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Rapid quantification of alcohol production in microorganisms based on nanostructure-initiator mass spectrometry (NIMS)

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A B S T R A C T

We described a mass spectrometry-based assay to rapidly quantify the production of primary alcohols directly from cell cultures. This novel assay used the combination of TEMPO-based oxidation chemistry and oxime ligation, followed by product analysis based on Nanostructure-Initiator Mass Spectrometry. This assay enables quantitative monitor both C5 to C18 alcohols as well as glucose and gluconate in the growth medium to support strain characterization and optimization. We find that this assay yields similar results to gas chromatography for isoprenol production but required much less acquisition time per sample. We applied this assay to gain new insights into *P. Putida*'s utilization of alcohols and find that this strain largely could not grow on heptanol and octanol.

1. Introduction

Synthetic biology-based tools are widely used to develop production approaches for biofuels and bioproducts [1,2]. These approaches often require extensive testing of thousands of different constructs to develop high efficiency and high titer microbial strains [3], for example to produce diverse products from glucose [4–6]. Higher alcohols with carbon chains ranging from C5 to C18 are important target products given their wide range of applications, from fuels to solvents to cosmetics [7]. However, a bottleneck to improving the alcohol-producing strains is the lack of a high throughput quantitative assay that has sufficient sensitivity for direct characterization of 96-wells plate-based cell cultures.

Gas chromatography-mass spectrometry (GC-MS)-based approaches are widely used to quantify alcohols with different carbon content (due to the low boiling points of these molecules) [8]. GC-MS can provide excellent sensitivity and high resolution needed for the separation and identification of alcohols. However, since GC-MS based analysis has a longer data acquisition time for each sample (usually 5–30 min/sample) and it cannot process multiple samples at the same time, it is considered a low throughput approach.

Fluorescence assays [9], on the other hand, can be high throughput for rapid automated testing of large sample libraries, but have low selectivity for a different type of alcohols [10] and typically only provide a measure of the total alcohol content. Here, we report the development of a high throughput mass spectrometry-based assay that has sufficient sensitivity to detect alcohols from cell culture and resolves C5-18 alcohols as well as glucose and gluconate. Our new assay is based on Nanostructure-Initiator Mass Spectrometry (NIMS), which is a novel laser desorption ionization technique developed by Siuzdak et al. [11]. Different class of molecules were found to have different ionization/desorption efficiency on NIMS surface. For example, peptides and fatty acids can be detected by NIMS without any derivatization. For glycans and steroids, however, spraying the NIMS surface with sodium or silver ions respectively is needed to facilitate the ionization/desorption of these two classes of molecules [12]. Recently, f-AuNPs has been developed as an agent for NIMS to facilitate the detection of both lipophilic and polar metabolites [13,14]. It is an exciting new development to allow comprehensive bioimaging by taking advantage of fluorinated nanoparticles [15,16]. Regarding to primary

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alcohols, there are no previous reports for their quantification on NIMS surfaces. When we tested C5 to C18 alcohols (0.5 μL of 4 mM alcohols in dodecane deposited directly on NIMS surface with an area size of 3 mm × 3 mm), we could not detect any desirable signals beyond the background for all alcohols tested. Typically, for the detection of fatty alcohols by mass spectrometry, chemical derivatization is needed to generate ionizable intermediates [17]. Since fatty alcohols could not be detected directly on NIMS surface, we decided to perform derivatization by taking advantage of the probe that we have developed before for quantitative glycan analysis [18]. As glycans could not be detected by NIMS without surface modification, a derivatization strategy was utilized. There are many reports about glycan labeling strategies and their use in identification and quantification [19]. We chose the formation of oxime adducts from glycans for their identification and quantification since the reaction conditions for oxime formation are typically very mild and compatible with aqueous solutions. In addition, the reaction is almost quantitative [20]. Following this strategy, we designed and synthesized a unique perfluorinated alkoxyamine probe for glycan analysis by NIMS. Under room temperature in acidic aqueous solution, the probe can form a stable oxime bond with the reducing ends of soluble glycans. The resulting oxime adducts can be easily quantified on NIMS surface by comparing the intensity ratio of the analytes with internal standard (e.g. ^{13}C glucose). We have successfully used this assay to quantify the various glycan products from the enzymatic hydrolysis of solid biomass (one example is shown in Fig. S1) [18]. Since our probe works specifically with compounds containing carbonyl function, primary alcohols only need to be converted to the corresponding aldehydes. Therefore, a two-step assay for quantifying alcohols was developed. Firstly, we used a mild chemical oxidation step to convert the primary alcohols to the corresponding aldehydes, which can react with perfluorinated alkoxyamine tags in the second step for direct characterization using Nanostructure-Initiator Mass Spectrometry (NIMS) [18]. Together with the high throughput platform we have already built for NIMS analysis of large GH enzyme library [21], this creates a platform that can quantitatively monitor both alcohols and glycans in the growth medium to support the characterization of microbial metabolism.

2. Experimental

2.1. Materials and instrumentations

All reagents were of the highest grade that could be obtained commercially. Water (18.2 MΩ·cm) was generated using a Barnstead Millipore Milli-Q water system and was used to prepare all buffers and aqueous solutions. All NIMS analyses were performed on NIMS chips, fabricated as described previously [22] using a Bruker UltrafleXtreme MALDI TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Chips were loaded using a modified standard MALDI plate. FlexControl and FlexAnalysis were used for acquisition and data analysis. Spectra were recorded in positive reflector mode. The laser has a spatial resolution of 10 μm. The instruments were calibrated using Anaspec Peptide Calibration mixture 1 (Anaspec, Fremont, CA). Data were acquired by summing up 3000 laser shots in 500 shot steps, randomly sampling 6 regions locations per spot. Sample spots were identified using grids and inscriptions made with a diamond-tip scribe prior to sample deposition.

2.2. Microbial production of isoprenol in microtiter plates

All strains and plasmids used in this study are listed in Table S1. *Escherichia coli* DH1 strains were used for isoprenol production. The production was performed in 24-well deep-well microtiter plates (rectangular, V-shaped well bottom, Axygen Scientific P-DW10ML24CS). For *E. coli* isoprenol production, the starter cultures of all production strains were prepared by growing single colonies in LB medium containing 30 μg/mL chloramphenicol and 100 μg/mL carbenicillin at 37 °C with 200-rpm shaking for overnight. The starter cultures were diluted to an optical density (OD_{600nm}) of 0.1 in 2 mL MOPS EZ-Rich defined medium (Teknova, CA, USA) or M9-MOPS medium [23] containing 10 g/L or 20 g/L glucose, 30 μg/mL chloramphenicol, 100 μg/mL carbenicillin, and 0.5 mM IPTG in the 24-well plates sealed with sterile film (Sigma-Aldrich, St. Louis, MO). The cell cultures were incubated in rotary shakers (200 rpm) at 30 °C for 48 h. The statistical software GraphPad was used to analyze the experimental data and to perform the unpaired *t*-test.

2.3. Isoprenol quantification by gas chromatography (GC)

For isoprenol quantification, an aliquot of cell culture samples was extracted by an equal volume of ethyl acetate containing 1-butanol (30 mg/L) as an internal standard. The mixture of ethyl acetate and cell culture was vigorously mixed for 15 min and then centrifuged at 21,130 g for 3 min. The ethyl acetate layer was collected and appropriately diluted in ethyl acetate containing 1-butanol (30 mg/L). 1 μL was analyzed by gas chromatography – flame ionization detection (GC-FID, Thermo Focus GC) equipped with a DB-WAX column (15-m, 0.32-mm inner diameter, 0.25-μm film thickness, Agilent, USA), the oven temperature program was as follows: starting at 40 °C, a ramp of 15 °C/min to 100 °C, a ramp of 40 °C/min to 230 °C and held at 230 °C for 3 min.

2.4. Fitness study of n-alcohols for Pseudomonas putida KT2440

P. putida KT2440 strains were grown in LB medium overnight and diluted into 0.2 mL M9 minimal medium [24] containing 10 g/L glucose (1%, w/v) and 1 g/L n-alcohols in a Corning flat-bottom 96-well transparent plate. The initial optical density at 600 nm (OD_{600}) was set to 0.01. Cell growth in 96-well plates was monitored using an automated reader, shaker, and incubator (Tecan-F200pro) at 30 °C for 24 h. The statistical software GraphPad was used to analyze the experimental data and to perform the unpaired *t*-test.

2.5. Microbial production of dodecanol

Details for plasmids and producing strains, and dodecanol production run in a BioLector microbioreactor can be found in the previous publication [25].

2.6. NIMS assay

a) Oxidation solution was prepared as follows:

In a 4 mL glass vial was added TBACl (10 mg), NCS (60 mg), and TEMPO (5.0 mg), followed by the addition of dichloromethane (3 mL). For the quantification of dodecanol and other related fatty alcohols ranging from 1-octanol to 1-octadodecanol, an Eppendorf™ tube (0.2 mL) was added 50 μL of pH 8.6 buffer solution, 50 μL of the above oxidation solution, and 50 μL of fatty alcohols containing dodecane overlay. The resulting mixture was shaken at room temperature for 4 h.

For the quantification of 3-methyl-3-buten-1-ol (isoprenol),1-pentanol, 1-hexanol and 1-heptanol, an Eppendorf™ tube (0.2 mL) was added 50 μL of pH 8.6 buffer solution, 50 μL of the above oxidation solution and 50 μL of cell culture samples. The resulting mixture was shaken at room temperature for 1.5 h.

b) The oxime tagging reaction was performed as follows:

To an Eppendorf™ tube (0.2 mL) was added 6 μL of pH 1.3 glycine buffer (100 mM), 4 μL of organic solvents (prepared by mixing methanol:acetonitrile:aniline = 1:2:0.1), 0.5 μL of ^{13}C glucose (5 mM aqueous solution), 1 μL of aminooxy alkyl probe (100 mM in a mixture of methanol: water = 1 : 1). For the dodecanol (and other fatty alcohols ranging from 1-octanol to 1-octadodecanol) quantification assay, 3 μL of

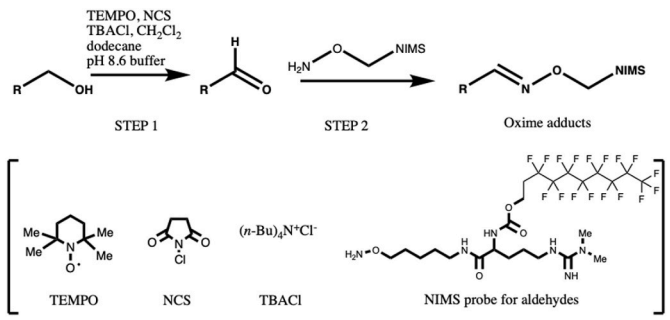

Fig. 1. Two-step Reactions: 1) TEMPO-based oxidation of primary alcohols; 2) Aldehyde captured using NIMS probe.

the dodecane layer from the above oxidation reaction was used. For the 3-methyl-3-buten-1-ol (isoprenol), 1-pentanol, 1-hexanol and 1-heptanol quantification assay, 2 μL of the dichloromethane layer were used. The resulting mixture was incubated at room temperature for 12 h.

3. Results and discussion

3.1. Assay development

To enable direct characterization of cell cultures we focused on establishing mild alcohol oxidation methods, which also need to be compatible with aqueous solutions. After several unsuccessful trials with nanoparticles-based oxidation of alcohols in an aqueous solution (e.g., polyoxometalate cluster-based oxidation [26]; magnetic Fe_3O_4 nanoparticles-based oxidation [27]) and using biological oxidation with commercially available alcohol dehydrogenase [28], we found that a modified phase transfer reaction performed at room temperature worked well for our application. As shown in step 1 (Fig. 1), TEMPO (2,2,6,6-Tetramethylpiperidine 1-oxyl) based heterogeneous biphasic reactions can selectively convert primary alcohols to the corresponding aldehyde by using dichloromethane as the organic phase and catalytic amounts of TEMPO and N-chlorosuccinimide (NCS) as the stoichiometric oxidants [29]. Tetrabutylammonium chloride (TBACl), a quaternary ammonium salt, was included as a phase-transfer reagent to facilitate the migration of a reactant from one phase into another phase where the reaction occurs. Subsequently, the aldehyde products generated from the first step are conjugated with an alkyloxy amine probe [18], and the resulting mixture is spotted directly on the NIMS surface for mass spectrometry analysis.

3.2. Isoprenol production in E. Coli, as determined by NIMS analysis of growth media under different media conditions

We first used this method to quantify 3-methyl-3-buten-1-ol (isoprenol) production in *E. coli* strains under two growth conditions (see SI for details), rich medium (EZ) and minimal medium (M9) where the NIMS assay was performed by using cell culture samples directly with ^{13}C glucose as the internal standard, whereas the GC analysis was performed after extraction with ethyl acetate. Although NIMS assays used similar sample volume compared to GC-MS assays, they are completed in a few seconds/sample vs. 7–8 min/sample for GC-MS (the comparison

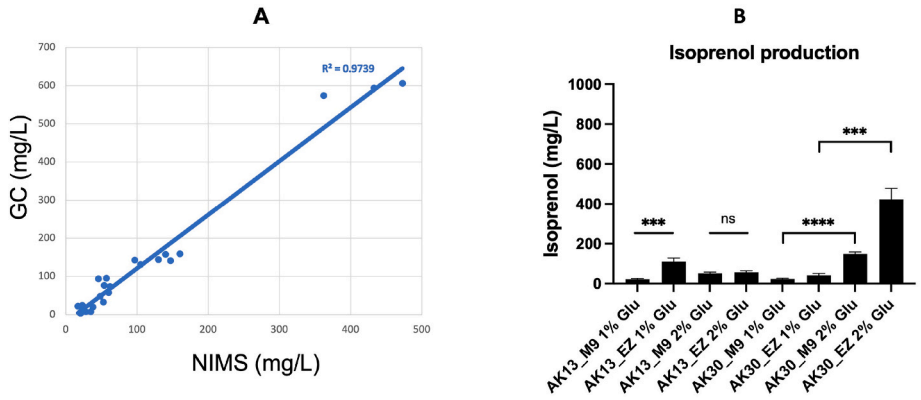

Fig. 2. (A) GC-NIMS results correlation for 3-methyl-3-buten-1-ol (isoprenol) (B) Isoprenol production by *E. coli* strains (AK13, lower producer; AK30, higher producer), as determined by NIMS analysis of growth media under different media conditions. ns, not significant; ***, $P < 0.001$; ****, $P < 0.0001$.

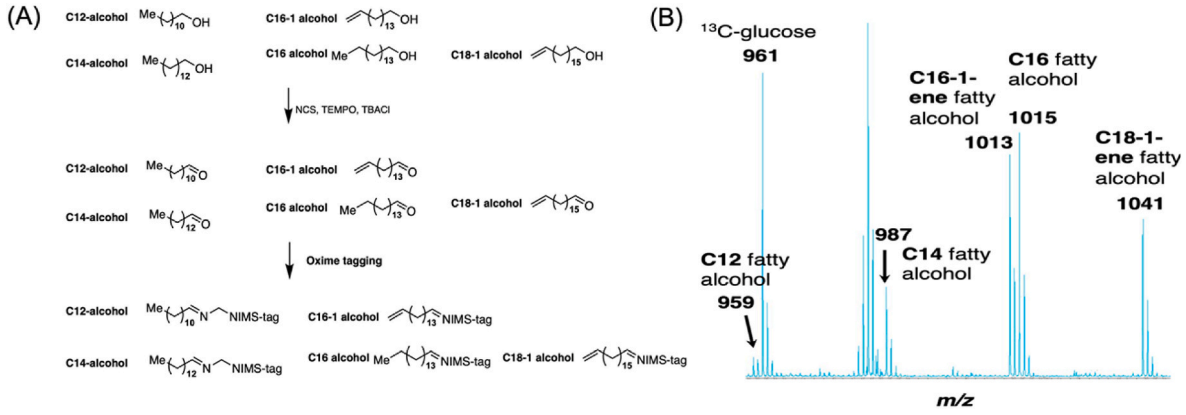

Fig. 3. (A) Chemical transformations of various fatty alcohols to form detectable oxime adducts by NIMS. (B) Representative mass spectral signatures for quantification of fatty alcohols.

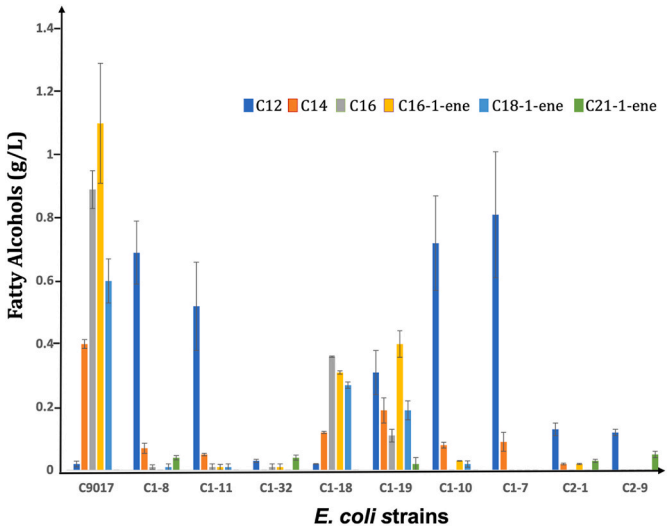

Fig. 4. Fatty alcohols production (Experiments were performed in triplicate and error bars represent the standard deviation).

was made based on the acquisition time). We observe consistent results between the two assays as shown in Fig. 2A. In addition, we observe that the CV across strains for the NIMS assay is <20%. As shown in Fig. 2B, we observe differences in isoprenol production between strains and medium conditions. The lower-producing strain (AK13) produced more isoprenol in the rich medium than in the minimal medium ($p < 0.05$) at 1% glucose conditions. However, glucose concentration played a more important role in the higher-producing strain (AK30) after deleting acetate pathway genes ($p < 0.05$), which verified that acetate accumulation was a limiting factor during isoprenol production [23].

3.3. Fatty alcohol quantification by NIMS

We next used this NIMS-based assay to study fatty alcohol production in engineered *E. coli* strains, that are known to be high-titer dodecanol (C12) producers that are also capable of producing longer-chain fatty alcohols (C14–C18) [30], Dodecane was used as a solvent overlay during fatty alcohol production for in situ extraction. As shown in Fig. 3A, various fatty alcohols in the sample can undergo oxidation followed by alkoxyamine ligation to form oxime adducts, which can be detected and quantified by NIMS. Fig. 3B is the mass spectra obtained for a sample containing various fatty alcohols and the intensity ratio of fatty alcohols v. s. that of ^{13}C glucose (peak m/z 961) is used for quantification of each individual fatty alcohol.

Consistent with previous results obtained using GC-MS [25,30], we observed that C1-7, C1-8, C1-10, and C1-11 strains are the highest producers of 1-dodecanol (C12) among all tested strains, but C9017, C1-18, and C1-19 strains can produce higher levels of tetradecanol (C14), hexadec-15-en-1-ol (C16-1-ene), C16 (1-hexadecanol), octadec-17-en-1-ol (C18-1-ene) (Table S2). Different product distribution profiles for each mutant are displayed in Fig. 4. Since biosynthetic fatty alcohols typically contain multiple alcohol components (saturated, unsaturated, different chain lengths, etc.), our assay provides the identification and quantification of different types of alcohol components simultaneously (Fig. 4), which greatly improves the efficiency to quantify alcohol distribution.

3.4. Fitness study of n-alcohols for Pseudomonas putida KT2440

With the thriving of the synthetic biology industry, one critical need is to establish a database for the fitness response to any products of interest for different host strains. Thus, it is highly desirable to develop one automation-compatible analytic method that can collect various metabolic information in a high throughput format. Here, we demonstrated the use of our method in a fitness study of n-alcohols for *P. putida* KT2440, which is an emerging microbial host due to its capability of catabolizing broad carbon sources and high tolerance to xenobiotics [31]. We used it to examine n-alcohols use by *P. putida* KT2440. As shown in Fig. 5 A-B, *P. putida* grew on C5, C6, C9, and C10 n-alcohols, but failed to grow on C7 and C8 n-alcohols after 24-h cultivation, suggesting a less tolerance of *P. putida* with these two alcohols. Using our NIMS assay we found high levels of depletion for most alcohols (from C5 to C10) at the end of the cultivation (Table S3). In addition, we used the oxime-NIMS method [18] to analyze glucose and gluconate remaining in the culture. As expected, there is more glucose and gluconate present when the cells are grown on C7 and C8 alcohols which may inhibit growth (Fig. 5C). Given that n-alcohols are considered to be catabolized through an oxidation to their associated acids followed by the β-oxidation in *P. putida* [32], the lower tolerance on C7 and C8 n-alcohols may be attributed to a lower dehydrogenase activity or higher toxicity on the transitional chain length from short to medium-long chain n-alcohols, such as C7 and C8 n-alcohols [33]. Further studies are needed to understand the lower tolerance of C7 and C8 n-alcohols, such as to investigate whether their catabolic products are inhibitors of *P. putida*.

4. Conclusion

In summary, we have developed a new platform for screening primary alcohols using NIMS. We found that the NIMS-based assay provides comparable results to GC-MS for analysis of both short and long-chain primary alcohols. We used this approach to characterize n-alcohols use by *P. putida* and learned that this strain has dramatically different responses to C7 and C8 alcohols vs. other alcohols. Given the ability of this assay to directly quantify alcohols from 96-well plate

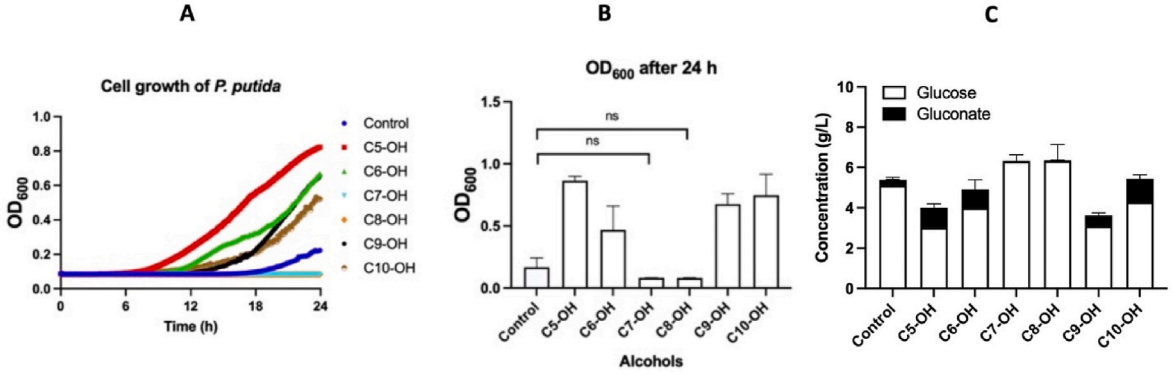

Fig. 5. Fitness study of n-alcohols for Pseudomonas putida KT2440. (A) Cell growth; (B) OD600 after 24 h ns, not significant; (C) Concentration of glucose and gluconate after 24 h. Experiments were performed in triplicate, and error bars represent the standard deviation.

cultures, it suggests this approach has significant potential for high-throughput screening of alcohol-producing and alcohol-consuming microbial strains. Potential applications may extend this method to discovery-based studies, such as fitness responses of alcohols, high-throughput screening of mutant libraries, as well as biomedical applications in compound screening and drug discovery.

Authors' contributions

K.D., X.W. and T.R.N conceived the study. K.D., X.W., T.S.L. and T.R.N. designed the experiments. K.D., N.I. and M.R. performed the NIMS analysis. X.W., P.O. and J.K. prepared the cell culture samples. K.D., X.W., T.S.L. and T.R.N. wrote the manuscript. B.A.S., P.D.A. and A.K.S. discussed, revised, and checked the manuscript. All author reviewed the manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2022.114997.

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