2009):

$$DE(\%) = \frac{W_1 - (W_3 + W_h + W_b)}{W_1} \times 100$$

Enzyme assay

Total cellulase activity of inoculated and uninoculated extracts was determined in terms of filter paper units (FPU) (Mandels and Andreotii, 1976). Aliquots of appropriately diluted extracts as enzyme source was added to Whatman No.1 filter paper strip (1 x 6 cm; 50 mg) immersed in one millilitre of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU activity) was defined as the amount of enzyme releasing 1 µmol of reducing sugar from filter paper per ml per min. The content of soluble protein in the extracts was also estimated with bovine serum albumin as a standard (Lowry et al., 1951).

Statistical analysis

The core experiments were repeated three times independently, and the results were represented as mean ±SD. The mean values for population density were transformed to logarithmic form and data were subjected to ANOVA followed by DMRT ($P \leq 0.05$). Correlation analysis between microbial growth and enzymes and proteins production was analyzed using statistical analysis system (SPSS, version 13).

RESULTS

Dehulling properties

The yields of different milled fractions of pigeon pea thus treated with CEs of *A. oryzae* of various incubation periods in the study along with their corresponding controls and their moisture contents at the time of dehulling are shown in Table 1. Increase in yield of percent dehulled grains was directly proportional to the increase of incubation period of CEs. Maximum dehulled grains were achieved by 12-day CE to the tune of 73% with least amount of undehulled kernels and fines (6.6 and 6.5%, respectively). Undehulled grain yield was significantly low ($P \leq 0.05$) with treatments of CE to control samples (with the range of 6.5-9.6% and 15.5-17.7% respectively). Yield of fines and hulls in all treatments are in the range of 6.3-9.3 and 10.9-13.9% respectively. Pigeon pea pre-treated with wheat bran and pigeon pea husk based CEs yielded higher amounts of dehulled kernels (with the range 71-73%) as compared to corresponding control samples (with the range 62-64%) with minimal cotyledon loss (6-8%) during milling. The CE treatments are also statistically significant ($P \leq 0.05$) to the controls. Overall, pigeon pea husk based CE performed better than wheat bran at every instance.

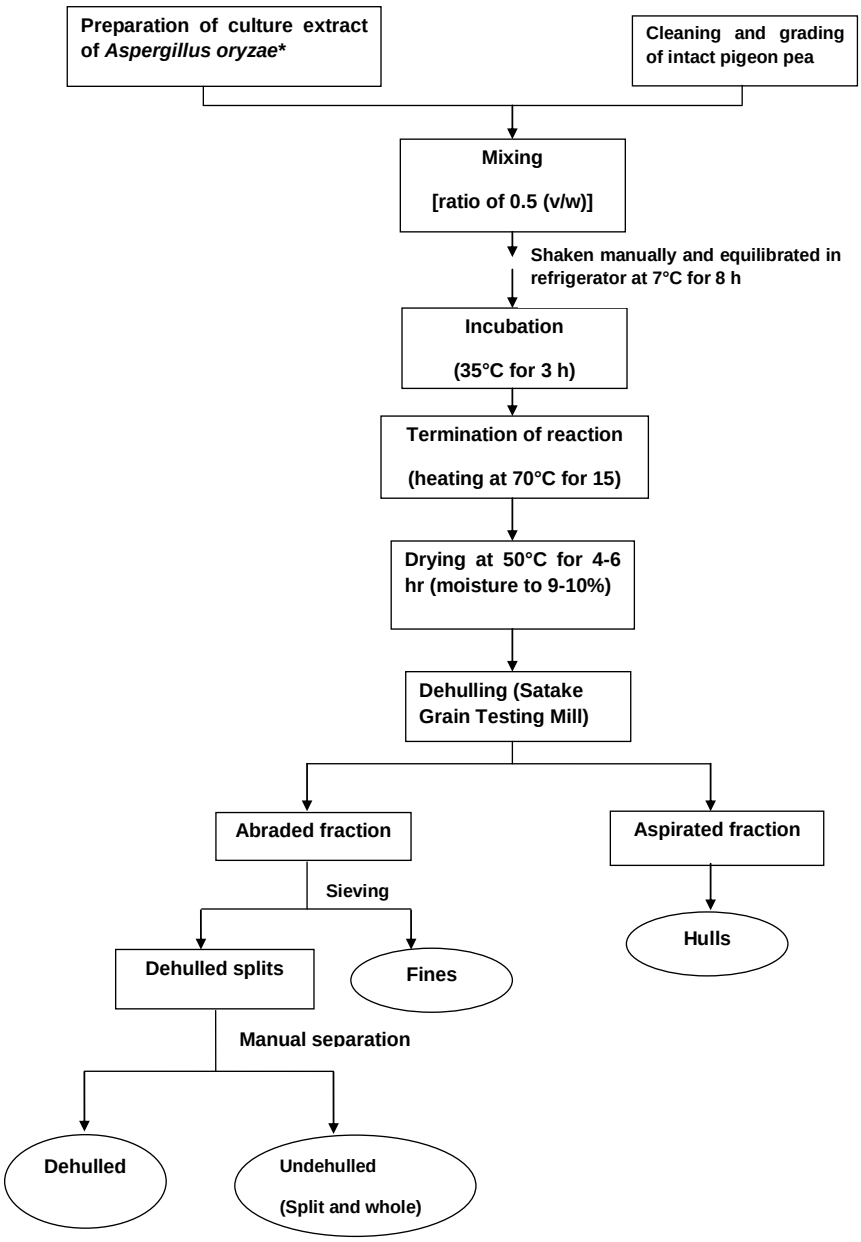

Figure 1. Flow diagram for dehulling and separation of dehulled fractions. *Culture extracts prepared from wheat bran and pigeon pea husk based media.

Table 1. Comparative analysis of the yield of milled fractions of pigeon pea pre-treated with wheat bran and pigeon pea husk based culture extracts of *A. oryzae* of various incubation periods (3-day, 6-day, 9-day, 12-day)*.

Dehulling treatment	Moisture content** (%)	Dehulled grains (%)	Undehulled grains (%)	Fines (%)	Hulls (%)
Wheat bran					
Control	9.7	61.9±0.5^{d}	17.7±0.8^{a}	9.3±0.4^{a}	10.9±0.9^{d}
3-day	9.0	70.8±0.8^{b}	9.6±0.7^{c}	8.0±1.9^{abc}	11.5±0.5^{cd}
6-day	9.0	71.4±0.4^{b}	7.8±0.8^{def}	7.8±0.6^{abc}	12.9±0.6^{ab}
9-day	9.2	70.8±0.5^{b}	8.9±0.8^{cd}	7.1±0.5^{abc}	13.1±0.8^{ab}
12-day	9.9	72.9±0.9^{a}	6.6±0.4^{fg}	6.5±0.6^{bc}	13.9±0.7^{a}

Table 1. Contd

Pigeon pea husk					
Control	9.9	64.0±0.7^{c}	15.5±0.5^{b}	8.8±0.3^{ab}	11.4±0.3^{cd}
3-day	9.0	71.5±0.7^{b}	8.3±0.7^{d}	8.0±2.1^{abc}	12.2±0.7^{bc}
6-day	9.2	71.4±0.4^{b}	7.9±0.9^{de}	7.8±2.1^{abc}	12.8±0.8^{ab}
9-day	9.0	73.1±0.8^{a}	6.5±0.5^{g}	6.3±1.0^{c}	14.0±0.7^{a}
12-day	9.9	72.8±0.2^{a}	6.8±0.5^{efg}	6.5±1.0^{bc}	13.8±0.7^{a}

Mean values bearing different superscript in the same column are significantly different ($P \leq 0.05$) according to Duncan's multiple range test; *Results are mean ± standard deviation of three determinations. **Moisture content (w.b.) at the time of dehulling expressed as an average of three determinations.

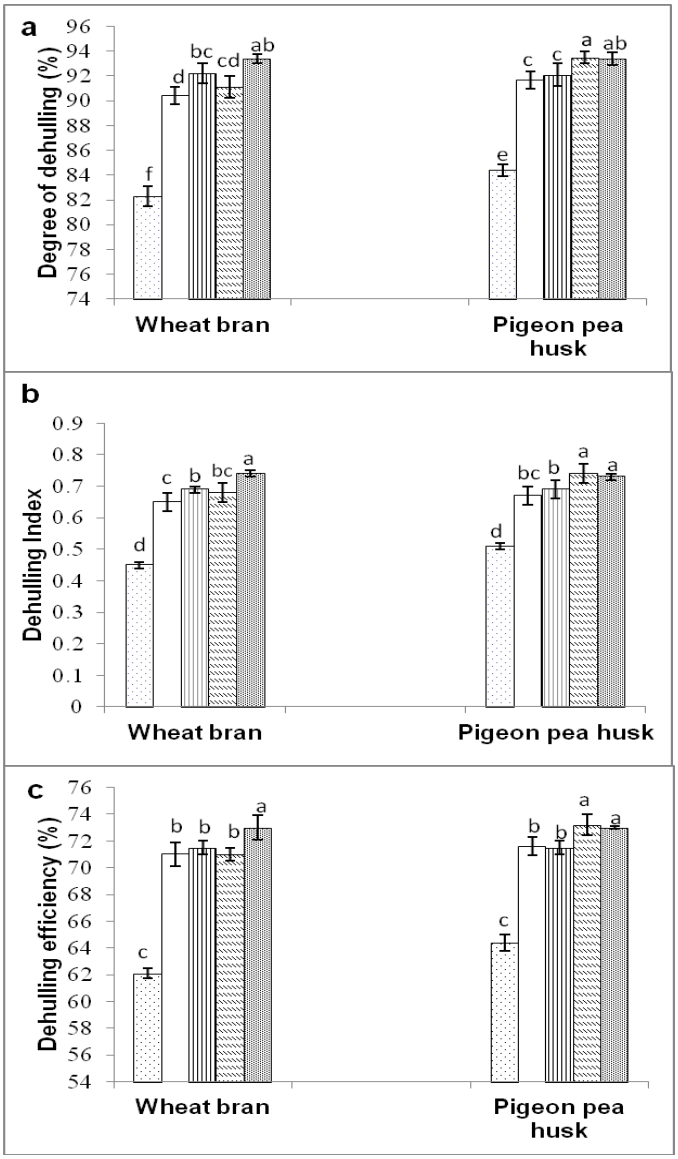

Figure 2. Dehulling properties of pigeon pea pre-treated with wheat bran and pigeon pea husk based culture extracts of *Aspergillus oryzae* of various incubation periods (▫= control, ▭= 3-day, ▥ = 6-day, ▨ = 9-day, ▩= 12-day). Bars represent ± SD of mean (of three replicates). Histograms with a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Statistically ($P \leq 0.05$) 9-day old pigeon pea husk based CE is optimum to achieve maximum dehulled grains (73%) with minimum fines (6.3%) among all the treatments under study. The highest DD (93.4%) and DI (0.74) in wheat bran based culture extract were observed in 12-day old CE (Figure 2). Since higher amount of dehulled kernels were obtained in this treatment, the DE (73%) was also significantly higher ($P \leq 0.05$) than corresponding control (62.1%). Statistically significant increase in DD, DI and DE of wheat bran CE treated pigeon pea were observed as compared to corresponding control. However, treatments between different CE incubation periods are not significant at all ages except 12-day at 5% level of probability. Pigeon pea husk based CE treatment also resulted in higher DD as compared to its corresponding control (Figure 2). However, no significant ($P \leq 0.05$) effect among the various ages of pigeon pea husk based CE were recorded. Increase in DD, DI and DE was progressive upto 9-day old CE which declined at the later age. However, the effect of 9-day old pigeon pea husk CE on DD, DI and DE of pigeon pea was at par with 12-day old pigeon pea husk CE at 5% level of probability. Least DD, DI and DE were attained by 3-day old CE (in the range of 90.4-91.7%, 0.65-0.67 and 71-71.6% respectively). Pigeon pea husk based CE recorded maximum dehulling efficiency (73.2%) by 9-day incubation period while wheat bran achieved the maximum (73%) by 12-day incubation period. Never-theless, statistically insignificant effect exists between the treatments of wheat bran and pigeon pea husk CEs.

Fungal growth and cellulase activity

Growth of *A. oryzae* in terms of population density increased with progressive increase of incubation period with a range 7.1-7.7 log CFU/ml (Figure 3). Of the two lignocellulosic agro-wastes in the study, wheat bran supported maximum growth of *A. oryzae.* However, statistically ($P \leq 0.05$) growth was not significant with the increase of incubation period. The cellulase activity in wheat bran and pigeon pea husk media increased concomitantly with the rise in population density of *A. oryzae* as incubation period advanced (Figure 4).

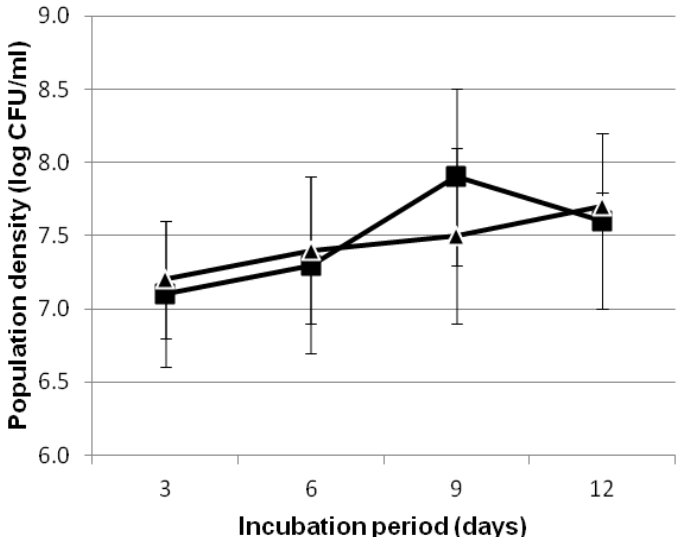

Figure 3. Change of population density of *A. oryzae* in wheat bran (■), and pigeon pea husk (▲) media during different incubation periods. Bars represent ± SD of mean (of three replicates).

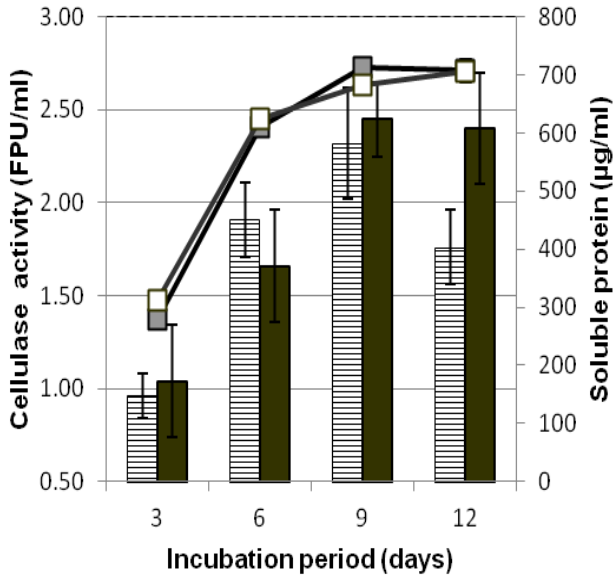

Figure 4. Change of cellulase activity and total protein content during fermentation of *A. oryzae* in wheat bran and pigeon pea husk based media. Histograms represent cellulase activity in wheat bran (▭) and pigeon pea husk (■), line graphs represent total protein content in wheat bran (▭) and pigeon pea husk (▭) media, and bars represent ± SD of mean (of three replicates).

Maximum cellulase activity of about 2.3 FPU per millilitre of wheat bran based CE and 2.5 FPU per millilitre of pigeon pea husk based CE was attained at 9-day interval. The extracellular soluble protein content in the CE of two media also increased concomitantly with the incubation period with maximal values ranging from 705-712 µg/ml. No growth, cellulase activity and protein content were detected in both uninoculated media

Table 2. The correlation analysis between *A. oryzae* growth and cellulase and soluble protein production in culture extract.

Parameters	Correlation coefficients
Growth x cellulase	0.882*
Growth x soluble protein	0.875*
Cellulase x soluble protein	0.973**

*Correlation is significant at the 0.05 level (2-tailed); **correlation is significant at the 0.01 level (2-tailed).

(corresponding controls). The correlations between microbial growth at varied incubation period and enzyme production were analyzed (Table 2). For cellulase and soluble protein in CE, correlation coefficient was 0.882 and 0.875, respectively ($P<0.05$). Cellulase activity showed high correlation coefficients to protein content. This was 0.973 ($P<0.01$). The results indicated a significant positive correlation between incubation period and enzyme production in CEs.

DISCUSSION

Strong bonds of gums and mucilage existing between hull and cotyledon are attributed to the hard dehulling nature of pigeon pea (Ramakrishnaiah and Kurien, 1983; Kurien and Ramakrishnaiah, 1985). Several findings in literature reveal the biochemical variations in proportion and structural arrangement of non starch polysaccharides (NSP) and glycoproteins in the seed coat, gums/mucilages and cell wall of legumes (Ramakrishnaiah and Kurien, 1983; Ryden and Selvendran, 1990; Swamy et al., 1991; Showalter, 1993; Stolle-Smits et al., 1995; Cosgrove, 1997; Stolle-Smits et al., 1997; Bravo et al., 1999; Wood et al., 2014). Pre-treatments are generally required to loosen the pulse seed coats including pigeon pea (Singh, 1995). Traditionally vegetable oil treatment (up to 1%) is used in commercial mills in Indian sub continent to loosen the husk of pulses that are difficult to mill (Sokhansanj and Patil, 2003). The recovery of dehulled kernels in commercial mills averages about 75% (Kulkarni, 1989; Kurien, 1981). Comparable yield (73%) of dehulled kernels of pigeon pea by applying CEs of *A. oryzae* was obtained in laboratory process in this study. This finding is also at par with dehulled kernel recovery (71.3 to 73.9%) in pigeon pea when tested with enzyme consortium (Deshpande et al., 2007). However, variable results of pigeon pea dehulled kernel recovery were documented when tested with different enzymes (namely xylanase- 58.9% and protease-78.4%) individually (Sreerama et al., 2009). This may be attributed to the incomplete accessibility of NSP or proteins for hydrolysis by diverse enzymes in isolation due to the nature of substitutes, presence of phenolic compounds such as flavonoids or lignin of lower molecular weight as reported in lima beans and chickpeas (Kannenburg and Allard, 1964; Knights, 1989). However, enzyme consortium enables attaining dehulled kernels to desired level by concerted hydrolytic action (of different enzymes in group) on the complex NSP/proteins in a cascade thus loosening the husk from cotyledon efficiently. CE of *A. oryzae* is a natural milieu of multiple hydrolytic enzymes of commercial value (Pandey et al., 1999). Moreover tempering of the pigeon pea seeds with 0.5 (v/w) with CE for three hours during enzyme reaction has provided a grain moisture content of around 25%. This may have resulted in a moisture gradient within the seed (a relatively high moisture content seed coat as compared to a relatively drier cotyledon). Moisture gradient facilitates the partial hydrolysis of NSP and proteins located in the interface between the seed coat and cotyledon by enzymes (Sreerama et al., 2009). Hence DI value of CE treated pigeon pea (0.74) is higher than the maximum DI value reported for pigeon pea (0.67) with steam treatment at 97°C followed by drying at 120°C (Opoku et al., 2003).

The efficacy of enzymes in dehulling process is a function of moisture content of grain, chemical composition of seed (husk and intermediate gums/mucilages), incubation temperature, incubation period and enzyme concentration (Sangani et al., 2014). But more importantly, it is essential to search enzyme(s)/enzyme consortium with broad spectrum hydrolytic capacity to loosen seed coats of wide variety of pulses having varied cell wall composition genetically. Consideration of universal enzyme consortium in this regard shall not only be unrealistic but also expensive. The only viable option is to search for microorganism(s) producing multiplicity of hydrolytic enzymes suited for pulse dehulling. We selected *A. oryzae* and used its multi-enzyme laden CEs to hydrolyse the NSP/glycoproteins at the interface of hull and cotyledon. Moreover, *A. oryzae* secretes different enzymes (namely cellulases, *β*-glucosidase, protease, lipase, *α*-galactosidase, *β*-galactosidase, *α*-amylase, glucoamylase, etc) at varied proportion in response to the nutrients in its growth medium (Pandey et al., 1999). Optimization of enzyme production by controlling fermentation temperature, pH, nutrients (carbon, nitrogen) and fermentation period is quite achievable. The cellulolytic activity under study is comparable to that of the most well studied fungus, *Trichoderma reesei* whose wild type or mutant cells in free status produced cellulase within a range of 1-2 FPU/ml on various media (Domingues et al., 2000). The production of cellulase by wild type cells of *Bacillus pumilus* (Kotchoni and Shonukan, 2002) and *Cellulomonas biazotea* (Rajoka et al., 1998) and *Trichoderma aureoviride* (Zaldivar et al., 2001) in liquid did not exceed 1.5 U/ml. The overall trend of dehulled kernel yield of pigeon pea under the influence of CEs of *A. oryzae* was 12-day > 9-day> 6-day> 3-day> control. The best period for the production of enzyme by *A. oryzae* was the 12-day (73%), but the 9-day observed a decline in cellular growth. The results suggested that enzyme production was

not growth dependent. This finding is in conformity with the earlier reports (Cho et al., 2002; Purwanto et al., 2009; Darah et al., 2013). Influence of fermentation periods of CEs was positively correlated to DD, DI and DE. However, statistically insignificant (P<0.05) decline occurred after the 9th day in case of pigeon pea husk based CE. Slight fall of hydrolytic enzymes at later growth phase is the plausible reason for the decline in dehulling properties. Insignificant (P<0.05) results within the uninoculated controls in relation to all dehulling parameters were noticed. Dehulled kernel recovery of controls was the least ranging from 62-64%. Absence of enzymes in the control extract is the reason for this effect. This is hence evident from this work that natural enzymes secreted by *A. oryzae* in CE were vital to loosen the husk upon improving the dehulling properties of pigeon pea. Pigeon pea husk based CE was better performer than wheat bran in improving the dehulling properties of pigeon pea which we report for the first time. Relatively high protein (18%) and low cellulose (10%) content in wheat bran to pigeon pea husk (2 and 22% respectively) may have brought variability in production of enzymes in respective CE (Schwarz et al., 1988; Pandey et al., 1999; Prasad et al., 2011). Relatively high cellulase content and comparable protein content of pigeon pea husk to wheat bran is the probable reason for the better performance. This variable nutrients content in the lignocellulosic wastes per se offers a probable scope for harnessing microbial potential to the best in improving the dehulling of pulses.

Conclusion

Low cost enzymes or enzyme consortia are the utmost need when opting for enzyme-assisted pulse milling on commercial scale. Naturally produced multi-enzymes from agro-waste based culture extract of effective *A. oryzae* are advantageous option in this regard. This fungus can produce vital enzymes of commercial value namely cellulases, *β*-glucosidase, protease, lipase, *α*-galactosidase, *β*-galactosidase, *α*-amylase, glucoamylase, etc. The prospects of low cost enzyme is that it can economize the pulse dehulling cost by replacing costly vegetable oil (used traditionally on commercial lines at present) while keeping the natural shape of decorticated split cotyledon of pigeon pea intact. Enzyme-assisted dehulling of pulses may also be potential to improve the cooking quality of pulses which is lacking in oil-assisted pre-milling process. Moreover, the technology of CE can be utilized in both manual and automated dehulling process effectively. The technical drudgery of preparing, mixing and storing of CE is relatively less than pure enzyme in dehulling process. The results indicated that microbial density in progressive incubation period directly correlates to increase in enzyme production and increase of dehulled pigeon pea with reduced undehulled grains and fines subsequently. Here, pigeon pea husk based CE performed well than wheat bran based CE with regards to dehulling properties including dehulling efficiency. It can be summarized from this study that CEs from different lignocellulosic substrates have different potential to influence the dehulling process of pigeon pea. Moreover, the optimum incubation period for enzyme production by *A. oryzae* is a source of activated enzyme to improve dehulling properties of pigeon pea effectively.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Disruption of negative regulators (*SP_nsdA* and *SP_nsdB*) in *Streptomyces peucetius* causes doxorubicin overproduction

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Pathway-specific regulatory genes generally participate in the secondary metabolites-related biosynthesis process. The genes *nsdA* and *nsdB* were reported to have a negative effect on the production of actinorhodin, prodigiosin and calcium-dependent antibiotic from *Streptomyces coelicolor*. In this study, we searched for similar genes in the *Streptomyces peucetius* genome, the doxorubicin producer. Amino acid sequence similarity between SC_NsdA and SP4635 (SP_NsdA) was 88.1%, and between SC_NsdB and SP1750 (SP_NsdB) was 78.4%. High performance liquid chromatography (HPLC) analysis revealed that the disruption of *SP_nsdA* and *SP_nsdB* significantly increased doxorubicin production by 2.07 and 1.74-fold, respectively. The *SP_nsdA* and *SP_nsdB* disruption mutants produced more yellow pigment and early aerial mycelium than did the original wild-type strain. These results show that *SP_nsdA* and *SP_nsdB* negatively affected doxorubicin production and morphological differentiation in *S. peucetius*.

Key words: Doxorubicin, gene disruption, negative regulator, secondary metabolite, *Streptomyces peucetius*.

INTRODUCTION

Streptomycetes are Gram-positive, soil-dwelling bacteria, and are widely recognized as producers of various secondary metabolites. Streptomycetes produce about 75% of commercially and medically useful antibiotics, and their antibiotic biosynthesis is mediated by several types of pathway-specific regulators (Champness, 2000).

Regulatory genes that are required for antibiotic biosynthesis act as positive and/or negative elements in antibiotic production. Identification and inactivation of repressor genes have proven effective in overproduction

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of clinically important drugs. Secondary metabolites with clinical value have been overproduced in significantly higher amount through the combined application of genetic engineering and strain improvement techniques. Pathway-specific regulatory genes like *actII-orf4*, *redD*, *cdaR* and *mmyR* regulate the antibiotic-related biosynthetic genes in *S. coelicolor* (Bibb, 1996), and other global regulators such as *bldA* (Fernandez-Moreno et al., 1991), *bldB* (Eccleston et al., 2002), *bldD* (Elliot et al., 1998) and *bldG* (Bignell et al., 2000) perform the highest-level of regulation and affect both morphological and physiological differentiation (Chater, 1993, 2001). In other cases, some regulatory genes containing *absA1-absA1* (Anderson et al., 2001; Ryding et al., 2002), *cutS-cutR* (Champness et al., 1992), *phoR-phoP* (Sola-Landa et al., 2003) and *tcrA* (Liu and Yang, 2006) are pathway-specific repressors that regulate antibiotic production in a negative way since their mutation or deletion results in the overproduction of antibiotics. *S. coelicolor* is a genetically well-characterized strain that can produce four types of antibiotics such as actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic (CDA), and methylenomycin (Mmy). Among the various regulatory genes, especially SCO5582 (*nsdA*) and SCO7252 (*nsdB*) in *S. coelicolor* were identified by gene disruption as a gene negatively affecting antibiotic production and sporulation (Li et al., 2006; Wang et al., 2009; Zhang et al., 2007).

Doxorubicin (DXR) was first isolated from *Streptomyces peucetius* subsp. *caesica* ATCC27952, a mutant strain derived from *S. peucetius* ATCC29050 (Arcamone et al., 1969) and it is commonly used in the treatment of a wide range of cancers including bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, multiple myeloma and others (Alfaro et al., 2013). Increasing DXR production is very important because the chemical synthesis of DXR is a tedious process and also it is chemically labile. On the other hand, multistep reactions requiring electrophilic bromination limited yield of DXR during its semi-synthetic production using daunorubicin (DNR) (Lown, 1993). Although a number of organisms produce DXR, *S. peucetius* ATCC27952 is the only organism reported to produce DXR (Grein, 1987). Therefore, the generation of industrial strain of *S. peucetius* ATCC27952 for DXR production is important. In this study, we found two genes, SP4635 and SP1750, in *S. peucetius* ATCC27952 which are orthologous genes of *nsdA* and *nsdB* in *S. coelicolor*, respectively. We studied the level of DXR production and morphological differentiation by gene disruption in *S. peucetius*.

MATERIALS AND METHODS

Bacterial strains, culture conditions and vectors

Escherichia coli were grown in Luria-Bertani (LB) broth (Difco) and maintained on LB agar medium at 37°C. *S. peucetius* were grown in R2YE (50 ml, 5% sucrose, 0.02% potassium sulfate, 1% magnesium chloride, 1% glucose, 0.5% yeast extract, and 0.01% Difco casamino acid) and maintained on R2YE agar medium at 28°C. DNA manipulation was carried out in *E. coli* XL1-Blue (Stratagene). pGEM-T easy vector (Promega, USA) was the routinely used cloning vector for DNA manipulation, and pKC1139 was used as *E.coli-Streptomyces* shuttle vector for gene inactivation.

DNA manipulation

We compared the genome of *S. peucetius*, and found two genes (SP4635 and SP1750) which have high sequence similarity with *nsdA* and *nsdB* in *S. coelicolor*. For disruption of SP4635 (*SP_nsdA*) and SP1750 (*SP_nsdB*) in *S. peucetius*, the upstream and downstream fragments (SP4635U, SP4635D, SP1750U and SP1750D) were amplified by polymerase chain reaction (PCR). The primer sequences were as follows: SP4635UF (5'-GTC GAG CTG GGC CTC GAT GAG GTC-3'), SP4635UR (5'-TCT TCT AGA ACC GGA GGG TCA GAC-3'), SP4635DF (5'-GGT TCT ACT CGT ACG ACC GGT TCG-3'), SP4635DR (5'-GCG GAA TTC GAC GAT CCG CAT TCC-3'), SP1750UF (5'-ACA AGC TTC TGC AG ATA CGC CC CA-3'), SP1750UR (5'-AAT CTA GAC GGC CGG ACT CAT CGA-3'), SP1750DF (5'-GTT CTA GAA ACC CGC CTC TTC GAG-3') and SP1750DR (5'-GGG AAT TCG GCT CGA GGG-3'). PCR was carried out under the following conditions: denaturation at 94°C for 7 min, and in each cycle, annealing at 55-65°C for 1 min and polymerization at 72°C, denaturation at 94°C for 1 min, for total of 30 cycles and finally gap filling at 72°C for 7 min.

Construction of recombinants

Amplified DNA fragments of *SP_nsdA* and *SP_nsdB* were cloned into the pGEM-T easy vector and then transformed into *E. coli* XL1-Blue. The upstream fragment of SP4635U was digested with *Hin*dIII and *Xba*I, and the downstream fragment of SP4635D was digested with *Xba*I and *Eco*RI; then they were cloned into pKC1139. The upstream fragment of SP1750U and the downstream fragment of SP1750D were digested and cloned as mentioned above. For the final construct, *SP_nsdA* and *SP_nsdB* recombinants were digested with *Xba*I and then ligated with the fragment of the thiostrepton resistance gene (1.0 kb) obtained from pIBR25 (Thuy et al., 2005), thereby resulting in pOJH0117 and pOMK1228. All these plasmid were confirmed by enzyme digestion and PCR sequencing. pOJH0117 and pOMK1228 were transformed into the *E. coli* ET12567 used as demethylation host, and then finally transformed into the wild type strain of *S. peucetius* ATCC 27952 (Flett et al., 1997).

DNA sequence accession number

The nucleotide sequences of *SP_nsdA* and *SP_nsdB* reported in this paper have been deposited in the NCBI nucleotide sequence database under accession numbers KF500401 and KF500402, respectively.

Transformation into *S. peucetius*

The protoplast transformation and the selection of thiostrepton-resistant transformants were performed using previously described methods (Jnawali et al., 2011). Wild-type *S. peucetius* ATCC 27952 strain was cultured in a 50-ml R2YE medium for 36 h at 28°C. The culture broth was transferred to a 50-ml tube and washed with 10.3% sucrose. The protoplasts were generated by incubating the mycelia at 37°C for 55 min with the addition of 3 ml of lysozyme (5 mg/ml). The recombinant DNA was transformed into *S. peucetius*, and the

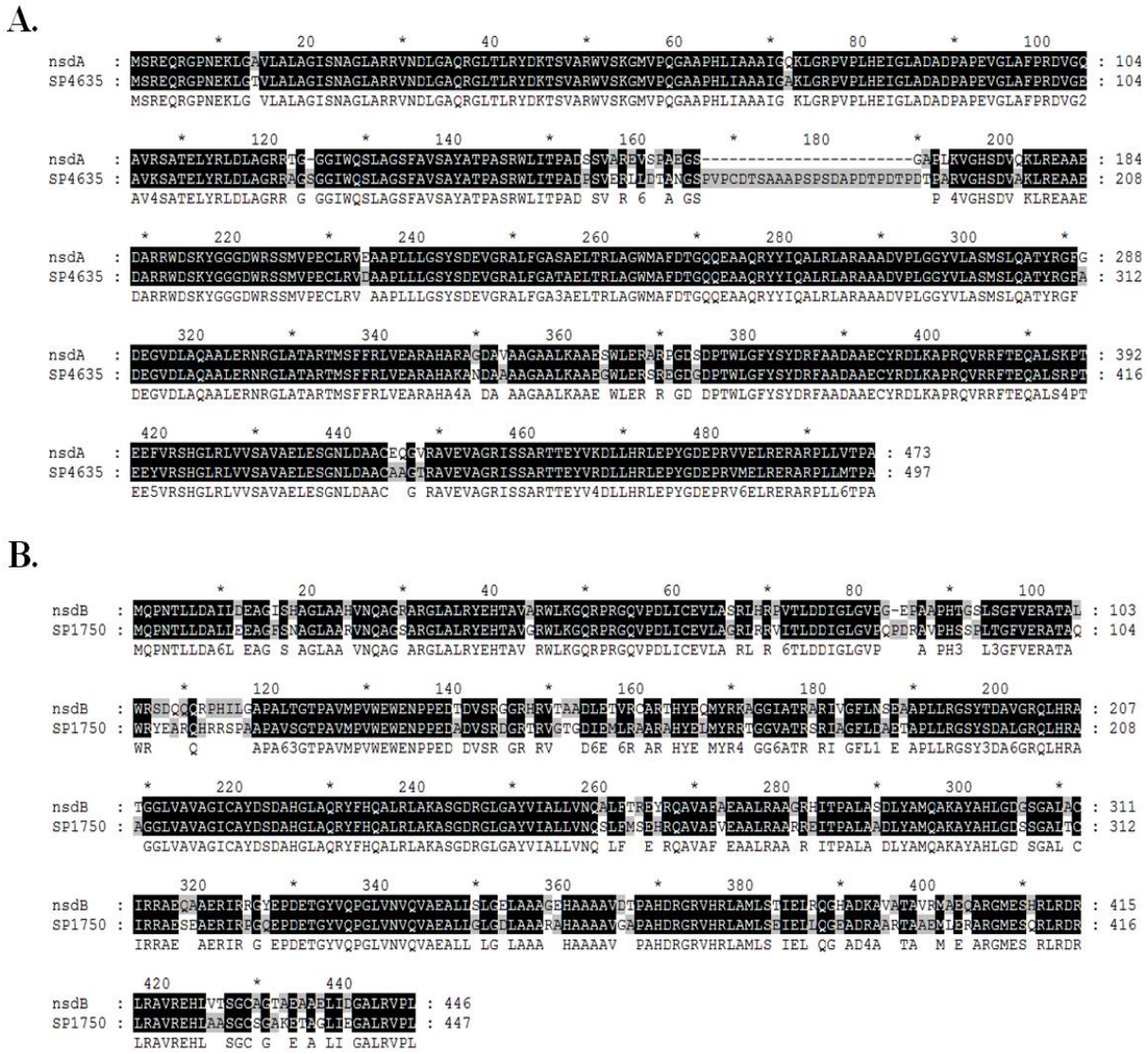

Figure 1. Multiple sequence analysis of SP4635 and SP1750. A. NsdA from *S. coelicolor* and SP4635 (SP_NsdA) from *S. peucetius*; B. NsdB from *S. coelicolor* and SP1750 (SP_NsdB) from *S. peucetius*.

protoplasts were mixed with plasmid DNA. 200 µl of 40% (w/v) polyethylene glycol 1,000 (PEG, Merck-Shuchardt) solution was promptly added, followed by brief centrifugation to remove PEG; the protoplasts were resuspended in protoplast buffer, and each plate was overlaid with 3 ml of soft agar (0.4%) containing 40 ug/ml thiostrepton. All the transformants were confirmed by PCR sequencing.

HPLC

The *S. peucetius* wild type and mutant strains were extracted with two volumes of $CHCl_3$:CH_3OH (9:1). The extract was dried under reduced pressure using a rotary evaporator and reconstituted in 1.5 ml of methanol. A 15-µl aliquot of the extract was analyzed by HPLC using a reversed-phase C18 column with a mixture of 100% acetonitrile (solvent B), distilled water (solvent A, pH 2.34 by trifluoroacetic acid) and sodium sulfate (1.327 g/L) for 71 min, with a flow rate of 1 ml/min by the following method: 0-50 min (0-100% B), 50-60 min (100% B), and 60-70 min (100% A). Peaks were monitored using a UV absorbance detector at 254 nm. DXR was used as a control.

RESULTS

Sequencing analysis

'*nsdA*' and '*nsdB*' are known as the negative transcriptional regulatory genes in *S. coelicolor*. According to the multiple sequence alignment result, SP4635 (SP_NsdA) showed 88.1% amino acid identity with 'NsdA' of *S. coelicolor*, and SP1750 (SP_NsdB) showed 78.4% amino acid identity with 'NsdB' of *S. coelicolor* (Figure 1). We proposed these genes have a negative effect on the production of DXR in *S. peucetius*, and designed primers for functional characterization of these genes.

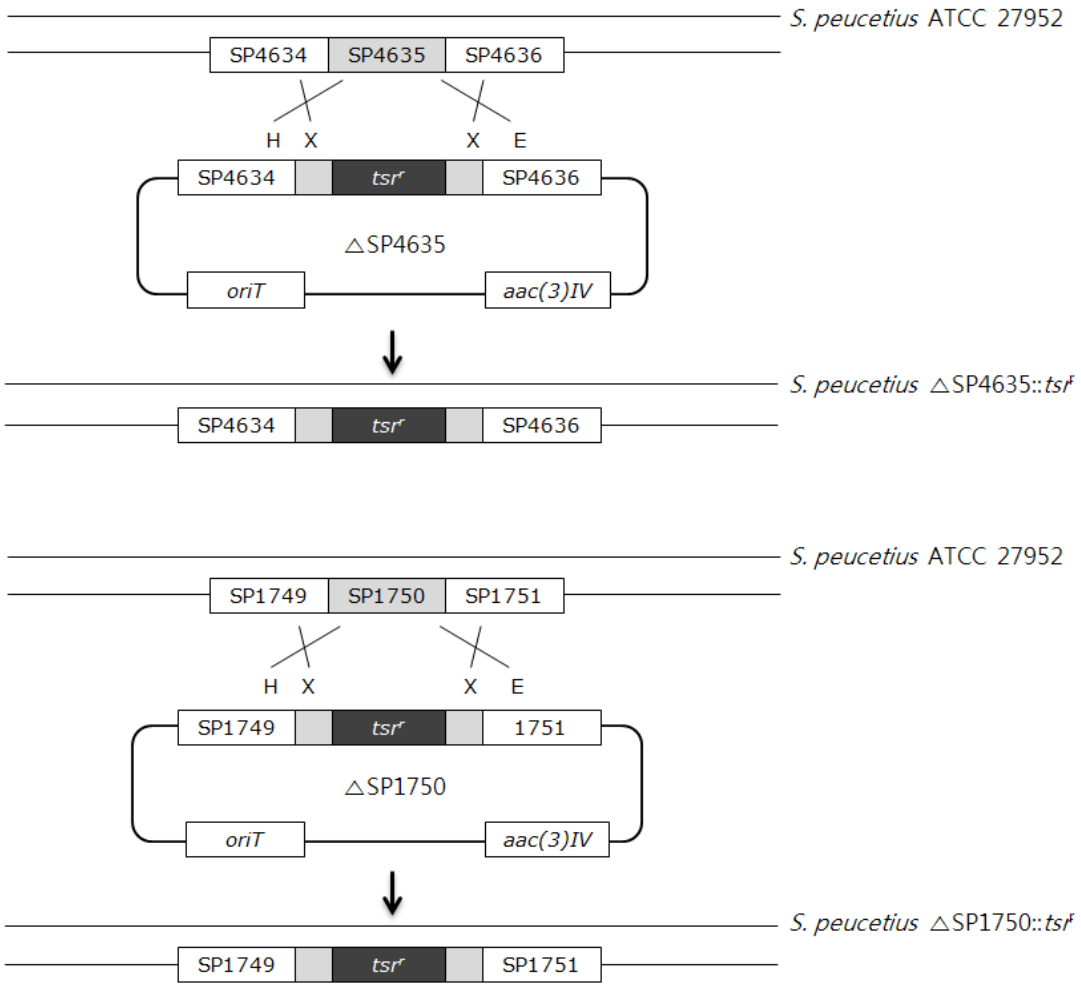

Figure 2. Scheme for gene disruption plasmid of pOJH0117 and pOMK1228. An internal DNA fragment of SP4635 upstream was inserted into the *Hin*dIII and *Xba*I sites of pKC1139 and that of SP4635 downstream was inserted into the *Xba*I and *Eco*RI of pKC1139. Thiostrepton resistance gene was inserted at the center of SP4635 upstream and SP4635 downstream, resulting in recombinant plasmid pOJH0117. pOMK1228 was also constructed similarly.

Construction of pOJH0117 and pOMK1228 for gene disruption

For disruption of the genes, SP4635 and SP1750, a total of 4 oligonucleotides were used, and PCR was performed as shown in materials and methods section. Finally, obtained PCR products such as SP4635U (1.3 kb), SP4635D (1.1 kb), SP1750U (1.7 kb), and SP1750D (1.6 kb) were cloned into a T-vector. All cloning was confirmed by restriction enzyme reaction and PCR sequencing (data not shown), then *E. coli* transformation was performed. After the PCR product was purified from the T-vector clone, it was again cloned into pKC1139 used as *E. coli-Streptomyces* shuttle vector. For the selection of final transformant, thiostrepton resistance gene (*tsr*^{r}) obtained from pIBR25 (Thuy et al., 2005) was inserted between upstream and downstream fragment as shown in Figure 2. Finally, pOJH0117 (*SP_nsdA* knock-out plasmid) and pOMK1228 (*SP_nsdB* knock-out plasmid) were constructed, and all these constructions were confirmed by PCR sequencing (data not shown).

Phenotype of *SP_nsdA* and *SP_nsdB* disruption transformants

After pOJH0117 and pOMK1228 were transformed into *S. peucetius*, we carried out plate assay to investigate the morphological differentiation between the wild-type and mutant strains (*S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750). Both mutants were different from the wild-type strain with respect to the growth rate and extent of mycelium. When cultured on an R2YE medium at 28°C, both the mutants began to grow aerial mycelium about 1 day earlier than the wild-type strain, and after 2 days, they also produced a yellow pigment, which is the color of

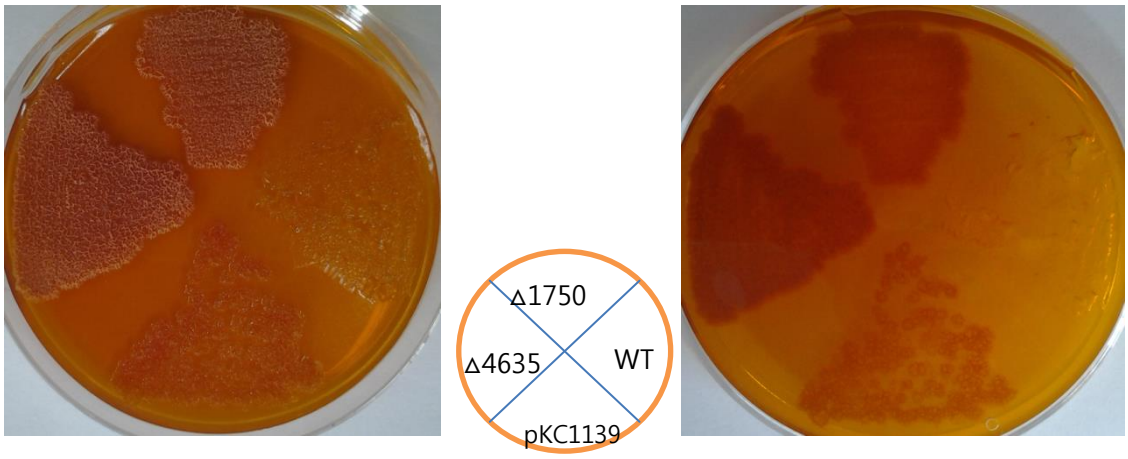

Figure 3. Morphological differenciation between wild-type and mutants strains. Incubation on R2YE agar plate (Apr^r, Tsr^r) for 3 days.

DXR. As expected, wild-type strain did not grow on this plate because it does not have resistance to apramycin and thiostrepton (Figure 3). On the other hand, the wild-type strain produced DXR upon longer incubation, but, both mutants rapidly produced more amount of DXR than the other mutant that only had a vector (Figure 4B). These results indicate that SP4635 and SP1750 play a negative role in morphological differentiation and production of DXR in *S. peucetius*.

Analysis of the enhanced DXR production

HPLC analyses were carried out to assess the quantitative change in DXR production. At first, we investigated the growth pattern of *S. peucetius.* For studying the growth rate of *S. peucetius*, it was grown on R2YE liquid medium at 28°C. After 3 days, the OD value of *S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750 dropped, while that of the wild-type strain and the mutant that only had a vector kept on increasing (data not shown). This result also indicated that SP4635 and SP1750 negatively regulate DXR production because DXR can inhibit cell growth due to its toxicity. In other words, *S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750 produced more amount of DXR and inhibited cell growth as described below. After this, we extracted DXR from the wild-type and mutants strains. We carried out HPLC analysis using extracted samples (Figure 4A). Standard DXR was detected at about 9.5 min and also desired peaks were detected at the same time from mutant and wild-type extracts. Both *S. peucetius* ΔSP4635 and *S. peucetius* Δ1750 mutants produced DXR about 2.07 and 1.74 times, respectively as compared to *S. peucetius* wild-type (Figure 4B). Using the complementary experiments, same wild-type level of DXR production was recovered from both mutants (data not shown). These results showed that SP4635 and SP1750 have a negative effect on DXR production in *S. peucetius*.

DISCUSSION

In this paper, *SP_nsdA* and *SP_nsdB* were identified in *S. peucetius*, and they shared a high degree of sequence identity with the well-known proteins, NsdA (SCO5582) and NsdB (SCO7252), which negatively control the secondary metabolite production and morphological differentiation in *S. coelicolor* (Li et al., 2006; Uguru et al., 2005; Zhang et al., 2007). Wang et al. (2009) also reported that disruption of *nsdA* increased milbemycin A4 and nanchangmycin about 1.5- and 9-fold in *S. binchengensis*. SP_NsdA/SP_NsdB belong to a protein family containing a domain of unknown function, DUF921, which has so far been found in *S. coelicolor* and *S. griseus* (Marchler-Bauer et al., 2003), and they also have a tetratricopeptide repeat (TPR)-like domain, which may mediate protein-protein interactions (D'Andrea and Regan, 2003). The TPR-like motifs are degenerate 34 amino acid sequences identified in common streptomyces, which are Gram-positive bacteria that can produce many secondary metabolites. However, they do not contain additional DNA binding domains, and it is speculated that they possibly contain undefined domains that play a role in DNA binding. Alternatively, they might also interact with certain transcriptional regulators to control production of secondary metabolites.

The disruption of *SP_nsdA* and *SP_nsdB* resulted in higher production of DXR in *S. peucetius*, suggesting their negative effect on DXR biosynthesis. In general, DXR production is limited because DNR, the precursor of DXR, is produced commercially by semi-synthesis rather than by purification from *S. peucetius*. Therefore, this study could be helpful for industrial production of DXR.

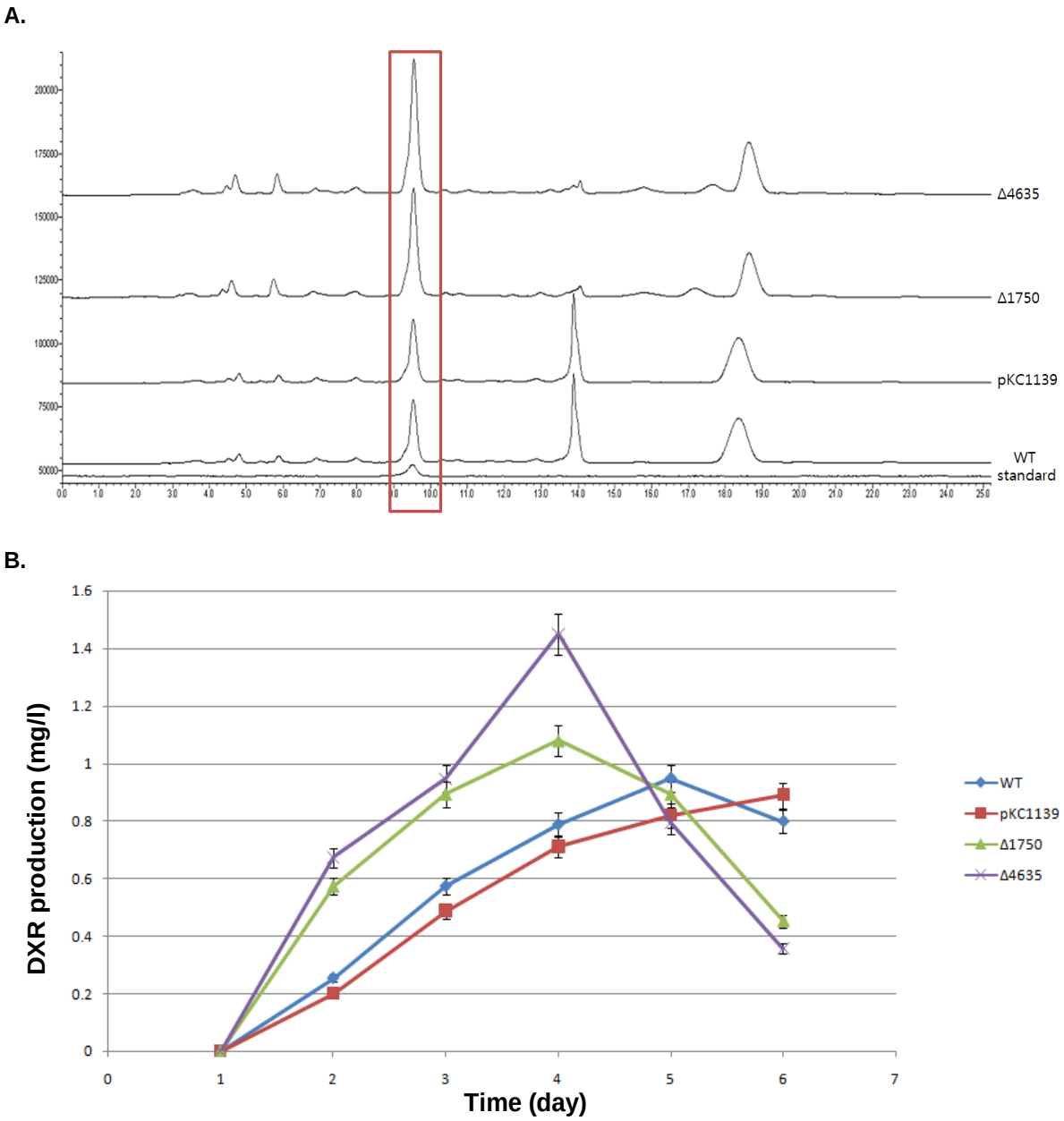

Figure 4. Comparison of DXR production. A. HPLC analysis of DXR production between the wild-type and mutants strains. B. Comparison of DXR production during 6 days. After 4 days, DXR yield from *S. peucetius* ΔSP4635 and *S. peucetius* ΔSP1750 was higher than that from wild-type and pKC1139-inserted mutant.

The fact that our group could secure the genome of *S. peucetius* indicates that new transcription factors, including pathway specific regulators and their corresponding factors for DXR biosynthesis, will be identified, which could be potential in enhancing the yield of DXR.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Analysis of arsenic species in realgar bioleaching solution by capillary zone electrophoresis

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Realgar was bioleached by *Acidithiobacillus ferrooxidans* BY-3. A capillary zone electrophoresis (CZE) method was developed to analyze arsenic species in realgar bioleaching solution. This way can simultaneously separate arsenic compounds including arsenite (iAs^{III}), arsenate (iAs^{V}), monomethylarsenic (MMA^{V}) and dimethylarsenic acid (DMA^{V}). Borate and cetyltrimethylammonium bromide (CTAB) were selected to compose a background electrolyte (BGE). Identification and quantification of the arsenic species at µg/ml levels was possible by use of direct UV detection at 195 nm. The limits of detection and quantification for the targeted analytes were in the range of 3.10–12.00 and 10.34–39.83 µg/ml, respectively. The intraday precision was in the range of 1.45–2.06% for migration times and 3.14–4.28% for peak areas, while interday precision was in the range of 2.24–3.20% for migration times and 6.46–7.11% for peak areas, respectively. Spiked recoveries at three levels were in the range of 84.92–101.67%. Results indicated that the bioleaching solution of realgar only exists in organic arsenic, the components were identified to be arsenite and arsenate.

Key words: Realgar, bioleaching, *Acidithiobacillus ferrooxidans*, arsenic species, capillary zone electrophoresis.

INTRODUCTION

Realgar, also called red arsenic or *Xiong-Huang* (China), has poor solubility in water. It contains more than 90% of arsenic disulfide (As_2S_2) or tetra-arsenic tetra-sulfide (As_4S_4) and small quantities of arsenic trioxide (As_2O_3) (Liu et al., 2008). The medical use of realgar has been traced back to thousands of years ago for various diseases. In recent years, realgar was believed to have antibiotic, anti-viral and anti-tumor effects (Wang et al., 2008; Xu et al., 2006). Notably, realgar has been effectively applied to treat chronic myelocytic leukemia (CML) and acute promyelocytic leukemia (APL) (Zhang et al., 2008).

Realgar was extracted using several methods to improve clinical efficacy. For example, realgar can be extracted with elutriation method and alkali solvent before clinical use (National Pharmacopoeia, 2005; Wu et al., 2004). With the increase of solubility, the toxicity will increase while the efficiencies of extracts will be low. Therefore, we used bioleaching technologies to deal with medicinal realgar so as to obtain a new reconstituent

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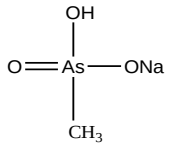
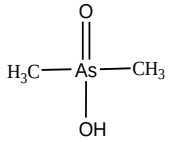
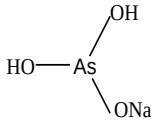
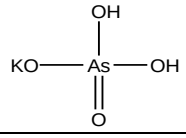
Table 1. Arsenic compound of interest.

Chemicals with symbolic name	Structure	λmax(nm)	pKa
MMA^V		192	3.6 8.2
DMA^V		194	6.2 9.3
iAs^{III}		197	9.2 13.5
iAs^V		192	2.3 6.9 11.4

which is expected to possess increased efficiency and decreased toxicity.

Bioleaching is a process for dissolution of metals from their mineral source through the use of bacteria to convert insoluble metal sulfides (CoS, PbS and ZnS) into water-soluble metal sulfates ($CoSO_4$, $PbSO_4$ and $ZnSO_4$). Realgar is a mineral containing both sulfur and arsenic, and it has been reported that arsenic was bioleached form realgar (Zhang et al., 2007). Although realgar bioleaching solution had better therapeutic effect than arsenic trioxide for hepatoma, lung cancer and leukocythemia *in vivo/vitro* by inducing apoptosis, the active substances had not been identified (Zhang et al., 2008). We expect that the bacterial realgar bioleaching solution contain iAs^{III} species and methylated arsenic species.

For the elucidation of active substances in realgar bioleaching solution, different arsenic compounds were analyzed in a recent study. It has been reported that high-performance liquid chromatography (HPLC) with a variety of detection methods including inductively coupled plasma mass spectrometry (ICP-MS), hydride generation atomic fluorescence spectrometry (HG-AFS) (Ammann, 2011; Campillo et al., 2008; Cui et al., 2004; Gonzalez-Contreras et al., 2011; Hata et al., 2007; Jaafar et al., 2007; Kirby et al., 2004; Raab et al., 2004; Tlustos et al., 2004; Yang et al., 2012; Zhao et al., 2011), hydride generation inductively coupled plasma mass spectrometry (HG-ICPMS) can be used for separating arsenic compounds (Inoue et al., 1999). Compared with HPLC, capillary electrophoresis has been demonstrated to be a simple and powerful separation technique for arsenic. And it has advantages of many separation modes, high selectivity and sensitivity, small quantities and doses of reagent and sample dosage.

The aim of this work was to develop a rapid CZE method for the simultaneous determination of inorganic and organic arsenic compounds including iAs^{III}, iAs^V, MMA^V and DMA^V, and was validated to determine parameters such as linearity, detection and quantification limit, precision, recovery and stability. The method was also successfully applied to analyze the arsenic species from leachate by bioleaching of realgar using *Acidithiobacillus ferrooxidans* BY-3.

MATERIALS AND METHODS

Realgar and arsenic standards

Realgar (As_2S_2, 99.1% in purity), was obtained from Shimen County, Hunan Province, China. Disodium tetraborate decahydrate (≥98% in purity) was purchased from Tianjin Chemical Reagent Factory (China). Dimethylarsenic acid [$(CH_3)_2AsO(OH)$, DMA^V] and potassium arsenate (KH_2AsO_4, iAs^V) were purchased from Sigma Co. Ltd. (America). Sodium monomethyl-arsonate ($CH_4AsNaO_3{\cdot}(3/2)H_2O$, MMA^V) was purchased from Shanghai Quandao Scientific Trade Co. Ltd. (China). Arsenic trioxide (As_2O_3) was purchased from Beijing Chemical Reagent Factory (China). All other reagents were of analytical or HPLC grade. Arsenic sample solutions were prepared by dissolving in Milli-Q water to form the stock solutions with concentrations of 1 mg/ml. Before storage, all stock solutions were filtered through a 0.22 μm nylon filter membrane and degassed in ultrasonic bath for 15 min. The molecular structures, maximum UV absorbance and p*K*a values of the arsenic analytes are shown in Table 1 (Sun et al., 2004).

Bioleaching experiments and preparation of realgar bioleaching solution

Bioleaching of realgar was carried out in 500 ml flasks with 200 ml

of the 9 K medium (with 1 g of sterile sulfur powder per liter) containing 0.2 g of realgar powder sample with an initial pH of 2.0 (Kutschera et al., 2007). Each flask was inoculated with *A. ferrooxidans* BY-3 (CCTCC- M203071) suspension at 10% (v/v). The flasks were incubated at 150 rpm, 28°C, with a pH of 2.0. The experiment lasted for 30 days.

The bacterial cultures were centrifuged at 2000 rpm for 15 min to remove the precipitation, and then the supernatant were filtered through a 0.22 μm nylon filter membrane. The filtrate is the realgar bioleaching solution, and injected directly after degassing by sonication.

Instruments and conditions

All CZE separations were performed on a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA, USA). This system was equipped with a reversible-polarity power supply (0 to (±30) kV) and a photo-diode-array detector. The system was controlled by a 32 Karat system and version 5.0 for data collection and analysis. The separations were carried out on an uncoated fused-silica capillary (60 cm × 75 μm i.d.) with a detection window located 10 cm from its extremity. A built-in temperature control system was designed to maintain separation temperature and minimize Joule heat generated, thus enabling the CZE system to use a high electric field and to achieve very low band dispersion. The detection wavelength was set at 195 nm. The separation voltage was set at -20 kV and the optimized buffer was composed of 20 mM borate/0.5 mM CTAB at pH 9.5 by titrating with 0.1 M NaOH. The BGE was prepared daily and vortexed for 2 min, filtered through 0.45 μm filter membrane and degassed for 15 min. Before first use, a new capillary was rinsed with 1 M HCl for 5 min, followed by water for 2 min, 0.1 M NaOH for 10 min, water for 2 min and running buffer for 15 min. Samples were injected by applying a pressure of 0.5 psi for 5 s (1 psi = 6894.76 Pa).

RESULTS AND DISCUSSION

Development of capillary zone electrophoresis

Effect of the borate concentration

In general, ion migration velocity, separation, column efficiency and peak shape in CZE are sensitive to changes in BGE characteristics. In this paper, to verify the effect of buffer concentration on migration behavior, the running buffer containing 0.5 mM CTAB and borate with concentrations from 20 to 50 mM at pH 9.5, applied voltage of -17.5 kV was investigated to separate arsenic species. Figure 1 shows the effects of different borate concentrations on the electrophoretic separation of arsenic species. Obviously, retention time, peak height and peak area (sensitivity) were affected by the borate concentration. With increasing borate concentration, migration became slower. This may be due to the higher Joule heating caused by increased conductivity of the buffer and decreases in electroosmotic flow (EOF), zeta-potential and ion migration velocity at the capillary wall-solution interface with increasing ion strength. Considering the Joule heats, migration times, column efficiency, peak shape and resolution of real samples, 20 mM borate containing 0.5 mM CTAB was selected as BGE.

Effect of the buffer pH value

The electrophoretic mobility of the arsenic compounds is strongly dependent on their p*Ka* value as shown in Table 1 and the pH of the running electrolyte solution since arsenic compounds contain acidic groups in their chemical structure. This fact is especially important in the case of arsenic compounds showing several p*Ka* values. These arsenic compounds can be present in different ionized forms depending on the pH of buffer, and therefore it is possible to change their electrophoretic mobility. The effects of different BGE pH values from 9.0 to 10.5 with a 0.5 (±0.02) -step on the electrophoretic separations of arsenic species is shown in Figure 2 When pH value was below 9.5, the baseline separation of anions including iAs^{V2-}, MMA^{2-}, DMA^{2-}, iAs^{III2-} was achieved, however, the detection sensitivity was lower than that at pH 9.5; when pH value was above 9.5, iAs^{V2-}, iAs^{III2-} had lower sensitivity than that at pH 9.5; when pH value was above 10.0, MMA^{2-}, DMA^{2-} and iAs^{V2-} had low separation and sensitivity; Whole migration became much slower with increase of the BGE pH because pH alters the charges and thus electrophoretic mobilities. Although in the above pH range each arsenic analyte should be at the same ionization degree: iAs^{V2-}, MMA^{2-}, DMA^{2-} and iAs^{III2-}, migration profiles were different. The migration order was iAs^{V2-}, MMA^{2-}, DMA^{2-} and iAs^{III2-} since the EOF increases with increase zeta potential. Furthermore, the zeta potential is directly proportional to charge-mass ratio (charge-mass ratio: $iAs^{III2-} > DMA^{2-} > MMA^{2-} > iAs^{V2-}$). In contrast, satisfactory separation and sensitivity were obtained at pH 9.5, indicating that the buffer capacity was high enough that local pH and conductivity did not change as a result of sample injection and separation. Finally, a BGE of pH 9.5 was selected.

Effect of separation voltage

The inorganic and organic arsenic species investigated are acidic compounds which are not totally dissociated under normal conditions. The dissociation degree of the species, which affects ionic charge and electrophoretic mobility, depends on the pH of the running electrolyte solution and the p*Ka* of the species. Under normal conditions (the capillary surface is negatively charged and positive voltage is applied) the EOF transports, firstly, the undissociated forms of the arsenic compounds toward the cathode and the partially dissociated anionic compounds then follow. In the electropherograms, only anionic species with lower electrophoretic mobilities than the magnitude of the EOF can be observed. Although, increasing the buffer pH results in increased electroosmotic mobility, the electrophoretic mobilities of the arsenic compounds also increase, because the compounds are more dissociated. A complete separation is obtained by use of a negative voltage, and the direction of the EOF can be reversed. In this case, the electrophoretic

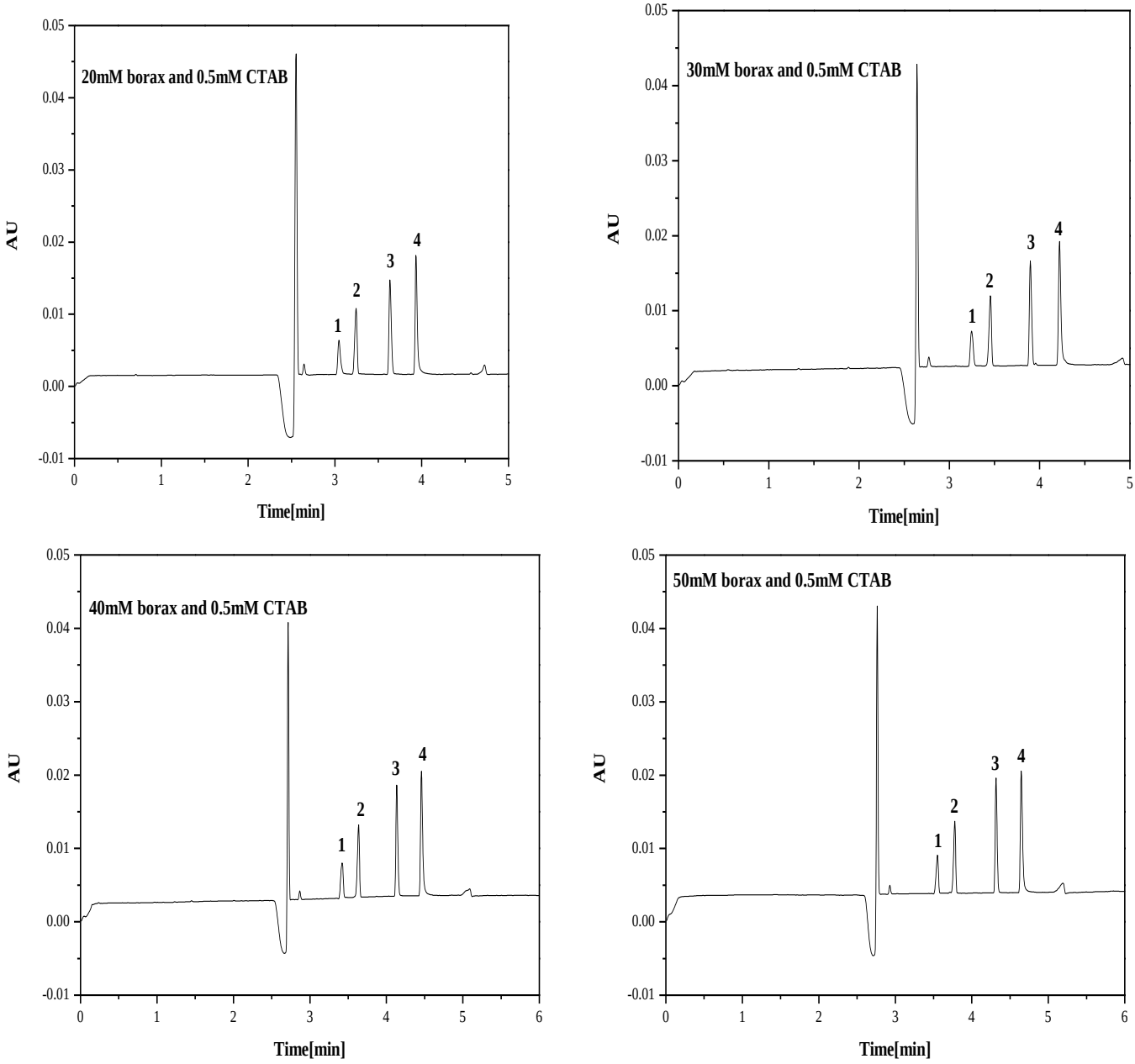


Figure 1. Electrophoregram obtained from the four arsenic compounds at different borate concentration. sample, 250 µg/ml iAs^{V2-}, 250 µg/ml MMA^{2-}, 250 µg/ml DMA^{2-}, 250 µg/ml iAs^{III2-}; buffer pH 9.5; injection pressure, 0.5 psi for 5 s; detection wavelength, 195 nm; voltage, -17.5 kV; temperature, 25°C; Peaks: (1) iAs^{V2-}; (2) MMA^{2-}; (3) DMA^{2-}; (4) iAs^{III2-}.

and electroosmotic mobilities are oriented in the same direction-towards the anode, reversal of the EOF was achieved by addition of 0.5 mM CTAB to the running buffer. The effect of applied negative voltage on migration times and separation efficiencies was also investigated with separations in the voltage range from -20 to -15 kV with a 2.5-step at pH 9.5. Figure 3 shows that the migration times decreased but the detection sensitivity did not improve with increasing voltage while the baseline noise decreased. This may be attributed to the higher Joule heating and lower viscosity of the buffer with increasing voltage. Thus -20 kV was selected as the separation voltage.

Choice of detection wavelength

The iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V} have absorption maxima at wavelengths between 190 and 205 nm (Table 1). These species can be detected by direct UV detection,

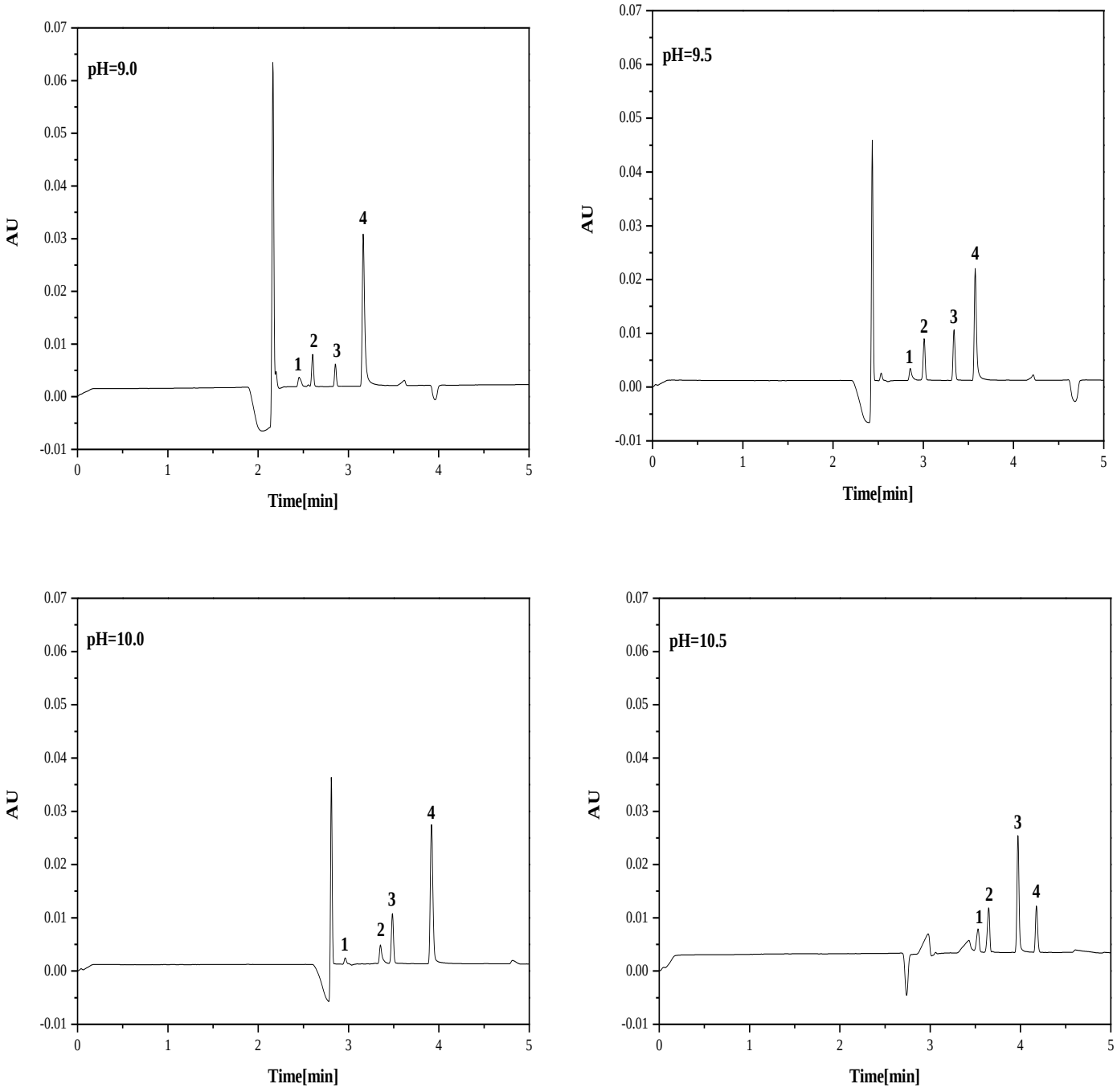


Figure 2. The effects of BGE pH on the electrophoretic separation of arsenic compounds. Buffer, 20 mM borate, containing 0.5 mM CTAB; Sample, 250 µg/ml iAs^{V2-}, 250 µg/ml MMA^{2-}, 250 µg/ml DMA^{2-}, 250 µg/ml iAs^{III2-} ; injection pressure, 0.5 psi for 5 s; detection wavelength , 195 nm; voltage, -17.5 kV; temperature, 25°C; Peaks: (1) iAs^{V2-}; (2) MMA^{2-}; (3) DMA^{2-}; (4) iAs^{III2-}.

because solutions of borate buffer are more UV transparent. To select an optimum detection wavelength, a wavelength range of 195-205 nm was tested. Figure 4 shows the electrophoregrams of iAs^{V2-}, MMA^{2-}, DMA^{2-} and iAs^{III2-} obtained using 20 mM borate, pH 9.5 and containing 0.5 mM CTAB at all tested detection wavelengths. With increase in the detection wavelength from 195 to 205 nm, the sensitivity of detection decreased while the baseline noise increased. After considering the detection sensitivity and baseline noise, 195 nm was thus selected as the detection wavelength.

In BGE of pH 9.5, consisting of 20 mM borate and 0.5 mM CTAB, the separation condition were 195 nm detection wavelength, injection pressure 0.5 psi for 5 s,

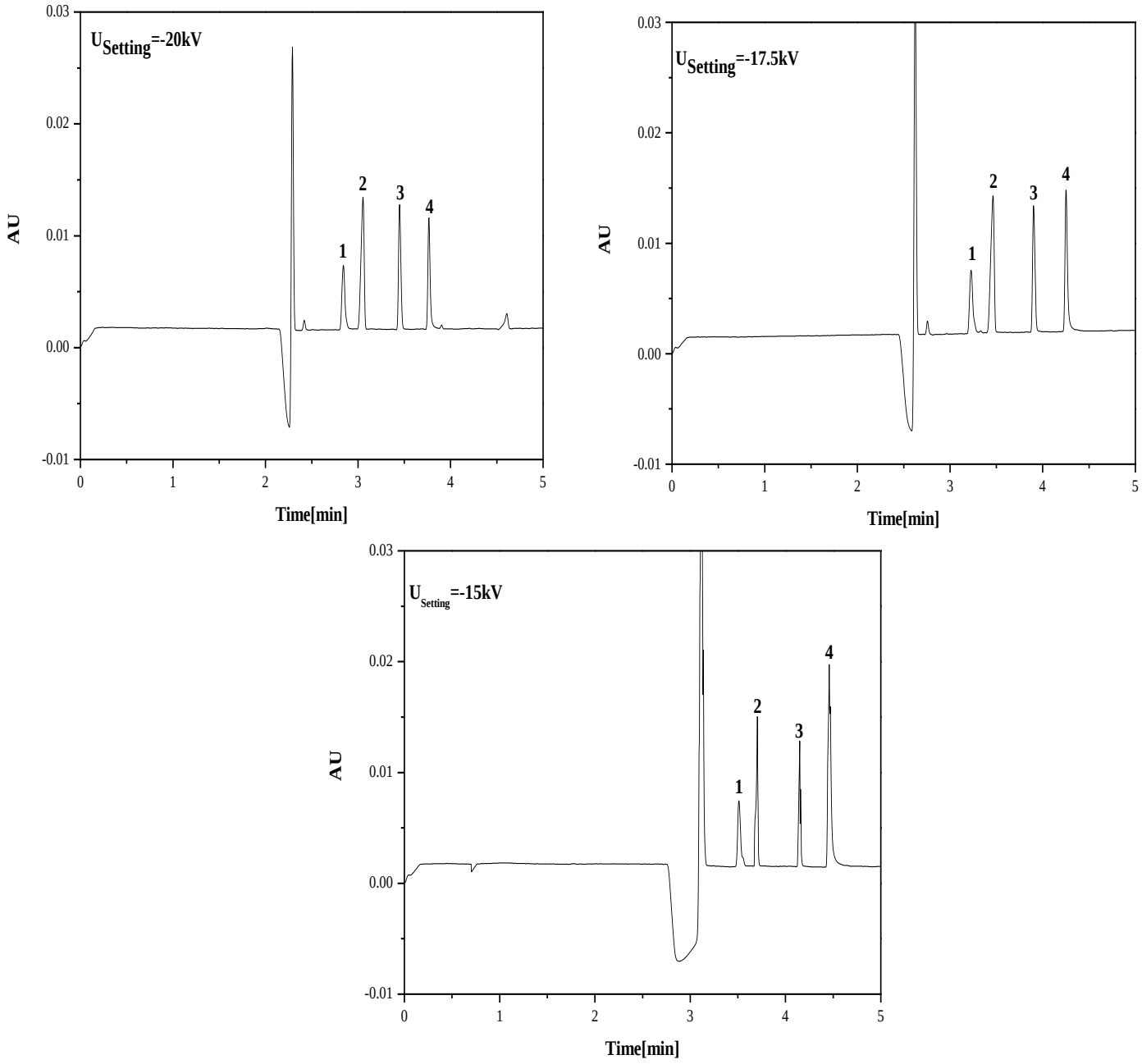


Figure 3. The electrophoregrams was obtained at different voltage. Buffer, 20 mM borate and 0.5 mM CTAB; pH 9.5; Sample, 576 µg/ml iAs^{V2-}, 192 µg/ml MMA^{2-}, 192 µg/ml DMA^{2-}, 40 µg/ml iAs^{III2-}; injection pressure, 0.5 psi for 5 s; temperature, 25°C. Peaks: (1) iAs^{V2-}; (2) MMA^{2-}; (3) DMA^{2-}; (4) iAs^{III2-}.

and -20 kV separation voltage, a baseline level resolution of four arsenic compounds was achieved (Figure 5).

Calibration curve and validation of the method

With 20 mM borate and 0.5 mM CTAB BGE at pH 9.5, the calibration curves were calculated by analyzing the iAs^{V2-}, MMA^{2-}, DMA^{2-} and iAs^{III2-} as y = a+bx, where x was peak area, and y was the concentration. Five concentration points were used to prepare the calibration curve for each compound. The linear regression equation of the calibration curve and correlation coefficient (r) are shown in Table 2. The limit of detection (LOD) is the lowest concentration of the analyte in a sample that can be detected, equal to 3 s, where s is the sample standard deviation of 10 independent sample blanks fortified at lower concentrations, measured one each. The limit of

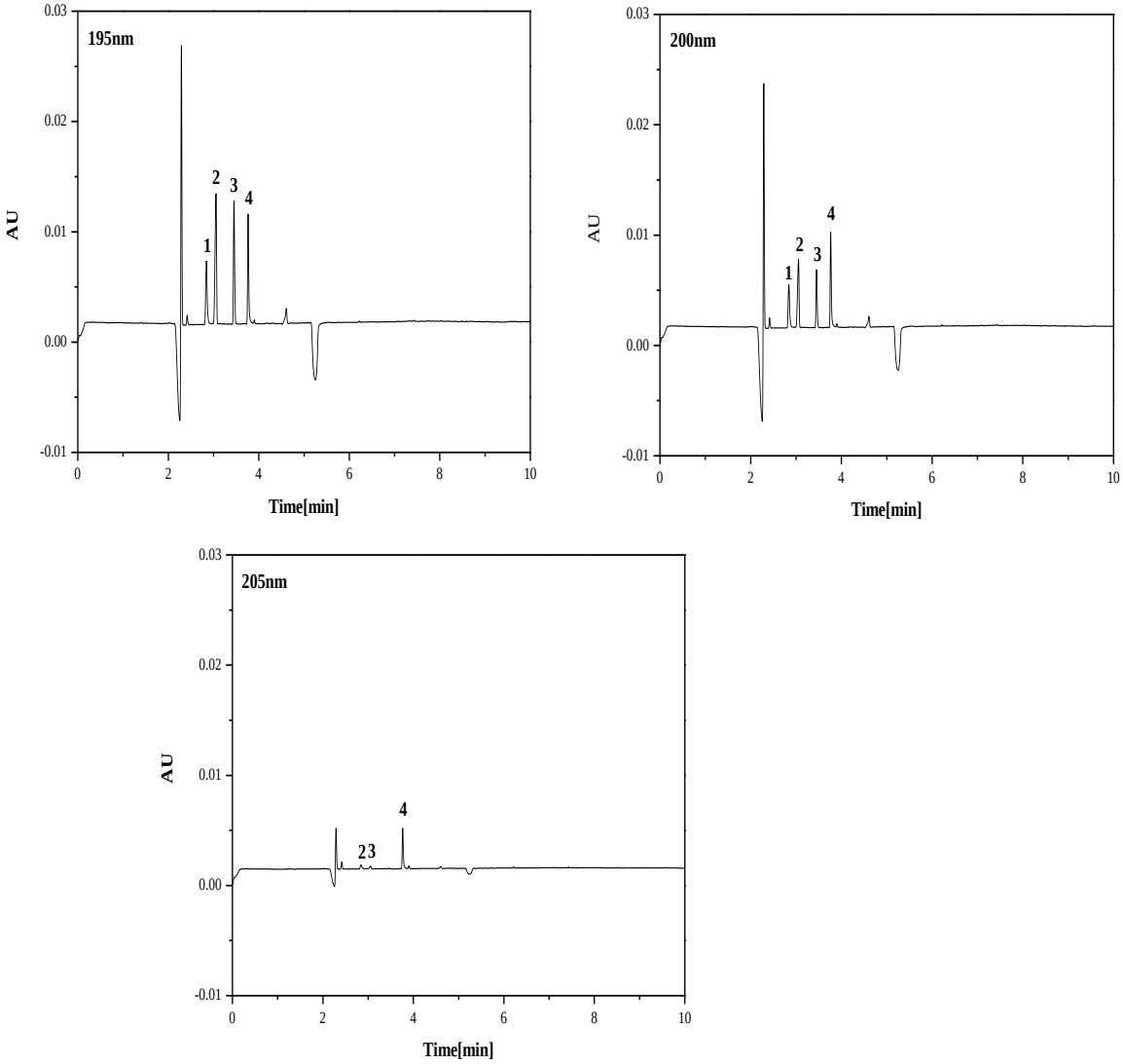


Figure 4. The electrophoregrams was obtained at different detection wavelengths. Buffer, 20 mM borate, pH 9.5, containing 0.5 mM CTAB; Sample, 650 µg/ml iAs^{V2-}, 160 µg/ml MMA^{2-}, 160 µg/ml DMA^{2-}, 30 µg/ml iAs^{III2-}; injection pressure, 0.5 psi for 5 s; voltage, -20 kV; temperature, 25°C. Peaks: (1) iAs^{V2-}; (2) MMA^{2-}; (3) DMA^{2-}; (4) iAs^{III2-}.

quantification (LOQ) is the concentration of the fortified sample that can be quantified with less than 20% variation in precision, according to this rule, LOD and LOQ were also displayed in Table 2. Table 3 shows that the intraday precision expressed as relative standard deviations (R.S.D.%) with respect to migration time and peak area from five successive injections for the different analytes was found to be 1.45-2.06% for migration times and 3.14 - 4.28% for peak areas. The interday precision found by analyzing different analytes on three successive days was found to be 2.24 - 3.20% for migration times and around 6.46 -7.11% for peak areas. The deviation of the retention time observed in the intraday tests was lower than the deviation observed in the interday tests. This is logical, since small changes when the running buffer is prepared can affect the retention time of the analytes. The spiked recoveries were 84.92, 96.88, 101.67 and 88.65% for iAs^{V}, DMA^{V}, MMA^{V} and iAs^{III}.

Identification of arsenic species in the realgar bioleaching solution

In the first phase of bioleaching, the powder of realgar was insoluble in culture. After 15 days, a portion realgar was soluble and the precipitation was observed in the flask. After 30 days, the realgar powders were all soluble. Then the cultures were used for preparation of realgar bioleaching solution.

It has been reported that the main components in the

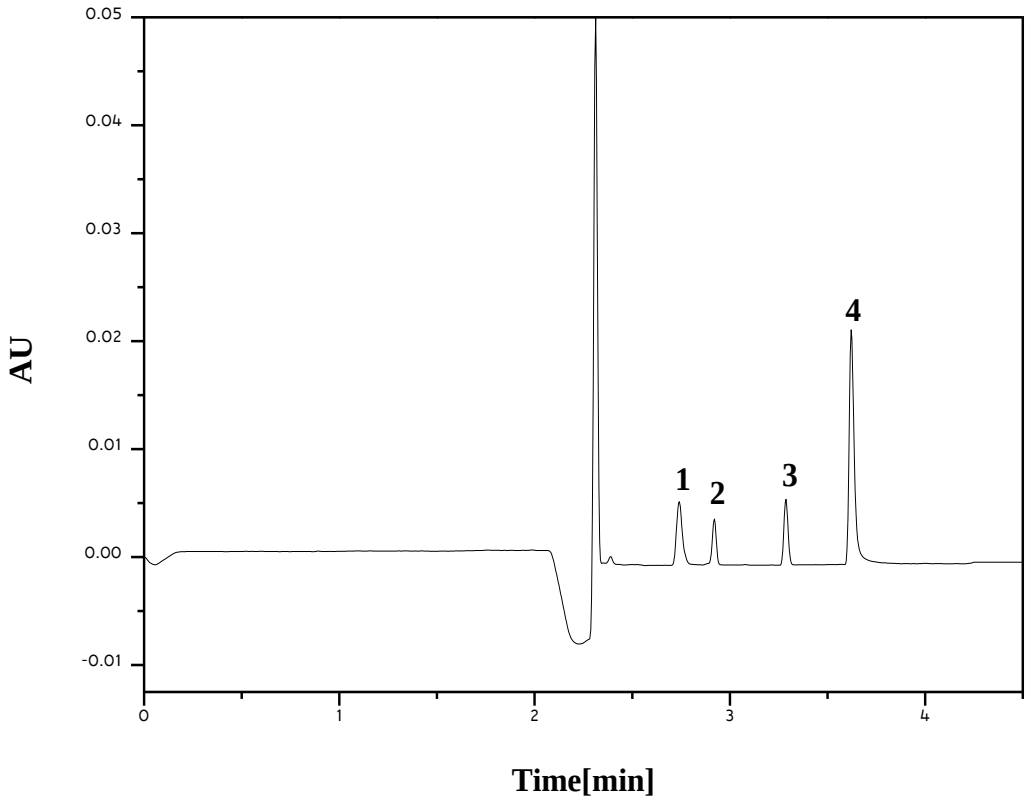


Figure 5. The electrophoregrams of arsenic compounds from standard mixture. Buffer, 20 mM borate, pH 9.5, containing 0.5 mM CTAB; Sample, 500 µg/ml iAs^{V2-}, 120 µg/ml MMA^{2-}, 130 µg/ml DMA^{2-}, 250 µg/ml iAs^{III2-}; injection pressure, 0.5 psi for 5 s; voltage, -20 kV; temperature, 25°C. Peaks: (1) iAs^{V2-}; (2) MMA^{2-}; (3) DMA^{2-}; (4) iAs^{III2-}.

Table 2. Linearity range (µg/ml), calibration line and detection limits for arsenic compounds.

Analyte	Linearity range (µg/ml)	Calibration line		Detection limits	
		Regression equation[a]	r	LOD (µg/ml)[b]	LOQ (µg/ml)[b]
iAs^{V}	50-500	y = 0.0412x + 4.3121	0.99982	12.00	39.83
MMA^{V}	12-120	y = 0.0378x + 4.4839	0.99975	3.13	10.46
DMA^{V}	13-130	y = 0.0155x - 4.7362	0.99930	3.88	12.94
iAs^{III}	25-250	y = 0.0027x – 2.473	0.99972	3.10	10.34

a. y and x stand for the peak area and the concentration (µg/ml) of the analytes, respectively. b. The LOD was defined as the concentration where the signal-to-noise ratio is 3 and the LOQ was defined as the concentration where the signal-to-noise ratio is 5.

Table 3. Precision and recovery of the analytical method.

Analyte	Intraday, R.S.D.(%)(n=5)		Inter-day, R.S.D.(%)(n=5)		Recovery, R.S.D. (%)
	Migration time	Peak area	Migration time	Peak area	(n=5)
iAs^{V}	1.45	3.66	2.24	6.46	84.92
MMA^{V}	1.61	4.28	2.54	6.57	96.88
DMA^{V}	1.87	4.27	2.76	6.74	101.67
iAs^{III}	2.06	3.14	3.20	7.11	88.65

alkali extracts of realgar were iAs^{III2-} and iAs^{V2-} (Wu et al., 2004). However, the determination of inorganic and organic analytes in real samples by CZE with direct ultraviolet detection was challenging since the

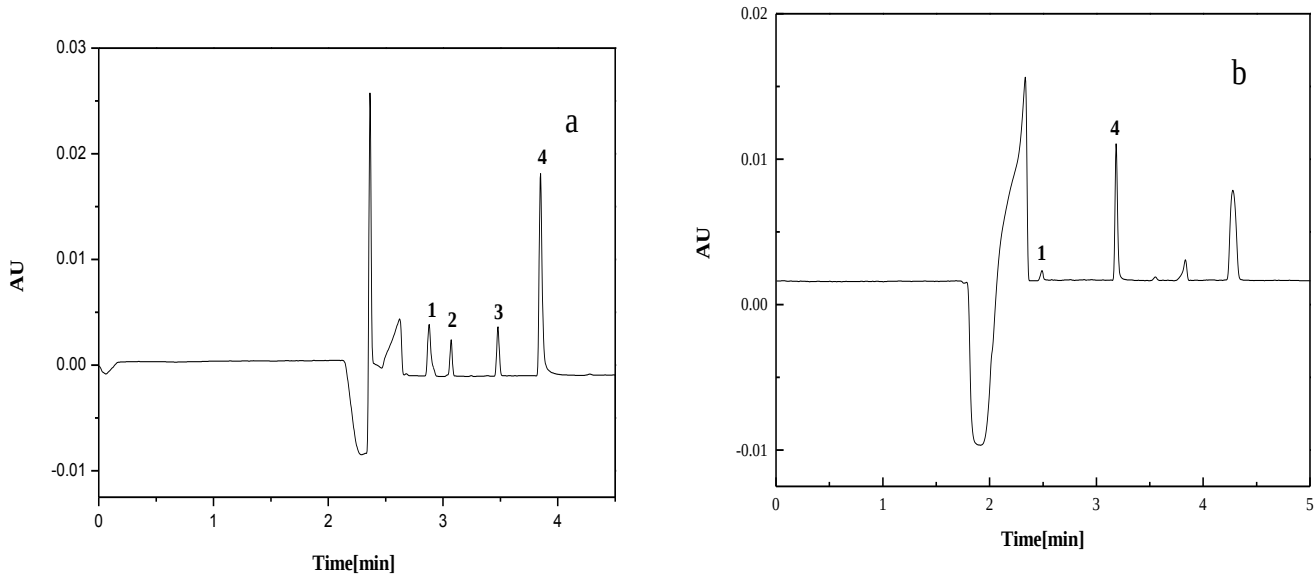


Figure 6. The electrophoregrams of (a) realgar bioleaching solution by adding appropriate standard, and (b) bio-leachate of realgar. Buffer, 20 mM borate and 0.5 mM CTAB; pH 9.5; injection pressure, 0.5 psi for 5 s; detection wavelength, 195 nm; voltage, -20 kV; temperature, 25°C. Peaks: (1) iAs^{V2-}; (2) MMA^{2-}; (3) DMA^{2-}; (4) iAs^{III2-}.

components of realgar bioleaching solution were unknown. The realgar bioleaching solution was analyzed using the established CZE method, the corresponding electrophoregrams indicated that iAs^{V2-} and iAs^{III2-} were the main components. Figure 6 shows that no signal peak was detected except iAs^{V2-} and iAs^{III2-} when compared with the electrophoregrams of the realgar bioleaching solution by adding appropriate standards. Using the established calibration curves, the concentrations of iAs^{V2-} and iAs^{III2-} in realgar bioleaching solution were 102.28 and 136.36 µg/ml as As. So far, this work is the first report on using CZE method with direct UV BGE to analyze the realgar bioleaching solution, adding valuable information for studying the components in the bioleaching of realgar.

Conclusion

A CZE method was developed and used to analyze arsenic species in realgar bioleaching solution produced by *A. ferrooxidans* BY-3. The CZE with direct UV detection showed excellent suitability for the simultaneous separation and determination of the inorganic and organic arsenic compounds using the borate as BGE. The reliability of the technique has also been verified by analysis of linearity, repeatability, reproducibility and sensitivity. The arsenic species in realgar bioleaching solution produced by *A. ferrooxidans* were iAs^{V2-} and iAs^{III2-}, and their concentrations were found to be 102.28 and 136.36 µg/ml, respectively.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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